Physico-Chemical Methods in Drug Discovery and Development

**Edited by
Zoran Mandić**

A CIP catalogue record for this book is available from
the National and University Library in Zagreb under 804310

Published by:
IAPC Publishing, Zagreb, Croatia, 2012

Zoran Mandić, editor
Physico-Chemical Methods in Drug Discovery and Development
Copyright © 2012, IAPC. All rights reserved

No part of this book may be reproduced in any form or by any means without written permission from the publisher. No part of this book may be transmitted or translated into machine or other languages.

The efforts have been made to publish reliable and accurate data as much as possible, but the authors and the editor cannot assume responsibility for the validity of materials or the consequences of their use.

ISBN 978-953-56942-0-5

Printed in Zagreb, Croatia

IAPC Publishing is a part of International Association of Physical Chemists

CONTENTS

PREFACE	xi
ACKNOWLEDGMENTS	xiii
LIST OF CONTRIBUTORS	xv

Chapter 1

PHYSICOCHEMICAL PROFILING IN DRUG RESEARCH AND DEVELOPMENT	1
1.1. INTRODUCTION	3
1.2. THEORETICAL BACKGROUND	6
1.2.1. The physical-chemistry of drug action	6
1.2.2. Physicochemical parameters	8
1.3. METHODS FOR PHYSICOCHEMICAL PROFILING.....	25
1.3.1. pK_a determination.....	25
1.3.2. $\log S$ determination	32
1.3.3. $\log P$ determination	37
1.4. CASE STUDIES.....	41
1.4.1. pK_a determination	41
1.4.2. $\log S$ determination	44
1.4.3. $\log P$ determination	49
1.5. OUTLOOK.....	52
Acknowledgement.....	52
REFERENCES	52

Chapter 2

APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE MEASUREMENTS OF LIPOPHILICITY AND BIO-MIMETIC BINDING PROPERTIES IN DRUG DISCOVERY	61
2.1. INTRODUCTION.....	63
2.2. THEORETICAL BACKGROUND OF HPLC	65
2.2.1. Direct measurement of lipophilicity by chromatographic methods	68
2.2.2. Measurements of lipophilicity using isocratic chromatographic retention	70
2.2.3. Measurements of lipophilicity using gradient chromatographic retention.....	73
2.2.4. pH dependence of lipophilicity and reversed phase chromatographic retention	74
2.3. APPLICATION OF BIO-MIMETIC HPLC STATIONARY PHASES	79
2.3.1. Immobilised human serum albumin (HSA) stationary phase.....	79
2.3.2. Immobilised α -1-acid-glycoprotein (AGP) stationary phase	81
2.3.3. Immobilised artificial membrane (IAM) stationary phase.....	83
2.4. GENERAL EXPERIMENTAL DESCRIPTION OF FAST GRADIENT HPLC ANALYSIS OF LIPOPHILICITY, PROTEIN AND PHOSPHOLIPID BINDING OF DRUG DISCOVERY COMPOUNDS.....	84
2.4.1. Measurements of chromatographic hydrophobicity indices at three different pHs	84
2.4.2. Measurement of HSA binding by HPLC retention	85
2.4.3. Measurement of AGP binding by HPLC retention	87
2.4.4. Measurement of IAM binding by HPLC retention.....	89
2.5. ANALYSIS OF THE HPLC BASED LIPOPHILICITY, PROTEIN BINDING AND PHOSPHOLIPID BINDING DATA	90
2.5.1. Analysis of the effect of charge	90
2.5.2. Analysis by the solvation equation	93
2.6. APPLICATIONS OF BIO-MIMETIC HPLC PROPERTIES TO MODEL DRUGS <i>IN VIVO</i> DISTRIBUTION	95
2.6.1. Modelling steady state volume of distribution	98
2.6.2. Modelling unbound volume of distribution	103
2.6.3. Dependence of unbound volume of distribution on HSA and IAM binding ...	104
2.6.4. General structure-property relationships for lead optimisation	107
2.7. ALTERNATIVE METHODS.....	108
2.7.1. Methods based on the principle of frontal chromatography.....	108
2.7.2. Methods based on solid particles.....	111
2.7.3. Methods based on equilibrium dialysis and ultra-filtration.....	112
REFERENCES.....	113

Chapter 3

THE IMPORTANCE OF AND DIFFERENT APPROACHES TO PERMEABILITY DETERMINATION.....121

3.1. INTRODUCTION	123
3.1.1. Permeability and the biopharmaceutical classification system	123
3.1.2. The rationale for permeability determinations in drug discovery	126
3.2. THEORETICAL BACKGROUND OF PAMPA.....	127
3.2.1. pH partition hypothesis.....	128
3.2.2. The PAMPA assay	129
3.2.3. Definition of permeability	130
3.2.4. Advanced transport model for PAMPA.....	131
3.2.5. Permeability terms	134
3.2.6. Permeability hydrodynamic model.....	134
3.2.7. Permeability-pH dependence	135
3.3. COMPARISONS OF DIFFERENT PAMPA METHODS.....	135
3.3.1. Lipid models	136
3.3.2. Experimental procedures	140
3.4. APPLICATIONS OF PAMPA DATA TO PREDICT AND/OR MODEL <i>IN VIVO</i> DATA.....	151
3.4.1. Corrections for paracellular transport	152
3.4.2. Absorption model.....	154
3.4.3. Correlation between <i>in vitro</i> permeability and the absorbed fraction of drugs in humans	154
3.4.4. Prediction of the absorbed fraction of amphoteric drugs in humans	155
3.5. CONCLUDING REMARKS.....	158
Acknowledgements.....	159
REFERENCES	159

Chapter 4

PROTEIN-LIGAND INTERACTIONS.....	165
4.1. INTRODUCTION.....	167
4.2. OVERVIEW OF DRUG DISCOVERY.....	168
4.3. PROTEIN-LIGAND BINDING EQUILIBRIA, THERMODYNAMICS AND KINETICS	169
4.4. NON-COVALENT INTERACTIONS IN PROTEIN-LIGAND BINDING	172
4.4.1. Hydrogen bonding.....	173
4.4.2. Ionic or electrostatic interactions.....	173
4.4.3. Van der Waals interactions.....	173
4.4.4. Hydrophobic interactions	173
4.5. BIOPHYSICAL METHODS FOR CHARACTERISING PROTEIN-LIGAND INTERACTIONS.....	174
4.5.1. Isothermal titration calorimetry (ITC)	174
4.5.2. Surface Plasmon Resonance (SPR).....	175
4.5.3. Optical waveguide grating (OWG).....	178
4.5.4. Spectroscopic methods – Nuclear Magnetic Resonance (NMR)	178
4.5.5. Spectrometric methods – Mass Spectrometry (MS)	180
4.6. BRIEF CASE STUDIES EXEMPLIFYING THE USE OF BIOPHYSICAL METHODS	181
4.6.1. DNA gyrase	181
4.6.2. Beta-site APP cleaving enzyme 1 (BACE-1)	183
4.6.3. MEK protein kinase	184
4.7. FUTURE TRENDS FOR BIOPHYSICAL APPROACHES.....	185
4.8. SUMMARY	186
REFERENCES.....	186

Chapter 5

NMR SPECTROSCOPY FOR STUDYING INTERACTIONS OF BIOACTIVE MOLECULES	189
5.1. INTRODUCTION	191
5.2. LIGAND-PROTEIN INTERACTIONS	203
5.3. LIGAND-DNA, LIGAND-RNA AND LIGAND MEMBRANE INTERACTIONS	209
5.4. STRUCTURAL ANALYSIS OF LIGANDS	214
5.5. STRUCTURAL ANALYSIS OF PEPTIDES AND PROTEINS	215
5.6. MACROLIDE-RIBOSOME INTERACTIONS	217
Acknowledgement.....	220
REFERENCES	220

Chapter 6

CHIRAL SEPARATION FOR ENANTIOMERIC DETERMINATION IN THE PHARMACEUTICAL INDUSTRY	233
6.1. INTRODUCTION	235
6.2. ENANTIOMERS, DIASTEREOMERS, RACEMATES	236
6.3. REQUIREMENTS FOR CHIRAL SEPARATION	237
6.4. THE TYPES OF MOLECULAR INTERACTIONS.....	237
6.4.1. Chiral separation through hydrogen bonding.....	237
6.4.2. Chiral separation through inclusion compounds.....	243
6.4.3. Charge transfer.....	259
6.4.4. Chiral separation through a combination of charge transfer, hydrogen bonding and electrostatic interactions.....	260
6.4.5. Ligand exchange	270
6.4.6. Mixed types of interactions.....	273
6.5. CONCLUSION.....	319
REFERENCES	319

Chapter 7

THE IMPORTANCE OF CHOOSING THE OPTIMAL SOLID FORM OF A DRUG	350
7.1. INTRODUCTION.....	352
7.2. THERMODYNAMICS	355
7.2.1. Polymorphs.....	355
7.2.2. Hydrates.....	360
7.3. FORM SCREENING.....	366
7.3.1. Screening for Salts/Co-Crystals.....	366
7.3.2. Screening for Polymorphs	369
7.3.3. Types of polymorph screens.....	372
7.4. CHARACTERISATION.....	374
7.4. 1. X-ray diffraction (XRD).....	374
7.4.2. Raman spectroscopy	376
7.5. CONCLUSION	378
ABBREVIATIONS	381
REFERENCES.....	382

Chapter 8

THERMAL ANALYSIS AND CALORIMETRIC METHODS APPLIED TO PHARMACEUTICAL SOLID FORMS	386
8.1. INTRODUCTION.....	388
8.2. BASIC PRINCIPLES OF THERMAL METHODS AND THEIR IMPORTANCE IN PHARMACEUTICAL ANALYSIS	389
8.2.1. Thermal analysis methods.....	389
8.2.2. Microcalorimetry	389
8.2.3. Overview of applications.....	389
8.3. FACTORS TO BE CONSIDERED IN THE INSTRUMENTATION	392
8.3.1. Heat flux differential scanning calorimetry (DSC) and power compensation DSC.....	392
8.3.2. Thermogravimetry.....	398
8.4. WHAT ARE THE AREAS OF APPLICATION FOR THE STUDY OF SOLID FORMS?	401
8.4.1. Thermodynamic data	401
8.4.2. Purity determination.....	409
8.4.3. Kinetic aspects.....	411

8.4.4. Sorption isotherms and hydrates	414
8.4.5. Combined techniques	417
8.4.6. Isothermal microcalorimetry and solution calorimetry	423
8.5. SOME EXAMPLES OF APPLICATIONS	425
8.5.1. The selection of the right form	425
8.5.2. Sensitivity of DSC.....	426
8.5.3. Disappearing solvates	427
8.5.4. Selection of the best form considering the pharmaceutical environment.....	427
8.6. CONCLUSION	429
REFERENCES	429

Chapter 9

NEAR INFRARED SPECTROSCOPY IN DRUG DISCOVERY AND DEVELOPMENT PROCESSES	432
9.1. INTRODUCTION	434
9.2. NIRS AND IMAGING: THEORY AND INSTRUMENTATION.....	435
9.2.1. General introduction.....	435
9.2.2. Characteristics of NIRS	436
9.2.3. NIR instrumentation	437
9.3. CHEMOMETRICS AND VALIDATION OF NIR METHODS	438
9.3.1. Development and validation of NIR methods	438
9.3.2. Mathematical preprocessing of spectroscopic data	442
9.3.3. Principal component analysis (PCA)	443
9.3.4. Pattern recognition.....	444
9.3.5. Regression methods	446
9.4. QUALITATIVE ANALYSES BY NIRS	447
9.4.1. Identification and qualification	447
9.4.2. Polymorphism.....	449
9.4.3. Other applications.....	449
9.5. QUANTITATIVE ANALYSES BY NIRS.....	450
9.5.1. Physical parameters.....	450
9.5.2. Polymorphs determination.....	451
9.5.3. Moisture determination	451
9.5.4. Content determination.....	452
9.6. ON-LINE CONTROL BY MEANS OF NIRS.....	455
9.6.1. Powder Blending	455

9.6.2. Granulation.....	457
9.6.3. Drying.....	458
9.6.4. Crystallinity and polymorphism	458
9.6.5. Coating.....	459
9.6.6. Biotechnology	459
9.7. APPLICATIONS OF NIR SPECTRAL IMAGING.....	461
9.7.1. General use of NIR chemical imaging for pharmaceutical applications	461
9.7.2. Tablet composition analysis with NIR imaging	461
9.7.3. Application of NIR imaging to process optimization	462
9.8. CONCLUSION	464
REFERENCES.....	464
INDEX.....	481

PREFACE

Improving human health and preventing illness and various diseases is one of the major areas of human endeavour in contemporary science. Huge amounts of money are invested in drug research both in academic institutions and pharmaceutical companies. Discovery of new biologically active chemical entities, their promotion in clinical candidates and drug molecules, as well as design of efficient drug formulations and their improvement are long-term tasks requiring a multi-disciplinary approach. Throughout the research process scientists are faced with challenges where important decisions have to be made and crucial actions have to be taken in order to keep pace with ever-increasing demands from the pharmaceutical market and pharmaceutical management.

Physical chemistry underlies most of the tools that medicinal chemists have at hand to assist them in their research. The vast armada of physico-chemical methods and techniques available enable fast and accurate measurements of specific parameters facilitating identification and selection of drug candidates. However, appropriate applications of physico-chemical techniques or their combinations, the proper choice of the corresponding methods as well as accurate interpretation of the results relies not only on good knowledge of physical chemistry but also on open and active communication between scientists of various backgrounds.

This book gives a selection of topics related to the various methods commonly used in pharmaceutical research. The intention, on the one hand, is to provide a theoretical background of the particular technique in order to enable inexperienced readers to gain a general impression of the usefulness of the field. On the other hand, a lot of practical examples will provide scientists, who encounter these methods either in the pharmaceutical research process or in academic institutions, with enough useful information for the successful application of these methods in their everyday work.

ACKNOWLEDGMENTS

This book would not be possible without efforts and time the authors and co-authors spent in writing their valuable contributions. Their willingness to freely share their knowledge and experience cannot be appreciated enough. Most of the authors participated and delivered lectures at the series of *World Conferences on Physico-Chemical Methods in Drug Discovery and Development* organized by *International Association of Physical Chemists*. I am very much indebted to the people and staff of the *International Association of Physical Chemists* for their financial and technical support in both organization of the mentioned conferences and the preparation of this book.

Special thanks go to people and staff of PLIVA Ltd. Although PLIVA underwent some serious re-organizations and was recently acquired by TEVA, its determination to support not only its own research facilities but also the research potential outside its "borders" remained constant. I would especially thank Biserka Cetina-Čižmek, Ernest Meštrović and Katica Lazarić whose warm support significantly helped the publication of this book.

Vesna Gabelica Marković provided a long-term valuable support which is greatly appreciated.

I am very grateful to Dražen Čavuzić who critically read some parts of the book and gave valuable suggestions.

Last but not least, I wish to express my gratitude to Alex Avdeef. His energy, positive attitude and willingness to support our work boosted our determination to keep going.

Zoran Mandić

LIST OF CONTRIBUTORS

Klara Dégardin, F. Hoffmann – La Roche Ltd., Basel, Switzerland

Robert A.W. Dryfe, School of Chemistry, University of Manchester, UK

Danièle Giron, ex Novartis-Pharma & University of Basel, Switzerland,
Consultant, France

Nelu Grinberg, Chemical Development, Boehringer Ingelheim Pharmaceuticals,
Inc., Ridgefield, CT US

Rolf Hilfiker, Vice President, Head of Department Solid-State Development,
Solvias AG, Kaiseraugst, Switzerland

Geoff Holdgate, AstraZeneca, Discovery Sciences, Macclesfield, Cheshire, UK

Tomislav Jednačak, Department of Chemistry, Faculty of Natural Sciences,
University of Zagreb, Croatia

Predrag Novak, Department of Chemistry, Faculty of Natural Sciences,
University of Zagreb, Croatia

Su Pan, Analytical Research & Development, Pharmaceutical Research Institute,
Bristol-Myers Squibb Company, NJ, US

Yves Roggo, F. Hoffmann – La Roche Ltd., Basel, Switzerland

Kin Y. Tam, AstraZeneca, Mereside, Macclesfield, Cheshire, UK

Krisztina Takács-Novák, Department of Pharmaceutical Chemistry,
Semmelweis University, Budapest, Hungary

Michel Ulmschneider, F. Hoffmann – La Roche Ltd., Basel, Switzerland

Klara Valko, CSC-Analytical Chemistry, GlaxoSmithKline, Stevenage, UK

Matěj Velický, School of Chemistry, University of Manchester, UK

PHYSICOCHEMICAL PROFILING IN DRUG RESEARCH AND DEVELOPMENT

Krisztina Takács-Novák

Contents

1.1. INTRODUCTION	3
1.2. THEORETICAL BACKGROUND	6
1.2.1. The physical-chemistry of drug action	6
1.2.2. Physicochemical parameters	8
1.2.2.1. Ionization (pK_a).....	8
1.2.2.2. Solubility ($\log S$).....	17
1.2.2.3. Lipophilicity ($\log P$).....	20
1.3. METHODS FOR PHYSICOCHEMICAL PROFILING.....	25
1.3.1. pK_a determination.....	25
1.3.1.1. Potentiometric method	26
1.3.1.2. UV/pH titration.....	27
1.3.1.3. Other methods.....	28
1.3.1.4. Co-solvent method.....	30
1.3.1.5. Decision tree for method selection	31

1.3.2. log <i>S</i> determination	32
1.3.2.1. Methods for determination of kinetic solubility	32
1.3.2.2. Methods for determination of equilibrium solubility	33
1.3.2.2.1. Saturation shake-flask method (SSF)	33
1.3.2.2.2. Potentiometric methods	34
1.3.2.2.3. μ DISS method	35
1.3.2.2.4. High throughput methods	35
1.3.2.3. Special applications	36
1.3.3. log <i>P</i> determination	37
1.3.3.1. Shake-flask (SF) method	37
1.3.3.2. Potentiometric method	38
1.3.3.3. Indirect log <i>P</i> measurement methods	39
1.3.3.4. High throughput methods	39
1.3.3.5. Decision tree for method selection	40
1.4. CASE STUDIES	41
1.4.1. pK_a determination	41
1.4.2. log <i>S</i> determination	44
1.4.3. log <i>P</i> determination	49
1.5. OUTLOOK	52
Acknowledgement	52
REFERENCES	52

1.1. INTRODUCTION

The purpose of drug research is to develop effective, safe, and high quality new medicines to treat diseases where no drugs or otherwise nonoptimal ones are available. This activity is very complex, lengthy, expensive, and risky. Since drug research became industrialized, the highest level of scientific and technological knowledge has been applied during the given era. Fundamentally, the industry uses and puts into the practice the newest scientific results as early as possible thus drug research itself becomes the driving force for the development of new theories, technologies, and methods [1].

Taking a look back at the history of drug research, one can recognize on the long way of the evolution of the present system some milestones, paradigm-changes which resulted in considerable development in its age (Figure 1.1). In the '60s of the last century, the formerly used traditional methods (such as the extraction of active compounds from medicinal plants; random screening, trial-error method; side-effect observation; serendipity, *etc.*) more or less have been replaced or at least extended by the new strategy of *rational drug design*. Its first application was the Quantitative Structure-Activity Relationships (QSAR) analysis introduced by C. Hansch [2] and based on the accumulated knowledge of structure-activity relationships. The rational drug design was completed with the application of 3D molecular modeling, theoretical and computational chemistry (Computer Aided Drug Design, CADD) and proved to be a more effective tool than previous ones in the discovery and optimization of new active molecules. The appearance and fast expansion of *high throughput screening* (HTS) and *combinatorial chemistry* in the '90s have greatly enhanced the number of active compounds found [3]. The latest paradigm-change was provoked by the human genome project and the increased number of potential targets identified by genomics. However, these changes in the research strategy did not mean that former methods were completely neglected, indeed a majority of them are an inherent part of drug research. Each method has its appropriate use and importance in it.

Since the first recognitions of structure-activity relationships, medicinal chemists involved in drug research have been always paying outstanding attention to those properties of drugs which determine their pharmacological action. The knowledge of solubility, ionization ability and lipophilicity of drug candidates provides useful information about the expectable pharmacokinetic properties

and gives synthetic chemists adequate tools to improve them by modifying the structural moieties of the molecule [4].

Figure 1.1. Strategies in drug research

In the past, however, the main focus of drug research was first devoted almost exclusively to the pharmacodynamic aspects of the biological activity and only later in the development phase were the pharmacokinetic properties examined. This has led to a high attrition rate of compounds. In the late '80s studies reported two prominent reasons of drug candidate failure: the poor biopharmaceutical properties (*e.g.* low bioavailability) and safety. Pharmaceutical companies have made initiatives to shift the physicochemical profiling of compounds earlier in the drug discovery process [5].

Currently, drug research is usually divided into two main phases: (1) *discovery* phase, which involves the target identification, hit discovery, lead selection and optimization; and (2) *development* phase in which preclinical and clinical studies are conducted (Figure 1.2). The role and timing of the physicochemical characterization has considerably changed. The new strategy applied since the '90s is based on a parallel optimization of efficacy and prognostic profiling of drugability. This required a new mentality: to break down the wall between the discovery and development phases and to migrate from sequentially assessing efficacy and drugability to the parallel process; to evaluate the therapeutic and drug-like features together [6].

Figure 1.2. Drug research process

Good pharmaceutical properties, besides the efficacy, mean good absorption and distribution, chemical and metabolic stability (appropriate bioavailability) and low toxicity.

For optimization of drug-like properties, physicochemical parameters are simple and cheap tools in the early phase of drug research. Determination of properties relevant to biological activity of drugs such as ionization, solubility, lipophilicity and permeability is called *physicochemical profiling* (Kerns *et al.* 2001.) [7].

It is distinguished from the more complex term *pharmaceutical profiling* which involves the investigation of integrity, stability, metabolic properties (*e.g.* CYP 450 inhibition), transporter effects and drug-drug interactions as well (Figure 1.3) [8].

Figure 1.3. Pharmaceutical profiling vs. physicochemical profiling

The present chapter focuses on only three parameters of physicochemical profiling (pK_a , $\log S$, and $\log P$) while Chapter 3 is dedicated to the role and determination of membrane permeability. The traditional non-automated, time- and material-consuming methods developed in the past for physicochemical profiling are not suitable in discovery for the measurement of the drastically increased number of new chemical entities (NCE). Nowadays, such early physicochemical determinations must be material-saving, HT, and reasonably reliable. Several excellent commercial instruments have been developed for this purpose, which are miniaturized, automated, and adapted to high-throughput technologies [9].

The first comprehensive overview of physicochemical profiling was reported by P. Taylor in 1990 [10]. The progressive development achieved in the next decade is surveyed in A. Avdeef's book: *Absorption and Drug Development: Solubility, Permeability and Charge State* [11]. This book can be considered as the most competent and detailed compilation of advanced knowledge required by physical chemists involved in drug development. Numerous reviews summarized the state-of-the-art of new HT experimental techniques [12-15], the most recent was

published by Y. Henchoz *et al.* [16]. So, various literature sources are available for all who would expand their understanding of physicochemical profiling according to their need.

The aim of this chapter is to provide: (i) a concise summary of theoretical background; (ii) a comparison of different experimental methods and approaches; (iii) an introduction to ample, useful, and practical examples. The case studies taken from more than 30 years of experiences of the author are intended to provide help to physical chemists in the right method selection and measurement of difficult molecules.

1.2. THEORETICAL BACKGROUND

1.2.1. The physical chemistry of drug action

Drug action is a consequence of several chemical and biological processes in which binding to the receptor (pharmacodynamic phase) is essential. Besides this however, the pharmacokinetic processes have also fundamental importance in the biological activity. The active ingredient of a drug must separate from the applied pharmaceutical dosage form, must dissolve in body fluids and permeate through biological membranes to reach the receptor site. Following the receptor response, the active compound dissociating from the binding site generally undergoes metabolism and is excreted from the body. These liberation, absorption, distribution, metabolism, excretion (LADME) features are mainly determined by the physicochemical properties of drugs, namely by ionization, solubility and lipophilicity.

Biological membranes are the main physiological permeation barriers to be crossed by drugs. Structurally, they have a lipid bilayer resulting from the orientation of amphiprotic lipids (phospholipids, glycolipids, sphingomyelin) and cholesterol in the aqueous medium. This bilayer has some of the properties of a two-dimensional fluid (fluid-mosaic membrane model) in which individual lipid molecules can diffuse rapidly in the plane of their monolayer (lateral mobility) but cannot easily pass to the other monolayer. An important observation is that phospholipids are asymmetrically distributed in the membrane. Generally, the outer (extracellular) half of the bilayer comprises mainly zwitterionic lipids (phosphatidylcholine and phosphatidylethanolamine), whereas the inner (intracellular) part contains negatively charged lipids (*e.g.*, phosphatidylserine). Different proteins that induce transporter, signal transduction, or metabolic functions are integrated into the lipid bilayer [17,18]. The biological membranes are apolar barriers, where the relative permittivity inside is extremely low ($\epsilon \sim 2$). It has long been assumed that most drugs use transcellular transport and pass these barriers by passive diffusion which is favorable only for unionized, lipophilic compounds. There are several other mechanisms of permeation. Active transport is ligand-mediated by different transporters for compounds. Paracellular permeation exists between the cells for smaller, more polar compounds.

Some compounds are transported by endocytosis, when the molecules are engulfed by the membrane and move through the cell in these membrane-enclosed vesicles. For further detailed information, the reader is encouraged to review specialized resources [18,19].

The pH-partition hypothesis [20] provides a good model for the passive transport of ionizable molecules with sufficient lipophilicity. Figure 1.4 shows a schematic representation of the transport of a basic (B) (*e.g.* papaverine, chlorpromazine, *etc.*) and an acidic (HA) (*e.g.* acetylsalicylic acid, ibuprofen, *etc.*) molecule. In the extracellular aqueous medium, the ratio of ionized ($[BH^+]$ or $[A^-]$) and unionized ($[B]$ or $[HA]$) forms is dependent on the actual pH of the given compartment and the pK_a of the compound. The uncharged, neutral species has much higher lipophilicity than its charged (ionic) form, thus it can permeate through the lipid membrane even if being present as a minor component. In medicinal chemistry this species is called the “*transport form*”. Entering into the intracellular aqueous medium, another ionization process takes place resulting in the ionized form again which generally interacts with the target and is referred to as the “*receptor form*”. The amount of the transport form present at the membrane surface depends on its solubility. Molecules must be in solution in order to diffuse into the membranes, however low solubility can be a limiting factor of permeation. Permeability as a determinant kinetic parameter of transport is discussed in Chapter 3.

Figure 1.4. Transport and receptor forms of an acid and a base

The concept derived from the pH-partition theory that “only neutral molecules permeate membranes” started to be questioned from the mid ‘90s because of an increasing body of experimental evidence supporting ion-partitioning into artificial membranes like liposomes [21]. This was interpreted with electrostatic interactions and hydrogen bonding between the charged group of compounds

and ionized polar head group of phospholipids in the “pH priston model” [22]. Recently, S. Krämer and coworkers [23] reviewed the mechanisms underlying lipid bilayer permeation. They proposed a kinetic “flip-flop model” based on a three-step mechanism, namely the partitioning into one lipid layer, translocation (flip-flop) to the opposite lipid layer and partitioning into the aqueous phase. According to this model, the permeation of a charged species could be the result of occasionally occurring trans-membrane translocation of charged compounds. It was concluded that membrane permeation is more complex than expected from a simple diffusion model and pH-partition hypothesis.

Another possible mechanism of transport for ionized, hydrophilic compounds is carrier-mediated active transport. The increasing number of different uptake transporters discovered in the past 15 years highlights the importance of the role of active transport in membrane permeation of drugs which may be underestimated. Their physiological function is to deliver the necessary nutrients and other endogenous biochemical compounds having low lipophilicity for passive diffusion to the cell. Several drugs were found to be the substrate of different specific transporters like oligopeptide (PEPT1: captopril, enalapril, ampicillin, acyclovir), organic anion (OATP1: fexofenadine, enalapril, temocaprilat), organic cation (OCT1: metformin, famotidin), or nucleoside, *etc.* [18,24]. The efflux transporters (P-glycoprotein, P-gp; breast cancer resistance protein, BCRP; multidrug resistance protein, MRP2) assist in the movement of compounds out of the cell as they protect the cell from potentially toxic xenobiotics. This outward transport has a negative effect on the pharmacokinetics of some compounds. The activity of efflux transporters is very intensive in the blood-brain barrier and some tumor cells resulting in multidrug resistance. Binding to the transporters is determined by the chemical structure of compound. Similar moieties to the natural substrate, a large number of H-bond acceptors (N + O atoms), and high molecular weight ($M_w > 400$) appear to increase the likelihood of P-gp efflux [25].

Physicochemical properties influencing the fate of a drug in the body are described by the thermodynamic equilibrium constants. Below, we summarize the fundamentals of pK_a , $\log S$, and $\log P$ terms.

1.2.2. Physicochemical parameters

1.2.2.1. Ionization (pK_a)

Drugs are multifunctional compounds. A great majority of them contain one or more ionizable (acidic or basic) functional groups. In aqueous solutions, ionizable compounds exist in different ionization (charged or uncharged) states depending on their strength of acidity or basicity and the pH of the solution.

Definitions, terms

The ionization constant (or acid dissociation constant), K_a , is used to characterize the acid-base chemistry of a molecule generally expressed as a negative logarithm: $-\log K_a = pK_a$. In medicinal chemistry, it is common to use pK_a for both acids

and bases. In aqueous solutions, the pK_a scale spans from 0 to 14. The stronger the acid, the lower is its pK_a value. The opposite is true for bases; a higher pK_a value means stronger basicity [26].

Figure 1.5. The pK_a scale in aqueous medium

Some examples for the most frequently occurring acidic and basic functional groups in drugs are listed in Tables 1.1 and 1.2.

Equations 1.1-1.4 show the ionization equilibria and the relevant thermodynamic ionization constants using general symbols: HA for acid, B for base, XH for diprotic ampholyte molecule.

$$pK_a = pH + \log \frac{[HA]}{[A^-]} \quad (1.1c)$$

Table 1.1. Some important acidic functional groups in drugs

group	name	pK _a	example (pK _a)
sulphonic acid	0-1	metamizole
carboxyl	2-7	benzoic acid (3.98)
enol	2-6	piroxicam (2.33)
tetrazole	4-5	valsartan (4.8)
sulphonimide	5-6	tolbutamide (5.3)
N-aryl-sulphonamide	6-8	sulfadimidine (7.49)
lactam	7-8	barbital (7.9)
phenol	9-11	acetaminophen (9.63)
thiol	8-11	captopril (9.8)

Table 1.2. Some important basic functional groups in drugs

group	name	p <i>K</i> _a	example (p <i>K</i> _a)
guanidine	13-14	debrisoquine (13.0)
aliphatic primary amine		noradrenaline (8.5)
aliphatic secondary amine	8-11	ephedrine (9.6)
aliphatic tertiary amine		lidocaine (7.9)
aromatic primary amine		benzocaine (2.4)
aromatic secondary amine	2-5	tetracaine (2.4)
aromatic tertiary amine		chloropyramine (2.0)

$$pK_a = pH + \log \frac{[BH^+]}{[B]} \quad (1.2c)$$

$$pK_{a1} = pH + \log \frac{[XH_2^+]}{[XH]} \quad pK_{a2} = pH + \log \frac{[XH]}{[X^-]} \quad (1.3c, 1.4c)$$

In certain research articles, preference is given to the use of the ionization rather than the proton association process and the term protonation constant, K_p , particularly in coordination chemistry [27]. The relationship between them is reciprocal where $K_a = 1/K_p$, or $pK_a = \log K_p$. For a monoprotic compound this relationship is evident, but may not be clear regarding molecules with more than one ionizable group. Below, we describe the ionization processes of a triprotic compound (like amoxicillin) from both points of view: dissociation (molecule releases the proton) and association (molecule gains the proton).

$$\begin{aligned} \log K_{p1} &= 9.6 \\ \log K_{p2} &= 7.4 \\ \log K_{p3} &= 2.4 \end{aligned}$$

Dissociation

Protonation

$$K_{a1} = \frac{1}{K_{p3}} \quad K_{a2} = \frac{1}{K_{p2}} \quad K_{a3} = \frac{1}{K_{p1}} \quad (1.8a, 1.8b, 1.8c)$$

$$pK_{a1} = \log K_{p3} \quad pK_{a2} = \log K_{p2} \quad pK_{a3} = \log K_{p1} \quad (1.9a, 1.9b, 1.9c)$$

Ionization microconstants

The equilibria above characterize the dissociation/protonation of the molecule at the molecular level, so called macroscopic level, using ionization *macroconstants*. Ionization macroconstants quantitate the overall acidity/basicity of the molecule, but cannot be assigned to individual proton binding sites of multiprotic molecules. Ionization *microconstants* are the terms which describe the proton binding ability of the individual functional groups and are useful in calculating the pH-dependent concentrations of microspecies (named microspeciation) [28]. In the past decade, the microspeciation of several drug molecules was published [e.g. 29-31].

The macroscopic and microscopic protonation scheme of a diprotic molecule using norfloxacin as a model is shown in Figure 1.6. For simplicity, K denotes the protonation macroconstants and k is used for microconstants. The superscript denotes the functional group is protonating in a given process, the subscript (if any) shows the already protonated group and N and C refer to the piperazine nitrogen and the carboxylate group, respectively. There are two possible alternative routes of protonation. From the most basic anionic form (X^-) the carboxylate group first accepts a proton resulting in the chargeless (XH^0) form, then a secondary amine group protonates producing the cation (XH_2^+) (lower route). The other pathway of protonation is conducted through the formation of a zwitterion ($XH^\pm$) due to the protonation of an amino group first. The chargeless and zwitterionic forms are chemically different microspecies (they bear the proton on different binding sites) having the same stoichiometric composition (one proton is accepted), so they are protonation isomers.

Figure 1.6. The protonation macro- and micro-equilibria of norfloxacin

The relationships between the macro- and microconstants are the following:

$$K_1 = k^C + k^N \quad K_1 K_2 = k^C k_C^N = k^N k_N^C \quad (1.10a, 1.10b)$$

$$\frac{1}{K_2} = \frac{1}{k_C^N} + \frac{1}{k_N^C} \quad (1.10c)$$

Once macro- and microconstants are known, the mole fraction of each species can readily be calculated and the pH-dependent distribution of macro- and microspecies can be constructed. Figure 1.7 shows the distribution of different protonation forms of norfloxacin against the pH and indicates the predominance of the zwitterionic form over the chargeless microspecies. However, it is also visible that their concentration at the iso-electric point pH is commensurable and both forms are present in a significant amount.

Figure 1.7. Distribution curve of the 4 microspecies of norfloxacin as a function of pH

The microspeciation of a triprotic molecule [32,33] is more complicated, containing 8 microspecies. The total protonation process can be depicted by 12 microconstants as demonstrated in case of amoxicillin in Figure 1.8. The O, N, C sub- or superscripts of the k microconstant refer to the three proton binding sites, namely phenolate, amino, and carboxylate groups, respectively. The relationships between the macro- and microconstants are the following:

$$K_1 = k^O + k^N + k^C \quad (1.11)$$

$$K_1 K_2 = k^O k_O^N + k^O k_O^C + k^N k_N^C = k^N k_N^O + k^C k_C^O + k^C k_C^N \quad (1.12)$$

$$K_1 K_2 K_3 = k^O k_O^N k_{O,N}^C = k^N k_N^O k_{O,N}^C = \dots \quad (1.13)$$

The theory and practice of proton microspeciation based on NMR-pH titration and data in the literature on complete microspeciation of small ligands including drugs have recently been surveyed [34].

Figure 1.8. Protonation macro- and micro-equilibria of triprotic amoxicillin

Temperature and ionic strength

The ionization constant as a thermodynamic parameter is temperature-dependent. For the precise determination of pK_a , experiments must be conducted under controlled constant temperature. In practice, the common reference value is 25 °C and only few data are available measured at 37 °C. The change in pK_a upon an increase of temperature from 25 °C up to the physiological temperature of 37 °C is dependent on the given molecule. Generally the change in the pK_a of acids is less, while bases are more sensitive to temperature change [26]. The approximate average value of temperature dependence is known as $\delta pK_a/\delta T$: 0.02-0.03, which means 0.24-0.36 ΔpK_a values between 25 and 37 °C. If the pK_a of a compound falls into the pH range 1.5-8 (the pH gradient present in the human gastrointestinal tract), then even a relatively small difference may lead to poor *in*

vitro-in vivo correlations. For a better interpretation of the cellular transport mechanism of such molecules, the biorelevant pK_a value is particularly useful. A prediction method for this value based on a 2D structure and pK_a at 25 °C was proposed very recently [35].

The ionic strength of the medium also affects the pK_a value. It is common to measure at constant ionic medium, generally at $I = 0.15$ M adjusted by KCl or NaCl corresponding to the physiological level. Frequently, a different ionic medium is used or data calculated to zero ionic strength using the Debye-Hückel theory are also published, thus it is always necessary to report the ionic strength and temperature of a pK_a measurement.

Importance of pK_a in medicinal chemistry

The degree of ionization at a given pH can be calculated once the pK_a is known. As a rule of thumb, at $pH = pK_a$ 50 % of the compound is ionized and 50 % is in the unionized form, while at $pH = pK_a \pm 2$ predominance of one species becomes 99 %. For example, an acid is present in 99 % at $pH = pK_a - 2$ as unionized (HA) and at $pH = pK_a + 2$ as ionized (A^-) (the opposite case applies to a base).

The ionization state determines the transport properties, thus its precise calculation allows the estimation of ADME features. With the knowledge of the pK_a value, the proportion of the transport form can be calculated at any physiologically important pH values. Regarding ampholyte compounds, the pK_a values are useful to calculate the iso-electric point or the pH at which a molecule has the lowest solubility and highest lipophilicity. Since solubility, lipophilicity, and permeability are pH-dependent properties, the pK_a value of a new molecule must be determined in advance to the log S , log P and permeability measurement.

Ionic interactions play a fundamental role in the receptor binding of ionizable molecules. An ionic bond is the strongest non-covalent binding type. The electrostatic attraction of opposite charges directs the molecule to the receptor surface and electrostatic complementarity with the receptor is a prerequisite of any drug action.

Antiarrhythmic drugs (class I: Na^+ -channel antagonists) serve as a good example of how pK_a affects drug action. These drugs are weak bases with most having pK_a values ranging from 7.5 - 9.5. At the physiological pH of 7.4 they exist in an equilibrium mixture consisting of both the free base (B) and protonated (BH^+) cationic form. In compounds with the $pK_a > 9$ (like procainamide, mexiletine, propafenone), the presence of the receptor form exceeds 90 % which is favorable for the binding to the sodium-channel. However, for compounds in which the $pK_a < 8$ (like quinidine, lidocaine) this ratio is much less favorable (Table 1.3). Lidocaine ($pK_a = 7.96$) has a stronger electrophysiologic effect in ischemic than normal myocardial tissue. This potentiation has, in part, been attributed to the increase in H^+ concentration (lower pH) within the ischemic areas of the heart. Acidosis increases the portion of receptor form of the drug (Table 1.3) and consequently the proportion of Na^+ -channels occupied by the BH^+ of lidocaine [36].

Table 1.3. Ionization state of antiarrhythmic class I drugs at normal and ischemic tissue

compound	pH = 7.4 (normal tissue)		pH = 6.4 (ischemic tissue)	
	BH ⁺ , %	B, %	BH ⁺ , %	B, %
procainamide	98.4	1.6	99.9	0.1
mexiletin	98.2	1.8	99.8	0.2
quinidine	76.0	24.0	99.0	1.0
lidocaine	78.4	21.6	97.3	2.7

1.2.2.2. Solubility (log S)

Solubility is a molecular property which determines the maximal concentration of a solute in a given solvent. The aqueous solubility depends on the polarity of a molecule and varies with the pH for ionizable compounds. Solubility can be described by different parameters and a vast variety of terms and symbols are used to express the solubility data of compounds, hence below we summarize the most common basic definitions.

Definitions, terms

Equilibrium (or thermodynamic) solubility (S) is the concentration of a compound in a saturated solution when a solid is present and the solution and solid are at equilibrium. This value is constant at a given pressure and temperature and characteristic for a given compound.

For ionizable molecules, further terms are distinguished. *Intrinsic solubility (S₀)* is the equilibrium solubility of a free acid (HA) or free base (B) form of an ionizable compound at a pH where it is fully unionized. With respect to ampholytes, this refers to the neutral (chargeless) form (XH) which exists at the iso-electric (i.e.) point pH. *Effective solubility (S_{pH})* is the equilibrium solubility of an ionizable compound at a pH where both unionized and ionized forms are present. It is also denoted as *apparent* (or total) solubility and defined at a particular pH as the sum of the concentrations of all compound species dissolved in an aqueous solution.

The *solubility of salt form* of an ionizable compound (*S_{salt}*) can be derived from the *solubility product (K_{sp})*. For a monoprotic acid or base:

$$S_{\text{salt}} = \sqrt{K_{\text{sp}}}$$

where, $K_{\text{sp}} = [\text{A}^-] [\text{Y}^+]$ for an acid and $K_{\text{sp}} = [\text{BH}^+] [\text{X}^-]$ for a base, Y⁺ and X⁻ represent the counter-ion in the salt.

Recently, a new term called *kinetic solubility (S^{APP})* was introduced in early drug discovery. It is the concentration of a solution of an examined compound at the moment when the first precipitation of the solid is observed in an experiment where a small volume of 10-20 µg/ml dimethyl sulfoxide (DMSO) stock solution is added to aqueous buffer. This parameter is not a thermodynamic physico-chemical constant because the system does not reach an equilibrium state. Generally, S^{APP} is higher than the equilibrium solubility since there is no need to overcome the crystal lattice forces by aqueous solvent once the compound has

been dissolved in DMSO. Kinetic solubility data are mainly used for ranking the molecules in the early stages of discovery and cannot replace the determination of the true equilibrium constant later in the development phase.

The above solubility parameters can be expressed in various concentration terms like: g/100 ml; g/ml; mg/ml; $\mu\text{g/ml}$ or mol/L (M); mmol/L (mM); $\mu\text{mol/L}$ (μM), *etc.* For better comparability, the logarithm of solubility term ($\log S$) is frequently used and can be obtained from M or μM concentration. Preference for the $-\log S$ term is found in the literature in order to avoid negative numbers for low solubility compounds. However, it may be somewhat confusing because the aforementioned term yields higher values meaning lower solubility.

Solubility is affected by many factors, such as temperature, pressure, pH, ionic strength of aqueous media, purity of a sample, crystal form, particle size, polymorphism, *etc.* The effect of these factors have been comprehensively discussed in classic [37,38] and new books [11,18]. Here, we focus only on the pH dependency of solubility.

Solubility-pH profile

The solubility of ionizable compounds varies with the pH. They are more soluble in the charged than in the unionized form. When a molecule exists only in the monomer state, its pH-dependent equilibrium solubility is derived from the Henderson-Hasselbalch (HH) equations (Equations 1.1c-1.4c). The HH relationship for a monovalent acid, base, and (diprotic) ampholyte molecule can be derived from solubility and ionization equilibria as follows where, by convention $[\text{HA(s)}] = [\text{B(s)}] = [\text{XH(s)}] = 1$, and $[\text{A}^-]$, $[\text{BH}^+]$, $[\text{X}^-]$, $[\text{XH}_2^+]$ are expressed using Equations 1.1b, 1.2b, 1.3b.

acid:

$$\text{HA(s)} \rightleftharpoons \text{HA} \quad (1.14)$$

$$S_0 = \frac{[\text{HA}]}{[\text{HA(s)}]} = [\text{HA}] \quad (1.15)$$

$$S_{\text{pH}} = [\text{A}^-] + [\text{HA}] \quad (1.16)$$

$$S_{\text{pH}} = \frac{K_a [\text{HA}]}{[\text{H}^+]} + [\text{HA}] \quad (1.17)$$

$$S_{\text{pH}} = [\text{HA}] \left(\frac{K_a}{[\text{H}^+]} + 1 \right) \quad (1.18)$$

$$S_{\text{pH}} = S_0 \left(10^{(-\text{p}K_a + \text{pH})} + 1 \right) \quad (1.19)$$

$$\log S_{\text{pH}} = \log S_0 + \log(1 + 10^{(\text{pH} - \text{p}K_a)}) \quad (1.20)$$

base:

$$S_0 = \frac{[B]}{[B(s)]} = [B] \quad (1.22)$$

...

$$\log S_{pH} = \log S_0 + \log(1 + 10^{(pK_a - pH)}) \quad (1.23)$$

diprotic ampholyte:

$$S_0 = \frac{[XH]}{[XH(s)]} = [XH] \quad (1.25)$$

$$S_{pH} = [X^-] + [XH] + [XH_2^+]$$

...

$$\log S_{pH} = \log S_0 + \log(1 + 10^{(pK_{a1} - pH)} + 10^{(pH - pK_{a2})}) \quad (1.26)$$

Figure 1.9 shows the characteristic solubility-pH profile (a plot of $\log S_{pH}$ vs. pH) for an acid (a), base (b), and diprotic ampholyte (c).

Figure 1.9. Solubility-pH profile of (a) an acid, (b) a base and c) a diprotic ampholyte

The HH relationship can be used to predict the pH-dependent equilibrium solubility of drugs when the pK_a and $\log S_0$ values of a compound are known. It is a frequent practice in drug research to convert the experimentally measured intrinsic solubility value to equilibrium solubility at a physiological relevant pH in order to estimate its expected behavior.

The validity of the HH relationship has been widely investigated and certain deviations were found [39,40]. They were interpreted with the influence of different molecular interactions such as aggregation and micelle formation [39,41]. Recently, a revisit of the HH relationship concerning organic bases confirmed the validity provided if highly precise pK_a and $\log S_0$ values were used for its generation [42] (see also Section 1.4.2).

Importance of $\log S$ in medicinal chemistry

The aqueous solubility of compounds receives considerable attention in drug development, because this is a key molecular property for the gastrointestinal absorption of orally administered drugs. Further on, in biological activity tests compounds must be in solution otherwise false, erroneous data can be obtained. Low solubility is detrimental from both pharmacokinetic and pharmacodynamic points of view. Determination of aqueous solubility is an inevitable part of physicochemical profiling in drug research. Its importance has grown since the Biopharmaceutical Classification System (BCS) was first proposed by G. Amidon in 1995 [43]. This classification uses four classes to categorize drugs based on their solubility and intestinal permeability (class 1: high solubility + high permeability; class 2: low solubility + high permeability; class 3: high solubility + low permeability; class 4: low solubility + low permeability). For class 1 molecules, the rate-limiting factor of intestinal absorption is the rate of dissolution, low solubility in class 2 molecules, while low permeability in class 3 is rate-limiting. In class 4, both properties are unfavorable for oral administration, and no *in vitro-in vivo* correlation can be expected.

To improve the *in vitro-in vivo* correlation, the measurement of solubility is recommended for biomimetic media as well. There is growing evidence that in the intestine, the presence of bile acids and other components such as lipids can alter (usually increase) the intrinsic solubility of (lipophilic) compounds. Two physiologically relevant media developed by Dressmann *et al.* [44] are used. These are the fasted-state simulated intestinal fluid (FaSSIF) and the fed-state simulated intestinal fluid (FeSSIF) having pH 6.5 and 5.0, respectively, and contain different amounts of sodium taurocholate, lecithine and salts [44].

1.2.2.3. Lipophilicity ($\log P$)

The more fundamental property governing the fate of a drug in the body is undoubtedly the lipophilicity. This molecular property represents the affinity of a molecule for a lipophilic environment. It is most commonly described by the logarithm of partition coefficient ($\log P$) between two immiscible solvents, one is an organic apolar (*e.g.* octanol) and the other an aqueous polar (buffer solution) [45].

Besides P , other symbols have been used in the literature such as P_{ow} , K_{ow} , K_p , PC , etc., however, we use the terminology widely accepted in medicinal chemistry.

Two types of partition parameters are distinguished: the true partition coefficient (P) and the distribution coefficient (D or in older literature P_{app}). Their definition and relationship are briefly summarized below.

Definitions, terms

The true partition coefficient (according to the *Nernst* law) refers to the partition of a single electrical species, and is expressed as an equilibrium concentration ratio of the same molecular form in both phases of the solvent system. This value is constant at a given temperature and pressure, independent of the pH and characteristic for the molecule. It can be derived for the neutral, monomeric form of a compound ($\log P^N$) (Equation 1.27) and theoretically can be also defined for the partition of an ionic form ($\log P^I$) (Equation 1.28), but later the value has orders of magnitude lower and in most of cases can be practically neglected.

$$P^N = \frac{[\text{unionized form}]_{\text{octanol}}}{[\text{unionized form}]_{\text{water}}} \quad (1.27)$$

$$P^N = \frac{[HA]_o}{[HA]_w} \quad P^N = \frac{[B]_o}{[B]_w} \quad P^N = \frac{[XH]_o}{[XH]_w} \quad (1.27a-c)$$

$$P^I = \frac{[\text{charged species}]_{\text{octanol}}}{[\text{charged species}]_{\text{water}}} \quad (1.28)$$

$$P^I = \frac{[A^-]_o}{[A^-]_w} \quad P^I = \frac{[BH^+]_o}{[BH^+]_w} \quad P^I = \frac{[X^-]_o}{[X^-]_w} \quad P^I = \frac{[XH_2^+]_o}{[XH_2^+]_w} \quad (1.28a-d)$$

The distribution coefficient of an ionizable compound refers to all species that are present in the solution (Equation 1.29). Since it is a pH-dependent term, the pH must be specified as D^{pH} .

$$D^{pH} = \frac{[\text{unionized} + \text{ionized species}]_{\text{octanol}}}{[\text{unionized} + \text{ionized species}]_{\text{water}}} \quad (1.29)$$

For monoprotic acid and base:

$$D^{pH} = \frac{[HA]_o + [A^-]_o}{[HA]_w + [A^-]_w} \quad D^{pH} = \frac{[B]_o + [BH^+]_o}{[B]_w + [BH^+]_w} \quad (1.29a-b)$$

For diprotic ampholyte:

$$D^{pH} = \frac{[X^-]_o + [XH]_o + [XH_2^+]_o}{[X^-]_w + [XH]_w + [XH_2^+]_w} \quad (1.29c)$$

with the assumption that the concentration of the ionic forms in the organic phase is much less than that of the neutral forms (*e.g.*, $[A^-]_o \ll [HA]_o$ and $[BH^+]_o \ll [B]_o$, *etc.*) and upon substituting the aqueous equilibrium concentrations from Equations 1.1b, 1.2b, and 1.3b, the relationships between P and D can be obtained. For simple molecules, these relationships are given below (Equations 1.30a-c) while interactions between more complicated multiprotic compounds can be found in the literature [46].

For monoprotic acid:

$$\log P^N = \log D^{pH} + \log(1 + 10^{(pH - pK_a)}) \quad (1.30a)$$

For monoprotic base:

$$\log P^N = \log D^{pH} + \log(1 + 10^{(pK_a - pH)}) \quad (1.30b)$$

For diprotic ampholyte:

$$\log P^N = \log D^{pH} + \log(1 + 10^{(pK_{a1} - pH)} + 10^{(pH - pK_{a2})}) \quad (1.30c)$$

Partition microconstants

Similarly to ionization microconstants, micro-log P (denoted as $\log p$) of a given microspecies of multiprotic compounds can also be defined [46]. This has particular significance in the case of ampholyte compounds where the most lipophilic species, the neutral (XH) form, is a composite from zwitterionic (XH[±]) and chargeless (XH⁰) microspecies. If they are present in solution in a commensurable amount (*e.g.*, norfloxacin in Figure 1.7) then the exclusive partitioning of the chargeless form can be expected into the lipophilic phase and micro-log P of XH⁰ microspecies may be the relevant lipophilicity parameter. Its calculation requires knowledge of the $\log D$ at iso-electric pH value, $\log D^{i.e.pH}$, and the protonation microconstants (k^C , k_C^N , k_N^C) as previously published [47].

$$\log p^0 = \log D^{i.e.pH} + \log \left(1 + \frac{1}{k^C [H^+]} + \frac{k_C^N}{k_N^C} + k_C^N [H^+] \right) \quad (1.31)$$

Lipophilicity-pH profile

The plot of $\log D^{pH}$ against the pH (lipophilicity-pH profile) of a compound (Equations 1.30a-c) can be derived from the HH relationships (Equations 1.1c-1.4c), provided that there is no ion-pair partition involved in the process. If such ion-pair partition exists, the profiles show a plateau at $\log D$ of values 3-4 orders lower (for acids at high pH, for bases at low pH) than as indicated in Figure 1.10.

The lipophilicity-pH profiles are useful to estimate the effective lipophilicity of a compound at physiologically relevant pH values and widely used in medicinal chemistry.

Figure 1.10. Lipophilicity-pH profile of (a) an acid, (b) a base, and (c) a diprotic ampholyte

Solvent systems for log P

The widely accepted reference solvent system for log P measurement is octanol/water proposed first by Hansch [45]. In this system, the two phases are isotropic. A buffer solution serves as the aqueous phase and *n*-octanol is used as a typical H-bond donor and acceptor organic solvent. This system is thought to model the essential properties of general biological membranes. The structure of water-saturated octanol became better understood in the '90s [48]. Inverted micellar aggregates are formed where water clusters are surrounded by about 16 molecules of octanol, with the polar OH groups pointing to the clusters and intertwined in a hydrogen-bonding network. The aliphatic tails form a hydrocarbon region with properties not too different from the hydrocarbon core of bilayers. Obviously, the octanol/water system cannot be a universal model for all types of membranes. In the past two decades, partition solvents other than octanol have been explored. Leahy *et al.* [49] proposed the "critical quartet" system consisting of octanol/water, chloroform/water, alkane/water and propylene glycol dipelargonate (PGDP)/water for the general modeling of membranes. Later, 1,2-dichloroethane (DCE) and cyclohexane were found useful organic solvents. Once log P has been measured both in alkane/water and octanol/water systems, the $\Delta \log P$ ($\log P_{\text{octanol}} - \log P_{\text{alkane}}$) can be calculated, and used as a simple parameter for the H-bond formation ability of a compound.

Recently, anisotropic systems such as liposomes (vesicles formed from phospholipid bilayers) were increasingly used to model membrane partitioning.

Liposome/water $\log P$ values are considered as $\log P_{\text{mem}}$ (membrane partition). An accumulation of $\log P_{\text{mem}}$ data show a significantly higher partitioning of ionic forms. Generally, charged species partition into membranes about 100 times more strongly than into octanol. The theory and practice of liposome/water lipophilicity were reviewed [5,50,51].

Importance of $\log P$ in medicinal chemistry

The $\log P$ is the oldest and most traditional physicochemical parameter used in medicinal chemistry. Lipophilicity is implicated in numerous biological events (such as transport, receptor binding *via* hydrophobic interactions, metabolic processes, storage in fat tissues, *etc.*). The $\log P$ value – concerning its information content – is much more than a simple number, because the same molecular interactions which exist between the compound and the biological environment results in this value. At the same time, $\log P$ is very easy to handle by chemists for comparison of molecules with different lipophilicity and estimating the expected transport behavior in the body.

Among the properties suggested by Lipinski, (known as “rule of 5”) one of the criteria for drug-likeness is that $\log P$ should be below 5 [52]. It seems to be a reasonable concept since 90% of marketed drugs have a $\log P$ value in the range of 0 – 5 (see Figure 1.11). From hydrophilic compounds ($\log P < 0$) good solubility, but poor absorption from the GI tract can be expected except for those which have active transport (such as for example ascorbic acid). Compounds with moderate lipophilicity ($\log P$ between 0 and 3) are optimal for oral administration due to a good balance of solubility and permeability. For good blood-brain barrier (BBB) penetration, the optimal $\log P$ value is about 2.

Figure 1.11. The $\log P$ scale of drugs

Highly lipophilic compounds ($\log P > 5$) are sparingly soluble in aqueous compartments, tend to accumulate in lipoidal parts and are also more sensitive to

metabolism. Extremely high lipophilicity may lead to strange pharmacokinetics, for example, amiodarone has $\log P = 7.37$ and half-life $t_{1/2}$: 25-30 days(!) (Figure 1.11). First, in 1987 Hansch called attention to the danger of exceedingly high lipophilic drug candidates and proposed the “minimal hydrophobicity” concept for the design of new compounds [53]. Since then, the unfavorable tendency of highly lipophilic drug production has not stopped, as new molecules in drug research are getting more lipophilic and less water-soluble [54].

1.3. METHODS FOR PHYSICOCHEMICAL PROFILING

Demands set up to the methods for physicochemical profiling are different in various phases of drug research. In the discovery phase, the drastically increased number of NCEs produced by combinatorial chemistry requires high throughput (HT), material saving, automated approaches, while less emphasis is placed on precision. A method for physicochemical profiling is considered HT when its capacity exceeds the measurement of 50 compounds/day [7]. Later, in the development phase reliable, precise data are needed which is why accuracy is more important and not the speed of the method.

This subchapter is dedicated to experimental methods used for the measurement of pK_a , $\log P$, and $\log S$ values and comparison of their capacity, accuracy, time, and material demand (see Tables 1.4-1.6). We focus here mainly on the practical aspects of their application, while the detailed theoretical background of the methods is out of the scope of this review. For this purpose, excellent basic books are recommended to readers [11,18,26,37].

1.3.1. pK_a determination

Potentiometry and UV spectroscopy are the commonly used standard methods of pK_a determination. Due to its simplicity and precision, potentiometry is the method of choice once the aqueous solubility of a compound reaches a minimum of 0.5 mM concentration in the entire pH range of the titration. For less soluble compounds, a good alternative tool is the UV/pH titration provided that the molecule has a pH-dependent UV spectrum. In this method, it is generally enough if the compound dissolves in a concentration of 10-500 μM depending on its molar absorptivity, ϵ . Both potentiometric and UV/pH titration methods are strongly supported commercially, and the available automated instruments such as the GLpKa and its follow up the Sirius T3 automated analyzers (Sirius UK) are widely used. In the recent years, capillary electrophoresis (CE) has proved to be a very powerful pK_a determination method, being more sensitive and less sample consuming [11,16]. Some other methods such as NMR/pH titration [55], CD/pH titration [56], and chromatographic technique [57] have also been applied for special cases, but so far have not become routine techniques.

1.3.1.1. Potentiometric method

Procedure. In potentiometric titration, the pH of a 1-5 mM solution of a sample is precisely measured with a carefully standardized combined glass electrode upon addition of small volumes of a strong acid (*e.g.* HCl) or base (*e.g.* KOH) volumetric solution. The measurement is performed in a stirring solution, under an inert gas atmosphere (argon or nitrogen) while the ionic strength of the solution is kept constant using an inorganic salt (*e.g.* 0.15M KCl), and the titration cell is thermostated usually at 25.0 ± 0.1 °C. Typical sample volume for titration is 5-15 ml, but measurement in as less as 1 ml solution has been reported [58]. The concentration of the titrant is generally 0.5 M in order to avoid considerable dilution upon titration. The potentiometric titration can be used as a *direct* approach for pK_a measurement, when the tested compound is a (relatively) strong acid/base to produce enough potential change (big jump) in the titration curve. Otherwise, the “*Calvin-Δml*” difference-titration is a useful and widely applied method. Here, the pK_a value is obtained from the difference between the titration curve of a tested compound and a “blank” titration (see below). This approach is a built in function in pK_a analyzers.

Calculation. The pK_a value can be calculated according to the HH equations (Equations 1.1c-1.4c). The pH is measured and the term $\log([\text{protonated}]/[\text{non-protonated}])$ is obtained from the mass balance of the titration data. In automated analyzers built in programs (*e.g.*, Refinement-Pro™) calculate the pK_a . First, the titration curve is converted to the Bjerrum plot (the average number of bound protons/molecule, $\bar{n}$ vs. pH), where the pK_a value is equal to the pH at $\bar{n} = 0.5$ (for a multiprotic compound: second pK_a at $\bar{n} = 1.5$, third at $\bar{n} = 2.5$, *etc.*). The obtained raw values are then further refined by a nonlinear least squares method. The adjustable parameters are the concentration of the material, acid/base error of pH measurement, carbon dioxide content, *etc.*

Accuracy. This method with the above experimental parameters allows the measurement of precise pK_a values in a range from 2 to 12 with a standard deviation $SD = \pm 0.01$ -0.03. By using a glass electrode of excellent quality, performing proper electrode calibration, excluding the presence of ambient carbon dioxide as much as possible, and accurately dispensing very small titrant volumes (0.01 ml or even smaller) potentiometry in aqueous solution can be applied to a concentration as low as 0.1 mM (according to some authors as low as 0.01 mM). Of course, the accuracy and reproducibility of titrations in such diluted solutions is much less ($SD = \pm 0.10$ -0.15). Similarly, the precision of the measurement decreases out of the pH range of 2-12.

Calibration. Electrode calibration is a fundamental step in pH-metric pK_a determination. A standardized “Four-parameter procedure” developed by Avdeef *et al.* [59] is widely used. A known concentration of HCl is titrated with KOH (from pH 1.8 to 12.2) under standard experimental conditions (see above). Data from this “blank” titration are used to convert the operational pH scale to the concentration scale ($p_cH = -\log [H^+]$) by a multi-parametric equation.

$$\text{pH} = \alpha + S \text{p}_c\text{H} + j_{\text{H}} [\text{H}^+] + j_{\text{OH}} K_{\text{w}} / [\text{H}^+] \quad (1.32)$$

The parameters are determined by a weighted nonlinear least-squares procedure. The intercept parameter α in aqueous solution mainly corresponds to the negative logarithm of the activity coefficient of H^+ at the working temperature and ionic strength. The j_{H} term corrects pH readings for the nonlinear pH response due to the liquid junction and asymmetric potentials in moderately acidic solutions, while the j_{OH} term corrects the high-pH nonlinear effect. Factor S accounts for the fact that a particular electrode may not have 100% Nernstian-slope and K_{w} is the ionization constant of water. Typical aqueous values of the adjustable parameters at 25 °C and 0.15 M ionic strength are: $\alpha = 0.08 \pm 0.01$, $S = 1.001 \pm 0.001$, $j_{\text{H}} = 1.0 \pm 0.2$, and $j_{\text{OH}} = -0.6 \pm 0.2$.

Advantages/drawbacks. Potentiometry is a simple, fast, and precise method for pK_{a} determination. The smallest practical volume of sample solution is about 5 ml. This requires 1.5 mg of sample for a compound with M_{w} 300 to achieve the 1 mM concentration which is ideal for titration. For reliable pK_{a} , 2–3 parallel measurements are necessary, so the sample consumption reaches 3–4.5 mg. A titration between pH 2–12 typically takes 20–40 min to perform. With an automated instrument (*e.g.* GLpKa) 30–40 titrations could be performed during one 24-h day [60]. So, the maximum capacity is about 10–12 compounds/day. This is a relatively low throughput. The main limitation of the application of this technique is the poor solubility of compounds. In such cases, the co-solvent method can be applied (see Section 1.3.1.4). Further on, it is difficult to handle impure or unstable compounds (*e.g.*, certain esters, diphenols, *etc.*).

1.3.1.2. UV/pH titration

Procedure. In spectrophotometric pK_{a} determination method the change in the UV spectrum upon ionization is registered. Such a pH dependent UV-spectrum is obtained if the ionizable group is near to the chromophore of the molecule. In traditional UV/pH titration two aliquots of typically 10–50 μM solutions of a sample are prepared in either 0.01 (or 0.001) M HCl or 0.01 (or 0.001) M NaOH, with the total ionic strength of 0.15 M. In one solution the molecule is fully ionized while in the other fully unionized. By mixing the two stock solutions under precise pH control, 5–6 solutions are prepared in a rather narrow pH range (± 0.6 unit) around the expected pK_{a} . Their absorbance is measured at a wavelength where the difference in the absorbance between the ionized and unionized form is the largest. Recently, this time-consuming process has been automated (GLpKa with a D-PAS attachment). In a titration cell, the solution of the sample is titrated across a pH range that includes the pK_{a} value(s) and multi-wavelength UV spectra registered at each pH with the help of a fiber optics dip probe immersed into the titration cell [60,61].

Calculation. In traditional UV/pH titration, the pK_{a} value can be calculated from the pH of the solution and the absorption data measured at a single wavelength using the HH Equations 1.1c–1.4c. The pK_{a} of a compound is obtained as an

average value calculated from the solution series. This method is applicable for the determination of a single pK_a , or multiple pK_a values if they are well separated (>1.5 pH units). In the D-PAS technique, target factor analysis (TFA) is used to deduce the pK_a value(s) of a sample from an absorbance matrix [60]. This technique is able to handle multiprotic molecules with overlapping protonation.

Accuracy. The precision of pK_a determination by traditional UV/pH titration does not reach that of pH-metry, where the standard deviation can vary between ± 0.05 - 0.10 . However, according to a recent validation study, the D-PAS technique with a $SD = \pm 0.02$ has similar precision to potentiometry [62].

Advantages/drawbacks. The spectrophotometric method is usually more sensitive than potentiometry. The measurements can be performed at lower sample concentration allowing the pK_a determination of less soluble compounds directly in aqueous medium, while for water-insoluble materials the co-solvent method can be easily applied. The D-PAS is a fast technique, one titration takes up 30 min and is sample conserving, usually 1-2 mg of sample is enough for 3 parallel measurements. One limitation of spectrophotometry is that if the distance between the ionization and the chromophore center is greater than three sigma bonds then the pH-dependent spectral shift will be too small for measurement. Another limitation is if the absorption maxima of the compound occurs at a low wavelength (< 230 nm) then background noise disruption increases considerably. Traditional UV/pH titration is a very slow, time-consuming process, while the capacity of the D-PAS technique is similar to potentiometry (10-12 compounds/day). UV/pH titration was used for determination of microconstants in several cases (*e.g.* repaglinide [63], moxifloxacin [64]) when the shift in the UV spectrum is due to the ionization of a given functional group.

1.3.1.3. Other methods

NMR/pH titration. NMR/pH titration can also be used for pK_a measurement based on the fact that the chemical shift of NMR-active nuclei is governed (among other factors) by the protonation state of ionizable groups. Since protonation decreases the local electron density, a selected nucleus in the vicinity of the ionizable site exhibits a different shift in the ionized and unionized states. A plot δ^{obs} vs. pH has a sigmoidal shape with an inflection point at $\text{pH} = pK_a$.

Generally, NMR/pH titrations have been carried out in aqueous solutions using D_2O as a solvent. Although glass electrodes operate properly in D_2O , a correction factor of 0.40 has to be added to the measured pH to get the true pD value. To avoid this correction, NMR/pH titration may be conducted in a solvent mixture of H_2O/D_2O (90/10 v/v) and the water peak has to be suppressed by an appropriate method. Frequently, the whole titration is performed in a single NMR-tube and the pH is measured with a long, thin glass electrode. This method has been extended for the measurement of low pK_a values (between 0 and 2), where potentiometry is no longer applicable. Since at such low pH a glass electrode has

significant acidity error, dichloroacetic acid was proposed as an NMR “indicator molecule” for *in situ* monitoring of the pH in strong acidic solutions [55]. The pK_a values of individual groups of large biopolymers have been reported as measured by NMR technique [65].

The main advantage of this technique compared to potentiometry is the capability of selective monitoring of ionization of a given functional group in multiprotic molecules with overlapping protonation. Thus, this methodology has become the chief approach of microspeciation as reviewed recently [34]. The acid/base profiling of imatinib [66] and cetirizine [33], measured by NMR/pH titration was reported.

Capillary Electrophoresis (CE). The application of CE for pK_a determination has been intensively growing in the past decade as reviewed [7,16,67,68]. The method utilizes the change in electrophoretic mobility of a compound with change in pH. The effective mobility (μ_{eff}) is measured at various pH values and pK_a is obtained from the plot of μ_{eff} vs. pH. The experimental conditions effect the pK_a determination such as buffer type and ionic strength, applied voltage, detection method, *etc.* are discussed as detailed by Henchoz *et al.* [16].

In this technique, the sample consumption is small (ng), and impure samples can be handled due to the separation upon the analysis. It is rather universal, since different detection methods can be coupled to CE [69]. The precision is good enough and agreement with other methods is acceptable, about ± 0.2 pK_a units in a range from 2 to 10, but can be much weaker (± 0.5) out of this pK_a range. The method is sensitive for several factors, among them temperature which is cardinal.

Today, CE is a good tool for high throughput pK_a measurement. The instrumentation is fully automated using a multiplex 96-channel CE with UV detection (CombiSep, Ames, USA) and more than 150 samples/day can be measured [70].

Spectral Gradient Analysis (SGA). To further increase the throughput of physicochemical profiling, a rapid pK_a determination method was developed and reported first as “pH-gradient titration” [71]. Later, after the launch of a commercial instrument (Profiler SGA, Sirius) it is referred to in the literature as the SGA method. In this technique, a pH gradient flow – very linear in time – is created by mixing appropriate acidic and basic buffers. The sample is injected into this pH gradient flow which passes through a diode array UV spectrophotometer and the spectra are registered. The pH is not measured but estimated from the time elapsed since the start of the gradient generation. The pK_a values are determined from changes in absorption as a function of pH. The calculation is based on either the first derivative plot of the absorption spectrum for samples with only a single pK_a (or well separated pK_a values) or on the TFA approach for compounds with weak spectral change or overlapping ionization [60]. The precision of the method is evidently lower than that of other methods, but results of a comprehensive validation study show good agreement with literature data [72]. The SGA method allows pK_a measurement within 4 min leading to high

throughput capacity. The present available automated instrument (Sirius T3) containing an autoloader module (robotic arm) utilizes four 48-position vial trays for samples. It enables the measurement of 240 compounds/day. Low solubility and low molar absorptivity may be limitations of the SGA method.

1.3.1.4. Co-solvent method

Determination of pK_a using the above discussed methods is often hindered by the low water solubility of the samples. It is a frequent problem today since the new molecules in drug research are less water-soluble and more lipophilic. For water insoluble compounds, the co-solvent method can be used. In this approach, the apparent ionization constants, p_sK_a values, are measured in different ratios of organic solvent/water mixtures. The aqueous pK_a value is obtained by extrapolation to zero organic content. The co-solvent method is primarily used in pH-metry, but it can be applied in UV-spectroscopy and CE techniques as well.

Many water miscible organic solvents have been used such as methanol (MeOH), ethanol (EtOH), propanol, DMSO, dimehtylformamide (DMF), acetone, and tetrahydrofurane (THF). Since most literature data have been accumulated for a MeOH/water solvent mixture and it is generally accepted that MeOH shows a solvation effect closest to water, MeOH is normally chosen as an organic solvent of choice [11,16,68].

Different extrapolation methods are known, but the Yasuda-Shedlovky (YS) extrapolation has proven to be the most reliable. Here, a linear correlation is established in a plot of $p_sK_a + \log [H_2O]$ vs. $a/\epsilon + b$, where $\log [H_2O]$ is the molar water concentration of the given solvent mixture, ϵ is the dielectric constant of the mixture, and a and b are the slope and intercept, respectively. The aqueous pK_a values can be obtained for $\log 55.5$ and $1/78.3$, the molar concentration and dielectric constant of pure water, correspondingly. The dielectric constant of MeOH/water mixtures is lower than that of water and the extent of ionization is suppressed, thus pK_a values of acids are shifted higher while those of bases are toward lower values. The slope of the YS relationship is positive for acids and negative for bases. The YS procedure offers many benefits over the traditional plot of p_sK_a vs. R_w (wt % of organic solvent) which often shows a “hockey-stick” or “bow” shape, sometimes at $R_w > 60$ wt % an S-shape curve. Proper electrode calibration using four parameter procedures in the solvent mixture is crucial [73].

According to a comprehensive validation study, the reproducibility and precision of the method, based on 431 separate titrations in the interval of 15-65 wt% MeOH content using 25 model compounds, was found to be good ($SD = \pm 0.05$). Extrapolation from a methanol-rich region (R_w : 40-60 wt %) gives an error in pK_a not greater than ± 0.2 for weak acids and ± 0.1 for weak bases [74].

Since not all compounds dissolve in a single organic solvent (*e.g.* methanol), a new multicomponent co-solvent system significantly improving the solubility of pharmaceutical compounds was recently developed for pK_a determination [75,76]. The mixture consists of an equal volume of MeOH, dioxane, and acetonitrile

(referred to as MDM) diluted in water to obtain the required co-solvent system. This system enables pK_a measurements by potentiometry (and also by UV/pH titration) for a wide range of poorly soluble compounds. Since solubility considerably increases in the MDM system, measurements can be performed in a lower proportion of organic solvent, thus the long-distance extrapolation can be avoided. The linearity of the YS relationship is valid up to 55 wt % MDM content. Validation based on 50 compounds showed good reproducibility ($SD = \pm 0.01-0.08$) and the agreement of pK_a values extrapolated by this method with values measured by other methods is very good (< 0.10 unit).

The SGA method has been extended with measurements in 20 wt % MDM content, and general calibration equations were set up for acids and bases ($pK_{a(\text{aqueous})} = a p_{sK_{a(20\%MDM)}} + b$), so a single point estimation may provide rapid aqueous pK_a values for water-insoluble compounds in the early phase of drug research [76].

1.3.1.5. Decision tree for method selection

The selection of a suitable method must be based on the properties of the compound tested. Figure 1.12 shows a simple decision tree for method selection used in the laboratory of the author [4].

Figure 1.12. Decision tree for method selection of pK_a measurement

Table 1.4. Methods for pK_a determination

Method	Sample			Throughput		Precision	Instrumentation
	amount, mg	solubility, mM	high purity	speed ¹	capacity ²		
potentiometry	3-5	> 0.5	necessary	20-30	10-12	high	GLpKa, SiriusT3 (Sirius, UK)
UV/pH titration							
traditional	1-2	> 0.01	necessary	6-8 hours	1	medium	pH-meter + spectrophotometer
automated	1-2	> 0.01	necessary	30	10-12	high	GLpKa + D-PAS, SiriusT3 (Sirius, UK)
NMR/pH titration	1-2	>0.5	not necessary	2-3 hours	2-3	high	NMR spectrometer
CE*							
single channel	<< 1	> 0.01	not necessary	30	20	medium	CE
multiplexed	<< 1	> 0.01	not necessary	30	150	acceptable	CePro 9600 (CombiSep)
SGA†	1	> 0.01	necessary	4	240	acceptable	Profiler-SGA, SiriusT3 (Sirius, UK)

* Capillary Electrophoresis

† Spectral Gradient Analysis

¹ min/comp.² sample/day

1.3.2. log *S* determination

Several methods have been developed for the measurement of both equilibrium and kinetic solubility including traditional and high throughput techniques. Excellent reviews [7,16,39,41] have surveyed the state-of-the-art techniques. Below, after a short summary of kinetic solubility methods, approaches for equilibrium solubility measurement are discussed focusing on good laboratory practice (GLP).

1.3.2.1. Methods for determination of kinetic solubility

Concerning the large number but small content, samples in the early phase of drug discovery are subjected to compound-saving and HT methods which are suitable for the measurement of kinetic solubility. In the *turbidimetric method* introduced by Lipinski *et al.* [77] small aliquots (0.5 µl) of DMSO stock solution are added at 1 min. intervals to aqueous buffers (originally, 2.5 ml of pH 7 phosphate buffer) until the compound precipitates from the solution reaching the maximal (but not yet the equilibrium) solubility. The turbidity caused by the precipitation is measured by light scattering in the 620-820 nm range with a UV detector. In *nephelometric* [78], *direct UV* [79] and *ultrafiltration-LC/MS* [80] methods the above principle is adapted to 96-well plate using different detectors (nephelometer, diode array UV and MS, respectively). In the two later methods,

the precipitate is separated from the solution by filtration (or centrifugation) before the concentration measurement. The ultrafiltration-LC/MS technique has the advantage of high sensitivity and the capability of handling impure samples. Commercially available instruments (Nephelostar, BMG; Nepheskan Ascent, Thermo Labsystem, μ SOL, p ION) use fully automated liquid dispensing systems and provide high capacity (measurement of 200-300 compounds/day). The presence of DMSO in the kinetic solubility experiments may considerably affect the results in a highly compound-dependent way, thus it is practical to keep the DMSO at a minimum level (less than 0.5%).

The main disadvantages of kinetic solubility measurements are the lack of standardization, poor reproducibility, and difficulties in the comparability of results.

1.3.2.2. Methods for determination of equilibrium solubility

1.3.2.2.1. Saturation shake-flask method (SSF)

The SSF method is the standard approach for the determination of equilibrium solubility which when properly performed provides high quality data. It is a simple but very time-consuming procedure and requires lots of manual work. The solution of the tested compound containing excess solid is prepared in aqueous buffer using a small (2-5 ml) glass vial. The heterogeneous system is capped and vigorously stirred at a chosen temperature (usually 25 °C or 37 °C) for a specified time (24, 48 h or longer) until the equilibrium has been reached. After that, the two phases (solid and liquid) are separated by sedimentation, centrifugation, or filtration. Upon diluting sample aliquots with the solvent, if necessary, the concentration of the saturated solution is measured by an appropriate method, most frequently by UV spectroscopy or HPLC. Despite the longevity of SSF use, there is no accepted standard way to carry out this method. Published solubility studies show great differences in the experimental conditions used, particularly concerning the time of equilibration, the method of phase separation, and the control of pH during the measurement [37,38,41].

Recently, in a comprehensive study published by Baka *et al.* [81] the most important experimental factors influencing the measured equilibrium solubility by the SSF were investigated (see some results in Section 1.4.2) and a standardized protocol was proposed for GLP [82]. The following conditions are suggested:

- the measurement must be carried out at controlled temperature with precision ± 0.1 °C,
- Sørensen phosphate buffer can be used between pH 3-7, while Britton-Robinson buffer can be used in a wider pH range from 2.5 to 11.5. HCl of appropriate concentration can be used below pH 2.5,
- the pH of the solution must be carefully controlled during the measurement, advisably before and after the equilibration,
- to avoid the difficulties in sampling, only a small (~ 5 -10 mg/5 ml) excess of solid should be present,
- a minimum of 24 h is necessary to reach the equilibrium, this time should consist of 6 h of stirring and 18 h of sedimentation, but in case of very sparingly soluble compounds longer stirring time may be necessary for equilibrium, so in the most rigorous

application of SSF, the required time of equilibration must be determined from compound to compound,

- the safest technique of phase separation is sedimentation which assures a heterogeneous system until equilibrium has been achieved; if an opalescent solution is formed then the phase separation can be done by centrifugation while the most erroneous filtration should be avoided (see results in Section 1.4.2 below),
- a compound existing in a meta-stable polymorph form can be transformed into a more stable one during the duration of solubility measurement, thus the analysis of the solid phase (by X-ray powder diffraction or thermo-gravimetric methods) at the end of the experiment is highly recommended.

Using the above listed conditions the equilibrium solubility of more than 50 compounds was determined with a standard deviation of less than 4% in our laboratory.

1.3.2.2.2. Potentiometric methods

The principle of the potentiometric methods is based on that characteristic shift of the titration curve caused by the precipitation of the unionized form of a compound from a solution. Potentiometric titration was introduced for equilibrium solubility measurement by Avdeef *et al.* [83-85]. The *dissolution template titration* (DTT) method uses pK_a and $\log P$ values as input parameters. $\log P$ is used to estimate the intrinsic solubility based on a Hansch-Yalkowsky type equation ($\log S_o = 1.17 - 1.38 \log P$). Using the pK_a and the estimated intrinsic solubility, the DTT procedure simulates the entire titration curve before the assay begins. This curve serves as a titration template (the optimal quantity of the tested compound for the titration is suggested by the simulation) and also as a guide for the right titration protocol (how the instrument dispenses the titrant and collects the pH data) in the course of the titration. The titration starts at pH values, where the compound is unionized and forms a suspension (solid material is present in the solution). The titrant is dispensed accurately and slowly into the slurry, to drive the pH of the solution in the direction of dissolution. Typically, a 3-10 h (sometimes longer) time frame is required for the entire equilibrium solubility data collection (20-50 pH points) [41]. The method, when performed with the pSOL titrator (pION, US), provides a precise solubility-pH profile without assuming a HH relationship and is much faster than the SSF method but still has a very low throughput.

The novel potentiometric procedure (CheqSol) has been developed recently for rapid measurement of solubility using the instrument called the GLpKa-D-PAS (Sirius, UK). In this method, the equilibrium solubility is actively sought by changing the concentration of the neutral (unionized) form of a compound by adding acid or base titrants and monitoring the rate of the change of pH, due to precipitation or dissolution in a process called "Chasing Equilibrium". In this method, the titration is started at pH value, where the compound is fully ionized and dissolved and performed toward the direction of pH where the unionized form precipitates. The turbidity of the solution caused by the precipitation is detected with a fiber optic dip probe. With this method both the kinetic solubility and the equilibrium solubility can be determined. The kinetic solubility value is

obtained from the concentration when the first precipitation of the unionized form appears in the solution, while the equilibrium solubility is obtained from actively seeking the equilibrium pH where an equal amount of the sample is precipitating and dissolving per unit of time [86]. The CheqSol method is faster (typically 30–60 min/compound) because the intrinsic solubility is determined instead of the entire solubility-pH profile. Then HH equation is used for the calculation of the approximate log *S*/pH profile. It was validated against the SSF method and excellent agreement of solubility results was found [87].

1.3.2.2.3. μ DISS method

A miniaturized rotating disk dissolution instrument, called μ DISS ProfilerPLUS (pION, US) has been developed for characterizing the intrinsic dissolution rate in early preformulation. This apparatus is also suitable for the measurement of equilibrium solubility of sparingly soluble compounds, provided enough material is used to maintain the saturation [41,88,89]. In this procedure, 5 mg of drug are compressed into pellets and inserted into a rotating disk carrier containing an embedded magnetic stir bar at its bottom. This assembly is placed into a glass vial filled with a small volume (1–3 ml) of aqueous buffer as the dissolution medium. The concentration is measured with a rapid *in situ* fiber optic UV (diode array) detector. The instrument employs six parallel dissolution vessels and eight channels of UV detectors which provide better capacity above the SSF method. Over the high precision, further advantages of this method are: (i) any polymorph changes during dissolution can be recognized and (ii) the longer incubation time needed to establish the true equilibrium of the most stable form of a solid may be evident in the dissolution curve [39].

1.3.2.2.4. High throughput methods

Some methods suitable for medium or high throughput determination of log *S* were also described. The *miniaturised shake-flask (MSF) method* developed by Glomme *et al.* [90,91] is a compound saving and fast method, thus it is frequently used in pharmaceutical companies. Typically, 0.1–0.2 mg solid powder is introduced to a specially designed filter chamber and a small (*e.g.* 2 ml) volume of aqueous buffer is added. Purpose-built filter caps are firmly attached and the vials are shaken at constant temperature for 24 h. The filter-containing cap compartments are then depressed to effect separation of the solid and the top compartment solutions are analyzed by fast gradient RP-HPLC. The throughput is just medium, as 20 compounds/week can be measured. The MSF method was further developed for HT measurements by Zhou *et al.* [92] where a 96-well plate is used as the source of the samples and DMSO stocks were evaporated via a GeneVac evaporator.

Those 96-well plate based HT methods (originally developed for kinetic solubility measurement), where the incubation time is long enough (*e.g.* μ SOL method, [39]) and the effect of DMSO content is eliminated, are also suitable for equilibrium solubility determination. Generally, in these modified-microplate methods the 24 h incubation time is adequate to reach the solubility equilibrium [39].

However, due to small volumes, the precise pH control during the measurement may be problematic. In the *lyophilized solubility assay* (LYSA) the sample is dispensed into a microtiter plate along with 10 mM DMSO solution then the organic solvent is removed by lyophilization and aqueous buffer is added. During a 24 h incubation period the plate is agitated by a shaking mechanism, then filtrated and the concentration is measured using a UV plate reader [93]. Another promising HT procedure is the *PASS* (*Partially Automated Solubility Screening*) method, where the compounds are suspended in heptane and dispensed into the plate wells, then heptane is evaporated before buffer is added [94].

1.3.2.3. Special applications

The methods described above have been applied for special purposes. Potentiometric titration, according to the CheqSol approach, has been reported to study the solubility of polymorphs. A new method named “potentiometric cycling for polymorph creation” (PC)², was developed to generate the most stable polymorph in aqueous solution [95]. It was applied to sulindac producing two polymorphs including a new, more stable one. It was found that their intrinsic solubility differ by a factor of seven, which is much larger than that of any earlier measured difference between polymorphs.

Table 1.5. Methods for log *S* determination

Method	Detection	LOD, μg/ml	Throughput		Precision	Instrumentation
			speed, min/comp.	capacity, samp./day		
for kinetic solubility						
turbidimetric	UV	5	15	50	low	
nephelometric	laser nephelometer	5	4	300	low	Nephelostar, Nepheloscan
direct-UV	UV	2·10 ⁻³	4	300	medium	μSOL
ultrafiltration-LC/MS	MS	0.1	6	200	medium	LC/MS
for equilibrium solubility						
SSF	UV; HPLC	1	36 hours	< 1	high	
Potentiometric						
DTT	pH-metry	5·10 ⁻³	3-10 hours	1-5	high	pSOL
CheqSol	pH-metry	0.1	30-60	10-15	high	GLpKa; SiriusT3
μDISS	UV	1	24 hours	6	high	μDISS Profiler <i>PLUS</i>
modified-plate HT						
MSF	UV; LC/MS	1	24 hours	3-100	medium	
μSOL	UV	0.1	18-24 hours	100	acceptable	μSOL
LYSA	UV	1	24 hours	100	acceptable	
PASS	UPLC	10 ³	24 hours	100	acceptable	

The biorelevant solubility values are more and more required in drug discovery and development (DD&D). An optimized 96-well HT UV solubility method was adapted to measure solubility of drugs in biorelevant media such as FaSSIF and FeSSIF solutions [96]. The method provides reliable data using a very small amount of sample and small volumes of the expensive FaSSIF/FeSSIF components. The μ DISS method was also found useful for measurement in biorelevant media and temperature [97]. The study has revealed that the majority of the tested drugs exhibited higher solubility in these media than in pure buffers.

1.3.3. $\log P$ determination

Since $\log P$ is the oldest parameter in physicochemical profiling, several well-established experimental methods are available for its determination. Vast amounts of literature have described the theory and practice of the used methods [e.g. 11,46,98-101]. Aspects from the GLP guide for $\log P$ measurements have also been published [98,101,102]. The recent reviews provide a comprehensive survey about the latest developments in HT techniques [6,9,14-16].

Two types of methods can be distinguished for $\log P$ determination: (i) the *direct* approaches, where $\log P$ is directly obtained from the measured data (shake-flask, stir-flask, filter chamber, dual-phase potentiometric, etc.) and (ii) *indirect* (chromatographic, CE) techniques, where the measured parameter has a linear relationship with $\log P$ and $\log P$ is calculated using calibration equations. In this chapter, the direct methods are overviewed, out of the indirect methods only TLC is presented, while others like HPLC, MECK, etc. used for $\log P$ measurement are discussed elsewhere in the book.

In order to facilitate the comparison of their capacity, Table 1.6 summarizes both types of methods.

1.3.3.1. Shake-flask (SF) method

The traditional SF method is the reference and most widely used approach of $\log P$ determination.

Procedure. In advance, the two phases (n-octanol or other useful partition organic solvent that is immiscible with water and aqueous buffer) must be mutually saturated with vigorous agitation then filtered or centrifuged. The tested substance is dissolved in the aqueous phase and introduced into an appropriate glass vial. Octanol (or other organic solvent) is added in a required volume and the system is shaken at a constant temperature for a period long enough for equilibrium to be achieved (generally 1 h). After separation of the phases by centrifugation, the concentration is measured using an appropriate method, mostly UV spectroscopy. Concerning the difficulties of the precise analytical work with octanol, it is a common practice to measure the concentration decrease in the aqueous phase by detecting the absorbance before and after the partition.

Accuracy, sources of the experimental error. The SF method is suitable for $\log P$ measurement in the range from -2 to 5 having a $SD < 0.05$, provided that optimal

experimental conditions are maintained. Many factors can affect the reliability of the measured $\log P$ values increasing the experimental error. One of them is undoubtedly the applied extreme phase ratio necessary in the case of lipophilic compounds ($\log P > 3$). According to our experiences, the highest phase ratio that can be used without a considerable increase in error is $R = 500$ (e.g. 50 ml aqueous buffer: 0.1 ml octanol). However, in the opposite case with hydrophilic compounds, when more octanol has to be used, the sampling from the lower aqueous phase may be problematic, thus it is advisable to remove the upper octanol layer before aliquots are taken. Glass and surface adsorption, formation of stable emulsions, and the presence of impurities in the sample have often influenced the results.

Advantages/drawbacks. The main advantage of the SF method is its simplicity, sufficient accuracy, and applicability to non-ionizable compounds. But it has some well-known shortcomings, such as being tedious and time-consuming, difficulties with maintaining a constant temperature during the whole procedure, requiring relatively high amounts of sample and solvent. The SF method cannot be used for UV inactive compounds unless alternative detection methods are employed, and so on.

1.3.3.2. Potentiometric method

Dual phase potentiometric titration using automated instruments has become the “gold standard” of $\log P$ determination (for ionizable compounds) in the past ten years [11,60,100]. It consists of two titrations of the tested compound. One is performed without the partition solvent and provides the aqueous pK_a value. The second is done using the same conditions but in the presence of a partition solvent (e.g. octanol) with intensive stirring upon titrant addition, while stopping it when the pH is measured. If the unionized form of the compound partitions into octanol then the two titration curves will be different, due to a shift (similar to what was discussed in the co-solvent pK_a method). From the dual-phase titration, the apparent p_oK_a value is obtained. $\log P$ is calculated from the differences in pK_a values and the phase ratio. A large shift indicates high lipophilicity (see also in Section 1.4.3).

This method allows the $\log P$ determination in a range from -2 to 6, with very high precision ($SD = \pm 0.01$). The agreement with the SF method is excellent according to validation studies [103,104]. However, it has limited capacity and cannot be used for compounds with pK_a out of the established measurable pH range. A further drawback is that only a limited phase ratio can be applied (in our practice with GLpKa: 20 ml water : 0.05 ml octanol for lipophilic compounds and 5 ml of water : 15 ml of octanol for hydrophilic compounds). A newly developed instrument (SiriusT3) has further increased efficacy and measurement is possible in as low as 1 ml aqueous phase.

1.3.3.3. Indirect log P measurement methods

Because of the drawbacks and limitations of direct methods, numerous alternative procedures have been developed and applied. Several micellar, microemulsion, vesicle electrokinetic chromatographic systems, and reversed-phase chromatographic methods (RP-TLC, RP-HPLC) can be used to estimate lipophilicity. Some excellent reviews on the use of separation methods for indirect log P determination have been published [e.g. 105-106].

Although RP-HPLC is more widely used technique for log P estimation [105], RP-TLC undoubtedly has some unique advantages, including use of less expensive laboratory equipment and being easy to perform. Simultaneous running of 15-20 compounds on one plate can significantly reduce the analysis time per compound. The method is based on the linear relationship between log P measured by the SF method and the logarithm of chromatographic retention expressed as the R_M value. RP-TLC has been successfully applied for log P measurement of highly lipophilic molecules using calibration equations obtained with structurally related compounds [e.g. 107-109]. Recently, a validated RP-TLC method was proposed for parallel estimation of the lipophilicity of chemically diverse neutral compounds or weak acids and bases [110]. To cover a wide range of lipophilicity, two optimized RP-TLC systems were used: one for moderate lipophilic compounds (log P = 0 - 3) and another for highly lipophilic molecules (log P = 3 - 6). Two chemically diverse sets of compounds were selected to set up the general calibration equations. The method was tested with 20 randomly selected drugs and good agreement with SF results were found ($SD < 0.15$). With automated sampling and imaging detection of the compounds the method can be regarded as a possible alternative for rapid and acceptable accurate estimation of lipophilicity of drug candidates in the early phase of DD&D.

1.3.3.4. High throughput methods

Attempts have been done to miniaturize the traditional SF method into a microtiter plate platform with robotic liquid handling and HPLC/UV [111] or LC/MS [112] detection. In these techniques the partitioning process is transferred to 96-deep well plates and after equilibration, the detector signal produced by a sample from the octanol phase is divided by the signal from the aqueous phase. In a review by Kerns [7], the critical elements of these methods are discussed. A commercially available automated plate based instrument for HT log P determination is called the AlogP (Analiza, US).

Regarding indirect (HPLC and MECK) log P methods, additional successful strategies were applied to increase the throughput and speed up the determination time [16]. For example, the use of short columns and a high flow rate in HPLC, usage of UPLC, and multiplexed MECK have been reported. These methods are surveyed in the Chapter 2 of this book.

1.3.3.5. Decision tree for method selection

The method selection strategy followed in the laboratory of the author is shown in Figure 1.13.

Figure 1.13. Decision tree for method selection of log *P* measurement

Table 1.6. Methods for log *P* determination

Method	log <i>P</i> range	sample amount, mg	Throughput		Precision	Instrumentation
			speed, min/comp.	capacity, samp./day		
direct methods						
shake-flask						
traditional	-2 ↔ 5	2-10	180-360	2	high	
automated (96-well plate platform)	-2 ↔ 5	1-5	10	100	acceptable	AlogP (Analiza Inc.)
potentiometric	-2 ↔ 6	1-5	60	20	high	GLp <i>K</i> _a , SiriusT3
indirect methods						
RP-TLC	0 ↔ 6	1-3	120	50	medium	
RP-HPLC	-1 ↔ 6	0.01	15	100	acceptable	
MEEKC	-1 ↔ 7	<< 1	15	150	acceptable	CePro 9600, MCE 2000

1.4. CASE STUDIES

In this chapter we present some useful examples of physicochemical profiling for the illustration of methods discussed above. Mainly, such problematic compounds have been selected where the routine measurement is difficult or hindered by certain reasons. The most frequent difficulties in the pK_a , $\log S$, and $\log P$ determination are the low solubility, instability, lack of UV activity and polymorph transition of the compound. The case studies introduced below can provide a possible template for the measurement of “difficult to measure” molecules.

1.4.1. pK_a determination

Case 1.

Sample: RG-1503

Method: co-solvent method; potentiometric titration in methanol/water system

Instrumentation: GLpKa (Sirius, UK)

The compound is a multiprotic molecule containing four ionizable groups (Figure 1.14a: A-D). The two piperazine N atoms and the pyridine N are proton accepting basic centers, while the arylsulfonamide moiety represents a proton releasing acidic group. The aqueous solubility of the compound is less than 0.5 mM and it has no useful pH-dependent UV spectrum in the pH range 3-10, thus neither potentiometry nor UV/pH titration in an aqueous medium can be used.

Figure 1.14. Co-solvent pH-metry for pK_a determination: (a) structure of the sample (RG-1503), (b) titration curves in different methanol/water mixtures, (c) Bjerrum plots, (d) YS extrapolation curves

The pK_a values were measured by potentiometry using the co-solvent method (see Section 1.3.1.4). Six titrations were performed in methanol/water mixtures (40-60 wt%) between pH 1.5 - 12.5 in ~ 1 mM concentration of the sample, at 0.15 M (KCl) ionic strength, at 25.0 ± 0.1 °C temperature, under N_2 atmosphere (Figure 1.14.b). From the obtained p_sK_a values, the aqueous pK_a values were calculated by YS extrapolation (Figure 1.14d).

Results: $pK_{a1} = 2.02 \pm 0.22$ (B group); $pK_{a2} = 3.03 \pm 0.09$ (C group);
 $pK_{a3} = 7.35 \pm 0.03$ (A group); $pK_{a4} = 11.40 \pm 0.09$ (D group).

Case 2.

Sample: nitrofurantoin

Method: co-solvent method; UV/pH titration in MDM/water system

Instrumentation: GLpKa + D-PAS (Sirius, UK)

Nitrofurantoin is a water-insoluble compound which has one acidic group and exhibits a pH-dependent UV spectrum. The pK_a value was measured in an MDM/water system because its solubility is high enough in this solvent mixture for the spectroscopic determination. A stock solution was prepared in 10 mM concentration with MDM, 50 μ l of this stock solution was used for the titration in 15 ml of 20-50 wt% MDM/water mixtures between pH 3-10, at 0.15 M (KCl) ionic strength, at 25.0 ± 0.1 °C temperature, under N_2 atmosphere. Figure 1.15.c shows the p_sK_a values used for YS extrapolation. The extrapolated aqueous pK_a value is: 6.87 ± 0.01 ($R^2 = 0.9958$), which is in good agreement with literature data measured by other methods [76].

Figure 1.15. Co-solvent UV/pH titration for pK_a determination:
 (a) structure of the sample (nitrofurantoin), (b) pH-dependent UV spectra,
 (c) apparent pK_a values in different MDM/water mixtures, (d) YS extrapolation

Case 3.

Sample: lisinopril
 Method: potentiometric titration in aqueous solution and in methanol/water system; NMR/pH titration
 Instrumentation: GLpKa + D-PAS (Sirius, UK); Varian Inova 600 MHz spectrometer (Palo Alto, CA)

Lisinopril is a tetraprotic compound having two acidic (carboxyl) and two basic (a primary and a secondary amine) groups. Figure 1.16a shows the ionization processes of the molecule. The dissociation of the two carboxyl groups is highly overlapping. The solubility of lisinopril (0.22 M) allows the determination of pK_a values by the standard potentiometric method in aqueous medium. However, the first dissociation constant falls into the low pH range which may cause uncertainty of the measurement.

For the characterization of the acid/base property of the molecule, three independent methods were applied: potentiometry in aqueous solution and in a methanol/water system, as well as NMR/pH titration. In aqueous medium, three titrations were carried out in a 2 mM concentration solution, between pH 1.8–12, at 0.15 M (KCl) ionic strength, at 25.0 ± 0.1 °C temperatures, under N_2 atmosphere. Since pK_{a1} value falls below the lower applicability limit (< 2) of pH-metric titration, the pK_a values were also measured using the co-solvent method.

Figure 1.16. Protonation scheme of (a) tetraprotic lisinopril, (b) distribution of macrospecies

The apparent pK_a values of the COOH groups obtained in 14-44 wt% methanol/water mixtures shifted up to the established measurable pH range, and a reliable aqueous pK_{a1} value could be obtained by YS extrapolation.

For the exact proton speciation of lisinopril, ^1H NMR/pH titrations with *in situ* pH measurements were carried out, using the most similar experimental conditions as possible in potentiometry. This method was useful to assign the constants to the functional groups: pK_{a1} and pK_{a2} belong to the COOH groups, pK_{a3} refers to the secondary amine ($-\text{NH}-$), and pK_{a4} shows the basicity of the primary amine ($-\text{NH}_2$) function.

The highly precise pK_a values were calculated as an average of the best two values obtained by independent methods (Table 1.7). These values were used to calculate the distribution curve of different protonated species of lisinopril against the pH (Figure 1.16b).

Table 1.7. The pK_a values of lisinopril measured by different methods

method	ionization constants			
	$pK_{a1} \pm SD$	$pK_{a2} \pm SD$	$pK_{a3} \pm SD$	$pK_{a4} \pm SD$
potentiometry	1.54 ± 0.05	3.10 ± 0.01	7.14 ± 0.01	10.74 ± 0.01
potentiometry in solvent mixtures	1.62 ± 0.01	3.21 ± 0.02	7.22 ± 0.03	10.75 ± 0.01
NMR/pH titration	1.63 ± 0.01	3.15 ± 0.01	7.12 ± 0.01	10.53 ± 0.03
average of the best two values	1.63 ± 0.01	3.13 ± 0.01	7.13 ± 0.01	10.75 ± 0.01

1.4.2. log S determination

Case 4.

Sample: hydrochlorothiazide

Method: SSF

Instrumentation: Radiometer PH220 pH meter; LAUDA M20S thermostat; Heidolph MR 1000 magnetic stirrer; JASCO V-550 UV/VIS spectrophotometer

Hydrochlorothiazide is a bivalent acid with pK_a values: 8.75 and 9.88. Its intrinsic solubility (S_o) value was measured at pH 6.0 using the SSF method [81].

First the S_o of the sample was measured according to a standard (literature) protocol with the following conditions. Buffer: Britton-Robinson (BR); solid excess: small amount; temperature: 25.0 ± 0.1 °C; equilibration time: 48 h stirring plus 24 h sedimentation; phase separation technique: sedimentation; concentration measurement: UV spectroscopy ($\lambda=271$ nm, $A^{1\%_{1\text{cm}}}$: 696); number of parallels: 6. Result: $S_o = 556 \pm 13.2$ µg/ml.

Next, different parameters of this protocol were examined, always one of the six parameters (buffer choice, amount of solid excess, temperature, time of stirring, time of sedimentation, phase separation technique) was varied while the other conditions were kept unchanged.

Effect of buffer solution. Three buffer solutions were used at pH 6.0. The results are shown in Figure 1.17a. The statistical analysis has indicated that S_o values in BR and Sørensen phosphate (I) buffers are in accordance, but the solubility in Sørensen citrate (II) buffer deviates significantly. The ionic strength of this later buffer is four times higher than that of BR or Sørensen I.

Effect of phase separation. Alternative techniques to sedimentation such as centrifugation and filtration were studied. 12 samples were centrifuged after 48 h stirring at 2000 rpm for 10 min, while 12 samples were filtered through 0.45 μm membrane filters. Results shown in Figure 1.17b are significantly different. The highest deviation is caused by filtration.

Effect of equilibration time. Figure 1.17c shows the experimental results obtained when (i) stirring time was changed from 30 min to 48 h followed by a 24 h sedimentation; and (ii) sedimentation time was changed from 1 h to 24 h, keeping the stirring time constant (48 h). From the results it can be concluded that the time of sedimentation plays a greater role in the development of equilibrium than the time of intensive agitation.

Effect of temperature. The solubility of hydrochlorothiazide increases with the temperature (Figure 1.17d). It is almost double at 37 °C than at 25 °C, which underlines the need for solubility determination at biomimetic temperature as well.

Figure 1.17. Effect of experimental conditions on the intrinsic equilibrium solubility of hydrochlorothiazide: (a) buffer solution, (b) phase separation technique, (c) stirring time (upper graph) sedimentation time (lower graph), (d) temperature

Case 5.

Sample: papaverine hydrochloride
 Method: SSF
 Instrumentation: Radiometer PH220 pH meter; LAUDA M20S thermostat;
 Heidolph MR 1000 magnetic stirrer; JASCO V-550 UV/VIS
 spectrophotometer

The solubility-pH profile of papaverine hydrochloride was determined in a wide pH range using a new (shorter) protocol derived from the SSF method: BR buffer, small solid excess, 25.0 ± 0.1 °C, 6 h stirring and 18 h sedimentation [42].

First, the intrinsic solubility of the sample was measured at high pH (11.71) and found $17 \mu\text{g/ml}$ ($\log S_0 = 1.70$ [$\log \mu\text{M}$]). Then the equilibrium solubility (S_{pH}) values at twelve different pH values between 0.06 and 8.02 were determined.

From $\log S_0$ and $\text{p}K_a$ (6.36) values the theoretical $\log S_{\text{pH}}/\text{pH}$ profile was generated by the HH equation. Figure 1.18 shows the excellent agreement between the experimental data points and the predicted HH curve. This shape is typical for a monovalent base, where the solubility increases with a decrease of the pH, as the free base starts to convert to the protonated form. At around pH 3, the solubility of the papaverine hydrochloride salt reaches the maximum (pH_{max}), which is limited by the solubility product. Between pH 2 and 3 there is a constant value for the salt solubility. Below pH 2 the solubility of the salt decreases due to the common ion effect, caused by chloride ions from HCl used to adjust the pH.

Figure 1.18. Solubility-pH profile of papaverine hydrochloride

This example proves that the HH equation can be used for the calculation of solubility at physiological important pH values once the intrinsic solubility and the pK_a value have been previously determined [42].

Case 6.

Sample: telmisartan

Method: SSF

Instrumentation: Radiometer PH220 pH meter; LAUDA thermostat; Heidolph MR 1000 magnetic stirrer; JASCO V-550 UV/VIS spectrophotometer

The solubility of telmisartan was measured in distilled water (ordinarily pH ~6) and at 37 ± 0.1 °C temperature (one of the conditions where solubility is required by the registration authorities). Solid material at 0.01g was added to 20 ml freshly boiled and cooled water and then the new (shorter) protocol derived from the SSF method was followed. Aliquots were taken out from the supernatant and the absorbance was measured without dilution at $\lambda = 295$ nm, in a cell with a 5 cm pathlength. The concentration was calculated using $A^{1\%}_{1\text{cm}} = 510$ measured separately prior to solubility measurement. From three parallel experiments, the solubility of telmisartan was found as low as $S_{\text{pH}} = 0.50 \pm 0.09$ µg/ml. The relatively high error ($SD = \pm 18\%$) is due to the very low solubility (the lowest value we could ever measure by the SSF method) and the occasionally formed supersaturated solution, from which small (invisible) particles precipitated in the cell upon absorbance measurement.

Case 7.

Sample: maprotiline

Method: SSF and CheqSol

Instrumentation: Radiometer PH220 pH meter; LAUDA thermostat; Heidolph MR 1000 magnetic stirrer; JASCO V-550 UV/VIS spectrophotometer and GLpKa + D-PAS

The precise intrinsic solubility of maprotiline base ($pK_a = 10.33$) - another very sparingly soluble compound - could not be determined by the SSF method. The result obtained from three separate measurements in BR buffer at pH 11.5 was $S_o = 8.05 \pm 3$ µg/ml. The reason for the extremely high experimental error ($SD = \pm 37\%$) is that a colloid, slightly opalescent solution (perhaps due to re-crystallization or supersaturation) was formed upon equilibration. This opalescence could be eliminated by neither filtration nor centrifugation. So, the SSF solubility result must be considered as an approximate value. Thus, the potentiometric method, namely the Chasing Equilibrium Solubility (CheqSol) was also applied. Maprotiline was added (2 mg) to 10 ml of 0.15 M KCl solution then pre-acidified with 0.5 M HCl to pH 2 where the compound was fully dissolved. This solution was titrated with 0.5 M KOH until the solution became cloudy, which indicated the precipitation of the free base form. The occurrence of precipitation was detected using a spectroscopic dip probe then the solution was quickly

brought close to equilibrium by adding very small amounts of acidic or basic titrants alternatively resulting in an oscillation between supersaturation and subsaturation. The Bjerrum plot of titration is shown in Figure 1.19. While the sample is fully dissolved, the experimental data fit well to the nonprecipitation theoretical curve (a). After precipitation, the points lie close to the precipitation theoretical curve (b). The precipitation point is used to calculate the kinetic solubility value. The intrinsic equilibrium solubility was determined from 40 data points with 8 zero pH gradient crossings.

The intrinsic solubility was obtained as average of 6 separate titrations, $S_0 = 5.8 \pm 0.3$ $\mu\text{g/ml}$. The low *SD* indicates the higher precision of the data and the advantage of the CheqSol method in this case.

Figure 1.19. Bjerrum plot of solubility determination of maprotiline by CheqSol method. (a) nonprecipitation theoretical curve, (b) precipitation theoretical curve

Case 8.

Sample: venlafaxine HCl
 Method: SSF
 Instrumentation: Radiometer PH220 pH meter; LAUDA M20S thermostat;
 Heidolph MR 1000 magnetic stirrer; JASCO V-550 UV/VIS spectrophotometer

Venlafaxine is a monovalent base ($pK_a = 9.6$), its hydrochloride salt can form different polymorphs. The solubility of two polymorph forms (I and II) was investigated at three pH values: 4.9 (unadjusted pH in distilled water), 8.9 (BR buffer), and 12 (0.001 M NaOH) at 37 ± 0.1 °C temperature using the SSF method. The results are summarized in Table 1.8. The salt solubility is higher than 50 % (g/100 ml) in the case of both polymorphs. The intrinsic solubility of venlafaxine

measured at pH 12 was also found to be the same for both I and II forms ($S_o = 460 \pm 10 \mu\text{g/ml}$).

The diffraction analysis of the solid phase filtered out at the end of the solubility measurement revealed that polymorphs I and II equally converted to the same crystal form of free base venlafaxine. This experience underscores the need for analysis of the solid phase after the equilibrium state has been reached.

Table 1.8. Solubility (g/100 ml) of two polymorph forms of venlafaxine hydrochloride at three pH values and at 37 °C temperature

venlafaxine hydrochloride	S_{pH} (pH 4.9)	S_{pH} (pH 8.9)	S_o (pH 12.0)
Form I	> 50	0.180	0.046
Form II	> 50	0.208	0.046

1.4.3. log *P* determination

Case 9.

Sample: chlorpromazine

Method: SSF

Instrumentation: LAUDA M20S thermostat; Hewlett-Packard 8452A UV/VIS spectro-photometer

Chlorpromazine is a very lipophilic monovalent base (pK_a : 9.24). The true log *P* value cannot be measured at high pH values (> 11.5) directly by the SF method because of the low solubility of the free base form of the compound at high pH. In such cases (which is typical among drugs), the log D^{pH} is measured at different pH values at which the molecule partially ionizes and dissolves better and then it is converted to the true log *P* using Equation 1.30b. The log D^{pH} values of chlorpromazine were measured at three pH values (7.4, 8.0 and 8.5) in BR buffer as the aqueous phase, using an R = 200 and 100 phase ratios (50 ml buffer : 0.25 ml octanol and 25 ml buffer : 0.25 ml octanol). We followed the standard protocol of the SF method: 1 h intensive shaking in a shaking thermostat; phase separation by centrifugation (730 g for 10 min). The absorbance of the aqueous phase before (A_o) and after (A_1) the partition was measured by spectroscopy at $\lambda = 254 \text{ nm}$. The apparent partition coefficient is calculated according to $D^{\text{pH}} = [(A_o - A_1)/A_1]R$ [104]. The lipophilicity-pH profile is shown in Figure 1.20.

Result: $\log P = 5.13 \pm 0.10$ ($n = 18$)

Figure 1.20. Lipophilicity-pH profile of chlorpromazine (points represent the experimentally measured $\log D^{\text{pH}}$ values)

Case 10.

Sample: deramciclane
 Method: potentiometric method
 Instrumentation: PCA 101 (Sirius, UK)

Deramciclane was an original anxiolytic molecule developed by EGIS (Hungary) in the late '90s, which unfortunately failed from clinical phase III. It is a sparingly soluble monovalent base ($\text{p}K_{\text{a}} = 9.61$). It has a very weak UV absorption (low specific absorptivity) thus lipophilicity determination by the SF method (as done in Case 9) is hindered. The $\log P$ value was measured by dual-phase potentiometric titration at 25.0 ± 0.1 °C temperature, under N_2 atmosphere. Six titrations were performed between pH 3 and 12, in a 1 mM concentration solution of the sample using 15 ml water and 0.05 ml octanol phase (Figure 1.21a). From these titrations the apparent $\text{p}K_{\text{a}}$ values (measured in the presence of octanol, p_oK_{a}) were obtained. The Bjerrum plot (Figure 1.21b) shows big shift toward lower pH values (typical for bases) which indicates high lipophilicity of the sample. The $\log P$ value is calculated according to the equation: $\log P = (10^{(\text{p}K_{\text{a}} - \text{p}_oK_{\text{a}})} - 1)/r$. The extreme (octanol/water) phase ratio ($r = 0.0033$) used here allowed lipophilicity measurement as high as $\log P = 5.90 \pm 0.02$ ($n = 6$). According to our experiences, this represents the upper limit of the pH-metric $\log P$ determination method [113].

Figure 1.21. pH-metric log P determination of deramciclone: (a) titration curves in the presence of different amounts of octanol, (b) Bjerrum plot

Case 11.

Sample: prostaglandin E_1 -ethylester (PGEE)
 Method: RP-TLC
 Instrumentation: RP-diC₁ silanized plates, Merck #5747; Camag micro sampler; Shimadzu CS-9301CP densitometer

PGEE is an example for molecules where classical, standard methods cannot be applied. Due to the lack of useful UV absorption (above $\lambda > 230$ nm) or an ionizable group, neither the SF nor pH-metry can be used. Log P was determined by a validated RP-TLC method.

Measurement was performed on 20 cm x 20 cm plates precoated with silanized silica gel GF₂₅₄ as the stationary phase and methanol/water (55:45) as the mobile phase. Before use, the plates were washed with methanol (ascending development), then dried and heated at 160 °C for 1 h. The samples (PGEE and calibration set) were dissolved in a 1 : 1 methanol/chloroform mixture (2 mg/ml) and 2 μ l was spotted on the plate. The chamber was saturated with the mobile phase for 30 min before use. After development the plates were dried and evaluated by densitometry.

The calibration curve was set up using seven compounds [114] and obtained from three parallel runs: $\log P = 3.508R_M + 0.968$ ($r = 0.995$, $n = 21$). The log P of PGEE was calculated with the help of this equation.

Result: $\log P_{TLC} = 4.02 \pm 0.05$ ($n = 3$).

1.5. OUTLOOK

Concerning the role of physicochemical profiling in the future, we can certainly predict that it remains an integrated part of drug research providing a simple, cheap, and fast tool for the estimation of ADMET parameters in the early stage of DD&D.

A higher level of automation (*e.g.* integration of several robotic platforms) and higher sensitivity of detection methods can be expected leading to the increase of the HT feature of the applied methods, but it must be synchronous with the improvement of the reliability of the data determined. Next to this, the cost-effectiveness will be the critical factor in the selection between methods having the same capacity.

We can anticipate the increasing application of biorelevant experimental conditions in physicochemical profiling. Standardization and validation of these biomimetic systems are obviously necessary in the near future.

The use of *in silico* methods will be growing if further development of computational approaches results in even more reliable data. For the *in silico* methods based on big databases the quality of the input of experimental values must be further improved.

Finally, more effective usage of physicochemical profiling in drug research can be promoted by including informative courses or seminars, for example, in higher education to strengthen this special field of medicinal chemistry in academia.

Acknowledgement

I would like to thank my colleague Gergely Völgyi, PhD for his experimental work, valuable suggestions, and help in preparing the figures. I also thank the Hungarian National Science Foundation (Grant No.: OTKA K 78102) for financial support.

REFERENCES

1. J. Wang, L. Urban. The impact of early ADME profiling on drug discovery and development strategy. *Drug Discovery World* Fall (2004) 73-86.
2. C. Hansch, P.P. Maloney, T. Fujita, R. Muir. Correlation of biological activity of phenoxyacetic acids with Hammett substituent constants and partition coefficients. *Nature* **194** (1962) 178-180.
3. G.K. Dixon, J.P. Major, M.J. Rice. , *High Throughput Screening: The Next Generation* Bios, Oxford, 2000.
4. K. Takács-Novák, G. Völgyi. Physicochemical profiling in drug research. *Magyar Kémiai Folyóirat* **111** (2005) 169-176 (Hungarian).
5. B. Testa, H. van de Waterbeemd, G. Folkers, R. Guy. , *Pharmacokinetic Optimization in Drug Research. Biological, Physicochemical, and Computational Strategies*, Wiley-VHCA, Zürich, 2001.

6. L. Di, E.H. Kerns, G.T. Carter. Drug-like property concepts in pharmaceutical design. *Current Pharmaceutical Design* **15** (2009) 2184-2194.
7. E.H. Kerns. High throughput physicochemical profiling for drug discovery. *Journal of Pharmaceutical Sciences* **90** (2001) 1838-1858.
8. L. Di, E.H. Kerns. Application of pharmaceutical profiling assays for optimization of drug-like properties. *Current Opinion in Drug Discovery and Development* **8** (2005) 495-504.
9. E.H. Kerns, L. Di. Automation in pharmaceutical profiling. *Journal of the Association for Laboratory Automation* **10** (2005) 114-123.
10. P.J. Taylor. Hydrophobic properties of drugs. In C. Hansch, P.G. Sammes, J.B. Taylor, H. Lennernäs. (Eds.), *Comprehensive Medicinal Chemistry* Vol. 4, Pergamon, Oxford, 1990, pp 241-294.
11. A. Avdeef. *Absorption and Drug Development: Solubility, Permeability and Charge State*. Wiley, New York, 2003.
12. A. Avdeef, B. Testa. Physicochemical profiling in drug research: a brief survey of the state-of-the-art of experimental techniques. *Cellular and Molecular Life Sciences* **59** (2002) 1681-1689.
13. E.H. Kerns, L. Di. Physicochemical profiling: overview of the screens. *Drug Discovery Today: Technologies* **1** (2004) 343-348.
14. H. Wan, A.G. Holmén. High throughput screening of physicochemical properties and in vitro ADME profiling in drug discovery. *Combinatorial Chemistry and High Throughput Screening* **12** (2009) 315-329.
15. J. Wang, S. Skolnik. Recent advances in physicochemical and ADMET profiling in drug discovery. *Chemistry and Biodiversity* **6** (2009) 1887-1899.
16. Y. Henchoz, B. Bard, D. Guillarme, P-A. Carrupt, J-L. Veuthey, S. Martel. Analytical tools for the physicochemical profiling of drug candidates to predict absorption/distribution. *Analytical and Bioanalytical Chemistry* **394** (2009) 707-729.
17. A. Pagliara, M. Reist, S. Geinoz, P-A. Carrupt, B. Testa. Evaluation and prediction of drug permeation. *Journal of Pharmacy and Pharmacology* **51** (1999) 1339-1357.
18. E. Kerns, L. Li. *Drug-like properties: concepts, structure design and methods: from ADME to toxicity optimization*. Elsevier, San Diego, 2008.
19. J.K. Seydel, M. Wiese. Drug-membrane interactions. Analysis, drug distribution, modeling. In: R. Mannhold, H. Kubinyi, G. Folkers. (Eds), *Methods and Principles in Medicinal Chemistry*. Wiley-VCH, Weinheim, 2002.
20. P.A. Shore, B.B. Brodie, C.A.M. Hogben. The gastric secretion of drugs: a pH partition hypothesis. *Journal of Pharmacology and Experimental Therapeutics* **119** (1957) 361-369.
21. R.P. Austin, A.M. Davis, C.N. Manners. Partitioning of ionizing molecules between aqueous buffers and phospholipid vesicles. *Journal of Pharmaceutical Sciences* **84** (1995) 1180-1183.
22. A. Avdeef, K. Box, J.E. Comer, C. Hibbert, K.Y. Tam. pH-metric logP. 10. Determination of vesicle membrane-water partition coefficients of ionizable drugs. *Pharmaceutical Research* **15** (1998) 209-215.

23. S.D. Krämer, D. Lombardi, A. Primorac, A.V. Thomae, H. Wunderli-Allenspach. Lipid bilayer permeation of drug-like compounds. *Chemistry and Biodiversity* **6** (2009) 1900-1916.
24. Y. Sai, A. Tsuji. Transporter-mediated drug delivery: recent progress and experimental approaches. *Drug Discovery Today* **9** (2004) 712-720.
25. R. Didziapertis, P. Japertas, A. Avdeef, A. Petrauskas. Classification analysis of P-glycoprotein substrate specificity. *Journal of Drug Targeting* **11** (2003) 391-406.
26. A. Albert, E.P. Serjeant. *The Determination of Ionization Constants*. 3rd ed. Chapman & Hall, London, 1984.
27. B. Noszál. Acid-base properties of bioligands. In K. Burger, (Ed): *Biocoordination Equilibria in Biologically Active Systems*. Ellis Horwood, Chichester, 1990, pp 18-55.
28. K. Takács-Novák, B. Noszál, I. Hermecz, G. Keresztúri, B. Podányi, G. Szász. Protonation equilibria of quinolone antibacterials. *Journal of Pharmaceutical Sciences* **79** (1990) 1023-1028.
29. K. Mazák, S. Hosztafi, Á. Rácz, B. Noszál. Structural and physicochemical profiling of morphine and related compounds of therapeutic interest. *Mini-Reviews in Medicinal Chemistry* **9** (2009) 984-995.
30. K. Kóczyán, G. Völgyi, J. Kökösi, B. Noszál. Site-specific acid-base properties of tenoxicam. *Helvetica Chimica Acta* **90** (2007) 1681-1690.
31. A. Dogan, E. Kilic. Tautomeric and microscopic protonation equilibria of some α -amino acids. *Analytical Biochemistry* **365** (2007) 7-13.
32. K. Takács-Novák, B. Noszál, M. Tőkés-Kövesdi, G. Szász. Acid-base properties of terbutaline in terms of protonation macro- and microconstants. *Journal of Pharmacy and Pharmacology* **47** (1995) 431-435.
33. A. Marosi, Z. Kovács, S. Béni, J. Kökösi, B. Noszál. Triprotic acid-base microequilibria and pharmacokinetic sequelae of cetirizine. *European Journal of Pharmaceutical Sciences* **37** (2009) 321-328.
34. Z. Szakács, M. Kraszni, B. Noszál. Determination of microscopic acid-base parameters from NMR-pH titrations. *Analytical and Bioanalytical Chemistry* **378** (2004) 1428-1448.
35. N. Sum, A. Avdeef. Biorelevant pK_a (37 °C) predicted from the 2D structure of the molecule and its pK_a at 25 °C. *Journal of Pharmaceutical and Biomedical Analysis* **56** (2011) 173-182.
36. H.H. Cocolas. Antiarrhythmic drugs. In J.N. Delgado, W.A. Remers. (Eds), *Wilson and Gisvold's Textbook of Organic Medicinal and Pharmaceutical Chemistry*. 9th edition, Lippincott, New York, 1991 pp. 546-555.
37. S.H. Yalkowsky, S. Banerjee. *Aqueous Solubility: Methods of Estimation for Organic Compounds*. Marcel Dekker, New York, 1992.
38. D.J.W. Gant, T. Higuchi. *Solubility Behavior of Organic Compounds*. Wiley, New York, 1990.
39. A. Avdeef. Solubility of sparingly-soluble ionizable drugs. *Advanced Drug Delivery Reviews* **59** (2007) 568-590.
40. C.A.S. Bergström, K. Luthman, P. Artursson. Accuracy of calculated pH-dependent aqueous drug solubility. *European Journal of Pharmaceutical Sciences* **22** (2004) 387-398.

41. A. Avdeef, D. Voloboy, A. Foreman. Dissolution-solubility: pH, buffer, salt, dual-solid, and aggregation effects. In B. Testa, H. van de Waterbeemd. (Eds): *Comprehensive Medicinal Chemistry II*. Vol. 5. ADME-TOX Approaches, Elsevier, Oxford, 2007 pp. 399-423.
42. G. Völgyi, E. Baka, K. Box, J.E. Comer, K. Takács-Novák. Study of pH-dependent solubility of organic bases. Revisit of Henderson-Hasselbalch relationship. *Analytica Chimica Acta* **673** (2010) 40-46.
43. G.L. Amidon, H. Lennernäs, V.P. Shah, J.R. Crison. A theoretical bases for a biopharmaceutical drug classification: The correlation of *in vitro* drug product dissolution and *in vivo* bioavailability. *Pharmaceutical Research* **12** (1995) 413-420.
44. J.B. Dressmann, M. Vertzoni, K. Goumas, C. Reppas. Estimating drug solubility in the gastrointestinal tract. *Advanced Drug Delivery Reviews* **59** (2007) 591-602.
45. A. Leo, C. Hansch, D. Elkins. The partition coefficients and their uses. *Chemical Reviews* **71** (1971) 525-616.
46. A. Avdeef. Assessment of distribution-pH profiles. In V. Pliska, B. Testa, H. van de Waterbeemd. (Eds), *Methods and Principles in Medicinal Chemistry*. Vol. 4. CVH, Weinheim, 1996 pp. 109-139.
47. K. Takács-Novák, M. Józán, G. Szász. Lipophilicity of amphoteric molecules expressed by the true partition coefficient. *International Journal of Pharmaceutics* **113** (1995) 47-55.
48. N.P. Franks, M.H. Abraham, W.R. Lieb. Molecular organization of liquid n-octanol: An X-ray diffraction analysis. *Journal of Pharmaceutical Sciences* **82** (1993) 466-470.
49. D.E. Leahy, P.J. Taylor, A.R. Wait. Model solvent systems for QSAR. 1. Propylene glycol dipelargonate (PGDP). A new standard for use in partition coefficient determination. *Quantitative Structure-Activity Relationships* **8** (1989) 17-31.
50. G.P. van Balen, C.A.M. Martinet, G. Caron, G. Bouchard, M. Reist, P-A. Carrupt, R. Fruttero, A. Gasco, B. Testa. Liposome/water lipophilicity: Methods, information content, and pharmaceutical application. *Medical Research Reviews* **24** (2004) 299-324.
51. S. Krämer. Liposome/water partitioning: theory and applications. In B. Testa, H. van de Waterbeemd, G. Folkers, R. Guy. (Eds), *Pharmacokinetic Optimization in Drug Research. Biological, Physicochemical, and Computational Strategies*, Wiley-VHCA, Zürich, 2001.
52. C.A. Lipinski, F. Lombardo B.W. Dominy, P.J. Feeney. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* **23** (1997) 3-25.
53. C. Hansch, J.P. Björkroth, A. Leo. Hydrophobicity and central nervous system agents: On the principle of minimal hydrophobicity in drug design. *Journal of Pharmaceutical Sciences* **76** (1987) 663-687.
54. G. Ferenczy, G.M. Keserű. Thermodynamics guided lead discovery and optimization. *Drug Discovery Today* **15** (2010) 919-932.
55. Z. Szakács, G. Hägele. Accurate determination of low pK values by ^1H NMR titration. *Talanta*, **62** (2004) 819-825.

56. H. Hegedűs, A. Gergely, P. Horváth, B. Noszál. Acid-base properties of biological phenyl-alkyl-amines, characterized by CD-pH titration. *Journal of Chemical Research Synopses* (1999) 306-307.
57. F.Z. Oumada, C. Ràfols, M. Rosés, E. Bosch. Chromatographic determination of aqueous dissociation constants of some water-insoluble nonsteroidal anti-inflammatory drugs. *Journal of Pharmaceutical Sciences* **91** (2002) 991-999.
58. K. Box, J.E. Comer. Using measured pK_a , logP and solubility to investigate supersaturation and predict BCS class. *Current Drug Metabolism* **9** (2008) 869-878.
59. A. Avdeef, J.J. Bucher. Accurate measurement of the concentration of hydrogen ions with a glass electrode: Calibrations using the Prideaux and other universal buffer solutions and a computer-controlled automatic titrator. *Analytical Chemistry* **50** (1978) 2137-2142.
60. J.E.Comer. High-throughput measurement of logD and pK_a . In P. Artursson, H. Lennernäs, H. van de Warterbeemd. (Eds), *Methods and Principles in Medicinal Chemistry*. Vol. 18. Wiley-CVH, Weinheim, 2003 pp. 21-45.
61. R.I. Allen, K. Box, J.E. Comer, C. Peake, K.Y. Tam. Multiwavelength spectrophotometric determination of acid dissociation constants of ionisable drugs. *Journal of Pharmaceutical and Biomedical Analysis* **17** (1998) 699-712.
62. K.Y. Tam, K. Takács-Novák. Multiwavelength spectrophotometric determination of acid dissociation constants: a validation study. *Analytica Chimica Acta* **434** (2001) 157-167.
63. Z. Mandić, V. Gabelica. Ionization, lipophilicity and solubility properties of repaglinide. *Journal of Pharmaceutical and Biomedical Analysis* **41** (2006) 866-871.
64. M-H. Langlois, M. Montagut, J-P. Dubost, J. Grellet, M-C. Saux. Protonation equilibrium and lipophilicity of moxifloxacin. *Journal of Pharmaceutical and Biomedical Analysis* **37** (2005) 389-393.
65. M. Tollinger, J.D. Forman-Kay, L.E. Kay. Measurement of side-chain carboxyl pK_a values of glutamate and aspartate residues in an unfolded protein by multinuclear NMR spectroscopy. *Journal of American Chemical Society* **124** (2002) 5714- 5717.
66. Z. Szakács, S. Béni, Z. Varga, L. Őrfi, G. Kéri, B. Noszál. Acid-base profiling of imatinib and its fragments. *Journal of Medicinal Chemistry* **48** (2005) 249-255.
67. Z. Jia. Physicochemical profiling by capillary electrophoresis. *Current Pharmaceutical Analysis* **1** (2005) 41-56.
68. S. Babić, A.J.M. Horvat, D. Mutavdžić Pavlović, M. Kaštelan-Macan. Determination of pK_a values of active pharmaceutical ingredients. *Trends in Analytical Chemistry* **26** (2007) 1043-1061.
69. S.K. Poole, S. Patel, K. Dehring, H. Workman, C.F. Poole. Determination of acid dissociation constants by capillary electrophoresis. *Journal of Chromatography A* **1037** (2004) 445-454.
70. S. Pang, J. Kenseth, S. Coldiron. High throughput multiplexed capillary electrophoresis in drug discovery. *Drug Discovery Today* **9** (2004) 1072-1080.
71. K. Box, J.E. Comer, P. Hosking, K.Y. Tam, L. Trowbridge, A. Hill. Rapid physicochemical profiling as an aid to drug candidate selection. In G.K. Dixon, J.S. Major, M.J. Rice (Eds), *High Throughput Screening: The Next Generation*. Bios, Oxford, 2000, pp. 67-74.

72. K. Box, C. Bevan, J.E. Comer, A. Hill, R. Allen, D. Reynolds. High-throughput measurement of pK_a values in a mixed-buffer linear pH gradient system. *Analytical Chemistry* **75** (2003) 883-892.
73. A. Avdeef, J.E. Comer, S.J. Thomson. pH-metric logP. 3. Glass electrode calibration in methanol/water, applied to pK_a determination of water-insoluble substances. *Analytical Chemistry* **65** (1993) 42-49.
74. K. Takács-Novák, K.Box, A. Avdeef. Potentiometric pK_a determination of water-insoluble compounds: validation study in methanol/water mixtures. *International Journal of Pharmaceutics* **151** (1997) 235-248.
75. K. Box, G. Völgyi, R. Ruiz, J.E. Comer, K. Takács-Novák, E. Bosch, C. Ràfols, M. Rosés. Physicochemical properties of a new multicomponent cosolvent system for the pK_a determination of poorly soluble pharmaceutical compounds. *Helvetica Chimica Acta* **90** (2007) 1538-1553.
76. G. Völgyi, R. Ruiz, K. Box, J.E. Comer, E. Bosch, K. Takács-Novák. Potentiometric and spectrophotometric pK_a determination of water-insoluble compounds: Validation study in a new cosolvent system. *Analytica Chimica Acta* **583** (2007) 418-428.
77. C. Lipinski. Computational and experimental approaches to avoiding solubility and oral absorption problems in early discovery. In R. Borchard (Ed), *Designing Drugs with Optimal in Vivo Activity after Oral administration*. Drew University Residential School on Medicinal Chemistry, Madison, NJ. 2000.
78. C. Bevan, R.S. Lloyd. A high throughput screening method for the determination of aqueous solubility by laser nephelometry in microtitre plates. *Analytical Chemistry* **72** (2000) 1781-1787.
79. A. Avdeef. High-throughput measurements of solubility profiles. In B. Testa, H. van de Waterbeemd, G. Folkers, R. Guy (Eds), *Pharmacokinetic Optimization in Drug Research. Biological, Physicochemical, and Computational Strategies*, Wiley-VHCA, Zürich, 2001. pp. 305-326.
80. T. Yamashita, Y. Dohta, T. Nakamura, T. Fukami. High-speed solubility screening assay using ultra-performance liquid chromatography/mass spectrometry in drug discovery. *Journal of Chromatography A* **1182** (2008) 72-76.
81. E. Baka, J.E. Comer, K. Takács-Novák. Study of equilibrium solubility measured by saturation shake-flask method using hydrochlorothiazide as model compound. *Journal of Pharmaceutical and Biomedical Analysis* **46** (2008) 335-341.
82. E. Baka. Good laboratory practice of equilibrium solubility measurements. *Acta Pharmaceutica Hungarica* **81** (2011) 18-28 (Hungarian).
83. A. Avdeef. pH-metric solubility. 1. Solubility pH profiles from Bjerrum plots. Gibbs buffer and pK_a in the solid state. *Pharmacy and Pharmacology Communications* **4** (1998) 165-178.
84. A. Avdeef, C.M. Berger, C. Brownell. pH-metric solubility. 2. Correlation between the acid-base titration and the saturation shake-flask solubility-pH methods. *Pharmaceutical Research* **17** (2000) 85-89.
85. A. Avdeef, C.M. Berger. pH-metric solubility. 3. Dissolution titration template method for solubility determination. *European Journal of Pharmaceutical Sciences* **14** (2001) 281-291.
86. M. Stuart, K. Box. Chasing equilibrium: measuring of the intrinsic solubility of weak acids and bases. *Analytical Chemistry* **77** (2005) 983-990.

87. K. Box, G. Völgyi, E. Baka, M. Stuart, K. Takács-Novák, J.E. Comer. Equilibrium versus kinetic measurements of aqueous solubility and the ability of compounds to supersaturate in solution. A validation study. *Journal of Pharmaceutical Sciences* **95** (2006) 1298-1307.
88. A. Avdeef, O. Tsinman. Miniaturized rotating disk intrinsic dissolution rate measurement: Effect of buffer capacity in comparisons to traditional Wood's apparatus. *Pharmaceutical Research* **25** (2008) 2613-2627.
89. K. Tsinman, A. Avdeef, O. Tsinman, D. Voloboy. Powder dissolution method for estimating rotating disk intrinsic dissolution rates of low solubility drugs. *Pharmaceutical Research* **26** (2009) 2093-2100.
90. A. Glomme, J. März, J.B. Dressman. Comparison of a miniaturized shake-flask solubility method with automated potentiometric acid/base titrations and calculated solubilities. *Journal of Pharmaceutical Sciences* **94** (2005) 1-16.
91. A. Glomme, J. März, J.B. Dressman. Predicting the intestinal solubility of poorly soluble molecules. In B. Testa, S.D. Krämer, H. Wunderli-Allenspach, G. Folkers (Eds), *Pharmacokinetic Profiling in Drug Research: Biological, Physicochemical and Computational Strategies*, Wiley-VCH, Weinheim, 2006. pp 259-280.
92. L. Zhou, L. Yang, L., S. Tilton, J. Wang. Development of a high throughput equilibrium solubility assay using miniaturized shake-flask method in early drug discovery. *Journal of Pharmaceutical Sciences* **96** (2007) 3052-3071.
93. A.T. Serajuddin. Salt formation to improve drug solubility. *Advanced Drug Delivery Reviews* **59** (2007) 603-616.
94. J. Alsenz, E. Meister, E. Haenel. Development of a partially automated solubility screening (PASS) assay for early drug development. *Journal of Pharmaceutical Sciences* **96** (2007) 1748-1762.
95. A. Llinàs, K. Box, J. Burley, R. Glen, J. Goodman. A new method for the reproducible generation of polymorphs: two forms of sulindac with very different solubilities. *Journal of Applied Crystallography* **40** (2007) 379-381.
96. B. Bard, S. Martel, P-A. Carrupt. High throughput UV method for the estimation of thermodynamic solubility and the determination of the solubility in biorelevant media. *European Journal of Pharmaceutical Sciences* **33** (2008) 230-240.
97. J. Fagenberg, O. Tsinman, N. Sun, K. Tsinman, A. Avdeef, C. Bergström. Dissolution rate and apparent solubility of poorly soluble drugs in biorelevant dissolution media. *Molecular Pharmaceutics* **7** (2010) 1419-1430.
98. A. Hersey, A. Hill, R. Hyde, D. Livingstone. Principles of method selection in partition studies. *Quantitative Structure-Activity Relationships* **8** (1989) 288-296.
99. L-G. Danielsson, Y-H. Zhang. Methods for determining *n*-octanol-water partition constants. *Trends in Analytical Chemistry* **15** (1996) 188-196.
100. J.E. Comer, K. Tam. Lipophilicity profiles: theory and measurement. In B. Testa, B., H. van de Waterbeemd, G. Folkers, R. Guy (Eds), *Pharmacokinetic Optimization in Drug Research. Biological, Physicochemical, and Computational Strategies*, Wiley-VHCA, Zürich, 2001. pp. 275-304.
101. J.C. Dearden, G.M. Bresnen. The measurement of partition coefficient. *Quantitative Structure-Activity Relationships* **7** (1988) 133-144.

102. K. Takács-Novák. Practical aspects of determination of partition coefficient according to GLP rules. *Acta Pharmaceutica Hungarica* **67** (1997) 179-191 (Hungarian).
103. B. Slater, A. McCormack, A. Avdeef, J.E. Comer. pH-metric logP. 4. Comparison of partition coefficients determined by shake-flask, HPLC and potentiometric methods. *Journal of Pharmaceutical Sciences* **83** (1994) 1280-1283.
104. K. Takács-Novák, A. Avdeef. Interlaboratory study of logP determination by shake-flask and potentiometric methods. *Journal of Pharmaceutical and Biomedical Analysis* **14** (1996) 1405-1413.
105. K. Valkó. Application of high-performance liquid chromatography based measurements of lipophilicity to model biological distribution. *Journal of Chromatography A* **1037** (2004) 299-310.
106. R. Kaliszan. QSRR: Quantitative structure-(chromatographic) retention relationships. *Chemical Reviews* **107** (2007) 3212-3246.
107. G.L. Biagi, A.M. Barbaro, A. Sapone, M. Recanatini. Determination of lipophilicity by means of reversed-phase thin-layer chromatography. I. Basic aspects and relationship between slope and intercept in TLC equations. *Journal of Chromatography A* **662** (1994) 341-361.
108. A. Pyka, M. Babuska. Lipophilicity of selected steroid compounds. I. Investigations on RP18W stationary phase by RP-HPTLC. *Journal of Liquid Chromatography and Related Technologies* **29** (2006) 1891-1903.
109. Z. Rozmer, P. Perjési, K. Takács-Novák. Use of RP-TLC for logP determination of isomeric chalcones and cyclic chalcone analogues. *Journal of Planar Chromatography* **19** (2006) 124-128.
110. G. Völgyi, K. Deák, J. Vámos, K. Valkó, K. Takács-Novák. RPTLC determination of logP of structurally diverse neutral compounds. *Journal of Planar Chromatography* **21** (2008) 143-149.
111. L. Hitzel, A.P. Watt, K.L. Locker. An increased throughput method for the determination of partition coefficients. *Pharmaceutical Research* **17** (2000) 1389-1395.
112. D.M. Wilson, X. Wang, E. Walsh, R.A. Rourick. High throughput logD determination using liquid chromatography-mass spectrometry. *Combinatorial Chemistry and High Throughput Screening* **4** (2001) 511-519.
113. K. Takács-Novák. Investigation of physicochemical properties of deramciclone (EGIS-3886), a new anxiolytic compound. *Acta Pharmaceutica Hungarica* **69** (1999) 123-127 (Hungarian).
114. K. Takács-Novák, P. Perjési, J. Vámos. Determination of logP for biologically active chalcones and chalcone analogs by RPTLC. *Journal of Planar Chromatography* **14** (2001) 42-46.

APPLICATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE MEASUREMENTS OF LIPOPHILICITY AND BIO-MIMETIC BINDING PROPERTIES IN DRUG DISCOVERY

Klara Valko

Contents

2.1. INTRODUCTION	63
2.2. THEORETICAL BACKGROUND OF HPLC.....	65
2.2.1. Direct measurement of lipophilicity by chromatographic methods	68
2.2.2. Measurements of lipophilicity using isocratic chromatographic retention	70
2.2.3. Measurements of lipophilicity using gradient chromatographic retention	73
2.2.4. pH dependence of lipophilicity and reversed phase chromatographic retention.....	74
2.3. APPLICATION OF BIO-MIMETIC HPLC STATIONARY PHASES.....	79
2.3.1. Immobilised human serum albumin (HSA) stationary phase	79
2.3.2. Immobilised α -1-acid-glycoprotein (AGP) stationary phase	81
2.3.3. Immobilised artificial membrane (IAM) stationary phase	83

2.4. GENERAL EXPERIMENTAL DESCRIPTION OF FAST GRADIENT HPLC ANALYSIS OF LIPOPHILICITY, PROTEIN AND PHOSPHOLIPID BINDING OF DRUG DISCOVERY COMPOUNDS.....	84
2.4.1. Measurements of chromatographic hydrophobicity indices at three different pHs	84
2.4.2. Measurement of HSA binding by HPLC retention	85
2.4.3. Measurement of AGP binding by HPLC retention	87
2.4.4. Measurement of IAM binding by HPLC retention.....	89
2.5. ANALYSIS OF THE HPLC BASED LIPOPHILICITY, PROTEIN BINDING AND PHOSPHOLIPID BINDING DATA	90
2.5.1. Analysis of the effect of charge	90
2.5.2. Analysis by the solvation equation	93
2.6. APPLICATIONS OF BIO-MIMETIC HPLC PROPERTIES TO MODEL DRUGS <i>IN VIVO</i> DISTRIBUTION	95
2.6.1. Modelling steady state volume of distribution	98
2.6.2. Modelling unbound volume of distribution	103
2.6.3. Dependence of unbound volume of distribution on HSA and IAM binding ..	104
2.6.4. General structure-property relationships for lead optimisation	107
2.7. ALTERNATIVE METHODS.....	108
2.7.1. Methods based on the principle of frontal chromatography.....	108
2.7.2 Methods based on solid particles.....	111
2.7.3. Methods based on equilibrium dialysis and ultra-filtration.....	112
REFERENCES.....	113

2.1. INTRODUCTION

One of the major concerns during the drug discovery process is that over 90% of potent candidate molecules fail during drug development. This is often due to the lack of efficacy in the clinical setting, unexpected side effects or unfavourable pharmacokinetics. It is widely accepted that unfavourable physico-chemical properties often contribute to these failures. Of the many recent reviews of the subject, one of the most well-known examples is the “Lipinski Rule of Five” [1], which indicates that solubility and permeability are the two critical properties for good oral absorption. Both solubility and permeability can be related to the size of the molecule, their lipophilicity and the number of H-bond donor and acceptor groups. Since then, numerous publications have suggested that solubility, lipophilicity, permeability and the acid/base character are important factors that should be optimised in a candidate molecule in order to improve chances of drug molecule success [2-7]. There are several *in silico* prediction tools available [8-10] for chemists to estimate these important molecular physico-chemical properties before they synthesize compounds. However, there is always a need to have measured values from direct experimentation as well, as clinical chemistry delves into new chemical property areas where the *in silico* prediction tools are less accurate.

In general, we require methodologies for the measurement of solubility, lipophilicity, permeability and the acid/base character that are inexpensive, not labour intensive, and require small amounts of compound to obtain good, quality data.

Therefore, there is a need for physico-chemical property measurements of early discovery compounds that can provide fit-for-purpose accuracy with a fast turnaround time on a reasonably large number of compounds. We also need to be able to use these measured data to build structure-property relationships that can guide the medicinal chemistry efforts to find the best compromise of potency and physico-chemical properties. These properties can also be used for ranking and selecting compounds for more expensive and time consuming *in vitro* and *in vivo* measurements. The large amount of measured physico-chemical property data can also be used to improve *in silico* prediction tools by re-training the models. The optimised properties would provide the best chance for the compound to become a successful drug with the desired bioavailability and pharmacokinetic properties with the smallest possible dose and chance of toxic side effects.

Early drug discovery compounds are usually new chemical entities for which we have very little information. Research compounds usually go through a generic High Performance Liquid Chromatography/Mass Spectrometry (HPLC/MS) analysis to determine the purity and identity of the compound before potency screening and further testing. HPLC technology is well adapted to cope with the analysis of large numbers of compounds. The fully automated procedure requires small amounts of compound and very little human intervention in providing the required information. It would be ideal if the same analysis technique could provide additional information other than purity and identity. It is well-known that in principle reversed phase retention is governed by the lipophilicity/hydrophobicity of compounds [11]; hence there is a possibility to obtain information about the compounds' lipophilicity at the same time as the quality control measurements are made. By altering the pH of the mobile phase and measuring the lipophilicity at various pHs via retention time we can provide a fast alternative to obtain information about the acid/base character of the compounds as well. The HPLC technique provides numerous advantages for the determination of the solubility of compounds. This involves the measurement of the concentration of the saturated solution which is in equilibrium with undissolved solid particles. The HPLC with UV detection, especially when it is coupled with mass spectrometry (MS), is one of the most popular generic methods that can be applied for fast, inexpensive and reasonably precise concentration determination. Thus, it is suitable to measure a compound's permeation through an artificial membrane. In conclusion, HPLC is very powerful not only as a separation and analytical method to determine purity and identity, but also as part of the methodology to determine various physico-chemical properties of drug discovery compounds such as lipophilicity, solubility, permeability and acid/base character. These properties can be used to build models for the estimation of the *in vivo* behaviour of the compounds and as selection criteria during the lead optimisation process. Applying these data in predictive models can help in the selection of molecules for further development at earlier stages of the lead optimisation process, thus reducing the chance of attrition in the clinical setting.

In this chapter the basic principles of chromatographic retention will be described along with how it can be used to derive various physico-chemical and binding properties of compounds. A short experimental description of the HPLC methodology will be provided for the determination of lipophilicity (including bio-mimetic lipophilicity), and the acid/base character of drug discovery compounds. Bio-mimetic chromatography as a powerful technique for measuring compound interactions with the major protein and phospholipids components in humans will also be explained. Finally, the application of the data will be demonstrated in various models used to predict human *in vivo* distribution.

2.2. THEORETICAL BACKGROUND OF HPLC

HPLC is an established technique dating back to the 1960's. Several research journals, such as the Journal of Chromatography, Journal of Liquid Chromatography, Journal of Chromatographic Sciences, and Chromatographia publish regularly about the theory and application of HPLC. There are several books published for beginners in the field, with only a few cited here [12,13], directing readers if they need more detailed knowledge about the principles of HPLC and running and troubleshooting HPLC experiments. Only the basic principles of HPLC that are necessary to understand the applicability of the method for physico-chemical measurements will be discussed here.

HPLC is a procedure where compounds are injected into a chromatographic system, which consists of a liquid mobile phase and a solid stationary phase. Compounds are separated due to differential migration in the chromatographic system depending on their interactions with the stationary and mobile phases. Components of a mixture injected into the system will move with the mobile phase but the speed of the component zones are different for each type of molecule in the mixture depending on their distribution between the mobile and stationary phases (see Figure 2.1).

Figure 2.1. Schematic principles of chromatographic separation process

The mobile phase content is monitored at the end of the chromatographic column usually by UV detection. The detector signal is recorded as a function of time measured from the injection of the sample which takes place at the beginning of the chromatographic column. This column contains the stationary phase in the form of small particles. The recorded signal as a function of time is called a chromatogram. The chromatogram can show several peaks depending on the number of components in the mixture injected onto the column. The peak retention time

(the time elapsed from the injection until the appearance of the maximum peak) is characteristic of the chemical nature of the components, while the peak area is proportional to the injected quantity. Thus, the chromatogram provides information both about the quantity and quality of the components in the mixtures. As the retention time depends upon the chromatographic conditions such as the type and composition of the stationary and mobile phases, the mobile phase flow rate, and the column dimensions, a known set of standard compounds should be analysed under identical conditions and then use the retention time itself for identification. Similarly, for quantification we need to inject a known amount of compound below a predetermined quantity and compare the peak area to the peak area of the unknown quantity of components in the samples. Nowadays, column dimensions of 30 to 50 mm in length and 2.1 to 3 mm diameter are used. The particle size of the stationary phase varies from 2 to 3 μm . The mobile phase flow rate is usually less than 1 ml per minute.

In early drug discovery it is very common to use generic reversed phase gradient [14,15] elution which involves silica stationary phase particles chemically bonded with C-18 or C-8 ligand chemistry or polymer particles and aqueous acetonitrile or methanol as the mobile phase. The organic phase concentration changes during the analysis from a weaker - low organic phase concentration to a stronger - high organic phase concentration, thus ensuring that even strongly bound lipophilic compounds are eluted from the stationary phase in a single run and within a reasonably short time (usually 2 to 5 minutes).

Besides separation and concentration determination, the technique has great potential to determine partition properties of compounds based on their chromatographic retention times. The chromatographic retention time is directly related to the compound's distribution between the mobile and stationary phases. The retention factor (k) determined from the retention time (t_R) and dead time (t_0) as $(t_R - t_0)/t_0$ is equal to the ratio of the average number of analyte molecules in the stationary phase to the average number of molecules in the mobile phase (Equation 2.1) during the elution process.

$$k = \frac{n_s}{n_m} = \frac{t_r - t_0}{t_0} \quad (2.1)$$

where k is the retention factor, n_s is the average number of molecules in the stationary phase, n_m is the average number of molecules in the mobile phase.

The retention factor, k , can be related to the chromatographic partition coefficient of the compound, K , between the mobile and the stationary phase, according to Equation 2.2.

$$\log k = \log K + \log \left(\frac{V_s}{V_m} \right) \quad (2.2)$$

where V_s/V_m is the volume ratio of the stationary phase and the mobile phase.

Equation 2 shows that we need to know the value of the volume ratio of the stationary and mobile phases (V_s/V_m) to be able to obtain the absolute value of the chromatographic partition coefficient ($\log K$).

Equations 2.1 and 2.2 form the theoretical basis of deriving partition data from chromatographic retention. Thus, partition coefficients can be obtained from time measurements instead of concentration determination. The retention time is independent of the compound concentration/amount injected into the chromatographic system, and it is the main indicator of a true partition process. Impurities do not affect the measurements as they are usually separated from the main component. Low solubility also does not affect the measurements. The above advantages are very important in early drug discovery, where there are a large number of diverse compounds requiring analysis.

The chromatographic technique is very versatile as various stationary phases can be used to provide various types of partition information. For example, normal paraffin hydrocarbons, immobilised octanol, and bio-mimetic phases such as immobilised artificial membrane (IAM), human serum albumin (HSA), and alpha-acid-glycoprotein (AGP) stationary phases can be used to reveal the compound's interaction/partition into a particular phase. The pH and polarity of the mobile phase can also be altered, which will affect compound interactions with the stationary phase too. The technique, therefore, is easily applicable for measurement of more than just octanol/water partitions. Since the advent of publications on chromatographic techniques [16-18] in the early 1970's, several hundred papers have been published about the applicability of reversed phase liquid chromatography for the determination of lipophilicity. From time to time, several review papers have summarised the major approaches [19- 21].

Dorsey and Khaledi [22] have discussed the theoretical differences between chromatographic lipophilicity and partitioning between bulk solvents. They have also discussed the thermodynamic basis for possible failures in comparing the partitioning of small molecules between bulk solvents and chromatographic mobile and stationary phases, as well as the partitioning of solutes into lipid layers and biological membranes. Biological partition of compounds in aqueous bi-phase systems, such as blood and various tissues certainly happens in close to equal volume ratios involving a large area of contact surfaces between the partitioning phases. Therefore the chromatographic partition process is similar to the biological distribution processes. Significant differences between bulk solvent partition and partition involving large surfaces may be observed mainly for surface-active compounds, such as amphiphilic and charged molecules with large hydrophobic moieties.

In spite of the obvious advantages of HPLC, and the possibility of using bio-mimetic phases which may prove to be better models for biological partitions, the collation of HPLC based lipophilicity data is not widespread. The major problem in providing a more easily measurable alternative to the industry standard of octanol/water partition measurements lies in the variety of chromatographic

stationary phases available and the lack of standardisation which make inter-laboratory comparison of the data very difficult. To overcome these difficulties, the chromatographic system needs to be calibrated using known standards that also allow the chromatographic retention data to be converted into bio-mimetic partition coefficients. The reproducibility of current commercially available stationary phases and the automation and reliability of modern HPLC instrumentation now make it possible to fully exploit the advantages of the technique to collate a large amount of lipophilicity data in a reproducible way. However, it also requires consensus for standardisation.

2.2.1. Direct measurement of lipophilicity by chromatographic methods

HPLC provides a generally applicable approach for the determination of compound concentration. The equilibration of the compound in octanol and water can take place in an auto-sampler vial, 96 well plates or specially designed devices. By applying a generic fast gradient method, the equilibrium concentration of the compound in the two phases can be determined in minutes without the need for method development for each compound. The proportion of the peak areas obtained from the aqueous and the octanol phases can directly provide partition coefficients by taking into account the volumes of the two partitioning solvents. The biggest advantage of this approach is that it provides a true thermodynamic octanol/water partition coefficient. The HPLC technique is used for automatic determination of the concentration of the compound in the two partitioning solvents. In this respect, the result is independent of the chromatographic conditions used. An approximate amount of the compound is dissolved first in the octanol/saturated buffered water and the peak area of the solution is used as the “standard” concentration, C_s , before partitioning. Various amounts of octanol are added to the aliquots of the sample solution, and the solutions are allowed to equilibrate before injecting again from the aqueous buffer layer, C_w . The decrease of the peak area, $C_s - C_w$, indicates the amount of compound that partitioned to the octanol phase. Thus, the partition coefficient, P , can be obtained by Equation 2.3:

$$P = \frac{C_s - C_w}{C_w} = \frac{A_s - A_w}{A_w} \quad (2.3)$$

where A_s is the peak area obtained from the aqueous buffer solution before equilibration with octanol, A_w is the peak area obtained from the aqueous buffer phase after equilibration with the octanol phase assuming we inject the same volume before and after the equilibration procedure [23]. Extra care has to be taken to make sure that the sample dissolves completely in the aqueous buffer before equilibration. Incomplete solubility during the equilibration provides false results for the partition coefficient. The volume of the two partitioning phases has to be significantly greater than the interface between the two solvents otherwise surface active compounds may accumulate at the interface and provide false partition coefficients. It is always advisable to repeat the measurements

using various volume ratios of the two partitioning phases (octanol and aqueous buffer) and various amounts of sample and check the reproducibility of the results. Figure 2.2 shows an example of the HPLC chromatograms obtained from an initial aqueous stock solution and subsequent injections of the same compound after equilibration with various amounts of octanol.

Figure 2.2. Chromatograms obtained from the initial aqueous buffer solution from a sample and after equilibration with various amounts of octanol

It is possible to use the chromatographic principle based on retention time measurements for the determination of octanol/water partition coefficients when we use octanol directly as the stationary phase and octanol saturated aqueous buffer as the mobile phase [24,25]. The difficulty of this approach is the immobilisation of octanol on the stationary phase surface, and keeping its volume constant during the elution process. Therefore, frequent calibration of the system is required. The chromatographic peaks are usually wide, which makes determination of retention time at the peak maximum variable, and the separation efficiency is low for possible impurities. The dynamic range of the precisely measurable octanol/water partition coefficients is small, as the octanol saturated mobile phase has low elution strength. In order to cover the wide range of octanol/water partition coefficients ($-1 < \log P < 5$) the volume ratio of the octanol phase and water has to be changed.

Several attempts have been made to apply commercially available stationary phases and search for mobile phase additives that make the chromatographic partition system similar to the octanol/water partition system [26,27]. These approaches have the advantage of using commercially available efficient stationary phases. However, it is not certain that these systems behave exactly like octanol/water, especially in the case of ionised solutes. Although good agreement has been found between the chromatographic and equilibrium octanol/water partition data, deviations can be expected, as a large surface between

the two partitioning phases and surface properties (surface activity, shape) can influence the interactions. For example, it was found [28] that negatively charged compounds did not show good correlation with the octanol/water distribution coefficients, even if the chromatographic partition was carefully tuned to model octanol/water partition.

2.2.2. Measurements of lipophilicity using isocratic chromatographic retention

HPLC is usually performed with high efficiency bonded reversed phase columns and today the commercially available columns are both robust and reproducible. Partitioning from aqueous/organic mobile phases into standard reversed phase (often C-18) HPLC stationary phases can be used as a direct measure of lipophilicity. In order to cover a wide range of lipophilicity, various concentrations of the organic solvent in the mobile phase must be used. Thus, it requires a preliminary estimate of the expected lipophilicity in order to choose the appropriate mobile phase composition. Several measurements at various mobile phase concentrations are needed for each compound resulting in longer processing time and data processing complications. To compare retention using different organic phase concentrations, they are extrapolated to the same condition, which is normally to zero organic solvent concentration. Equation 2.4 is used the most often for such an extrapolation.

$$\log k = S \varphi + \log k_w \quad (2.4)$$

Equation 2.4 shows the linear relationship between the organic solvent concentration, φ , and the logarithmic retention factor. The relationship is not linear for the full range of organic solvent concentrations, but it is a good approximation within the working limits of $-0.5 < \log k < 1.5$. A linear plot of measured $\log k$ versus φ provides the intercept ($\log k_w$), which is the extrapolated retention factor to zero organic phase concentration. In most cases, unless all the measurements are made on an identical HPLC column, it is also necessary to measure a standard set of compounds in order to calibrate the chromatographic system. Standardisation as suggested by the OECD guidelines [29], for example, is based on calibration using octanol/water $\log P$ values and the extrapolated $\log k_w$ values. However, this is not a rigorously correct approach for two reasons. Firstly, the $\log k$ versus organic phase concentration plot is non linear, therefore different $\log k_w$ values can be obtained for the same compound even when using the same column and instrument, when the $\log k_w$ are derived using a different set of mobile phase compositions. Also different $\log k_w$ values are obtained when acetonitrile or methanol is used as an organic modifier. Secondly, the reversed phase chromatographic system does not model well the octanol/water system for structurally diverse compounds.

It has been demonstrated [30] that the straight lines obtained by Equation 2.4 can cross each other (see Figure 2.3), hence, different lipophilicity rankings are obtained at different organic solvent concentrations.

Figure 2.3. The hypothetical straight lines obtained for five compounds by plotting the logarithmic retention factor ($\log k$) against the organic phase concentration in the mobile phase (ϕ). The dotted lines show the extrapolation to zero organic phase concentration ($\log k_w$)

The best correlation with the octanol/water partition coefficients is not necessarily achieved at the zero organic phase concentration, therefore the S value in Equation 2.4 can be used as a second independent variable together with the $\log k_w$ values (Equation 2.5).

$$\log P = aS + b \log k_w + c \quad (2.5)$$

where a , b and c are regression coefficients. The ratio of a to b gives an organic modifier concentration value, to which the $\log k$ values should be extrapolated to give the best statistical correlation to the $\log P$ values. In this way the chromatographic partition can be tuned to better model the octanol/water partition. With the improvements in stationary phases, where the masking of the free silanol groups is very successful, the S and $\log k_w$ values show quite good correlation, which means the same retention order can be obtained at various organic phase concentrations. Thus, the ability to tune the reversed phase chromatographic system to model the octanol/water partition system is greatly reduced.

The quotient of the intercept and slope in Equation 2.4 ($-\log k_w/S = \phi_0$) also shows better correlation with the $\log P$ values than $\log k_w$ [31] as is shown by Equation 2.6.

$$\log P = d\phi_0 + e \quad (2.6)$$

where d and e are regression coefficients.

The ϕ_0 is the x -coordinate of the intersection of the $\log k$ vs. ϕ lines as demonstrated in Figure 2.4.

Figure 2.4. The intersection points of the $\log k$ vs. ϕ lines for hypothetical compounds. The x-axis provides the ϕ_0 values of each compound that is defined as the isocratic chromatographic hydrophobicity index

The quotient (ϕ_0) is equivalent to the organic solvent concentration in the mobile phase that is required to get the compound retention time exactly twice that of the dead time, *i.e.* $\log k = 0$. The ϕ_0 value of a compound is defined as the chromatographic hydrophobicity index (CHI). It ranges from 0 to 100 % which also indicates an appropriate organic phase concentration by which the compound can be eluted from the column in a reasonably short time (retention time is double that of the dead time). Based on the data for over 500 compounds [31], an approximation can be made for the organic phase concentration in the mobile phase using acetonitrile (AcN) and methanol (MeOH) based on calculated octanol/water partition coefficients ($\log P$) of a compound using Equations 2.7 and 2.8.

$$\phi_0(\text{AcN}) = 16 \log P + 26 \quad (2.7)$$

$$\phi_0(\text{MeOH}) = 13 \log P + 42 \quad (2.8)$$

The advantage of ϕ_0 is that it does not involve an extrapolation to outside a measurable range as is often required to obtain the $\log k_w$ values. An important aspect of the isocratic CHI is that it has been derived from the retention data of compounds that were analysed at a mobile phase pH when the compounds were not ionised and the octanol/water partition coefficients also were obtained for the neutral form of the compounds. The pH dependence of lipophilicity and the reversed phase chromatographic retention will be discussed later in this chapter.

2.2.3. Measurements of lipophilicity using gradient chromatographic retention

A linear gradient increase of the organic solvent concentration in the mobile phase during a chromatographic run helps to elute compounds over a wide range of lipophilicity. In isocratic mode, several retention time measurements of the same compound are required to establish the CHI, while one measurement in gradient mode can cover compounds with approximately a five order of magnitude difference in partition coefficient. In order to maintain the resolution of the separation, slow organic phase gradient methods are used. Mutton [15] has pointed out that by increasing the flow rate and using short columns it is possible to reduce the gradient time without loss of separation efficiency and resolution. Usually, an acetonitrile gradient is used. The analysis time per compound can be reduced to 5 minutes. During a gradient run any point of the run time is equivalent to a particular organic solvent concentration. By knowing the dead volume and dwell volume of the HPLC system it is possible to estimate the organic phase concentration at the time point when the compound elutes from the column. During a fast organic phase gradient the solvent slope parameter S (see Equation 2.4) has a negligible influence on the gradient retention time. When the appropriate organic phase concentration reaches the column, each compound will travel at approximately the mobile phase velocity. For a first approximation, the retention time in a fast gradient run should be linearly related to the isocratic φ_0 values. It has been shown that the gradient retention times are in good correlation to the isocratically obtained φ_0 values using experimental data from 76 diverse drug molecules obtained from both isocratic and gradient elution [32]. The gradient retention times of a calibration set of compounds give a straight line when plotted against the φ_0 values. The slope and the intercept of the calibration line can be used to convert the gradient retention times to a gradient CHI that is suitable for inter-laboratory comparison and for building a database [32]. The CHI index measurement takes only 5 minutes using standard HPLC conditions and is a platform widely used by research chemists. The conditions used cover a 5-6 log P unit range of lipophilicity and simple data processing can be used to convert the gradient retention times to CHI values. The CHI values can also be projected to the widely used logarithmic value of the partition coefficient (log P) by linear conversion. It is important to note that the gradient retention times show a linear relationship to the logarithmic value of the isocratic retention factor. Equation 2.9 shows the CHI conversion to CHI log D while Equation 2.10 shows the CHI conversion to Chrom log D [33].

$$\text{CHI log } D = 0.054 \text{ CHI} - 1.467 \quad (2.9)$$

$$\text{Chrom log } D = 0.0654 \text{ CHI} - 2 \quad (2.10)$$

Kern *et al.* [34] used the gradient retention times of a calibration set of compounds with known log D octanol/water partition coefficients and converted the gradient retention times directly to octanol/water log D values. The gradient

retention times correlated approximately with the $\log D$ values using a Polaris C-18 column and acetonitrile gradient. Donovan and Pescatore [35] have reported the use of gradient methods to obtain a wide range of measured lipophilicity values from gradient retention times, but they use an octadecyl polymer column and a methanol gradient and claim their values to be close to octanol/water partition coefficients. The starting mobile phase pH is 7.4. The biggest advantage of this methodology is the provision of a lipophilicity parameter directly from the LC-MS compound integrity/purity measurements. In general, using gradient HPLC and C-18 phases, reproducible lipophilicity values can be obtained in a high throughput way while the perfect match with the traditionally used octanol/water partition coefficients is lost. It should be noted that there are significant differences between $\log D$ values derived from gradient retention times and the octanol/water $\log D$ values. For neutral molecules H-bond donor compounds generally show a lower chromatographic $\log P$ than the octanol/water $\log P$. Charged molecules however, tend to give higher $\log D$ values in the chromatographic system than octanol/water $\log D$ values (as will be discussed later). So, the conversion is pragmatic and it is used only for expressing the CHI scale (normally 0 to 100) as a $\log P$ scale (normally -1 to 5). CHI values of more than 200,000 research compounds have been collected at GlaxoSmithKline and proved to be useful in numerous lead optimisation projects. Camurri and Zaramella [36] have adapted a methodology for the MS detection of compounds during the gradient run. The MS detection makes it possible to determine CHI values for mixtures of compounds and in this way further reducing the analysis time. Equivalent selectivity for the fast gradient and the isocratic systems has been demonstrated by Du *et al.* [37]. The connection between the gradient and isocratic retention times has been discussed extensively by Snyder and utilised in DryLab software [38] that computes isocratic method development conditions based on a few carefully selected gradient measurements.

2.2.4. pH dependence of lipophilicity and reversed phase chromatographic retention

Drug molecules often contain ionisable groups which mean that their ionisation state depends on the pH of the environment. When the compound is ionised the lipophilicity drops dramatically and so does the octanol/water distribution coefficient. Ionised species do not partition into the octanol phase unless a suitable neutral ion-pair is formed. The Henderson-Hasselbalch equation describes the relationship between pH, the acid dissociation constant (pK_a) and percentage ionisation of compounds that forms the basis of the equation that describes the pH dependence of distribution coefficients ($\log D$) as shown by Equations 2.11a and 2.11b for acids and bases, respectively:

$$\log D_{(pH)} = \log P - \log \left[1 + 10^{(pH - pK_a)} \right] \quad (2.11a)$$

$$\log D_{(pH)} = \log P - \log \left[1 + 10^{(pK_a - pH)} \right] \quad (2.11b)$$

Figure 2.5 shows a typical $\log D$ vs. pH profile for an acid with a $pK_a = 8$, while Figure 2.6 shows the $\log D$ vs. pH profile for a base with a $pK_a = 8$.

Figure 2.5. Typical $\log D$ - pH profile for an acid ($pK_a = 8$)

Figure 2.6. Typical $\log D$ - pH profile for a base ($pK_a = 8$)

Equations 2.11a and 2.11b do not take into account the partitioning of the fully ionised form of the molecules which is normally 3 to 4 orders of magnitude smaller than the neutral form's partition coefficient.

Note that when 50% ionisation occurs (pH 8) the lipophilicity of the compound is only 0.301 (log 2) less than the lipophilicity of the neutral form. Canals *et al.* [39] investigated the gradient retention time vs. pH profile using acetonitrile gradients. The first problem with using acetonitrile gradients is that the mobile phase pH is influenced by the acetonitrile concentration and therefore it is continuously changing during the analysis. Canals *et al.* [39] have reported that the pH 2 starting mobile phase can increase up to nearly pH 5 when the acetonitrile concentration reaches 95 %. The high pH starting mobile phase pH dropped from pH 10.5 to pH 8.5 when the acetonitrile concentration changed from 0 to 95%. Interestingly, the neutral pH (pH 7.4) remained stable when an ammonium acetate buffer and acetonitrile gradient was used as shown in Figure 2.7.

However, the compound's pK_a values are also affected by the presence of the organic phase in the aqueous buffer. Figure 2.8 shows the log D vs. pH profile of 4-*tert*-butylpyridine obtained from the gradient CHI expressed as CHI log D , and the pH profile expected from the octanol/water partition system. The result of the study is that an empirical constant needs to be introduced for weak acids and weak bases into Equations 2.11a and 2.11b to fit the gradient retention time vs. pH profile obtained experimentally as shown by Equation 2.12.

$$gt_R = \frac{(gt_{R(HA)} 10^{S(pK_a - pH)} + gt_{R(A)})}{10^{S(pK_a - pH)} + 1} \quad (2.12)$$

Figure 2.7. The change of the buffer pH by mixing with various amounts of acetonitrile

Thus, there is a possibility to measure the gradient retention time, gt_R , of a compound using several starting mobile phase pHs and a fitting Equation 2.12 to the gt_R vs. pH plot and calculate the compound's pK_a . However, the accuracy of the pK_a determination depends on the number of experiments, that is, the number of pH's applied for the gradient retention time determination. So, even if we use a fast generic pH gradient that takes, for example, 4 min, it would take an hour to measure the pK_a of a compound using 15 experiments at different pHs. In order to have an approximate idea of the compound's acid/base character, in practice we use only three gradient retention time measurements: low pH (pH 2 - 3), physiological pH (pH 7.4) and high pH (pH 9-11). We calculate the CHI value by applying a simple algorithm, and then the compounds are grouped into six categories: acid, base, neutral, weak acid, weak base, and amphoteric (see Figure 2.9).

This simple algorithm is based on the comparison of obtained CHI values at the three different pHs. If the CHI values are the same (within 5 CHI units) at all three pHs, the compound is considered neutral at physiological pH. We have to be careful however, as zwitterionic molecules that are always ionised (either positive or negative or both) could behave as neutral. When the CHI value is significantly greater (more than 5 CHI units) at low pH, but the values are the same (within 5 CHI units) at pH 7.4 and high pH, then the compound is considered an acid, and we can be sure that it contains negatively charged species at physiological pH.

Figure 2.8. The theoretical CHI log D vs. pH profile (open circle by Equation 2.11b) and the experimental CHI log D vs. pH profile (full circles by Equation 2.12) for a weak base

Figure 2.9. Categorising compounds according to their acid/base character using the measurement of Chromatographic Hydrophobicity Index (CHI) at three pHs (acidic, neutral and basic)

When the same CHI values are obtained at low pH and pH 7.4, but values are significantly lower (more than 5 CHI units) at basic pH, then the compound is assigned as a weak acid and it can be considered neutral (unionised) at pH 7.4. When a compound has the same CHI value at pH 7.4 and basic pH, but the CHI is significantly lower at acidic pH, then the compound is assigned as a weak base, and it is considered as neutral (unionised) at pH 7.4. Basic compounds show significantly higher CHI values (more than 5 units) at higher pHs, and they are positively charged at physiological pH.

Another approach has been suggested by Kaliszan *et al.* [40,41] to determine the acid dissociation constant (pK_a) and lipophilicity of compounds using two gradient reversed phase chromatographic measurements. The first measurement should be carried out using a starting mobile phase pH at which the compound is not ionised (high pH for basic compounds, low pH for acidic compounds) and using an organic phase (MeOH or AcN) gradient. Based on the compound's retention time in the first experiment, the organic phase concentration that results in a retention factor greater than 10 should be calculated. The second experiment is a pH gradient that starts at high pH for basic compounds and reducing to low pH, and starting low pH for acidic compounds and increasing to high pH. The organic phase concentration should be kept constant. From the retention time of the second experiment the compound's pK_a can be calculated.

2.3. APPLICATION OF BIO-MIMETIC HPLC STATIONARY PHASES

One of the great advantages of using HPLC technology for measuring properties of compounds is that various types of stationary phases can be applied including ones that mimic biologically important constituents such as membranes and proteins. Protein stationary phases chemically bonded to silica particles have been originally developed for chiral separations [42]. There are two protein bonded commercially available stationary phases that are important not only for chiral separation, which include the human serum albumin (HSA) and alpha-1-acid-glycoprotein (AGP). These two proteins can be found in human and animal plasma. While plasma proteins contain around 40% HSA, the AGP concentration is much lower (1 – 3%). Although the AGP concentration in plasma is relatively low, it has great significance as it can bind basic (positively charged) drug molecules very strongly, while HSA binds the lipophilic and negatively charged compounds. The major component of the plasma proteins are immunoglobulins which bind compounds based on their lipophilicity regardless of whether they are negatively or positively charged.

2.3.1. Immobilised human serum albumin (HSA) stationary phase

Several authors presented good correlations between the logarithmic retention factors measured on the HSA stationary phase and plasma protein binding of drugs obtained by equilibrium dialysis or ultra-filtration methods [43–46]. The principles of using immobilised target bio-polymers to measure drug affinity by HPLC have been reviewed by Bertucci *et al.* [47]. However, Bucholz *et al.* [48] reported the lack of correlation between plasma protein binding and HPLC retention measured on HSA columns. There are two important aspects that should be taken into account when comparing the HPLC method with the equilibrium dialysis method for measuring compounds binding to plasma proteins. Firstly, the unit of the binding measure has to be comparable. Plasma protein binding is most often expressed as % bound or fraction unbound, while in HPLC the logarithmic retention factor measures the partition coefficient of the compound between the aqueous mobile phase and the protein stationary phase. The two measures are in a sigmoid relationship with each other as can be seen in Figure 2.10. As shown, the relatively large changes in log K result in minor changes in % bound at the high binding region. Both measures can be calculated from one another using Equations 2.13 and 2.14:

$$\% \text{ bound(HSA)} = \frac{k}{k+1} \quad (2.13)$$

$$\log K_{(\text{HSA})} = \log \left(\frac{\% \text{ bound(HSA)}}{100 - \% \text{ bound(HSA)}} \right) - \log [\text{HSA}] \quad (2.14)$$

where k is the chromatographic retention factor in the HSA stationary phase and K is the binding affinity constant. However, this equation is valid only for non-specific binding, when compound retention is independent of the injected amount and the overall binding can be modelled by partition. The chromatographic retention factor k is equivalent to the albumin partition coefficient only if the V_s/V_m equals 1 in Equation 2.2.

Figure 2.10. The sigmoid relationship between the logarithmic retention factor ($\log k$) on HSA stationary phase and % bound HSA

The affinity constant also depends on the HSA concentration, which may vary. Figure 2.10 also shows that the same binding affinity constant may represent a different value when it is expressed as % bound depending on various physiological (or pathological) concentrations of HSA in the plasma. Secondly, a typical mistake is to compare the value of the albumin binding to the value of plasma protein binding as plasma contains only 40% HSA, it is very likely that the compound to a certain extent binds to other plasma proteins too. The deviations are most often observed with basic compounds that are positively charged at physiological pHs and bind strongly to AGP, for example. Kratochwil *et al.* [49] rightfully compared the affinity constants obtained by chromatographic and equilibrium dialysis methods using purified albumin, not plasma. It is also important to keep in mind that the chromatographic method measures the proportion of the average number of moles of the drug in the mobile and the stationary phase during the dynamic equilibrium process. Thus, it measures not only the compounds binding to one particular binding site of the albumin, but many other non-specific binding sites as well. Thus, we cannot assume one to one binding between the albumin and drug molecules. In a methodical point of view it

is also important to imitate the physiological condition during the chromatographic retention measurements. The best approach is to measure at pH 7.4, the pH of the plasma, and use isocratic conditions preferably without using an organic modifier in the mobile phase. As it was found by Tiller *et al.* [44], the $k/(k+1)$ proportion derived from the retention factor multiplied by 100 gave very close values to the reported plasma protein binding % for a variety of drug molecules even when injected together in a mixture and the chromatographic peaks were identified by MS. Valko *et al.* [46] applied a fast iso-propanol gradient during the retention time measurements in order to reduce the analysis time for strongly bound compounds. The obtained correlation of the calibrated logarithmic retention time values showed acceptable correlation to the plasma protein binding data in the literature. The good correlation was endorsed by the study of Kratochwil *et al.* [49] when they compared chromatographic binding data with binding data obtained using only HSA, (and not pooled plasma) and equilibrium dialysis. However, the correlation using data obtained by Colmenarejo *et al.* [50] who used acetonitrile as an organic modifier in the mobile phase was not as good. This is possibly because the acetonitrile changes the natural three dimensional structure of the albumin and changes its binding characteristics. The practical recommendation of the HSA HPLC columns for chiral separation is to use a small percentage of isopropanol as an organic modifier that supports the hypothesis that under such conditions the naturally chiral selective binding sites are intact. Therefore, it is advisable to use an enantiomeric mixture of warfarin that binds to the major binding site of albumin to check the enantio-selectivity of the stationary phase as it often deteriorates after extensive use of the column.

2.3.2. Immobilised α -1-acid-glycoprotein (AGP) stationary phase

Similarly to the HSA stationary phase, the AGP stationary phase has been developed for chiral separations. The AGP stationary phase was studied by Jewell *et al.* [51] who concluded that the retention factors obtained on AGP columns did not show correlation to AGP binding data obtained by *in vitro* displacement experiments. It is not surprising as the displacement experiments measure compounds binding to a particular binding site of the protein where the probe molecule binds. The chromatographic approach based on retention time measurements indicates the compound's overall partitioning to the protein phase and as such includes specific and non-specific binding. Good correlation has been found between the AGP binding of known drugs obtained by ultra-filtration and chromatographic methods [52] as demonstrated in Figure 2.11.

Some of the positively charged compounds showed stronger binding by the HPLC based measurements than by ultra-filtration. A possible explanation could be that under the ultra-filtration conditions the positively charged compounds saturated the negatively charged specific binding site due to the higher proportion of the drug concentration relative to the protein.

Figure 2.11. The correlation of AGP binding obtained by ultrafiltration method and chromatographic retention time measurements on immobilised AGP HPLC stationary phase. The basic acidic and neutral compounds marked differently

The proportion of the drug concentration to the protein concentration in chromatography is much closer to the physiological condition. Kaliszan *et al.* [53] studied the binding behaviour of positively charged drugs on an immobilised AGP stationary phase, and proposed a funnel shaped binding site that binds positively charged compounds to the negatively charged narrow end of the funnel. As AGP contains a large proportion of a sialic acid moiety, its presence could explain the strong affinity to basic compounds.

There are other chromatographic methods such as frontal and displacement chromatography that can be used to probe a compound's interactions with proteins as reviewed by Oravcova *et al.* [54]. For a quick assessment and ranking of compounds binding to plasma proteins, the HPLC based retention time measurements on immobilised protein stationary phases are recommended. It is advisable to apply a calibration set of compounds for which the retention time measurements are frequently repeated in order to check the column performance and suitability, and to increase the day to day and instrument to instrument reproducibility of the data.

2.3.3. Immobilised artificial membrane (IAM) stationary phase

Pidgeon and Venkataram [55] patented a method for immobilising phospholipids on HPLC grade silica stationary phases. The immobilised phospholipids mimic the lipid environment of a fluid cell membrane on a solid matrix (see Figure 2.12 for illustration). Nowadays, various immobilised artificial membrane (IAM) HPLC stationary phases are commercially available. The IAM stationary phases are prepared by covalently bonding the phospholipids to an amino-propyl silica stationary phase. The remaining amino-propyl groups and free silanol groups are treated to reduce their secondary interactions of the compounds. The most frequently used IAM stationary phase contains covalently bonded phosphatidyl choline covering the silica particles at monolayer density. There are excellent reviews about the various commercially available IAM stationary phases and their applications for the determination of compound binding to phospholipids by Taillardat-Bertschinger *et al.* [56-57] and Giaginis and Tsantili-Kakoulidou [58]. Stewart *et al.* [59] reviewed the application of immobilised artificial membrane chromatography for modelling drug transport. Usually isocratic methods are suggested, incorporating a very low volume (15%) of acetonitrile in the mobile phase. Equation 2.2 is suggested for the derivation of membrane partition data from isocratic retention factors. Equation 2.4 can be applied for the extrapolation of retention factors ($\log k$) obtained with various concentrations of organic modifiers to a 100% aqueous mobile phase. Taillardat-Bertschinger *et al.* [57] reviewed the application of IAM chromatography in drug discovery in great detail. A gradient method has been developed and published [60] that applies acetonitrile gradient on IAM HPLC columns and provides a Chromatographic Hydrophobicity Index (CHI IAM). The gradient retention times are calibrated with data obtained from isocratic measurements. The measurements and ranking of the phospholipid binding of drug discovery compounds can be used for characterising and prioritising compounds for progression. These data have been successfully applied in various models to estimate compound *in vivo* distribution as described later in this chapter.

Figure 2.12. The similarity between the IAM stationary phase and a phospholipid bi-layer

2.4. GENERAL EXPERIMENTAL DESCRIPTION OF FAST GRADIENT HPLC ANALYSIS OF LIPOPHILICITY, PROTEIN AND PHOSPHOLIPID BINDING OF DRUG DISCOVERY COMPOUNDS

2.4.1. Measurements of chromatographic hydrophobicity indices at three different pHs

The CHI lipophilicity on C-18 stationary phases using three different mobile phases pHs were obtained as described earlier [61]. Typical HPLC conditions are as follows:

HPLC column:	Luna C(18)2 5 μ m 50 x 3 mm Phenomenex Ltd. UK
Mobile phase A1 (pH 7.4):	50 mM ammonium acetate pH 7.4
Mobile phase A2 (pH 10.5):	50 mM ammonium acetate, plus cc ammonia solution to adjust the pH to 10.5
Mobile phase A3 (pH 2):	0.01 M phosphoric acid
Mobile phase B:	acetonitrile (HPLC grade)
Flow rate:	1.0 ml/min
Gradient:	0 to 2.5 min 0 to 100% B 2.5 to 3 min 100% B 3.0 to 3.2 min 0% B 3.2 to 5 min 0% B

Any HPLC instrument that is able to produce fast gradient analysis, with a lowest possible dwell volume is suitable. UV-detection of the compound can be used when a UV chromophore is available in the analyte. As was highlighted previously, the calibration of the retention time is essential. Any set of compounds that covers a wide range of lipophilicity and chemical diversity can be used for calibration. However, for accurate data that is useful for inter-laboratory comparison, the constants for the gradient system should be determined by isocratic measurements. Alternatively one can use the calibration set of compounds presented in Table 2.1 and use the data given for retention time calibration. The gradient retention times of the compounds are converted to CHI values using the obtained slope and intercept values of the calibration plots. The CHI values can be converted to the octanol/water partition coefficient scale ($\log D$) using the formula in Equation 2.9. Table 2.1 shows the calibration set of compounds and their CHI values at three different pHs obtained from isocratic measurements with typical gradient retention time data.

Table 2.1. The calibration set of compounds for the chromatographic hydrophobicity index (CHI) lipophilicity determination at three different pHs [61].

Name	t_R / min. (pH 2)	CHI 2	t_R / min. (pH 7.4)	CHI 7.4	t_R / min. (pH 10.5)	CHI 10.5
Theophylline	1.12	6.3	1.15	18.4	1	4.97
Phenyltetrazole	1.38	17.9	1.24	23.6	1.29	15.98
Benzimidazole	1.78	42.2	1.41	34.3	1.58	30.61
Colchicine	1.83	43.9	1.67	42.0	1.70	43.86
Phenyltheophylline	1.97	51.7	1.79	51.2	1.39	21.48
Acetophenone	2.12	64.1	1.98	65.1	2.05	64.12
Indole	2.29	72.1	2.15	71.5	2.19	72.07
Propiophenone	2.37	77.4	2.23	77.4	2.29	77.42
Butyrophenone	2.55	87.3	2.43	87.5	2.46	87.33
Valerophenone	2.72	96.4	2.60	96.2	2.61	96.36

2.4.2. Measurement of HSA binding by HPLC retention

The conditions for generic HSA binding measurements [46] are as follows:

HPLC column: immobilised HSA 50 x 3 mm
(Chromtech or Chiral Technologies)
Mobile phase A: 50 mM ammonium acetate pH 7.4
Mobile phase B: iso-propanol (HPLC grade)
Flow rate: 1.8 ml/min
Gradient: 0 to 3.0 min 0 to 30% B
3.0 to 10.0 min 30% B
10.0 to 10.5 min 0% B
10.5 to 15.0 min 0% B

Any HPLC instruments that are able to deliver a high flow rate and operate up to 600 bar back pressure can be used. An elevated temperature of up to 40 °C can be used to reduce the backpressure. Iso-propanol is a viscous solvent and when the 30% gradient is applied the back pressure can be close to the operational limit of the HPLC instrument. It is possible to apply a lower flow rate, but the gradient slope should be kept the same, and the analysis time should be increased proportionally. UV detection is suitable for UV active compounds. The application of MS detection is very useful, as isomers and enantiomers often produce multiple peaks for which the identification is important. Column performance checks and calibration should be performed before the analysis. The calibration set of compounds can be dissolved separately in a concentration of 0.5 mg/ml in 50% iso-propanol and 50% pH 7.4 ammonium acetate solutions. The calibration set of compounds, their plasma protein binding percentage found in the literature and its linear conversion value ($\log K_{it}$), as well as typical retention

times, their logarithmic values, $\log K$ derived from the calibration curve and % binding data are listed in Table 2.2. Figure 2.13 shows a typical chromatogram of the warfarin. If the racemates are separated it is considered that the warfarin binding site of the HSA is intact. Figure 2.14 shows a typical calibration plot.

Table 2.2. Calibration set of compounds with their literature and typical measured chromatographic data obtained with the human serum albumin (HSA) column.
(Literature data were obtained from reference 46).

Name	PPB*, % (literature)	Linearised PPB*, % ($\log K$ lit)	t_{R} / min	$\log$ (t_{R} / min.)	$\log K$ (from calibration)	HSA binding, % (from calibration)
Nizatidine	35	-0.28	0.937	-0.03	-0.25	36.27
Bromazepam	60	0.17	1.2	0.08	0.09	55.95
Carbamazepine	75	0.46	1.63	0.21	0.52	77.66
Budesonide	88	0.83	1.837	0.26	0.69	83.85
Nicardipine	95	1.20	3.01	0.48	1.38	96.95
Indometacin	99	1.69	3.845	0.58	1.72	99.12
Piroxicam	94.5	1.16	2.821	0.45	1.29	96.06
Diclofenac	99.8	1.92	4.2	0.62	1.85	99.58
Flurbiprofen	99.96	1.98	4.3	0.63	1.88	99.68

*Plasma protein binding (PPB)

Figure 2.13. Typical chromatogram of racemic mixture of Warfarin.
Chromatographic conditions are described in the experimental section

Figure 2.14. Typical calibration plot obtained on the HSA column

The logarithmic value of the gradient retention times (gt_R) obtained from the HPLC experiments were plotted against the linearised values of the percentage of plasma-protein binding, % PPB (*i.e.*, $\log K$). The slope and intercept were then used to convert the gradient retention time values to $\log K$ values for a new compound. From the so obtained $\log K$ values, the estimated % protein binding was obtained by Equation 2.15.

$$\% \text{ bound(HSA)} = \frac{101 \times 10^{\log K}}{1 + 10^{\log K}} \quad (2.15)$$

2.4.3. Measurement of AGP binding by HPLC retention

The general experimental condition for the AGP binding measurements are as follows:

Column:	immobilised AGP 50 x 3 mm (Chromtech or Chiral technologies)
Mobile phase A:	50 mM ammonium acetate pH 7.4
Mobile phase B:	iso-propanol (HPLC grade);
Flow rate:	1.8 ml/min
Gradient:	0 to 2.5 min 0 to 25% B; 2.5 to 4.5 min 25% B; 4.5 to 4.6 min 0% B; 4.6 to 6 min 0% B

Instrumentation and detection techniques are the same as described above. Column performance checks and calibration should be performed before the analysis. The compounds used for the column calibrations were dissolved separately in a concentration of 0.5 mg/ml in 50% iso-propanol and 50% pH 7.4 ammonium acetate solutions. The calibration set of compounds, their AGP binding percentage found in the literature and its linear conversion value using Equation 2.1 ($\log K$ lit.), as well as typical retention times are listed in Table 2.3 with their logarithmic values. Figure 2.15 shows a typical calibration plot.

Table 2.3. The compounds and their literature and experimental alpha-acid-glycoprotein (AGP) binding data.

Compound	Binding, % (literature)	AGP ($\log K$ lit)	Exp t_R / min.	$\log$ (t_R / min)	$\log K$ (from calibration)	AGP, % (form calibration)
Nizatidine	36	-0.257	1.248	0.10	-0.25	36.33
Bromazepam	50	-0.009	1.601	0.20	0.03	52.23
Warfarin	88	0.831	2.877	0.46	0.76	86.01
Propranolol	86	0.758	2.997	0.48	0.81	87.59
Imipramine	91	0.959	3.167	0.50	0.87	89.02
Nicardipine	92	1.010	3.349	0.52	0.93	90.32
Chlorpromazine	92	1.010	3.975	0.60	1.15	94.34

Figure 2.15. Typical calibration plot for AGP binding measurements

2.4.4. Measurement of IAM binding by HPLC retention

The following conditions can be applied for the phospholipid binding measurements [60]:

Column: immobilised artificial membrane column
(Regis Analytical LTD) 150 x 4.6 mm
Mobile phase A: 50 mM ammonium acetate pH 7.4;
Mobile phase B: acetonitrile (HPLC grade);
Flow rate: 2.0 ml/min
Gradient: 0 to 2.5 min 0 to 70% B
2.5 to 3 min 70% B
3.0 to 3.2 min 0% B
3.2 to 5 min 0% B

Instrumentation and detection as described above. The column performance check and calibration should be performed before the analysis. The compounds used for the column calibrations are dissolved separately in a concentration of 0.5 mg/ml in 50% acetonitrile and 50% pH 7.4 ammonium acetate solution mixtures. The CHI (IAM) values for the calibration set of compounds have been measured using the isocratic mode [60] and are listed in Table 2.4. A typical calibration plot is shown in Figure 2.16. The CHI (IAM) values were converted to the traditional lipophilicity scale ($\log k$ IAM) using the formula below (Equation 2.16) which was obtained by plotting the isocratic $\log k$ values against the CHI (IAM) values [60].

$$\log k \text{ (IAM)} = 0.046 \times \text{CHI (IAM)} + 0.42 \quad (2.16)$$

The constants in Equation 2.16 were obtained by plotting the CHI values obtained from the gradient retention times against the $\log k$ values that were obtained from isocratic retention time measurements and extrapolated to the 100% aqueous mobile phase.

Table 2.4. The calibration set of compounds and their literature CHI (IAM) values [60]

Compound	typical retention time t_R / min.	CHI (IAM)	Log k (IAM)
Octanophenone	3.18	49.4	2.69
Heptanophenone	3.07	45.7	2.52
Hexanophenone	2.94	41.8	2.34
Valerophenone	2.79	37.3	2.14
Butyrophenone	2.58	32	1.89
Propiophenone	2.35	25.9	1.61
Acetophenone	2.04	17.2	1.21
Acetanilide	1.85	11.5	0.95
Paracetamol	1.62	2.9	0.55

Figure 2.16. Typical calibration curve for the IAM measurements

2.5. ANALYSIS OF THE HPLC BASED LIPOPHILICITY, PROTEIN BINDING AND PHOSPHOLIPID BINDING DATA

2.5.1. Analysis of the effect of charge

As described above, chromatographic techniques provide a measurement of a compound's interactions with a variety of stationary phases including proteins and phospholipids. It is important to investigate the relationships between these properties. Are we just measuring a compound's lipophilicity in a different way? We expect a minimum binding of a compound to each of the investigated stationary phases based on its hydrophobicity. However, the compound can bind even stronger to a particular stationary phase if it has additional interaction to it above the hydrophobic interactions. In order to detect the additional interactions we can use the octanol/water partition coefficients for comparison. From the seminal work of Hansch *et al.* [62], the octanol/water partition system is used to model a variety of biological partition processes; therefore it is important to understand the relationships between the bio-mimetic properties and other lipophilicity measures. Figures 2.17-2.18 show plots of HSA and IAM binding values as a function of CHI log D values using the data obtained for 70 known diverse drug molecules. Figure 2.19 shows the plot of the HSA binding as a function of IAM binding. We can see in Figure 2.17 that the more lipophilic compounds bind stronger to HSA, however there are several compounds that show even stronger binding than would be expected from their CHI log D values, especially compounds that are assigned as acid.

Figure 2.17. Typical plot of the HSA binding values against the CHI log D values for known drugs. Acidic, basic and neutral compounds marked differently. The line represents the expected minimum HSA binding based on the lipophilicity (CHI log D)

In Figure 2.18 similar trends can be observed for IAM binding. Compounds with a certain degree of lipophilicity (CHI log D) have an expected binding to IAM. However, many drug molecules show stronger binding to IAM than would be expected from the CHI log D values, especially bases. It is interesting to see the plot of HSA and IAM binding in Figure 2.19. It can be seen that the negatively charged compounds bind more strongly to HSA than IAM, while the positively charged compounds bind more strongly to IAM than HSA. We can therefore conclude that the presence of charge causes significant differences between a simple C-18 lipophilicity and IAM and HSA binding.

From these results, it is obvious that the octanol/water log D or CHI log D does not distinguish between the positive or negative charges. Whichever charge is present on the molecule it will reduce its ability to partition into the octanol or C-18 stationary phase. However, compounds with a positive charge bind strongly to phospholipids while the negatively charged compounds bind strongly to albumin types of proteins.

Figure 2.18. The plot of the IAM binding values and the CHI log D values for known drug molecules. Acidic, basic and neutral compounds marked differently. The line represents the expected minimum IAM binding based on the lipophilicity (CHI log D). It can be seen that negatively charged acids have lower than the expected minimum binding to IAM

Figure 2.19. The plot of HSA and IAM binding of known drug molecules. Acidic, basic and neutral compounds marked differently. The line represents the expected equivalent binding to IAM and HSA based on compound lipophilicity only, without additional binding interactions

2.5.2. Analysis by the solvation equation

It is worth investigating the similarities or differences of the binding of neutral compounds to albumin and phospholipids and comparing them to the octanol/water partition coefficients. The solvation equation approach can provide a tool for investigating the behaviours of neutral molecules in various water/organic phase partition systems.

The Abraham solvation equation model [63] suggests that molecular size, and a small number of polarity descriptors (H-bond donor/acceptor, dipolarity) are sufficient to describe compound partition behaviour. By measuring $\log k$ (or the extrapolated $\log k_w$) values for a set of probe molecules (minimum 25 compounds, preferably 40 – 60) with known molecular descriptors, such as excess molar refraction, E ; dipolarity/polarisability, S ; H-bond acidity, A ; H-bond basicity, B ; and molecular size, V , the solvation equation characteristic for the chromatographic partitioning system can be constructed using the multiple linear regression equation as shown below:

$$\log k = eE + sS + aA + bB + vV \quad (2.17)$$

The regression coefficients of the molecular descriptors (e , s , a , b , and v) are characteristics for the chromatographic partition system. Numerous equations have been published [63-68] for the characterisation of the various reversed phase of columns (C-18, perfluorinated hydrocarbons, cyclodextrin) using acetonitrile and methanol as an organic modifier in the mobile phase. The generally good statistical fit of the chromatographic retention data and the molecular descriptors derived from bulk organic solvent/water partition coefficients support the comparability of the two types of partition data. However, it should be noted, that the parameters of the solvation equation are often based on the retention data of relatively small, mono- or bi-functional molecules in unionised form. Du *et al.* [69] compared the linear solvation equations obtained for isocratic HPLC retention factors (using C-18 stationary phases and acetonitrile as an organic modifier in the mobile phase) and the octanol water partition coefficients. The major difference between the two partition systems is their sensitivity towards the H-bond acidity of the molecules. While the water saturated octanol phase easily accommodates compounds with H-bond donor groups, the chromatographic C-18 stationary phase has no such polar functionality. Therefore, H-bond donor functional groups decrease chromatographic retention and, consequently, chromatographic partition coefficients. The correlation between isocratic retention data (with C-18 stationary phases and aqueous acetonitrile mobile phases) and octanol $\log P$ can be significantly improved by taking into account a simple H-bond donor count (HBC) or the Abraham H-bond acidity descriptor (A) in the equations shown below:

$$\log P_{\text{oct}} = 1.91 \log k + 0.37 \text{HBC} + 0.72 \quad (n = 111; r = 0.962; sd = 0.272) \quad (2.18)$$

$$\log P_{\text{oct}} = 2.07 \log k + 1.09 A + 0.52 \quad (n = 111; r = 0.982; sd = 0.189) \quad (2.19)$$

The gradient chromatographic CHI values obtained for the unionised forms of the molecules (CHIN) have also shown an acceptable correlation with the octanol/water log P values when HBC or A are included [61]. Equations 2.20 and 2.21 show the relationships obtained for a training set of 86 known drug molecules.

$$\log P_{\text{oct}} = 0.047 \text{ CHIN} + 0.36 \text{ HBC} - 1.10 \quad (2.20)$$

$(n = 86; r = 0.943; s = 0.39; F = 336)$

$$\log P_{\text{oct}} = 0.054 \text{ CHIN} + 1.32 A - 1.88 \quad (2.21)$$

$(n = 86; r = 0.970; s = 0.29; F = 655)$

Solvation equations have been derived for various biological partition/distribution processes, such as blood/brain barrier distribution [70], oral absorption [71], and skin-penetration [72]. The solvation equations obtained for biological partition processes can be compared with the solvation equations obtained for organic solvent/water partition coefficients [73,74]. In order to compare the various partition systems, the coefficients of each molecular descriptor was divided by the volume coefficient (v), otherwise the scale or unit of the solute parameter distorts the proper comparison of the systems in terms of their sensitivity towards the molecular descriptors. Table 2.5 shows the relative coefficients of the molecular descriptors obtained for various biological, bio-mimetic, and organic solvent/water partitions.

Table 2.5. The relative coefficients of the molecular descriptors (E, S, A and B) for various biological, bio-mimetic and organic solvent/water partition systems.

System	e/v	s/v	a/v	b/v
$\log k_{\text{IAM}}$	0.37	-0.24	0.03	-1.10
CHI_{IAM}	0.42	-0.25	0.07	-1.08
$\text{CHI}_{\text{RP,AcN}}$	0.09	-0.24	-0.30	-0.98
$\log P_{\text{octanol}}$	0.15	-0.28	0.01	-0.91
Blood/brain	0.19	-0.69	-0.72	-1.28
HSA/buffer	0.02	-0.07	0.16	-1.21
AGP/buffer	0.46	-0.38	-0.33	-0.85
water/skin	0.00	-0.33	-0.35	-1.95

As we can see in Table 2.5, the biggest variation of the coefficients can be observed in the relative sensitivity of the systems towards the H-bond acidity of the compounds. The octanol/water system, the IAM and HSA binding are not sensitive to the H-bond acidity of the molecules. This means that molecules with H-bond donor groups easily partition into octanol and bind to the phospholipid membrane and albumin types of proteins. This can be explained by the fact that

these systems contain water or another H-bond acceptor moiety that accommodates the H-bond donor groups forming hydrogen bonding. These findings support the idea that the octanol/water partition system is a good model for biologically relevant partitioning of the compounds. However, we can see from Table 2.5 that compound distribution between the blood and brain is strongly affected by the H-bond donor groups and that the a/v coefficient has a negative value. This means that molecules with H-bond donor groups prefer the blood to the brain when partitioning between them. The reversed phase chromatographic system and the water/skin partitioning systems also have a negative coefficient for the H-bond acidity, but slightly less so than the blood/brain system. All systems have very similar coefficients towards the H-bond basicity of the molecules (large negative values), which means the compounds with H-bond acceptor groups prefer the more aqueous environments. We can observe reasonably large variations of the system's sensitivity towards the dipolarity and polarisability parameter (s/v) and the excess molar refraction (e/v), which indicate the differences in polarity (dipole moments) and heteroatom interactions.

The solvation equation approach is a valuable tool for comparing the similarities and differences between physico-chemical partitioning and biological partitioning of the molecules; moreover it can be used to select the best physico-chemical model systems for biological partitioning [75]. We have to remember that the above mentioned solvation equations do not incorporate the effect of ionisation of the molecules and only the data of the uncharged molecules should be used for generating these equations for system comparisons. Recent efforts have been published [76] that estimate the effect of the ionisation on the partitioning of the molecule.

We can conclude from these findings that the physicochemical water/organic solvent partition systems, and the bio-mimetic chromatographic partitioning systems can be used as simple measurements of compound behaviour that are able to imitate compound partitioning in a biological environment. However, it would be even more useful to use these measured parameters in models that describe compound behaviour *in vivo*.

2.6. APPLICATIONS OF BIO-MIMETIC HPLC PROPERTIES TO MODEL DRUGS *IN VIVO* DISTRIBUTION

There are several experimental approaches that are used to characterise the *in vivo* distribution of drug molecules and describe their pharmacokinetic and pharmacodynamic behaviour. The body can be divided into several compartments to which the drug molecules are distributed. The simplest model divides the body into two compartments, the plasma compartment and tissue compartment. The volume of distribution, V_d , is used as a hypothetical volume that reveals how extensive the compound distribution is into tissues from the plasma. It can be defined in two different ways and can be obtained from the given intravenous dose and the plasma concentration, C_{plasma} , as described in Equation 2.22.

$$V_d = \frac{Dose}{C_{plasma}} \quad (2.22)$$

As the plasma concentration is continuously changing with time as the compound distributes and then starts to eliminate, the so-called steady state volume of distribution, V_{dss} , is introduced, which considers a repeated dose that results in a steady state plasma concentration. Using the steady state volume of distribution and knowing the volume of the plasma compartment, V_p , we can rearrange Equation 2.22 to form Equation 2.23.

$$V_{dss} = \frac{Dose \times V_p}{A_p} \quad (2.23)$$

where A_p is the amount of drug in the plasma compartment. We can define an equilibrium constant, $K_{tissue/plasma}$, for the drug partitioning between the tissue and the plasma compartment as the tissue concentration divided by the plasma concentration, supposing that the drug distribution between these compartments are independent from the dose as shown by Equation 2.24.

$$K_{tissue/plasma} = \frac{\frac{Dose - A_p}{V_t}}{\frac{A_p}{V_p}} \quad (2.24)$$

V_t is the volume of the tissue compartment. Theoretically, the amount of drug in the tissue compartment is the difference between the dose and the amount of drug in the plasma compartment. Combining Equations 2.23 and 2.24, Equation 2.25 is obtained that expresses the drug's partition coefficient between the tissue and plasma compartment as a function of the steady state volume of distribution.

$$K = \frac{V_{dss} - V_p}{V_t} \quad (2.25)$$

Equation 2.25 shows that the *in vivo* steady state volume of distribution is proportional to the drug's partition coefficient between the tissue and the plasma compartment. The schematic illustration of the drug's partitioning *in vivo* is shown in Figure 2.20. As Figure 2.20 demonstrates, there is another equilibrium process that takes place between the free and bound compartments, which is also important from a pharmaco-dynamic point of view. Based on Brodie's free drug hypothesis [77], the steady state free concentration of the drug molecule in plasma and tissues should be the same when there is no permeability barrier for the drug between compartments and no active transport (efflux or influx) is taking place. The free plasma concentration of a drug can be obtained from the steady state unbound volume of distribution, V_{du} , according to Equation 2.26.

Figure 2.20. The schematic illustration of drug's partitioning *in vivo* between plasma and tissue compartment as well as the free and bound compartments in plasma and tissues

$$V_{du} = \frac{Dose}{C_{free, plasma}} \quad (2.26)$$

While the average plasma volume and tissue volume can be measured for a living system, it is much more difficult to measure or estimate the volume of the “free” compartment in plasma and tissues. Similar to the expression of $K_{tissue/plasma}$, we can have a partition coefficient for the drug between the free and bound compartment, $K_{bound/free}$, which is proportional to the steady state unbound volume of distribution, V_{du} . The *in vivo* V_{du} is normally calculated from the V_d and the fraction unbound in plasma, f_u , which can be obtained by equilibrium dialysis or ultra-filtration methods [78,79]. Thus, Equation 2.27 shows the expression of V_{du} from the steady state volume of distribution and the fraction unbound in plasma.

$$V_{du} = \frac{V_{dss}}{f_u} \quad (2.27)$$

The plasma compartment contains around 40% albumin (HSA), 3% alpha-1-acid-glycoprotein (AGP) and 57% of various immunoglobulins. The tissue compartment contains a large amount of phospholipids besides the various protein components. Thus, the most significant difference between the two compartments is the presence or absence of phospholipids and lipids in general. So, it can be assumed that the major force that drives drug molecules from the plasma compartment to the tissue compartment is their affinity to phospholipids relative to proteins.

2.6.1. Modelling steady state volume of distribution

The hypothesis described above can be investigated using the human clinical steady state volume of distribution and plasma protein binding data [80,81] of known drugs as shown in Table 2.6. Table 2.7 contains measured HSA and IAM binding data [80] using the bio-mimetic HPLC methodology described above. As already published and validated [80], the difference between a compound's affinity to phospholipids and albumin is the major driving force for the drug molecules to partition between the tissue and plasma compartments, therefore showing good correlation to the logarithmic value of the steady state *in vivo* volume of distribution ($\log V_{\text{dss}}$) as described by Equation 2.28.

$$\log V_{\text{dss}} = 0.131 \log K_{(\text{IAM})} - 0.267 \log K_{(\text{HSA})} - 0.305 \quad (2.28)$$

$$(n = 70; r = 0.80; sd = 0.40)$$

where n is the number of drug molecules listed in Tables 2.6 and 2.7, r is the multiple regression coefficient, and sd is the standard error of the estimate. The $\log K_{(\text{IAM})}$ and $\log K_{(\text{HSA})}$ values are obtained as the e power $\log k_{(\text{IAM})}$ and $\log k_{(\text{HSA})}$ values. Figure 2.21 shows the plot of the $\log V_{\text{dss}}$ values obtained from clinical studies for the 70 marketed drug molecules as a function of the estimated $\log V_{\text{dss}}$ values by Equation 2.28.

Table 2.6. The human clinical steady state volume of distribution, V_{dss} , plasma protein binding, PPB, and unbound volume of distribution, V_{du} , of a representative set of known drug molecules from the literature [80,81]

DRUG	Bound in plasma, %	$\log \frac{\text{PPB}}{101 - \text{PPB}}$	V_{dss} (L/kg)	$\log V_{\text{dss}}$	V_{du} (L/kg)	$\log V_{\text{du}}$
Acetaminophen	1	-2.00	1.0	-0.02	0.97	-0.01
Acyclovir	15	-0.76	0.6	-0.22	0.71	-0.15
Amoxicillin	18	-0.66	0.2	-0.67	0.26	-0.58
Ampicillin	18	-0.66	0.3	-0.54	0.35	-0.46
Amrinone	45	-0.09	1.3	0.11	2.36	0.37
Aspirin	49	-0.03	0.2	-0.80	0.31	-0.51
Betamethasone	64	0.24	1.4	0.15	3.89	0.59
Bromazepam	60	0.17	0.9	-0.04	2.28	0.36
Bumetanide	99	1.69	0.2	-0.70	20.0	1.30
Bupivacaine HCl	95	1.20	1.0	0.02	20.0	1.32
Ceftazidime	21	-0.58	0.2	-0.64	0.29	-0.54
Cephalexin	14	-0.79	0.2	-0.59	0.30	-0.52
Chlorpheniramine	70	0.35	3.0	0.48	10.0	1.00
Cimetidine	19	-0.64	1.0	0.00	1.23	0.09
Ciprofloxacin	40	-0.18	2.2	0.34	3.67	0.56
Clonazepam	86	0.76	3.2	0.51	22.9	1.36
Cytarabine	12	-0.87	2.5	0.40	2.84	0.45

DRUG	Bound in plasma, %	$\log \frac{\text{PPB}}{101 - \text{PPB}}$	V_{dss} (L/kg)	$\log V_{\text{dss}}$	V_{du} (L/kg)	$\log V_{\text{du}}$
Diazepam	99	1.69	1.1	0.04	110	2.04
Digitoxin	97	1.38	0.5	-0.27	18.10	1.26
Diltiazem	78	0.53	4.5	0.65	20.45	1.31
Diphenhydramine	63	0.22	4.5	0.65	12.16	1.09
Doxepin	83	0.66	24.0	1.38	141	2.15
Ethosuximide	1	-2.00	0.7	-0.15	0.72	-0.14
Felbamate	23	-0.53	0.8	-0.12	0.97	-0.01
Finasteride	90	0.91	1.1	0.04	11.0	1.04
Flumazenil	50	-0.01	0.7	-0.15	1.40	0.15
Ganciclovir	1	-2.00	1.1	0.04	1.11	0.05
Gemfibrozil	97	1.38	0.1	-0.85	4.67	0.67
Glipizide	98.4	1.58	0.2	-0.70	12.5	1.10
Haloperidol	92	1.01	18.0	1.26	225	2.35
Hydrochlorothiazide	40	-0.18	2.0	0.30	3.33	0.52
Imipramine	90	0.91	18	1.26	180	2.26
Indomethacin	99	1.69	0.3	-0.59	25.7	1.41
Isradipine	97	1.38	4.0	0.60	133	2.12
Ketoprofen	98.7	1.63	0.1	-0.96	8.46	0.93
Lorazepam	91	0.96	1.5	0.18	16.7	1.22
Methylprednisolone	78	0.53	1.2	0.08	5.45	0.74
Metoclopramide	40	-0.18	3.0	0.48	5.00	0.70
Metronidazole	10	-0.96	0.7	-0.13	0.83	-0.08
Nabumetone	99	1.69	0.8	-0.1	79.0	1.90
Naproxen	99.7	1.88	0.1	-1.0	33.3	1.52
Nicardipine	95	1.20	1.1	0.04	2.02	1.34
Nifedipine	96	1.28	0.8	-0.10	19.6	1.29
Nizatidine	35	-0.28	0.88	-0.11	1.20	0.08
Ondansetron	73	0.42	1.9	0.28	7.04	0.85
Phenobarbital	51	0.01	0.2	-0.82	0.31	-0.51
Phenytoin	89	0.87	0.6	-0.19	5.84	0.77
Phenytoin	89	0.87	0.6	-0.19	5.82	0.76
Piperacillin	18.5	-0.65	0.18	-0.74	0.22	-0.66
Piroxicam	94.5	1.16	0.1	-1.00	1.82	0.26
Prazosin	95	1.20	0.5	-0.3	10.0	1.00
Prednisolone	70	0.35	0.9	-0.07	2.83	0.45
Prednisone	75	0.46	1.0	0.00	4.00	0.60
Procainamide	16	-0.73	2.0	0.30	2.38	0.38
Propranolol	87	0.79	2.8	0.45	21.5	1.33
Quinidine	87	0.79	2.5	0.40	19.2	1.28

DRUG	Bound in plasma, %	$\log \frac{\text{PPB}}{101 - \text{PPB}}$	V_{dss} (L/kg)	$\log V_{\text{dss}}$	V_{du} (L/kg)	$\log V_{\text{du}}$
Quinine	94	1.13	1.5	0.18	25.0	1.40
Rifampin	89	0.87	1.0	-0.01	8.83	0.95
Saquinavir	98	1.51	10.0	1.00	500	2.70
Sulfamethoxazole	62	0.2	2.0	0.3	5.30	0.72
Terbutaline	20	-0.61	1.8	0.25	2.23	0.35
Tolbutamide	96	1.28	0.1	-1.00	2.50	0.04
Trazodone	93	1.07	1.0	0.00	14.3	1.15
Trimethoprim	37	-0.24	1.3	0.11	2.06	0.31
Verapamil	90	0.91	4.4	0.64	44.0	1.64
Warfarin	99	1.69	0.1	-0.85	14.0	1.15
Zidovudine	2.5	-1.6	1.4	0.15	1.44	0.16
Zolmitriptan	25	-0.48	7.0	0.85	9.33	0.97
Zolpidem	92	1.01	0.7	-0.17	8.00	0.93

Another model for the estimation of volume of distribution was published by Lombardo *et al.* [82]. They applied descriptors of compounds obtained by chromatographic measurements. The model equation contained the $\text{Elog } D$ parameter (a chromatographically determined lipophilicity of compounds [28]), a measured or calculated amount of the fraction ionised species at physiological pH and fraction unbound in plasma obtained by traditional methods. The model provides a good estimate for the *in vivo* volume of distribution for neutral and basic drugs only; acids were not predicted very well. As it was described earlier, the traditional lipophilicity measures of compounds produce low values for ionised species, regardless of the type of charge, positive or negative. However, the IAM and HSA binding are affected by the type of charge very differently. Positively charged compounds bind much stronger to phospholipids than albumin, thus the volume of distribution of positively charged compounds are large. The negative charge on the molecule has an opposite effect; it reduces the volume of distribution as the compound binds more strongly to albumin and accumulates in the plasma compartment. The introduction of the fraction ionised values of basic compounds in the model accounted for the effect of the positive charge. The effect of the negative charge was not accounted for in the model. Another model was published more recently by Siu *et al.* [83], who applied the bio-mimetic chromatographic retention using IAM stationary phases. As the IAM retention is very sensitive to the presence of positive charge on the molecule and negatively charged compounds do not bind very strongly to IAM, the model was applicable for basic, neutral and acidic compounds as well.

Figure 2.21. The plot of *in vivo* human clinical steady state volume of distribution and the estimated volume of distribution by Equation 2.28 for 70 known drug molecules

Table 2.7. The measured HPLC based membrane partition (IAM) and human serum albumin (HSA) binding for the known drug molecules [80].

DRUG	$\log k_{\text{(HSA)}}$	$\log K_{\text{(HSA)}}$	HSA, %	CHIAM	$\log k_{\text{(IAM)}}$	$\log K_{\text{(IAM)}}$
Acetaminophen	-0.79	0.45	14	3.97	0.6	1.83
Acyclovir	-1.25	0.29	5.38	-6.98	0.1	1.1
Amoxicillin	-0.71	0.49	16.42	6.31	0.71	2.03
Ampicillin	-0.58	0.56	21.17	6.26	0.71	2.03
Amrinone	0.28	1.32	66.14	15	1.11	3.03
Aspirin	-0.23	0.79	37.34	-1.73	0.34	1.41
Betamethasone	0.09	1.09	55.58	31.73	1.88	6.55
Bromazepam	0.32	1.38	68.46	28.45	1.73	5.63
Bumetanide	1.15	3.15	94.3	26.08	1.62	5.05
Bupivacaine HCl	-0.16	0.85	41.26	39.89	2.25	9.53
Ceftazidime	-1.07	0.34	7.87	-5.09	0.19	1.2
Cephalexin	-0.79	0.45	14	-2.27	0.32	1.37
Chlorpheniramine	0.18	1.2	60.95	47.19	2.59	13.34
Cimetidine	-0.58	0.56	21.17	16.59	1.18	3.26
Ciprofloxacin	-0.09	0.91	45.02	47.82	2.62	13.73
Clonazepam	0.51	1.66	77.11	34.77	2.02	7.54
Cytarabine	-0.38	0.69	29.8	-14.86	-0.26	0.77
Diazepam	1.08	2.94	93.22	37.41	2.14	8.51
Digitoxin	0.49	1.64	76.43	27.46	1.68	5.38

DRUG	$\log k_{(\text{HSA})}$	$\log K_{(\text{HSA})}$	HSA, %	CHIAM	$\log k_{(\text{IAM})}$	$\log K_{(\text{IAM})}$
Diltiazem	0.14	1.15	58.46	41.63	2.33	10.33
Diphenhydramine	0.08	1.09	55.39	44.57	2.47	11.82
Doxepin	0.67	1.95	83.15	52.31	2.83	16.89
Ethosuximide	-0.68	0.51	17.49	-5.37	0.17	1.19
Felbamate	0.33	1.39	68.67	24.62	1.55	4.72
Finasteride	0.45	1.57	74.73	38.86	2.21	9.09
Flumazenil	-0.58	0.56	20.86	18.37	1.26	3.54
Ganciclovir	-0.84	0.43	12.76	-11.62	-0.11	0.89
Gemfibrozil	1.29	3.64	96.08	32.89	1.93	6.91
Glipizide	1.2	3.32	95.01	21.05	1.39	4.01
Haloperidol	0.61	1.84	81.06	45.17	2.5	12.16
Hydrochlorothiazide	-0.26	0.77	35.61	15.93	1.15	3.17
Imipramine	0.67	1.95	83.15	54.14	2.91	18.37
Indomethacin	1.82	6.17	99.5	32.46	1.91	6.77
Isradipine	1.05	2.86	92.78	40.03	2.26	9.59
Ketoprofen	1.57	4.8	98.35	21.94	1.43	4.17
Lorazepam	0.96	2.62	91.1	37.29	2.14	8.46
Methylprednisolone	0.08	1.08	54.93	32.14	1.9	6.67
Metoclopramide	0.02	1.02	51.4	35.35	2.05	7.74
Metronidazole	-1.25	0.29	5.38	-3.32	0.27	1.31
Nabumetone	1.15	3.16	94.32	38.42	2.19	8.91
Naproxen	1.95	7.06	99.89	22.85	1.47	4.35
Nicardipine	1.07	2.93	93.16	45.86	2.53	12.55
Nifedipine	0.34	1.41	69.54	29.02	1.75	5.78
Nizatidine	-0.6	0.55	20.42	17.88	1.24	3.46
Ondansetron	0.52	1.69	77.67	37.15	2.13	8.41
Phenobarbital	-0.27	0.76	35.22	13.58	1.04	2.84
Phenytoin	0.47	1.6	75.47	31.55	1.87	6.5
Phenytoin	0.61	1.84	81.04	31.09	1.85	6.36
Piperacillin	-0.74	0.48	15.44	9.43	0.85	2.35
Piroxicam	1.47	4.36	97.71	6.75	0.73	2.08
Prazosin	0.59	1.8	80.37	31.62	1.87	6.52
Prednisolone	-0.12	0.89	43.58	27.99	1.71	5.52
Prednisone	-0.23	0.8	37.56	25.87	1.61	5
Procainamide	-0.27	0.76	35.22	19.92	1.34	3.8
Propranolol	0.29	1.33	66.51	45.12	2.5	12.13
Quinidine	0.51	1.66	76.95	44.95	2.49	12.03
Quinine	0.5	1.65	76.65	47.11	2.59	13.29
Rifampin	0.51	1.67	77.17	35.98	2.08	7.97
Saquinavir	1.22	3.38	95.24	43.46	2.42	11.23

DRUG	$\log k_{(\text{HSA})}$	$\log K_{(\text{HSA})}$	HSA, %	CHIAM	$\log k_{(\text{IAM})}$	$\log K_{(\text{IAM})}$
Sulfamethoxazole	-0.36	0.7	30.86	31.6	1.87	6.51
Terbutaline	-0.4	0.67	28.79	16.83	1.19	3.3
Tolbutamide	1.28	3.59	95.95	10.24	0.89	2.44
Trazodone	0.9	2.46	89.75	36.26	2.09	8.07
Trimethoprim	-0.19	0.82	39.49	20.77	1.38	3.96
Verapamil	0.53	1.7	77.94	41.96	2.35	10.49
Warfarin	1.49	4.45	97.86	19.9	1.34	3.8
Zidovudine	-0.84	0.43	12.76	1.27	0.48	1.61
Zolmitriptan	-0.36	0.7	30.61	29.71	1.79	5.97
Zolpidem	0.43	1.54	73.58	33.91	1.98	7.24

2.6.2. Modelling unbound volume of distribution

The unbound volume of distribution, V_{du} , which is proportional to the drug's distribution between the free and bound compartments, showed good correlation with the sum of the phospholipid and albumin binding as described by Equation 2.29.

$$\log V_{\text{du}} = 0.24 \log K_{(\text{HSA})} + 0.12 \log K_{(\text{IAM})} - 0.4 \quad (2.29)$$

($n = 70$; $r = 0.89$; $sd = 0.40$)

The plots of the values found in the literature along with calculated $\log V_{\text{du}}$ values are shown in Figure 2.22. The models described and validated by the data of 130 known drug molecules have been published recently [84].

Figure 2.22. The plot of the literature $\log V_{\text{du}}$ and the calculated $\log V_{\text{du}}$ values by Equation 2.29 for 70 known drug molecules

It is very important to note that the unbound volume of distribution is a good measure of the *in vivo* efficacious potential of the drug molecule as it is related to the proportion of the free concentration of the drug relative to the administered dose. The unbound volume of distribution is reduced by both albumin and phospholipids binding as expressed by Equation 2.29. The lipophilicity of the compound increases both types of binding. The presence of charge has a significant effect either on the IAM binding (positive charge) or the HSA binding (negative charge). Thus, reducing the octanol/water distribution coefficient ($\log D$) or reversed phase partition by introducing charge on the molecules will have very little effect on the unbound volume of distribution.

2.6.3. Dependence of unbound volume of distribution on HSA and IAM binding

Figures 2.23 and 2.24 show the plots of the logarithmic value of the unbound volume of distribution relative to compounds binding to HSA and IAM, respectively.

Figures 2.23 and 2.24 illustrate that in spite of some compounds having high HSA binding the unbound volume of distribution can still be low (mostly for acidic compounds), while high IAM binding has a great impact on the increase of the unbound volume of distribution.

Figure 2.23. The plot of the logarithmic unbound volume of distribution, $\log V_{du}$, as a function of HSA binding ($\log k_{HSA}$)

Figure 2.24. The plot of the logarithmic unbound volume of distribution, $\log V_{du}$, as a function of IAM binding ($\log k_{IAM}$)

These models are the first attempt for estimating parameters such as volume of distribution that can be obtained only from *in vivo* experiments by using simple *in vitro* bio-mimetic chromatographic retention time measurements. These models are able to accommodate the charged molecules; moreover they reveal that the presence of positive charge drives drug molecules towards the tissues, as positively charged compounds show extremely strong affinity to phosphatidyl choline relative to the albumin affinity. It is also well known that positively charged drugs usually have a high volume of distribution and strong tissue binding.

Negatively charged compounds bind strongly to albumin and usually have a very low volume of distribution. Obviously, these models are very crude as the *in vivo* system has been simplified and considered only as two compartments and each compartment is characterised only by one major constituent. The drug distribution *in vivo* will deviate from the expected distribution if there is a permeability barrier or active transport process is involved that prevents the drug molecule from obtaining thermodynamic equilibrium. Deviation of the drug distribution can be expected if a compound specifically binds to constituents in either compartment which are present in smaller quantities than albumin and phospholipids. The thermodynamic steady state equilibrium of the drug molecule in the *in vivo* compartments should also be independent from the applied dose, which means that the fraction unbound in plasma, or the volume of distribution is constant even when various amounts of drug is administered. When this is not the case, i.e. non-linear pharmacokinetics are observed, this suggests that the drug binds to specific binding sites that can be saturated by the therapeutically required dose of the drug. This situation can cause significant individual variations in the free plasma concentration in the clinical setting and can cause variable efficacy in different populations of the patients. This phenomenon has been

observed with drugs that bind specifically and strongly to AGP [85]. As the AGP concentration in the plasma can vary depending on age, gender and disease state [86], the same dose can produce very different free plasma concentrations in different patient populations, thus the efficacious dose has to be monitored for each patient. The measurement of AGP binding is very important in early drug discovery in order to avoid late stage failure due to variable or lack of efficacy in the clinic. The strong specific AGP binding of a compound can be suspected when the AGP binding is much stronger than expected based on the measured lipophilicity.

The physicochemical and bio-mimetic chromatographic partitioning systems are useful for measuring thermodynamic equilibrium or affinity constants of molecules. The volume of distribution even at steady state involves kinetic and rate processes as well. Thus, we have to keep in mind the other form of expressing the volume of distribution which is shown by Equation 2.30.

$$V_d = t_{1/2} \times \text{clearance} \quad (2.30)$$

When modelling the steady state volume of distribution we suppose that the plasma concentration is constant over a period of time as the dose is repeated according to the rate of elimination (clearance) and half life ($t_{1/2}$). The importance of V_{du} is gaining more and more attention, since the introduction of the “drug efficiency” concept [87]. Drug efficiency, $DRUG_{eff}$, is defined as the quotient of the free bio-phase concentration and the dose multiplied by 100. The aim is to achieve the highest free concentration of the drug with a minimum dose. When the free drug hypothesis can be applied, the free plasma concentration is assumed equal to the maximum free bio-phase concentration at the site of action, thus the unbound volume of distribution is in principle the reciprocal value of the drug efficiency. Thus, compounds with the lowest unbound volume of distribution have the highest drug efficiency. It means that modelling the unbound volume of distribution enables us to estimate the drug efficiency early in the drug discovery process, before the selection of a candidate molecule for further development. If the potency of the compound is known and we have an idea as to the number of receptors the drug molecule needs to occupy, then we can estimate the required dose to achieve 50% receptor/target occupancy *in vivo* by modelling the unbound volume of distribution.

Besides the drug's distribution between the two major compartments of the body, several attempts have been made to model blood/brain distribution, skin penetration and oral absorption using simple chromatographic measurements of the drug's properties. The IAM retention data of drugs were used to estimate brain to blood partition [88] and compound permeation through the intestinal wall [89]. There are several publications [90-93] about the use of bio-partitioning micellar chromatographic retention to mimic biological *in vivo* distribution processes. However, when the physico-chemical or bio-mimetic models are applied for the estimation of compounds *in vivo* behaviour it is very important to keep in mind the limitations of such models. Just because a statistically

acceptable correlation exists between the measured *in vitro* and *in vivo* data does not mean that there will be no outlier compounds from such correlations. We estimate the behaviour of the drug molecules *in vivo* based on physico-chemical properties ignoring all the possible biological processes, such as active efflux or transport. In spite of the limitation to only thermodynamic processes, these models are still very informative even if they are not predictive for the *in vivo* situation. They allow us to estimate the compound's behaviour when there is no active transport or efflux processes present. When discrepancies are observed between the estimated and measured *in vivo* distribution, research can focus on the biological processes that cause such discrepancies.

2.6.4. General structure-property relationships for lead optimisation

Another important application of the measured bio-mimetic and physicochemical properties is building structure-property relationships. These relationships enable medicinal chemists to alter the chemical structure and design molecules with desirable *in vivo* behaviour. These structure-activity studies reveal which part of the molecule interacts with the target receptor or enzyme, and which part can be modified to maintain the potency and improve the *in vivo* drug distribution. It is also very useful to develop *in silico* calculations to predict the physico-chemical properties of the molecules before synthesis. Based on a vast amount of accurately measured properties of discovery compounds, a large amount of information is available now for estimating the effect of certain substituents or properties on the compound's *in vivo* behaviour. The simplest approach is to compare the properties of pairs of molecules which differ only from one substitution. Thus, the effect of the substituents on certain properties can be estimated [94]. The solvation Equation 2. approach also can be used to estimate of effect of H-bond donor and acceptor groups, or the change in polarity on the biological distribution processes (see Table 2.5).

Table 2.8 summarizes the major structure–property relationships observed for the physico-chemical and bio-mimetic properties of drug discovery compounds.

Table 2.8. General structure–property relationships for various physico-chemical and bio-mimetic chromatographic distribution systems

Distribution system	Structural features
Reversed-phase HPLC retention	In general it is driven by a compound's lipophilicity; H-bond donor groups reduce partitioning to C-18 much stronger than the octanol/water lipophilicity; increase of molecular weight generally increases retention
Immobilised artificial membrane affinity (IAM retention)	It increases with increasing lipophilicity expressed by octanol/water partition; positively charged compounds have strong affinity to IAM and consequently compounds with high IAM binding are expected to have a large volume of distribution and strong tissue binding

Distribution system	Structural features
Serum albumin binding (HSA retention)	It increases with increasing lipophilicity expressed by octanol/water partition; H-bond donor groups do not reduce binding; negatively charged compounds bind very strongly; strong binding reduces the volume of distribution
Alpha-1-acid-glycoprotein binding (AGP retention)	It is increased with increasing lipophilicity; H-bond donor groups reduce binding; positive charge increases binding
Blood/brain distribution	It is increasing with lipophilicity; H-bond donor groups significantly reduce compounds partitioning into the brain
Water/skin partition	It increases with increasing lipophilicity; H-bond donor groups reduce skin penetration
Intestinal absorption	Hydrophilic and large compounds have limited absorption; optimum lipophilicity is required; both H-bond donor and acceptor groups reduce absorption
Volume of distribution	In general it increases with increasing lipophilicity; positive charge increases, negative charge decreases volume of distribution
Unbound volume of distribution	It increases with increasing lipophilicity of the uncharged molecule; H-bond donor groups do not affect the unbound volume; phospholipid binding has more pronounced effect on unbound volume of distribution than plasma protein binding

2.7. ALTERNATIVE METHODS

As discussed above, the HPLC retention times obtained using reversed phase and bio-mimetic stationary phases can be used to measure compound relevant properties for estimating *in vivo* behaviour. The lipophilicity, protein binding and phospholipid binding affect compound distribution between plasma and tissue compartments and determine the free concentration of the drug at the site of action. There are several other methods that can be used for similar purposes.

2.7.1. Methods based on the principle of frontal chromatography

The chromatographic methods described in this chapter are based on the most often used elution mode of chromatography. The frontal mode of chromatography provides another alternative for measuring compound interaction with stationary phases. Frontal chromatography is a mode of chromatography in which the sample is introduced continuously into the column. The sample components migrate through the column at different velocities and eventually break through as a series of fronts. Only the least retained component exits the column in pure form. Thus, they produce continuous detector signals (not peaks as in the elution mode) that form steps as shown in Figure 2.25. The exact shape of a breakthrough curve is mainly determined by the functional form of the underlying equilibrium isotherms of the sample components, but secondary factors such as diffusion and mass-transfer kinetics also have an

influence. When only one sample component is introduced the molecules occupy the strongest binding sites of the stationary phase first. When all such binding sites are occupied the molecules start adsorbing to the weaker binding sites. The shape of the detector response curve for the breakthrough process can be analysed and the binding strength of the compound to the various binding sites of the stationary phase can be calculated. The application of frontal chromatography for the determination of compound's binding to proteins has been discussed in more detail by Shibukawa *et al.* [95].

Figure 2.25. Typical chromatogram obtained by frontal chromatography

The method is useful to determine compound binding to various binding sites of the protein. It also provides information about the kinetic aspects of the binding. HPLC instrumentation can be used to carry out the analysis. The calculation of the binding constants requires knowledge of the exact amount of the protein on the stationary phase surface. The column cannot be used immediately for the measurement of another compound, as it needs regeneration to remove the absorbed molecules from the surface. Therefore, the methodology is not applicable at early stages of the drug discovery process when thousands of new compounds have to be characterised in a short period of time. A similar principle is applied to an instrument developed by Biacore™. The instrument measures the Plasmon resonance on a gold surface where various protein molecules can be immobilised. The sample molecules are introduced on the surface until they fully occupy the binding sites on the immobilised protein. The sample molecules are then slowly removed by washing the surface with buffer (normally at physiological pH). Figure 2.26 shows a typical detector response curve.

Figure 2.26. Typical Surface Plasmon Resonance (SPR) response obtained by Biacore™.

From the surface plasmon resonance (SPR) curve shown in Figure 2.26, not only can the binding strength be calculated, but also the onset and offset rates of the binding. Day and Myszka [96] described a procedure in detail for the characterization of compounds binding to the albumin major binding sites. Oravcova *et al.* [54] have reviewed the application of chromatographic principles in affinity chromatography and size exclusion chromatography. The advantages of these alternative techniques are that they provide more detailed insight into the drug – protein binding identifying the various binding sites on the proteins and the binding kinetics. The understanding of the binding mode and kinetics of a molecule to a protein is very important in the drug design point of view too, as it provides information to medicinal chemists about the structural motives that might be involved in the binding. The Biacore™ technique is very useful for studying drug – target interactions as very different types of molecules can be immobilised on the gold surface and to be studied by SPR. This technique is very versatile and can be applied at various stages of the drug discovery process. On the other hand, these methods do not provide information about the weaker non-specific binding sites. Moreover, the possible non-specific binding of the compound to the protein surface actually disturbs these measurements as the one to one association constant cannot be calculated precisely. The individual binding constants are very difficult to use to calculate the free and bound drug concentration *in vivo* where not only individual binding sites, but a large amount of other proteins are also present. Therefore, these alternative methods provide slightly different types of information and can be applied at a different stage of the drug discovery process.

2.7.2. Methods based on solid particles

Another methodology has been recently introduced using solid supported proteins and lipids (*Transil*TM technology) in order to improve the throughput of protein and lipid binding measurements [97,98]. Figure 2.27 demonstrates the basic principles of the method.

Figure 2.27. The application of solid supported protein and lipid binding using the *Transil*TM technology

The method is applicable at early stages of drug discovery to measure a large number of discovery compounds using robotics and the 96-well plate format. The compound is equilibrated with the protein or lipid immobilised on solid silica particles. After equilibration and filtration, the compound concentration (unbound) can be determined using a generic gradient reversed phase HPLC method. The measurement of the sample solution before equilibration with the solid particles can provide the standard chromatogram. The difference between the peak areas obtained before and after equilibration with the protein and lipid particles indicates the amount of bound compound. The total specific and non-specific binding is obtained by the method; hence, the data can be used directly in models such as volume of distribution. The commercially available PK-MAP software (Bayer AG, Germany) uses the input data obtained by the *Transil* methodology to estimate a compound's *in vivo* volume of distribution. Two disadvantages become apparent when comparing this method with the method based on chromatographic retention time. Firstly, one needs to use a chromatographic method twice in order to determine the concentration of the compound before and after the equilibration with the solid particles and calculate the binding constant. Secondly, as the method requires concentration determination, the error increases when the binding of the compound is high, and requires a very sensitive and accurate analytical technique to measure strongly bound compounds.

2.7.3. Methods based on equilibrium dialysis and ultra-filtration

The equilibrium dialysis method for the determination of plasma protein binding and tissue binding can be considered as the traditional gold standard method. The process involves separating the macromolecules, such as plasma proteins and tissue homogenate from low molecular weight compounds in the solution through a semi-permeable membrane as shown in Figure 2.28. The small molecule that is bound to the macromolecule will not be able to cross the membrane. The concentration of the unbound molecules will be the same on both sides of the membrane after equilibrium has been reached. Measuring the concentration of the small molecule in the compartment where no macromolecules are present provides the unbound concentration. The technique is suitable to measure compound binding to a complex biological mixture of proteins and tissue constituents even directly obtained from patients after *in vivo* administration of the drug molecule. Thus, the result gives a precise measure of the free drug concentration *in vivo*; this is the biggest advantage of the technique. It can be used in a high throughput manner using a validated rapid equilibrium dialysis device [99]. The device contains the two compartments separated by the membrane in a 96-well plate format.

As the binding measurement is based on the determination of the free concentration, the method gets less accurate at the high binding region. Depending on the accuracy and sensitivity of the concentration determination method (usually HPLC/MS), compounds that are more than 99% bound to the plasma or tissue cannot be differentiated. The binding equilibrium is distorted due to the dilution process which happens upon the addition of a buffer compartment on the other side of the membrane. The dilution factor has to be included in the calculations.

Figure 2.28. The schematic illustration of the equilibrium dialysis measurement.

The ultra-filtration method is based on a similar principle, but instead of the application of a semi-permeable membrane a filtration procedure is applied for the separation of the unbound compound from the protein bound compound [100]. The concentration of the unbound compound has to be determined in the filtrate. These methodologies can be applied at later stages of the drug discovery process. They provide information on the binding properties of the compounds that is relevant to *in vivo* circumstances; however it is more difficult to use this information to investigate structure – binding properties as it describes the compound binding to a complex mixture of proteins.

REFERENCES

1. C.A. Lipinski, F. Lombardo, B.W. Dominy, P.J. Feeny. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* **23** (1997) 3-25.
2. Avdeef. Absorption and drug development; Solubility, permeability and charge state. *Wiley-Interscience*, 2003.
3. P.D. Leeson, B. Springthorpe. The influence of drug-like concepts on decision-making in medicinal chemistry. *Nature Reviews in Drug Discovery* **6** (2007) 881-890.
4. K. Valko, D.P. Reynolds. High-throughput physicochemical and *in vitro* ADMET screening; A role in pharmaceutical profiling. *American Pharmaceutical review* **3** (2005) 83-100.
5. M.P. Gleeson, A. Hersey, D. Montanari, J. Overington. Probing the links between *in vitro* potency, ADMET and physicochemical parameters. *Nature Reviews in Drug Discovery* **10** (2011) 197-208.
6. E. Kerns, L. Di. Drug-like properties: concepts, structure design and methods: from ADME to toxicity optimization. *Academic Press* 2008.
7. H. Van de Waterbeemd, B.C. Jones. Predicting oral absorption and bioavailability. *Progress in Medicinal Chemistry*. Ed. F.D. King, Oxford, A.W. **41** (2003) 1-59.
8. ACDlogP, Advanced Chemistry Development, www.acdlabs.com.
9. clogP calculator, BioByte, www.biobyte.com.
10. logP calculator, Chemaxon, www.chemaxon.com.
11. Horvath, W. Melander, I. Molnar. Solvophobic interactions in liquid chromatography with non-polar stationary phases. *Journal of Chromatography A* **125** (1976) 129-156.
12. L.R. Snyder, J.J. Kirkland, J.W. Dolan. Introduction to modern liquid chromatography. *Wiley* (2010).
13. V.R. Meyer. Practical high-performance liquid chromatography. *Wiley* (2010)
14. F. Leroy, B. Presle, F. Verillon, E. Verette. Fast generic-gradient reversed-phase high-performance liquid chromatography using short narrow-bore columns packed with small nonporous silica particles for the analysis of combinatorial libraries. *Journal of Chromatographic Sciences* **39** (2001) 487-490.
15. Mutton. Fast generic HPLC methods. In *Handbook of analytical separations* Vol. 1. Separation methods in drug synthesis and purification. Ed. K. Valko, *Elsevier* (2000) 73-85.

16. J.M. Call. Liquid-liquid partition coefficients by high-pressure liquid chromatography. *Journal of Medicinal Chemistry* **18** (1975) 549-552.
17. D.A. Brent, J.J. Sabatka, D.J. Minick, D.J. Henry. A simplified high-pressure liquid chromatography method for determining lipophilicity for structure-activity relationships. *Journal of Medicinal Chemistry*, **26** (1983) 1014-1020.
18. D.J. Minick, D.A. Brent, J. Frenz. Modeling octanol-water partition coefficients by reversed-phase liquid chromatography. *Journal of Chromatography A* **461** (1989) 177-191.
19. E. Tomlinson. Chromatographic hydrophobicity parameters in correlation analysis of structure – activity relationships. *Journal of Chromatography A* **113** (1975) 1-45.
20. T. Braumann, G. Weber, L.H. Grimme. Quantitative structure-activity relationships for herbicides: Reversed-phase liquid chromatographic retention parameter, log *k_w* versus liquid-liquid partition coefficient as model of the hydrophobicity of phenylureas, s-triazines and phenoxycarbonic acid derivatives. *Journal of Chromatography* **261** (1983) 329-343.
21. R. Kaliszan. Quantitative structure –retention relationships. *Analytical Chemistry* **64** (1992) 619A-631A.
22. J.G. Dorsey, M.G. Khaledi. Hydrophobicity estimations by reversed-phase liquid chromatography: Implication for biological partition processes. *Journal of Chromatography A* **656** (1993) 485-499.
23. K. Valko. Measurements of physical properties for drug design in industry. In *Handbook of analytical separations* Vol. 1. Separation methods in drug synthesis and purification. Ed. K. Valko, Elsevier (2000) 535-583.
24. M.S. Mirrlees, S.J. Moulton, C.T. Murphy, P. Taylor. Direct measurement of octanol-water partition coefficients by high-pressure liquid chromatography. *Journal of Medicinal Chemistry* **19** (1976) 615-619.
25. S.H. Unger, J.R. Cook, J.S. Hollenberg. Simple procedure for determining octanol-aqueous partition, distribution and ionization coefficients by reversed-phase high-pressure liquid chromatography. *Journal of Pharmaceutical Sciences* **67** (1978) 1364-1367.
26. D.J. Minnick, J.H. Frenz, M.A. Patrick, D.A. Brent. A comprehensive method for determining hydrophobicity constants by reversed phase high-performance liquid chromatography. *Journal of Medicinal Chemistry* **31** (1988) 1923-1933.
27. F. Lombardo, M.Y. Shalaeva, K.A. Tupper, F. Gao, M.H. Abraham. Elog_{P_{oct}}: A tool for lipophilicity determination in drug discovery. *Journal of Medicinal chemistry* **43** (2000) 2922-2928.
28. F. Lombardo, M.Y. Shalaeva, K.A. Tupper, F. Gao. Elog_{D_{oct}}: A tool for lipophilicity determination in drug discovery. 2. Basic and neutral compounds. *Journal of Medicinal Chemistry* **44** (2001) 2490-2497.
29. M. Harnisch, H.J. Möckel, G. Shulze. Relationship between log *P_{ow}*, shake flask values and capacity factors derived from reversed-phase high performance liquid chromatography for n-alkylbenzenes and some oecd reference substances. *Journal of Chromatography A* **282** (1983) 315-332.
30. K. Valko. General approach for the estimation of octanol/water partition coefficient by reversed-phase high performance liquid chromatography. *Journal of Liquid Chromatography* **7** (1984) 1405-1424.

31. K. Valko, P. Slegel. New chromatographic hydrophobicity index (ϕ_0) based on the slope and the intercept of the log k' versus organic phase concentration plot. *Journal of Chromatography A* **631** (1993) 49-61.
32. K. Valko, C. Bevan, D. Reynolds. Chromatographic hydrophobicity index by fast-gradient RP-HPLC: A high throughput alternative to log P /log D . *Analytical Chemistry* **69** (1997) 2022-2029.
33. K. Valko. Measurements of lipophilicity and acid/base character using HPLC methods. In "Pharmaceutical profiling in drug discovery for lead selection". Eds. R. Borchardt, E. Kerns, AAPS (2004) Arlington, VA 127-182.
34. E.H. Kerns, L. Di, S. Petusky, T. Kleintop, D. Huryn, O. McConnell, G. Carter. Pharmaceutical profiling method for lipophilicity and integrity using liquid chromatography-mass spectrometry. *Journal of Chromatography B* **791** (2003) 381-388.
35. S.F. Donovan, M.C. Pescatore. Method for measuring the logarithm of octanol-water partition coefficient by using short octadecyl-poly(vinyl alcohol) high performance liquid chromatography columns. *Journal of Chromatography A* **952** (2002) 47-61.
36. G. Camurri, A. Zamarella. High-throughput liquid chromatography/mass spectrometry method for the determination of chromatographic hydrophobicity index. *Analytical Chemistry* **73** (2001) 3716-3722.
37. C.M. Du, K. Valko, C. Bevan, D. Reynolds M.H. Abraham. Rapid gradient RP-HPLC method for lipophilicity determination: A salvation equation based comparison with isocratic methods. *Analytical Chemistry* **70** (1998) 4228-4234.
38. J.W. Dolan, D.C. Lommen, L.R. Snyder. Drylab® computer simulation for high-performance liquid chromatographic method development II. Gradient elution. *Journal of Chromatography A* **485** (1998) 91-112.
39. Canals, K. Valko, E. Bosch, A.P. Hill, M. Roses. Retention of ionisable compounds on HPLC. 8. Influence of mobile-phase pH change on the chromatographic retention of acids and bases during gradient elution. *Analytical Chemistry* **73** (2001) 4937-4945.
40. R. Kaliszan, P. Haber, T. Baczek, D. Siluk, K. Valko. Lipophilicity and pK_a estimates from gradient high-performance liquid chromatography. *Journal of Chromatography A* **965** (2002) 117-127.
41. P. Wiczling, P. Kawczak, A. Nasal, R. Kaliszan. Simultaneous determination of pK_a and lipophilicity by gradient RP-HPLC. *Analytical Chemistry* **78** (2006) 239-249.
42. R. Kaliszan, T.A.G. Noctor, I.W. Wainer. Quantitative structure – enantioselective retention relationships for the chromatography of 1,4-benzodiazepines on a human serum albumin based HPLC chiral stationary phase: An approach to the computational prediction of retention and enantioselectivity. *Chromatographia* **33** (1992) 546-550.
43. T.A.G. Noctor, M.J. Diaz-Perez, I.W. Wainer. Use of human serum albumin-based stationary phase for high performance liquid chromatography as a tool for rapid determination of drug plasma protein binding. *Journal of Pharmaceutical Sciences* **82** (1993) 675-676.
44. P.R. Tiller, I.M. Mutton, S.J. Lane, C.D. Bevan. Immobilized human serum albumin: Liquid chromatography/mass spectrometry as a method of determining drug-protein binding. *Rapid Communications in Mass Spectrometry* **9** (1995) 261-263.
45. F. Beaudry, M. Coutu, N.K. Brown. Determination of drug-plasma protein binding using human serum albumin chromatography column and multiple linear regression model. *Biomedical Chromatography* **13** (1999) 401-406.

46. K. Valko, S.B. Nunhuck, C.D. Bevan, M.H. Abraham, D.P. Reynolds. Fast gradient HPLC method to determine compounds binding to human serum albumin. Relationships with octanol/water and immobilized artificial membrane lipophilicity. *Journal of Pharmaceutical Sciences* **92** (2003) 2236-2248.
47. C. Bertucci, M. Bartolini, R. Gotti, V. Andrisano. Drug affinity to immobilized target bio-polymers by high-performance liquid chromatography and capillary electrophoresis. *Journal of Chromatography B* **797** (2003) 111-129.
48. L. Buchholz, C.H. Cai, A. Cleton, J. Brodfuehrer, L. Cohen. Evaluation of the human serum albumin column as discovery screening tool for plasma protein binding. *European Journal of Pharmaceutical Sciences* **15** (2002) 209-215.
49. N. Kratochwil, W. Huber, F. Muller, M. Kansy, P.R. Gerber. Predicting plasma protein binding of drugs – revisited. *Current Opinion in Drug Discovery & Development* **7** (2004) 507-512.
50. G. Colmenarejo, A. Alvarez-Pedraglio, J-L. Lavandera. Cheminformatic models to predict binding affinities to human serum albumin. *Journal of Medicinal Chemistry* **44** (2001) 4370-4378.
51. R.C. Jewell, K.L.R. Brouwer, P.J.C. McNamara. α 1-acid glycoprotein high-performance liquid chromatography column (Enantiopac) as a screening tool for protein binding. *Journal of Chromatography* **487** (1989) 257-264.
52. Rapti. Evaluation of α 1-acid glycoprotein(AGP) binding measurements by high performance liquid chromatography & ultrafiltration Methods, *Project Report, Drug Discovery MSci*, School of Pharmacy, University of London, 2006.
53. R. Kaliszan, A. Nasal, M. Turowski. Binding site for basic drugs on alpha-1-acid glycoprotein as revealed by chemometric analysis of biochromatographic data. *Biomedical Chromatography* **9** (1995) 211-215.
54. J. Oravcova, B. Bohs, W. Lindner. Drug-protein studies trends in analytical and experimental methodology. *Journal of Chromatography B* **677** (1996) 1-28.
55. Pidgeon, U.V. Venkataram. Immobilized artificial membrane chromatography: Supports composed of membrane lipids. *Analytical Biochemistry* **176** (1989) 36-47.
56. Taillardat-Bertschinger, C. Marca Martinet, P.-A. Carrupt, M. Reist, G. Caron, R. Fruttero, B. Testa. Molecular factors influencing retention on immobilized artificial membranes (IAM) compared to partitioning in liposomes and n-octanol. *Pharmaceutical Research* **19** (2002) 729-737.
57. Taillardat-Bertschinger, P. A. Carrupt, F. Barbato, B. Testa. Immobilised Artificial membrane HPLC in Drug Research. *Journal of Medicinal Chemistry* **46** (2003) 655-665.
58. Giaginis, A. Tsantili-Kakoulidou. Alternative Measures of lipophilicity: From octanol/water partitioning to IAM retention. *Journal of Pharmaceutical Sciences* **97** (2008) 2984-3004.
59. B.H. Stewart, F.Y. Chung, B. Tait, C. John, O.H. Chan. Hydrophobicity of HIV protease inhibitors by immobilized artificial membrane chromatography: application and significance to drug transport. *Pharmaceutical Research* **15** (1998) 1401-1406.
60. K. Valko, C.M. Du, C. Bevan, D. Reynolds, M.H. Abraham. Rapid-gradient HPLC method for measuring drug interactions with immobilized artificial membrane: Comparison with other lipophilicity measures. *Journal of Pharmaceutical Sciences* **89** (2000) 1085-1096.
61. K. Valko, C.M. Du, C. Bevan, D.P. Reynolds, M.H. Abraham. Rapid method for the estimation of octanol/water partition coefficient ($\log P_{\text{oct}}$) from gradient RP-HPLC

- retention and a hydrogen bond acidity term ($\Sigma\alpha_2^H$). *Current Medicinal Chemistry* **8** (2001) 1137-1146.
62. Hansch, T. Fujita. ρ - σ - π analysis. A method for the correlation of biological activity and chemical structure. *Journal of American Chemical Society* **86** (1964) 1616-1626.
 63. M.H. Abraham. Scales of solute hydrogen bonding: Their construction and application to physicochemical and biochemical processes. *Chemical Society Reviews* **22** (1993) 73-83.
 64. M.H. Abraham, H.S. Chadha, G.S. Whitin, R.C. Mitchell. Hydrogen bonding. 32. An analysis of water-octanol and water-alkane partitioning and the $\Delta\log p$ parameter of seiler. *Journal of Pharmaceutical Science* **83** (1994) 1085-1100.
 65. A.Nasal, M. Sznitowska, A. Bucinski, R. Kaliszan. Hydrophobicity parameter from high-performance liquid chromatography on an immobilized artificial membrane column and its relationship to bioactivity. *Journal of Chromatography A*, 692 (1995) 83-89.
 66. M.H. Abraham, H.S. Cadha, R.A.E. Leiato, R.C. Mitchell, W.J. Lambert, R. Kaliszan, A. Nasal, P. Haber. Determination of solute lipophilicity, as $\log P(\text{octanol})$ and $\log P(\text{alkane})$ using poly(styrene-divinylbenzene) and immobilized artificial membrane stationary phases in reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* **766** (1997) 35-47.
 67. K. Valko, M. Plass, C. Bevan, D. Reynolds, M.H. Abraham. Relationship between the chromatographic hydrophobicity indices and solute descriptors obtained by using several reversed-phase, diol, nitrile, cyclodextrin and immobilised artificial membrane bonded high-performance liquid chromatography columns. *Journal of Chromatography A* **797** (1998) 41-55.
 68. C.M. Du, K. Valko, C. Bevan, D. Reynolds, M.H. Abraham. Characterizing the selectivity of stationary phases and organic modifiers in reversed-phase high performance liquid chromatographic systems by a general solvation equation using gradient elution. *Journal of Chromatographic Science* **38** (2000) 503-511.
 69. C.M. Du, K. Valko, C. Bevan, D. Reynolds, M.H. Abraham. Rapid method for estimating octanol-water partition coefficient ($\log P_{\text{oct}}$) from isocratic RP-HPLC and a hydrogen bond acidity term (A). *Journal of Liquid Chromatography & Related Technologies* **24** (2001) 635-649.
 70. M.H. Abraham, J.A. Platts in "The Blood/Brain Barrier and Drug Delivery to CNS". Editors D. J. Begley, M. W. Bradbury, J. Kreuter. Marcel Dekker, New York. NY 2000, 9-33.
 71. Y.H. Zhao, J. Le, M.H. Abraham, A. Hersey, P.J. Eddershaw, C.N. Luscombe, D. Butina, G. Beck, B. Sherborne, I. Cooper, J.A. Platts. Evaluation of human intestinal absorption data and subsequent derivation of a quantitative structure - activity relationship (QSAR) with the Abraham descriptors. *Journal of Pharmaceutical Sciences* **90** (2001) 749-784.
 72. M.H. Abraham, H.S. Chadha, R.C. Mitchell. The factors that influence skin penetration of solutes. *Journal of Pharmacy and Pharmacology* **47** (1995) 8-16.
 73. M.H. Abraham, M. Roses. Hydrogen bonding. 38. Effect of solute structure and mobile phase composition on reversed-phase high-performance liquid chromatographic capacity factors *Journal of Physical Organic Chemistry* **7** (1994) 672-684.

74. D.M. Cimpean, C.F. Poole. Systematic search for surrogate chromatographic models of biopartitioning processes. *Analyst* **127** (2002) 724-729.
75. M. Hidalgo-Rodriguez, E. Fuguet, C. Rafols, M. Roses. Estimation of biological properties by means of chromatographic systems: evaluation of the factors that contribute to the variance of biological – chromatographic correlations. *Analytical Chemistry* **82** (2010) 10236-10245.
76. M. Roses, D. Bolliet, C.F. Poole. Comparison of solute descriptors for predicting retention of ionic compounds (phenols) in reversed-phase liquid chromatography using the solvation parameter model. *Journal of Chromatography A* **829** (1998) 29-40.
77. B.B. Brodie, H. Kurtz, L.J. Schanker. The importance of dissociation constants in lipid solubility on influencing the passage of drugs into the cerebrospinal fluid. *Journal of Pharmacology and Experimental Therapeutics* **130** (1960) 20-25.
78. M.J. Banker, T.H. Clark, J.A. Williams. Development and validation of a 96-well equilibrium dialysis apparatus for measuring plasma protein binding. *Journal of Pharmaceutical Sciences* **92** (2003) 967-974.
79. S.S. Singh, J. Mehta. Measurement of drug-protein binding by immobilized human serum albumin-HPLC and comparison with ultrafiltration. *Journal of Chromatography B* **834** (2006) 108-116.
80. F. Hollosy, K. Valko, A. Hersey, S. Nunhuck, Gy. Keri, C. Bevan. Estimation of volume of distribution in humans from high throughput HPLC-based measurements of human serum albumin and immobilized artificial membrane partitioning. *Journal of Medicinal Chemistry* **49** (2006) 6958-6971.
81. F. Lombardo, R.S. Obach, F.M. DiCapua, G.A. Bakken, J. Lu, D.M. Potter, F. Gao, M.D. Miller, Y. Zhang. A hybrid mixture discriminant analysis- random forest computational model for the prediction of volume of distribution of drugs in human. *Journal of Medicinal Chemistry* **49** (2006) 2262-2267.
82. F. Lombardo, R.S. Obach, M.Y. Shalaeva, F. Gao. Prediction of Volume of distribution values in humans for neutral and basic drugs using physicochemical measurements and plasma protein binding data. *Journal of Medicinal Chemistry* **45** (2002) 2867-2876.
83. X. Sui, J. Sun, H. Li, Y. Wang, J. Liu, X. Liu, W. Zhang, L. Chen, Z. He. Prediction of volume of distribution values in human using immobilized artificial membrane partitioning coefficients, the fraction of compounds ionized and plasma protein binding data. *European Journal of Medicinal Chemistry* **44** (2009) 4455-4460.
84. K. Valko, S.B. Nunhuck, A.P. Hill. Estimating unbound volume of distribution and tissue binding by in vitro HPLC-based human serum albumin and immobilised artificial membrane – binding measurements. *Journal of Pharmaceutical Sciences* **100** (2011) 849-862.
85. T. Fournier, N.-N. Medjoubi, D. Porquet. Alpha-1-acid glycoprotein. Review. *Biochimica et Biophysica Acta – Protein Structure and Molecular Enzymology* **1482** (2000) 157-171.
86. M.K. Grandison, F.D. Boudinot. Age-related changes in protein binding of drugs. Implications for therapy. *Clinical Pharmacokinetics* **38** (2000) 271-290.
87. S. Braggio, D. Montanari, T. Rossi, E. Ratti. Drug efficiency: a new concept to guide lead optimization programs towards the selection of better clinical candidates. *Expert Opinion on Drug Discovery* **5** (2010) 609-618.

88. T. Salminen, A. Pulli, J. Taskinen. Relationship between immobilized artificial membrane chromatographic retention and the brain penetration of structurally diverse drugs. *Journal of Pharmaceutical and Biomedical Analysis* **15** (1997) 469-477.
89. J. Kotecha, S. Shah, I. Rathod, G. Subbaiah. Relationship between immobilized artificial membrane chromatographic retention and human oral absorption of structurally diverse drugs. *International Journal of Pharmaceutics* **333** (2007) 127-135.
90. L. Escuder-Gilabert, J.J. Martinez-Pla, S. Sagrado, R.M. Villanueva-Camanas, M.J. Medina-Hernandez. Bio-partitioning micellar separation methods: modelling drug absorption. *Journal of Chromatography B* **797** (2003) 21-35.
91. Y. Martin-Biosca, S. Torres-Cartas, R.M. Villanueva-Camanas, S. Sagrado, M.J. Medina-Hernandez. Biopartitioning micellar chromatography to predict blood to lung, blood to liver, blood to fat and blood to skin partition coefficients of drugs. *Analytica Chimica Acta* **632** (2009) 296-303.
92. L. Escuder-Gilabert, M. Molero-Monfort, R.M. Villanueva-Camanas, S. Sagrado, M.J. Medina-Hernandez. Potential of biopartitioning micellar chromatography as an in vitro technique for predicting drug penetration across the blood-brain barrier. *Journal of Chromatography B* **807** (2004) 193-201.
93. S. Torres-Cartas, Y. Martin-Biosca, R.M. Villanueva-Camanas, S. Sagrado, M.J. Medina-Hernandez. Biopartitioning micellar chromatography to predict mutagenicity of aromatic amines. *European Journal of Medicinal Chemistry* **42** (2007) 1396-1402.
94. A.G. Leach, H.D. Jones, D.A. Cosgrove, P.W. Kenny, L. Ruston, P. MacFaul, J.M. Wood, N. Colclough, B. Law. Matched molecular pairs as a guide in the optimization of pharmaceutical properties; a study of aqueous solubility, plasma protein binding and oral exposure. *Journal of Medicinal Chemistry* **49** (2006) 6672-6682.
95. Shibukawa, Y. Kuroda, T. Nakagawa. High performance frontal analysis for drug-protein binding study. *Journal of Pharmaceutical and Biomedical analysis* **18** (1999) 1047-1055.
96. Y.S.N. Day, D.G. Myszka. Characterizing a drug's primary binding site on albumin. *Journal of Pharmaceutical Sciences* **92** (2003) 333-343.
97. Loidl-Stahlhofen, T. Hartmann, M. Schottner, C. Rohring, H. Brodowsky, J. Schmitt, J. Keldenich. Multilamellar liposomes and solid-supported lipid membranes (TRANSIL): Screening of lipid-water partitioning toward a high-throughput scale. *Pharmaceutical Research* **18** (2001) 1782-1788.
98. J. Schuhmacher, C. Kohlsdorfer, K. Buchner, T. Brandenburger, R. Kruk. High-throughput determination of the free fraction of drugs strongly bound to plasma proteins. *Journal of Pharmaceutical Sciences* **93** (2004) 816-830.
99. N.J. Waters, R. Jones, G. Williams, B. Sohal. Validation of a rapid dialysis approach for the measurements of plasma protein binding. *Journal of Pharmaceutical Sciences* **97** (2008) 4586-4595.
100. J.B. Whitlam, K.F. Brown. Ultrafiltration in serum protein binding determinations. *Journal of Pharmaceutical Sciences* **70** (1981) 146-150.

THE IMPORTANCE OF AND DIFFERENT APPROACHES TO PERMEABILITY DETERMINATION

Kin Y. Tam, Matěj Velický and Robert A.W. Dryfe

Contents

3.1. INTRODUCTION	123
3.1.1. Permeability and the biopharmaceutical classification system	123
3.1.2. The rationale for permeability determinations in drug discovery	126
3.2. THEORETICAL BACKGROUND OF PAMPA.....	127
3.2.1. pH partition hypothesis.....	128
3.2.2. The PAMPA assay	129
3.2.3. Definition of permeability	130
3.2.4. Advanced transport model for PAMPA.....	131
3.2.5. Permeability terms	134
3.2.6. Permeability hydrodynamic model.....	134
3.2.7. Permeability-pH dependence	135
3.3. COMPARISONS OF DIFFERENT PAMPA METHODS.....	135

3.3.1. Lipid models.....	136
3.3.1.1. Egg lecithin	136
3.3.1.2. Dioleoylphosphatidylcholine	137
3.3.1.3. The double-sink method	137
3.3.1.4. <i>n</i> -Hexadecane	138
3.3.1.5. Mixture of lipids (biomimetic).....	138
3.3.1.6. Lipid/oil/lipid tri-layer membrane	138
3.3.1.7. Immobilised phospholipid vesicles	139
3.3.1.8. Lipid/cholesterol/octanol mixture.....	139
3.3.1.9. Three lipid-component model.....	140
3.3.1.10. Porcine brain tissue extract	140
3.3.2. Experimental procedures.....	140
3.3.2.1. pH and solution compositions	140
3.3.2.2. Detection	141
3.3.2.3. Hydrodynamics	142
3.4. APPLICATIONS OF PAMPA DATA TO PREDICT AND/OR MODEL <i>IN VIVO</i> DATA	151
3.4.1. Corrections for paracellular transport	152
3.4.2. Absorption model	154
3.4.3. Correlation between <i>in vitro</i> permeability and the absorbed fraction of drugs in humans	154
3.4.4. Prediction of the absorbed fraction of amphoteric drugs in humans.....	155
3.5. CONCLUDING REMARKS	158
Acknowledgements	159
REFERENCES.....	159

3.1. INTRODUCTION

The oral route remains the most preferable and popular way of administration for pharmaceuticals because of patient convenience. It offers the flexibility of using immediate or sustained release formulations. In developing new drugs, pharmaceutical companies generally focus their initial efforts on developing the drug as an oral candidate. However, if good oral absorption is difficult to achieve, other routes of administration, such as intravenous or intramuscular, may be developed as alternatives. Assuming metabolism and chemical degradation in the gastrointestinal (GI) tract are not significant, the rate and extent of absorption of a drug after oral administration depends on the concentration of the drug in the intestinal lumen and the permeability of the drug across the intestinal mucosa [1]. In many cases, especially when administered at a high dose, the drug concentration corresponds to the solubility of the drug in the lumen. Both solubility and permeability are dependent upon the structural characteristics of the drug, and are routinely determined in pharmaceutical research. The former is covered in Chapter 1 of this monograph. In this chapter, we will focus on the experimental aspects of permeability. First, we will briefly introduce drug permeability and absorption in the context of the biopharmaceutical classification system (BCS). Then, we will outline the rationale for permeability determination in the early phase of drug discovery. Particular emphasis will be placed on the different approaches based on artificial membranes for the determination of permeability.

3.1.1. Permeability and the biopharmaceutical classification system

Permeability and drug absorption are important factors governing the availability of a drug in the systematic circulation. Permeability refers to the flux of a drug across a membrane normalised with respect to the drug concentration at the membrane surface, while absorption generally refers to the movement of a drug into the bloodstream. The term absolute bioavailability offers a precise description of drug absorption, which could be defined as the fraction of an administered dose of drug that reaches the systemic circulation. When the dose is administered intravenously (iv), the bioavailability is 100 %. Figure 3.1 shows the typical plasma concentration time profiles obtained from an oral (po) or intravenous (iv) administration. For an orally administered dose (po), the absolute bioavailability (F) can be defined as [2]:

Figure 3.1. Plasma concentration as a function of time where the doses were administrated orally (po) or intravenously (iv)

$$F = \frac{\overline{AUC}_{PO}}{\overline{AUC}_{iv}} \quad (3.1)$$

where $\overline{AUC}$ represents the area under the curve normalised by the dose used by the respective route of administration. Taking hepatic first pass metabolism and gut wall metabolism into consideration, F may be expressed as [2]:

$$F = F_a \times F_h \times F_g \quad (3.2)$$

where F_a , F_h and F_g represent, respectively, the fraction absorbed from the intestinal lumen, the fraction which escapes hepatic first pass metabolism and the fraction which escapes gut wall metabolism.

Thus, drug molecules invariably need to permeate through intestinal epithelial layers and various cellular barriers, then distribute to their sites of action and elicit a pharmacological response. Drug movement across membranes can be classified as transcellular, paracellular and active transport. Figure 3.2 shows a schematic of these routes. Transcellular transport is a passive process and is most likely to occur for lipophilic drugs. Paracellular transport is also passive in nature but is regarded as a size-restricted process for small molecules. Active transport generally invokes transporter proteins that actively expel or take up a certain class of molecules. The most common and widely studied transporter is the P-glycoprotein (P-gp) efflux transporter, which can limit the oral bioavailability or central nervous system (CNS) penetration of certain types of drugs. Other transporter proteins that facilitate the uptake of drug molecules have been reported, for example the organic anion transporters [3]. It has been suggested that carrier-mediated and active uptake of drugs might account for the majority of

Figure 3.2. Routes of drug molecule transport across a membrane.

(1) Paracellular transport, (2) Transcellular transport, (3) Active transport:
(3a) carrier-mediated transport, (3b) efflux

membrane transport processes in biological systems [3]. A recent compelling study revealed that both passive processes and facilitated/carrier-mediated processes coexist and contribute to drug transport across biological membranes [4].

The biopharmaceutical classification system is a classification scheme provided by the U.S. Food and Drug Administration (FDA) for correlating *in vitro* drug properties (permeability and solubility) with *in vivo* performance (bioavailability) [5,6]. As shown in Table 3.1, drugs can be categorised into four basic groups according to their solubility and permeability. This classification system can be used to assess the biopharmaceutical risks of investigational new drugs. For drug molecules with an immediate release solid oral dosage form and properties fitting in class I, it may be possible to waive the requirement for *in vivo* bioavailability and bioequivalence studies for any new formulations in the future, provided that the dissolution rate of the new dosage form remains rapid.

Table 3.1. The biopharmaceutical classification system (BCS)

Class I High Solubility High Permeability	Class II Low Solubility High Permeability
Class III High Solubility Low Permeability	Class IV Low Solubility Low Permeability

In the assessment of drug permeability class, the absolute bioavailability in humans is the most obvious parameter to consider. For instance, high

bioavailability (*i.e.* > 90 %) generally implies high permeability. However, when high bioavailability cannot be achieved, the FDA recommends various experimental approaches to determine the permeability class. These include: (1) *in vivo* intestinal perfusion studies in humans; (2) *in vivo* or *in situ* intestinal perfusion studies using suitable animal models; (3) *in vitro* permeation studies using excised human or animal intestinal tissues; or (4) *in vitro* permeation studies across a cultured monolayer of animal (*e.g.* MDCK cells) or human epithelial cells (*e.g.* Caco-2 cells). It has been noted that *in vivo* or *in situ* animal models and *in vitro* methods using cultured monolayers of epithelial cells should be limited to the study of passively transported drugs. This is typically reflected by a linear (pharmacokinetic) relationship between the dose and measures of bioavailability, or the lack of concentration dependency on *in vitro* permeability across monolayers epithelial cells which are known to express efflux transporters (*e.g.* P-gp). The determination of permeability class is accomplished by a rank-order relationship using a sufficient number of model drugs tested using the same experimental approach. For example, a test drug may be regarded as highly permeable when its permeability is equal to or greater than that of a selected model drug with high permeability.

3.1.2. The rationale for permeability determinations in drug discovery

The BCS provides a rigorous framework to rationalise the interplay between the permeability/solubility and the *in vivo* performance (bioavailability) of a tested drug. As highlighted in Section 3.1.1, the recommended experimental approaches for determining permeability require the use of animal/human subjects or tissues. This would be viable for investigational new drugs in clinical development, as the project normally deals with a single active pharmaceutical ingredient. However, in the early stage of a discovery project, the primary focus is to develop and optimise a lead chemical series against a particular drug target of interest. Structural diversity is regarded as an important criterion to enable a thorough exploitation of the structure-activity relationship and pharmaceutical properties. Consequently, the direct and routine use of these experimental approaches in early stage drug discovery projects may exceed the available resources in some discovery organisations.

In 1998, Kansy *et al.* introduced the parallel artificial membrane permeability assay (PAMPA) [7]. Since then, the technique has received considerable attention in the pharmaceutical industry. PAMPA offers a simple physicochemical measure of passive permeability for research compounds. The throughput is higher than that of other *in vitro* cell-based permeability assays (*e.g.* Caco-2 or MDCK cell lines) and it is relatively cheap to run [8-10]. PAMPA is a useful tool in early phase discovery projects where permeability is seen to be an issue in the chemical series of interest. For instance, in combination with other *in vitro* assays, such as metabolic assessments, aqueous solubility and plasma protein binding, PAMPA can form a part of the testing cascade to evaluate the suitability of research compounds.

As the lead chemical series progresses towards the lead optimisation phase, it is of great interest to identify the most promising lead compounds to evaluate their pharmacokinetic and pharmacodynamic properties in pre-clinical animal models. Poor oral bioavailability could arise because of a number of reasons, including metabolic liability (hepatic metabolism), efflux transport or other active processes, gut wall metabolism, as well as suboptimal physical properties such as poor solubility or poor permeability. PAMPA data helped to assess the permeability of the test research compounds in the absence of other detrimental factors such as metabolism and efflux. Other aforementioned *in vitro* assays could be carried out in parallel as part of the testing cascade. This holistic approach allows the discovery project to spot issues quickly, triggers the design and synthesis of the next round analogues to moderate any identified issues and helps with the prioritisation of the most promising lead compounds for pharmacokinetic studies, with the goal of reducing the use of live animals on those compounds that are unlikely to show a reasonable level of oral exposure.

In the next two sections, we will turn our attention to the theoretical aspect of PAMPA, followed by a brief discussion on different PAMPA models developed since 1998. Finally, in Section 3.4, we will highlight two examples on the use of PAMPA data in pharmaceutical research.

3.2. THEORETICAL BACKGROUND OF PAMPA

For an orally administered dose, the absorption of the drug predominately occurs in the small intestine. The pH of the GI tract ranges from about 1 in the stomach to 6.5 in the small intestine, whereas bloodstream pH is about 7.4. According to the pH partition hypothesis (see Section 3.2.1), it is thought that only neutral species transfer across the lipophilic intestinal epithelial layers. Generally speaking, a neutral drug tends to be less soluble in a water-based environment than an ionised drug of a similar size. To circumvent this problem, an ionisable centre can be introduced. On the other hand, a large fraction of ionised species results in higher solubility in the aqueous phase (GI tract or bloodstream), but decreases the rate of drug transfer across the intestinal epithelial layers. In the case of a monoprotic drug, the fraction of neutral species, f_N , can be calculated using the Henderson-Hasselbalch equation:

$$f_N = \frac{1}{(1 + 10^{\pm(\text{pH} - \text{p}K_a)})} \quad (3.3)$$

where the sign in the exponent is + for acids and – for bases. $\text{p}K_a$ represents the acid dissociation constant. Table 3.2 shows the equations for calculating the neutral fraction and ionised fraction for monoprotic and diprotic drugs.

Table 3.2. Equations for calculating the neutral and ionised fractions for ionisable drugs

Charge type	Acid dissociation constant	Equations for calculating the neutral (f_N) ^a and ionised ($f_i(+)$, cation; $f_i(-)$, anion) ^b fractions
Monoprotic acid	pK_a	$f_N = \frac{1}{(1 + 10^{(pH - pK_a)})}$ $f_i(-) = 1 - f_N$
Monoprotic base	pK_a	$f_N = \frac{1}{(1 + 10^{-(pH - pK_a)})}$ $f_i(-) = 1 - f_N$
Diprotic acid	$pK_{a,1}, pK_{a,2}$ ($pK_{a,2} > pK_{a,1}$)	$f_N = \frac{1}{(1 + 10^{(pH - pK_{a,1})} + 10^{(2pH - pK_{a,1} - pK_{a,2})})}$ $f_i(-) = 1 - f_N$
Diprotic base	$pK_{a,1}, pK_{a,2}$ ($pK_{a,2} > pK_{a,1}$)	$f_N = \frac{1}{(1 + 10^{-(pH - pK_{a,2})} + 10^{-(2pH - pK_{a,1} - pK_{a,2})})}$ $f_i(+) = 1 - f_N$
Diprotic ampholyte/zwitterion	$pK_{a,1}, pK_{a,2}$ ($pK_{a,2} > pK_{a,1}$)	$f_N = \frac{1}{(1 + 10^{-(pH - pK_{a,1})} + 10^{(pH - pK_{a,2})})}$ $f_i(+) = \frac{1}{(1 + 10^{(pH - pK_{a,1})} + 10^{(2pH - pK_{a,1} - pK_{a,2})})}$ $f_i(-) = \frac{1}{(1 + 10^{-(pH - pK_{a,2})} + 10^{-(2pH - pK_{a,1} - pK_{a,2})})}$

^a This represents the sum of the fraction of the neutral and zwitterionic species for a diprotic zwitterion

^b These represent the sum of the fraction of single and double charged species for a diprotic acid or base

3.2.1. pH partition hypothesis

The commonly acknowledged pH partition hypothesis described by Jacobs in 1940 assumes that only the neutral fraction of a weak electrolyte can cross the cell membrane [11]. This hypothesis was extended to transport across biological membranes by Shore *et al.*, who published work on the gastric secretion of drugs [12]. This report studied the appearance of drug molecules in the gastric juices of dogs after they had been administered intravenously. As the drug concentration changed with its dissociation constant, it was deduced that only the non-ionised fraction of the drug molecule could be absorbed through cell membranes. This assumption was applied to drug transport across the intestinal epithelium by the

same research group [13] and was also tested in alternative systems such as diffusion through polymeric membranes [14]. The pH partition hypothesis is a good rule of thumb for the permeation/partitioning of drugs. It has, however, been criticised for being an oversimplification and for neglecting the fact that ionised species also permeate biological membranes although at a much slower rate than non-ionised species [15]. A number of reports in the 1990s showed evidence of partitioning of the ionised form of drug molecules to phosphatidylcholine bilayers [16-18]. In addition, paracellular transport through tight junctions in the intestinal epithelia was shown to be more common for cationic than other forms of drug molecules [19]. Similar observations were made for the epithelial monolayer in the Caco-2 assay, where significant transport of the ionised fraction was found when the neutral fraction concentration dropped to less than 10 % [20].

3.2.2. The PAMPA assay

The PAMPA method is based on passive diffusion of a drug molecule between two aqueous compartments, the donor and the acceptor, separated by an artificial membrane. The assay was originally designed as a model to mimic transcellular transport of orally administered drugs across the intestinal epithelium. The schematic of the assay concept is shown in Figure 3.3. The membrane is soaked with a lipid solution in a non-polar organic solvent. The drug is present in the donor compartment at an appropriate concentration (usually tens to hundreds of μM). The PAMPA is typically carried out in a commercial 96-well microtitre plate (acceptor) and a filter plate where the filter is coated with a lipid solution (donor). This enables the study of a large set of compounds in one experimental batch. A drug molecule, originally present in the aqueous donor compartment, diffuses across the hydrophobic membrane into the aqueous acceptor compartment. The drug concentrations in both compartments are then detected by an appropriate analytical technique and permeability is determined.

Figure 3.3. Schematic diagram of the PAMPA method

Different PAMPA models have been developed over the last decade. These will be discussed in Section 3.3. In the next section (Section 3.2.3), we will direct our attention to the theoretical aspect of permeability.

3.2.3. Definition of permeability

Permeability is regarded as a rate parameter which can be broadly defined as the flux of material (normalised against the concentration) transported across a defined surface. This may be exemplified using *in vivo* intestinal perfusion studies in humans. In this approach, a local anaesthetic is applied to an awake human subject, and a multichannel tube is introduced into the intestine [21]. With the multichannel tube, two small balloons are inflated, 10 cm apart, to create a closed segment, which is rinsed and then perfused with a marker compound and the test drug. The human jejunal effective permeability, P_{eff} , is inferred from the loss of the test drug from the perfusate, which can be described as below:

$$P_{\text{eff}} = \frac{Q_{\text{in}}(C_{\text{in}} - C_{\text{out}})}{C_{\text{out}} \times 2\pi r l} \quad (3.4)$$

where Q_{in} represents the volume flow rate of the perfusate entering the segment. C_{in} and C_{out} represent, respectively, the concentrations of the test drug entering and leaving the segment. The symbols r and l represent, respectively, the radius and length of the segment. Note that the units of permeability are expressed as cm/s.

In cases of *in vitro* permeation studies using excised intestinal tissues or monolayers of cultured human epithelial cells (*e.g.* Caco-2), the experimental setup is conceptually the same as PAMPA, as shown in Figure 3.3. Calculations of the effective permeability, P_e , may be made according to the following equation [22]:

$$P_e = \frac{dM}{dt} \frac{1}{A \times C_D(0)} \quad (3.5)$$

where dM/dt represents the steady-state appearance rate of the test drug in the acceptor compartment (in units of mol/s). A is the exposed membrane area (in units of cm²) and $C_D(0)$ is the initial drug concentration in the donor compartment (in units of mol/cm³). Again, the units of permeability are in cm/s.

Equations 3.4 and 3.5 assume the unidirectional transport of the test drug from the intestinal lumen to the bloodstream, and from the donor to the acceptor, respectively. Mass transport in the opposite direction is ignored. These are generally regarded as one-way flux equations, and are valid under sink conditions. In the PAMPA method, sink conditions are not always maintained and drug retention in the membrane may introduce errors in the measurement. Avdeef has summarised various transport models with different limitations regarding reverse flux and membrane retention [23]. In particular, the commonly used two-way flux equation in PAMPA methods could be used to described the

acceptor compartment concentration ($C_A(t)$) as a function of time (t), which is written as follows [24]:

$$C_A(t) = \frac{M - M_{\text{mem}}}{V_D + V_A} + \left(C_A(0) - \frac{M - M_{\text{mem}}}{V_D + V_A} \right) \exp \left(-P_e A \left(\frac{1}{V_D} + \frac{1}{V_A} \right) t \right) \quad (3.6)$$

where M and M_{mem} represent, respectively, the total quantity of the drug in the system (in mol), and the sample loss to the membrane. The symbols V_D and V_A represent, respectively, the donor and acceptor volumes.

3.2.4. Advanced transport model for PAMPA

In this section, we present an advanced transport model for the PAMPA method [25]. In addition to reverse flux and membrane retention which were dealt with by the two-way flux equation, this enhanced model takes into account the initial lag-time, τ_{LAG} , (steady-state establishment across the donor-membrane-acceptor system). Correction for the lag-time is important as neglecting this factor leads to an underestimation of the permeability of lipophilic molecules (and an overestimation of the permeability of hydrophilic molecules). This will be discussed in Section 3.3.2.3.

The distribution of the drug in the donor-membrane-acceptor system is depicted in Figure 3.4 for (a) an infinitely stirred and (b) a well-stirred system. In this example, we assume the distribution coefficient of the drug compound to the

Figure 3.4. Schematic diagram of the concentration profile across a donor-membrane-acceptor tri-layer for the case of: (a) zero UWL thickness, (b) non-zero UWL thickness [25].

membrane phase (K_d) to be 2. For the infinitely stirred case (Figure 3.4a), the *unstirred water layer* (UWL) is virtually non-existent, while for the well-stirred case, the concentration profiles are bent in close proximity to the membrane-liquid interfaces due to the presence of the UWL. Both cases assume time $\geq \tau_{LAG}$ and therefore a linear concentration distribution within the membrane. Fick's first law is considered valid in such cases.

The following relationships were used in the model derivation:

(i) *Fick's first law applied to a homogeneous membrane:*

$$J(t) = P_e^{D \rightarrow A} C_D(t) - P_e^{A \rightarrow D} C_A(t) \quad (3.7)$$

where $J(t)$ is the time-dependent flux of the solute across the membrane and $P_e^{D \rightarrow A}$ and $P_e^{A \rightarrow D}$ denote the effective permeability coefficients for the donor-to-acceptor and acceptor-to-donor transport, respectively. Note that these permeability terms may differ if there is a pH gradient between the donor and acceptor phases. Finally, $C_D(t)$ and $C_A(t)$ are the time dependent bulk concentrations of the solute in the donor and acceptor compartments, respectively.

(ii) *Diffusive flux at the membrane/donor interface:*

$$J(t) = \frac{-V_D}{A} \frac{dC_D(t)}{dt} \quad (3.8)$$

(iii) *Mass balance:*

$$V_D C_D(0) = V_D C_D(t) + V_A C_A(t) + V_m C_m(t) \quad (3.9)$$

where V_D , V_A and V_m are the volumes of the donor, acceptor and membrane, respectively, A is the membrane area, $C_D(0)$ is the initial bulk solute concentration in the donor compartment and $C_m(t)$ is the time-averaged solute concentration within the membrane.

(iv) *Finally, the expression for fractional membrane retention R_f is defined as:*

$$R_f = 1 - \frac{V_D C_D(t) + V_A C_A(t)}{V_D C_D(0)} \quad (3.10)$$

By combining Equations 3.9 and 3.10, we obtain the mass balance equation containing the fractional membrane retention R_f :

$$V_D C_D(0) = V_D C_D(t) + V_A C_A(t) + R_f V_D C_D(0) \quad (3.11)$$

In the case of the infinitely stirred system with zero UWL thickness, the effective permeability coefficients can be expressed as follows:

$$P_e^{D \rightarrow A} = P_m^{D \rightarrow A} = \frac{D_m K_d^D}{h} \quad (3.12)$$

$$P_e^{A \rightarrow D} = P_m^{A \rightarrow D} = \frac{D_m K_d^A}{h} \quad (3.13)$$

where D_m is the diffusion coefficient of the solute within the membrane and $P_m^{D \rightarrow A}$ and $P_m^{A \rightarrow D}$ are the membrane permeability coefficients of the solute for donor-to-acceptor and acceptor-to-donor flux, respectively. K_d^D and K_d^A are the solute distribution coefficients between the membrane-donor and membrane-acceptor, respectively, and h is the membrane thickness. Combining Equations 3.7, 3.8, 3.11, 3.12 and 3.13, we obtain a linear ordinary differential equation:

$$\frac{dC_D(t)}{dt} + aC_D(t) - b = 0 \quad (3.14)$$

where:

$$a = \frac{AP_e^{D \rightarrow A}}{V_D} \left(1 + \frac{K_d^A}{K_d^D} \frac{V_D}{V_A} \right), \quad b = \frac{AP_e^{D \rightarrow A}}{V_A} \frac{K_d^A}{K_d^D} C_D(0)(1 - R_f) \quad (3.15), (3.16)$$

The differential equation, Equation 3.14, can be solved to obtain the analytical solution for times $\geq \tau_{LAG}$:

$$\ln k = -a(t - \tau_{LAG}) \quad (3.17)$$

where k is a function of the measured solute concentration in the acceptor compartment:

$$k = \frac{C_D(0)(1 - R_f) - \frac{b}{a} - \frac{V_A}{V_D} C_A(t)}{C_D(\tau_{LAG}) - \frac{b}{a}} \quad (3.18)$$

where $C_D(\tau_{LAG})$ is the solute bulk concentration in the donor compartment at time $t = \tau_{LAG}$.

Assuming (1) $K_d^D \approx K_d^A$ and (2) $\tau_{LAG} \approx 0$, Equation 3.17 could be simplified to the two-way flux equation (Equation 3.6). When the pH values of the donor and the acceptor compartments differ, K_d^D and K_d^A are not the same for ionisable molecules. In such cases, they could be determined from independent shake-flask partition experiments [26]. The lag-time correction will be discussed in Section 3.3.2.3, together with experimental data. In a mathematical sense, τ_{LAG} could be regarded as a constant of integration, so it could be treated as any time in the course of the experiment where the above derivations hold. In practice, τ_{LAG} should be chosen as the time at which the function of Equation 3.17 starts to exhibit linear behaviour. From the slope of the $\ln k$ vs. time plot (i.e. the constant a defined in Equation 3.15), one directly obtains the effective permeability value, as in the case of the two-way flux equation (Equation 3.6).

3.2.5. Permeability terms

The scenario shown in Figure 3.4a can only be regarded as an ideal situation where stirring is carried out at infinite speed, and the UWL thickness reduces to virtually zero. The solute concentrations in the bulk donor (acceptor) solution and at the donor-membrane (acceptor-membrane) interface are therefore the same. In reality, there is always a contribution of the UWL to the permeation, and the bulk solute concentration in the donor compartment is always higher than at the donor-membrane interface, as depicted in Figure 3.4b (likewise, the bulk solute concentration in the acceptor is always lower than the acceptor-membrane interface concentration). Therefore, what one measures is the *effective* permeability coefficient consisting of two independent terms: membrane permeability, P_m , corresponding purely to transport through the membrane (no UWL contribution), and unstirred water layer permeability, P_u , corresponding to transport through the two unstirred water layers adjacent to the membrane. The effective permeability can be described as the inverse of the total resistance to passive transport across the tri-layer UWL(donor)-membrane-UWL(acceptor) system and is broken down into the following two terms [23,27]:

$$\frac{1}{P_e} = \frac{1}{P_u} + \frac{1}{P_m} \quad (3.19)$$

where P_u is the combined permeability of both the donor and acceptor UWLs and P_m is the membrane permeability. In analogy to electrical circuits, the inverse permeability terms can be regarded as resistances, which are additive in series.

3.2.6. Permeability hydrodynamic model

Approximate relationships between the UWL permeability and stirring rate have been used in previous Caco-2 and PAMPA studies [27,28]:

$$P_u = K_p \omega^\alpha \quad (3.20)$$

where ω is the angular velocity of stirring, α is the hydrodynamic exponent and K_p is a constant composed of the aqueous diffusion coefficient of the solute D_{aq} , the aqueous kinematic viscosity ν and the geometrical factors of the permeation cell. The explicit form for the case of transport to the surface of a rotating-disc was found by Levich [29]. The unstirred water layer thickness, δ_u , can be expressed as follows:

$$\delta_u = 1.61 D_{aq}^{1/3} \nu^{1/6} \omega^{-1/2} \quad (3.21)$$

The Levich solution applied to the single UWL permeability yields:

$$P_u = 0.62 D_{aq}^{2/3} \nu^{-1/6} \omega^\alpha \quad (3.22)$$

In pharmaceutical applications, α is often treated as an empirical value, ranging from 0.7 to 1.0, and is usually determined by the best fit in UWL permeability analysis [27-28,30]. Combining Equations 3.21 and 3.22, which yields $\alpha = 0.5$, we

have a relationship between the unstirred water layer thickness and unstirred water layer permeability:

$$P_u = \frac{D_{aq}}{\delta_u} \quad (3.23)$$

Equation 3.23 is an analogue to Equations 3.12 and 3.13 where the membrane permeability, membrane diffusion coefficient and the membrane thickness are substituted for the UWL permeability, aqueous diffusion coefficient and the UWL thickness, respectively. Combining Equations 3.19 and 3.22, the $P_e - \omega$ dependency can be written as follows [31-34]:

$$\frac{1}{P_e} = \frac{1}{0.62 D_{aq}^{2/3} \nu^{-1/6} \omega^\alpha} + \frac{1}{P_m} \quad (3.24)$$

Measuring P_e at two or more different stirring rates allows extrapolation to the infinite angular velocity, where $P_u = 0$ and $P_e = P_m$, which yields the membrane permeability value.

3.2.7. Permeability-pH dependence

For ionisable compounds, the membrane permeability (P_m) depends on pH. Equation 3.25 defines the pH independent intrinsic permeability P_0 , *i.e.* the permeability of the neutral species, assuming that the pH partition hypothesis [12] is valid:

$$P_m = P_0 f_N \quad (3.25)$$

where f_N is the fraction of the neutral species, which is defined in Table 3.2. Combining Equations 3.19 and 3.25, we can write the following relationship:

$$\frac{1}{P_e} = \frac{1}{P_u} + \frac{1}{P_0 f_N} \quad (3.26)$$

Note that f_N is a function of pH and the $pK_a(s)$ of the drug. If two or more P_e values are measured at different pH values (for example within ± 2 pH units from the pK_a), one should be able to deduce the P_0 , and P_u values using Eq- 3.26 [27,35].

3.3. COMPARISONS OF DIFFERENT PAMPA METHODS

Since the introduction of PAMPA by Kansy *et al.* [7], the technique has evolved considerably. These advancements can be loosely classified as follows:

- Lipid models.
- Experimental procedures, including the control of hydrodynamics and solution compositions.
- Applications of PAMPA data to predict and/or model *in vivo* data in pharmaceutical research.

In most cases, the goal for improving/modifying lipid models and/or experimental procedures is to enhance the predictive/modelling power of PAMPA data. Hence, these three categories are somehow inter-related. In this section, we seek to focus on methodologies (*i.e.* lipid models and experimental procedures). In the next section, we will then turn our attention to selected examples of applying PAMPA data in pharmaceutical research.

3.3.1. Lipid models

Different research groups have developed lipid models for use in PAMPA. These are summarised in Table 3.3, and described in the following subsections.

Table 3.3. Summary of the PAMPA lipid models described in this chapter

Lipid model	Composition ^a	Filter ^b	Solvent	Agitation
Egg lecithin [7]	10 % Egg lecithin	PVDF	<i>n</i> -Dodecane	None
Dioloylphosphatidylcholine [24]	2 % DOPC	PVDF	<i>n</i> -Dodecane	Shaking
Double-sink method [23,36-37]	20 % Phospholipid mixture	PVDF	<i>n</i> -Dodecane	Individual well stirring
<i>n</i> -Hexadecane [35]	100 % <i>n</i> -Hexadecane	Polycarbonate	<i>n</i> -Dexadecane	Shaking
Mixture of lipids (biomimetic) [40]	0.8 % PC, 0.8 % PE, 0.2 % PS, 0.2 % PI, 1 % CHO	PVDF	1,7-Octadiene	None
Lipid/oil/lipid tri-layer membrane [43]	Neat DOPC	PVDF	Small amount of hexadecane	None
Immobilised phospholipid vesicles [45]	Neat phospholipid vesicles	Cellulose ester filters	None	Shaking
Lipid/cholesterol/octanol mixture [46]	1.7 % Egg PC, 2.1 % CHO, 96.2 % octanol	cellulose nitrate-acetate mixture filter	Octanol	Flow
Three lipid-component model [48]	2.6 % PS18:1, 0.9 % PC18:1, 1.5 % CHO	PVDF	<i>n</i> -Dodecane	None
Porcine brain tissue extract [49]	2 % Porcine brain extract	PVDF	<i>n</i> -Dodecane	None

^a DOPC – Dioleoylphosphatidylcholine, PC – phosphatidylcholine, PE – phosphatidylethanolamine, PS – phosphatidylserine, PI – phosphatidylinositol, CHO – cholesterol, PS18:1 – 1,2-dioleoylsn-glycero-3-[phospho-*L*-serine], PC18:1 – 1,2-dioleoyl-sn-glycero-3-phosphocholine (PC18:1)

^b PVDF – Poly(vinylidene fluoride)

3.3.1.1. Egg lecithin

This model refers to the lipid system reported in the PAMPA paper by Kansy *et al.* [7]. The artificial membrane was a hydrophobic filter (PVDF filter from Millipore; pore size 0.22-0.45 µm) impregnated with a 10 % solution of egg lecithin in *n*-dodecane. The selection of egg lecithin was to mimic the phospholipid composition of mammalian membranes. Permeation experiments were performed at pH 6.5 and 7.4 (same pH values for both donor and acceptor) on a selected number of

marketed drugs, over 15 hours without stirring. The measured PAMPA flux was shown to correlate with the percent human absorption values via a hyperbolic function.

Kansy's PAMPA model forms the basis for subsequent developments. Most of these new models vary in terms of the compositions of the lipid solution or filter materials with the aim to enhance PAMPA data, and/or to mimic the transport processes other than that of the intestinal epithelial cell membrane (*e.g.* blood-brain barrier).

3.3.1.2. Dioleoylphosphatidylcholine

This was the first commercially available PAMPA lipid model developed by Avdeef's group [24], based on a 2 % solution of a synthetic phospholipid, dioleoylphosphatidylcholine (DOPC), dissolved in *n*-dodecane. Membrane retention was considered via the two-way flux equation. Solution agitation during the permeation studies was accomplished by orbital shaking. In addition, LC-MS and UV detection methods were compared, and the resultant permeability data were shown to be highly correlated. Development by the same group has subsequently led to an improved formulation, the double-sink method, which is described in the next section (Section 3.3.1.3).

3.3.1.3. The double-sink method

The double-sink PAMPA method (DS-PAMPA) has subsequently superseded the 2 % DOPC model [23,36-37]. In particular, Avdeef has demonstrated that the PAMPA data obtained using the DS-PAMPA model correlates well with several absorption-related parameters [36,38-39]. Selected examples will be highlighted in Section 3.4.

DS-PAMPA uses a pH gradient between the donor and acceptor solution (usually 5.0-7.4 and 7.4, respectively), with the addition of chemical scavengers to the acceptor to mimic the presence of serum proteins in blood. Anionic surfactant, such as sodium lauryl sulfate (SLS), at a concentration of 35 mM (*c.a.* the saturated micelle concentration at room temperature) is used as chemical scavenger [23]. The effect of SLS is generally more pronounced in basic and neutral compounds, while the permeation of acidic compounds do not change significantly [23], suggesting electrostatic interactions between the anionic surfactants and the permeating compounds may have a role in the transport mechanism. This results in double-sink conditions that suppress reverse-flux permeation from the acceptor to the donor compartment and shorten the experimental time. The lipid solution consists of a 20 % phospholipid mixture in *n*-dodecane. A further advantage of this PAMPA model is the introduction of individual well stirring, which enables the unstirred water layer (UWL) to be controlled on an empirical basis [27]. With this model, the assay time can be shortened considerably by lowering the UWL thickness (and therefore resistance against diffusion). This is particularly useful for lipophilic molecules, where the membrane permeability (P_m) is comparable to the unstirred water layer permeability (P_u).

3.3.1.4. *n*-Hexadecane

Faller *et al.* introduced a lipid model based on hexadecane (HDM-PAMPA, in the absence of any phospholipids), impregnated on a thin (10 μm) polycarbonate filter of low porosity, in order to better resemble the thickness of bilayer membranes found in living cells [35]. However, the absence of any phospholipids implies that the membrane could be much less permeable than the membrane used in DS-PAMPA and other models (see below). Similar to the case of the DOPC model (Section 3.3.1.2), the sandwich plate is constantly shaken during the permeation assay. Permeability-pH profiles have been determined for a set of model drugs. It has been shown that HDM permeability (the maximum value within the pH range of pH 4-8) provides the best correlation with the absorbed fraction in humans. The correction for the effects of the unstirred water layer was discussed and analysed in light of the permeability-pH dependence (Equation 3.26).

3.3.1.5. Mixture of lipids (biomimetic)

Sugano *et al.* used a mixture of various lipids dissolved in 1,7-octadiene to mimic the content of mammalian cell walls in a so-called “bio-mimetic” PAMPA (BM-PAMPA) [40]. Specifically, the lipid solution consists of 0.8 % phosphatidylcholine (PC), 0.8 % phosphatidylethanolamine (PE), 0.2% phosphatidylserine (PS), 0.2 % phosphatidylinositol (PI) and 1.0 % cholesterol (CHO). It has been shown that the permeability data obtained at pH 5.5 and/or 6.5 (but not at pH 7.4) provided a good prediction of the dose fraction absorbed in humans for a set of marketed drugs. The permeation study was carried out without agitation. In a follow-up study [41], a larger set of drug molecules was investigated using the BM-PAMPA model. The measured permeability data were modified using the Renkin function [42] to account for paracellular transport. The modified permeability data were found to give a good prediction of passive intestinal absorption [41].

3.3.1.6. Lipid/oil/lipid tri-layer membrane

Chen *et al.* reported a new procedure to prepare artificial membranes that contain a reduced amount of organic solvent [43]. It was hypothesised that the excess amount of organic solvents used in other lipid models might lead to compound retention within the membrane. The artificial membrane is formed on PVDF by three coating steps. First, 1 μL of *n*-hexadecane is dispersed into PVDF, with hexane as a carrier to form the middle oil layer. DOPC, pre-dissolved in hexane, is then coated on either side of the membrane. Due to the volatile nature of hexane, it evaporates immediately after coating, thus leaving the lipid layers exposed on the surface. A set of drug molecules has been tested using this new lipid model, and the results appeared to outperform two other lipid models (the 2% DOPC model and DS-PAMPA) in terms of predicting/correlating the percent absorbed in humans and Caco-2 permeability. It has been noted that molecules such as antipyrine, caffeine, ketoprofen, metoprolol and naproxen are not under-

predicted by this model, while they are under-predicted by the other two lipid models. These molecules are relatively small ($MW < 270 \text{ g mol}^{-1}$), so it would be interesting to see if this good correlation holds for a larger set of drug-like molecules.

The lipid/oil/lipid tri-layer is commercially available as a pre-coated PAMPA plate (marketed by BD Biosciences). Avdeef *et al.* have recently reported an evaluation of this pre-coated PAMPA plate [44]. It is interesting to note that the high permeability of antipyrine is reproducible across a range of pH values. However, this could be due to the fragile thin membrane, which contains leaky water pores. Avdeef *et al.* have estimated the void volume in the PVDF filter to be about $2.6 \mu\text{L}/\text{well}$ [44]. It is plausible that the $1 \mu\text{L}$ hexadecane volume used in the pre-coated plates may not be enough to fully plug the inner volume of the filter. As a result, some water channels may form under gradient-pH conditions, leading to higher measured permeability values for relatively small polar molecules.

3.3.1.7. Immobilised phospholipid vesicles

Flaten *et al.* have developed a novel procedure to directly deposit a tight barrier of liposomes on a filter support without the use of an inert solvent such as hexadecane [45]. The preparation of the membrane involves a multi-step extrusion-centrifugation procedure. Egg phosphatidylcholine was used for preparing the liposomes. The permeation study was carried out from 5 hours up to 12 hours (with shaking to control the UWL thickness). A set of drug molecules has been evaluated in this model, alongside other lipid models, such as DS-PAMPA and BM-PAMPA. Membrane retention was found to be surprisingly low in this model, presumably due to the absence of any organic solvent in the membrane. It has been shown that the permeability data obtained from the liposome model correlate well with the percent absorbed in humans. Moreover, the performance of this model appears to be better than the other two PAMPA models, which may offer a useful model for further evaluation. However, the complex nature of the membrane preparation procedure may limit its routine use in other laboratories.

3.3.1.8. Lipid/cholesterol/octanol mixture

Corti *et al.* evaluated different lipid mixtures and filter membranes to develop the optimal lipid model [46]. Permeation experiments were carried out in a flow-through diffusion cell, using a reference model compound, naproxen. It has been shown that, with a lipid mixture containing 2.1 % cholesterol, 1.7 % egg phosphatidylcholine and 96.2 % octanol impregnated in a cellulose nitrate-acetate mixture filter, the measured PAMPA permeability matches exactly with the Caco-2 permeability. The lipid model has been applied to a set of structurally diverse drugs [47]. The measured permeability values were found to be correlated well with the percent absorbed in humans.

3.3.1.9. Three lipid-component model

Polli *et al.* have recently reported the evaluation of a three lipid-component model [48]. In brief, the lipid solution consists of 2.6 % 1,2-dioleoylsn-glycero-3-[phospho-L-serine] (PS18:1), 0.9 % 1,2-dioleoylsn-glycero-3-phosphocholine (PC18:1) and 1.5 % cholesterol in *n*-dodecane. Five microlitres of this solution were impregnated into a PVDF filter to prepare the artificial membrane. As PS18:1 is an anionic lipid, the model is referred to as A-PAMPA, where the letter A denotes anionic. A-PAMPA was designed to mimic the lipid composition of the enterocyte plasma membrane. This lipid model has been applied to a set of drugs and the permeability values were compared with the corresponding Caco-2 permeability values. It has been shown that the A-PAMPA model has correctly classified highly permeable drugs, while the classification has not always been correct for drugs of low permeability.

3.3.1.10. Porcine brain tissue extract

Di *et al.* applied 2 % porcine brain tissue extract dissolved in *n*-dodecane as their model lipid [49]. The system was tested using a set of commercial drugs and research compounds. The permeation study was carried out without agitation. It was shown that the model was able to differentiate CNS+ (high brain penetration) from CNS- (low brain penetration) compounds with a high success rate.

Avdeef and Tsinman undertook a study to compare the permeability data obtained from the DOPC-, HDM- and DS-PAMPA models [50]. Forty drug molecules were considered in their investigation. It was shown that PAMPA permeability values consistently ranked in magnitude according to: DS > DOPC > HDM. This has been rationalised by the fact that the higher lipid content in DS-PAMPA may help the permeation process by offering a source of hydrogen bonding with the membrane phase. In combo models based on Abraham descriptors (α – solute H-bond acidity; β – solute H-bond basicity) were developed to correlate the intrinsic permeability values (P_0) between the HDM and DS models, as well as the DOPC and DS models. As far as we are aware, no systematic comparative study on different lipid models has been reported in the open literature. It would be interesting to extend the comparison as carried out by Avdeef and Tsinman [50] to other recently developed lipid models using a representative set of drug molecules.

3.3.2. Experimental procedures

3.3.2.1. pH and solution compositions

PAMPA studies are normally carried out in aqueous buffer systems. Both the donor and acceptor compartments contain aqueous buffer at selected pH values (Figure 3.3). For instance, the pH of the donor and acceptor could be adjusted to 6.5 and 7.4 to mimic the physicochemical conditions in the small intestine and bloodstream, respectively. Some investigators prefer to determine the pH-

permeability profiles [35] by changing the donor and acceptor pH values to mimic the pH changes along the small intestine. This approach provides more information on permeability changes in different pH environments, which would be useful for mechanistic studies on the permeation process. In cases where the solubility of the drug molecules is low, it would be possible to introduce a small percentage of co-solvent into the assay buffer to avoid compound precipitation during the assay [40]. However, care must be taken in terms of the affects of the co-solvent on the permeability values. It is sensible to repeat the study with several different co-solvent compositions to rationalise the effects on the permeability values.

As mentioned in Section 3.3.1.3, the double-sink method introduces a chemical scavenger to the acceptor, which is another variant in solution composition. This suppresses the back-flux permeation from the acceptor to the donor compartment and shortens the experimental time.

3.3.2.2. Detection

The methods of detection of the permeating compound vary from ultraviolet (UV) spectrophotometry to more selective liquid chromatography/mass spectrometry (LC/MS) methods [24]. One of the reasons for the selection of LC/MS is mainly the large number of “difficult” molecules, which are unsuitable for UV analysis because of poor sensitivity. The high-throughput screening PAMPA method allows *ex situ* analysis of the donor and acceptor plates. At a particular time point, the donor-acceptor sandwich is disassembled and quantified to determine the concentration of the permeating compound. To generate multiple time point data, the PAMPA experiment can start with identical, multiple copies of donor-acceptor plate sandwiches, each of which could be disassembled and quantified at selected time points.

Recently, a rotating permeation cell was developed to investigate the physico-chemical aspects of drug permeation [25]. It employs *in situ* UV measurements of the solute combined with stirring of both the acceptor and donor compartments in a system with defined symmetric geometry. The acceptor pH is kept at 7.4; the donor pH is varied between 3.0 and 10.0. The standard permeation time is as short as 20-30 min, and stirring rates in the range of 200-1500 rpm are used (see Figure 3.5 for the experimental schematic). The chosen membrane model comprised of 1.5 % dioleoylphosphatidylcholine and 0.5 % stearic acid dissolved in 1,9-decadiene and impregnated on a hydrophobic poly(vinylidene fluoride) (PVDF) filter. Despite the fact that the cell only consists of a single permeation channel, the *in situ* UV measurement and the well-controlled stirring in both the donor and acceptor compartments enable the device to be used for the study of drug permeation under controlled hydrodynamic conditions and to investigate the time-dependent transport of the compound observed *in situ*. The *in situ* time dependency of the permeability data as determined using this rotating permeation cell will be discussed in the next section (Section 3.3.2.3).

Figure 3.5. Schematic diagram of the permeation cell used for in situ UV measurements:

1. rotation controller
2. paddle
3. glass tube (donor compartment)
4. spacer disc
5. PTFE cogwheel tightly connected to the glass tube
6. PVDF membrane
7. acceptor solution,
8. UV source optical path
9. quartz window
10. quartz lens
11. fibre optic cable
12. PTFE acceptor cell
13. steel pad
14. steel pad screw [25]

3.3.2.3. Hydrodynamics

In some PAMPA implementations (see Table 3.3), the permeation study is carried out in a static environment where the compounds under investigation are allowed to diffuse from the donor to the acceptor wells over a period of 10-15 hours. Some PAMPA practitioners appear to follow these static experiment protocols. For the study of lipophilic molecules under these conditions, it is not uncommon to see an effective permeability of about $(15-30) \times 10^{-6}$ cm/s. It is now recognised that this is due to the resistance of unstirred water layers which becomes a dominant factor in measurement of effective permeability (see Equation 3.19). For instance, in an unstirred PAMPA assay, the UWL thickness can be as great as 2000-4000 μm [23]. Since the UWL thickness in the human small intestine is assumed to be 30-100 μm [51], the unstirred permeation assay does not correctly match *in vivo* conditions.

Individual well stirring technique

Early pioneering work by the Avdeef [24] and Faller [35] groups soon identified this issue, and attempted to reduce the thickness of the unstirred water layers by gentle shaking of the donor-acceptor sandwich. Apparently, shaking is not a very efficient method of agitation, as the acceptor wells are situated in a confined space, which hinders convection. The Avdeef group has subsequently reported an individual well stirring technique in plate-based permeability measurements [27]. The stirring speeds were reported to vary from 49-622 rpm. This technique has been evaluated using a set of drug molecules. The hydrodynamic analysis of stirred PAMPA measurements using Equation 3.22 revealed a value of 0.709 for the hydrodynamic exponent, α .

Rotating permeation cell

Using the rotating permeation cell as shown in Figure 3.5, Velický *et al.* have carried out detailed permeation studies on several model drug molecules, including warfarin and verapamil under different donor pH values, concentrations and stirring rates [25]. Figure 3.6 shows the concentration vs. time plots and $\ln k$ vs. time plots (Equation 3.17) of warfarin and verapamil. The slope, a , of the $\ln k$ - time function where time $> \tau_{LAG}$ was used to calculate the effective permeability, P_e , using Equation 3.15. Here, the lag-time τ_{LAG} , was determined as follows: the $\ln k$ - time dependence data were fitted with a second order polynomial (usually within $0 < \text{time} < 400$ s) and a linear function (usually time > 700 s). The time

Figure 3.6. Example of concentration-time plots for (a) warfarin (309 nm) at donor/acceptor pH 6.5/7.4, (b) verapamil (280 nm) at donor/acceptor pH 7.4/7.4. Derived $\ln k$ -time plots based on Equation 3.17 are shown for (c) warfarin and (d) verapamil [25]

where the two fitted equations intersected was calculated as the lag-time τ_{LAG} . As shown in Figure 3.6c-d, warfarin exhibits a more linear $\ln k$ - time response than verapamil; the plot of the latter only becomes linear in the middle of the time course. The lag time for warfarin is very close to zero, but for verapamil, the lag times were determined as 1552 s and 1003 s at stirring rates of 250 rpm and 600 rpm, respectively. Given this unusual transient behaviour of verapamil, additional molecules were studied to gain further insight.

Lag-time analysis

Lag-time, τ_{LAG} , is regarded as the time at which the permeation system, consisting of the donor, membrane and acceptor, reaches a steady-state and the permeation is driven only by the solute concentration gradient between the bulk donor and acceptor. Mathematically, in the initial transient period, permeation follows a complex pattern of solute partitioning to the membrane and diffusion according to Fick's second law:

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} \quad (3.27)$$

where c is the solute concentration, t is time, x is the position and D is the diffusion coefficient (aqueous and/or in the membrane). After time $t = \tau_{LAG}$ when the steady-state across the donor-membrane-acceptor system has been established, the permeation can be approximated by Fick's first law:

$$J = -D \frac{\partial c}{\partial x} \quad (3.28)$$

where J is the diffusive flux and the other symbols have the same meaning as indicated above. As the system reaches the steady-state, $\ln k$ becomes linear with time. Before the steady-state is reached, $\ln k$ shows a non-linear dependence on time which can be approximated with a parabolic function. The $\ln k$ vs. time plots of four drug molecules, including propranolol, midazolam, quinine and verapamil, are shown in Figure 3.7. It can be seen that the lag-time of verapamil is longer than the actual time of measurement. Careful examination of the permeation profile shows that the steady-state was not completely reached within the 20 minute experiment. Reports on *in vitro* permeation across lipid bilayers and supported liquid membranes have quoted lag-time values in the range of 1-30 min [52-53]. As shown in Table 3.4, the lag-times of propranolol, midazolam, quinine and verapamil as determined in this study are consistent with the literature. It has been reported that the intestinal residence time is generally between 1-3 h [54]. For the drug molecules investigated in this study, the lag-times are well within the intestinal residence time, suggesting that the initial transient phase of permeation is unlikely to be an issue hindering drug absorption *in vivo*. However, for lipophilic molecules where the doses are low, the expected longer lag-times could be an issue and might potentially lead to unexpectedly poor absorption in pre-clinical and/or clinical studies.

Figure 3.7. Permeation $\ln k$ – time plots of propranolol, quinine, verapamil and midazolam (donor/acceptor pH 6.5/7.4 and stirring rate 280 rpm) showing the different lag-times as vertical bars. The initial donor concentrations were 320, 255, 210 and 80 μM for propranolol, quinine, verapamil and midazolam, respectively

Table 3.4. Lag-time and physicochemical properties of propranolol, quinine, midazolam and verapamil.

Drug	$MW / \text{g mol}^{-1}$	$(\log K_d^D)^a$	$(D_{aq} / 10^{-6} \text{cm}^2 \text{s}^{-1})^b$	$(\tau_{LAG} / \text{s})^c$
propranolol	259.34	1.96 ± 0.01	4.70	781 ± 47
quinine	324.42	0.86 ± 0.00	4.21	782 ± 19
midazolam	325.78	1.96 ± 0.01	4.20	1036 ± 35
verapamil	454.60	1.43 ± 0.01	3.57	1416 ± 78

^a Membrane/aqueous buffer drug distribution coefficient determined using the shake-flask method at aqueous pH 6.5 and 22°C.

^b Aqueous diffusion coefficient calculated using Equation 3.35

^c Lag-time values determined by extrapolation of the polynomial and linear segment of $\ln k$ – time dependence measured at donor/acceptor pH 6.5/7.4 and a stirring rate of 280 rpm.

Lag-time dependence on stirring rate

Stirring decreases the unstirred water layer thickness and thus increases the measured effective permeability. Figure 3.8 shows the $\ln k$ time profiles of propranolol obtained at stirring rates of 60, 80, 110, 160 and 280 rpm. Similar to the permeability terms, there are two separate components contributing to the overall lag-time value. One component, $\tau_{LAG,m}$, arises from the partitioning and loading of a solute to the membrane, whereas the other term, $\tau_{LAG,u}$, arises due to solute transport through the unstirred water layer adjacent to both sides of the membrane.

Figure 3.8. Permeation $\ln k$ - time plots of propranolol (225 nm) at donor/acceptor pH 7.4/7.4

The observed lag-time, τ_{LAG} , is a sum of the two, which can be described by the following equation:

$$\tau_{\text{LAG}} = \tau_{\text{LAG}, u} + \tau_{\text{LAG}, m} \quad (3.29)$$

For the membrane loading process, the lag-time can be approximated from Fick's second law as [55-56]:

$$\tau_{\text{LAG}, m} = \frac{h^2}{6D_m} \quad (3.30)$$

where h represents membrane thickness and D_m membrane diffusion coefficient. Using a similar relationship, where thickness and the membrane diffusion coefficient are replaced with UWL thickness, δ_u , and the aqueous diffusion coefficient, D_{aq} , for the UWL dependent term, the following relationship is obtained:

$$\tau_{\text{LAG}} = K_\tau \frac{\delta_u^2}{D_{\text{aq}}} + \frac{h^2}{6D_m} \quad (3.31)$$

where K_τ is an empirical constant. The aqueous term in Equation 3.31 can be further expressed as a function of the stirring rate using Equation 3.32:

$$\tau_{\text{LAG}} = 2.6 K_\tau D_{\text{aq}}^{-1/3} \nu^{1/3} \omega^{-1} + \frac{h^2}{6D_m} \quad (3.32)$$

Lag-time values measured for propranolol permeation at several different stirring rates are plotted in Figure 3.9. The graph shows the linear dependence of

τ_{LAG} on the inverse angular velocity of stirring, $1/\omega$, for propranolol at donor/acceptor pH 6.5/7.4. According to Equation 3.32, the intercept of this dependence should be equal to $\tau_{LAG,m}$. Substituting the membrane thickness (assumed to be 125-200 μm due to excess organic solvent immobilised on the membrane filter) and the membrane diffusion coefficient (calculated from Equation 3.12 and averaged over the values measured at different donor pH 5.5-10.5) to Equation 3.30 yields a membrane lag-time values, $\tau_{LAG,m}$, in the range 113-290 s. This is in a good agreement with the experimental value of 236 ± 2 s (Figure 3.9).

Figure 3.9. Dependence of the lag-time on the inverse angular velocity of stirring for propranolol permeation at donor/acceptor pH 6.5/7.4

Lag-time dependence on the concentration gradient

Figure 3.10 shows the effects of the concentration gradient on lag-time for permeation studies on propranolol. The experiments were carried out at donor/acceptor pH 7.4/7.4 and stirring rates of 60, 110 and 280 rpm. The initial drug concentration in the donor solution, $c_D(0)$, was varied from 30 to 300 μM . As shown in Figure 3.10, the lag-time was found to decrease with an increase in the concentration gradient between the donor and acceptor phase. It is apparent that a larger concentration gradient generates a larger driving force for mass transport and therefore leads to a shorter lag-time to reach the steady-state. This observation underlines the importance of unified inter-laboratory permeation assay standards in cases where the analytical permeability model does not allow for the lag-time.

Figure 3.10. Lag-time dependence on the initial donor concentration of propranolol at donor/acceptor pH 7.4/7.4

Hydrodynamic analysis

Figure 3.11 shows the hydrodynamic analysis on effective permeability as a function of the stirring rate (Equation 3.24) for warfarin and verapamil. Our data revealed that, for warfarin, the ideal hydrodynamic exponent, $\alpha = 0.5$, provides a good fit, while for verapamil, an α value of 1 is required. It was noted that the hydrodynamic exponent α describes the permeation sensitivity to stirring. With lower values of α , transport becomes membrane-limited; conversely, a higher α value implies diffusion-limited permeation, and is sensitive to stirring. We have extended the study to a larger set of 31 drug molecules. As shown in Figure 3.12, α correlates with the lipophilicity of the molecule (the membrane/buffer distribution coefficient at pH 6.5) via the following empirical polynomial equation:

$$\alpha = 0.0125(\log K_d)^3 + 0.0424(\log K_d)^2 + 0.0732 \log K_d + 0.0905 \quad (3.33)$$

where K_d represents the membrane/buffer distribution coefficient at pH 6.5.

Permeability-pH profiles

Figure 3.13 shows the permeability-pH profiles of warfarin and verapamil. Permeation data obtained under unstirred conditions were included (filled black square symbols in Figure 3.13). The intrinsic permeability coefficient (P_0) was calculated by least-squares analysis for all the stirring rates shown (0, 250, 400, 600, 1000 and 1500 rpm) via Equation 3.26. The intrinsic permeability was approximately constant for all stirring rates, as expected, and the average value is plotted as a dashed line. The hydrodynamic membrane permeability coefficient extrapolated from the dependence of the effective permeability on stirring rate via Equation 3.24 was plotted for each pH as a filled black diamond symbol. This is consistent with the membrane permeability coefficient, P_m , calculated from the

Figure 3.11. Dependence of the inverse of effective permeability on the stirring rate for (a) warfarin at pH 6.5/7.4, (b) verapamil at pH 7.4/7.4. The warfarin and verapamil effective permeability values are plotted against $\omega^{-0.5}$ and $\omega^{-1.0}$, respectively [25]

P_o value using Equation 3.25 (plotted as a solid curve). Finally, the hydrodynamic UWL permeability coefficient, P_u , was calculated separately for each stirring rate and pH using Equation 3.19 (dashed curves in Figure 3.13).

Figure 3.12. Dependence of α on the membrane/buffer distribution coefficient for the pH 6.5/7.4 permeation experiment of 31 studied drug molecules. The 31 drug molecules are: acetaminophen, antipyrine, atenolol, betamethasone, cefixime, cephalothin, cetirizine, chlorpheniramine, chlorthalidone, colchicines, diclofenac, eprosartan, fexofenadine, gatifloxacin, metolazone, midazolam, nafcillin, naproxen, norfloxacin, oxybutynin, pindolol, propranolol, pyridoxine, quinine, risperidone, salicylic acid, theophylline, tolbutamide, verapamil, warfarin and zopiclone [65]

Warfarin is a weak acid, with $pK_a = 4.82$ [23]. In pH range 6.5–8.0, it is predominantly ionised, and shows little change in effective permeability according to stirring rate (Figure 3.13a). This implies that permeation is membrane-limited and the UWL is very thin, giving little resistance to drug diffusion. In the pH range of 3.5–6.5, the permeability separation with stirring rate increases and permeation becomes diffusion-limited, largely affected by the increasing UWL thickness. In contrast, verapamil is a weak base with $pK_a = 9.07$ [23]. As shown in Figure 3.13b, the effective permeability is more dependent on stirring rate, and is much higher across the entire pH range than that of warfarin. The difference between the unstirred permeability value and membrane permeability coefficient is more than 1.7 log units at a donor pH of 6.5. In other words, the UWL thickness under these conditions is estimated to be $3830 \mu\text{m}$ (using Equations 3.19 and 3.23, and D_{aq} is given in Table 3.4), which yields a UWL resistance far greater than that of the membrane. This suggests that the determination of effective permeability using a static PAMPA method would lead to problematic results, and precise control of hydrodynamics is essential to delineate the effects of UWL.

Figure 3.13. Permeability-pH profiles of a) warfarin and (b) verapamil [25]

3.4. APPLICATIONS OF PAMPA DATA TO PREDICT AND/OR MODEL *IN VIVO* DATA

As described before, PAMPA permeability data have been applied for the prediction of percent absorbed in humans (Sections 3.3.1.1, 3.3.1.4-3.3.1.8). In these studies, marketed drugs with a known percent absorbed in humans were investigated in the respective lipid models. The permeability data were reported to be correlated with the data on the percent absorbed in humans. Except for the DOPC (Section 3.3.1.2) [24], DS-PAMPA (Section 3.3.1.3) [26,36-37], *n*-hexa-

decane (Section 3.3.1.4) [35], immobilised phospholipid vesicles (Section 3.3.1.7) [45] and lipid/cholesterol/octanol (Section 3.3.1.8) [46] models, permeation studies were carried out without agitation. Readers are directed to the relevant publications for further details.

The presence of the unstirred water layer in a PAMPA study could have a profound effect on the accuracy of the data, particularly for lipophilic molecules. In Section 3.3.2.3, we described the rotating permeation cell and the individual well stirring technique, which offer precise control of the hydrodynamics in the donor/acceptor compartments. Moreover, we have discussed approaches (Section 3.2) to disentangle the unstirred water layer permeability (P_u) from the effective permeability (P_e). In this section, we will focus our attention on these two techniques to build correlations between *in vitro* permeability and the absorbed fraction of drugs in humans.

A simple passive diffusion permeability model would not be expected to accurately predict the intestinal absorption of smaller hydrophilic drugs, since the paracellular route of absorption would not be adequately addressed. Sugano *et al.* [41] reported the use of a calculated contribution based on the Renkin function [42] to account for transport via the paracellular route. In the present study, this approach will be utilised to enhance the permeability data, and will be discussed in Section 3.4.1. The plug flow absorption model as developed by Amidon *et al.* [57] will be used to correlate the *in vitro* permeability (corrected for paracellular transport and unstirred water layer permeability) and the data on the absorbed fraction in humans (Section 3.4.2).

In Section 3.4.3, the rotating permeation cell will be applied to study a set of 26 marketed drugs (acidic, basic and neutral drugs). In Section 3.4.4, the individual well stirring technique in conjunction with the DS-PAMPA model will be applied to study a set of 33 amphoteric drugs. Drugs where the first pass hepatic clearances are low to moderate, with published human absolute bioavailability data spanning low to high values, were deliberately selected in these studies. In the modelling of the data, the absorbed fraction was used, which is derived from human bioavailability data corrected for first pass hepatic clearance, to avoid erroneous correlation with permeability. Care was taken to include drugs with moderate to good aqueous solubility to ensure that the absorption process was not solubility limited. In other words, the selected drug molecules generally fall within BCS classes I and III. These conditions provided the basis for a rigorous assessment of the correlation between the *in vitro* drug permeability coefficient and literature bioavailability determined *in vivo*.

3.4.1. Corrections for paracellular transport

Adson *et al.* [19,28] have proposed a general expression for paracellular permeability, which is written as follows:

$$P_p = \left(\frac{\varepsilon}{\delta} \right) \cdot D_{aq} \cdot F \left(\frac{r}{R} \right) \cdot \left[f_{(\pm/0)} + f_{(+)} \cdot \frac{\kappa \cdot |\Delta\phi|}{1 - e^{-\kappa|\Delta\phi|}} + f_{(-)} \cdot \frac{\kappa \cdot |\Delta\phi|}{e^{+\kappa|\Delta\phi|} - 1} \right] \quad (3.34)$$

where (ε/δ) represents the porosity-pathlength ratio (the porosity, ε , is the relative surface area of the junction opening divided by the total epithelial surface area, and the pathlength, δ , represents the thickness of the restricted-junction domain times the tortuosity of the paracellular route). D_{aq} ($\text{cm}^2 \text{ s}^{-1}$), is the aqueous diffusivity, which was estimated at 25 °C using the empirical function based on molecular weight as follows [23,58-60]:

$$\log D_{aq} = -4.14 - 0.46 \log MW \quad (3.35)$$

$F(r/R)$ is the Renkin molecular sieving function, which is defined as [42]:

$$F \left(\frac{r}{R} \right) = \left(1 - \left(\frac{r}{R} \right) \right)^2 \times \left(1 - 2.104 \left(\frac{r}{R} \right) + 2.09 \left(\frac{r}{R} \right)^3 - 0.95 \left(\frac{r}{R} \right)^5 \right) \quad (3.36)$$

The symbol r is the solute hydrodynamic radius and R is the pore radius, which was calculated using the Sutherland-Stokes-Einstein spherical-particle equation [58]:

$$r = \left(0.92 + \frac{21.8}{MW} \right) \frac{k_B T}{6\pi\eta D_{aq}} \quad (3.37)$$

where k_B is the Boltzmann constant, T is the absolute temperature, η is the dynamic viscosity of the solvent (0.00893 poise for water at 25°C).

The square-bracket term in Equation 3.34, which is referred as $E(\Delta\phi)$, is a function of the electrical potential drop, $\Delta\phi$ (mV), across the junction created by negatively-charged residues (carboxylate and phosphate) lining the junctional pores, where $f(\pm/0)$, $f(+)$ and $f(-)$ are the concentration fractions of the neutral/zwitterionic, cationic and anionic forms of the drug, respectively. $\kappa = F/(k_B T N_A)$, where N_A is Avogadro's number and F is the Faraday constant. The term in the brackets, $E(\Delta\phi)$, is 1 for neutral molecules, about 1.4 for cations and 0.7 for anions. In the present study, the parameters $R = 12.9 \text{ Å}$, $\varepsilon/\delta = 0.78 \text{ cm}^{-1}$ and $\Delta\phi = -30 \text{ mV}$ were taken from Avdeef [58] who derived these parameters by a re-analysis of the Caco-2 permeability data of several paracellular markers published by Adson *et al.* [19].

The membrane permeability, P_m , obtained from the *in vitro* permeation study (from Equation 3.19 and Equations 3.22 or 3.23, depending on the experimental technique used) is corrected for paracellular transport (using Equation 3.34) as follows:

$$\frac{1}{P_e^{\text{INT}}} = \frac{1}{P_u} + \frac{1}{S \times P_m + P_p} \quad (3.38)$$

where P_e^{INT} and P_u represent, respectively, the effective permeability (corrected for paracellular transport) and the permeability term due to the unstirred water

layer permeability adjacent to the intestinal epithelial layer. P_p is calculated by Equation 3.34, while P_u is calculated using Equation 3.23 by assuming an unstirred water layer thickness (δ_u) of 200-500 μm [61-62]. The variable S is a scaling factor. The choice of the scaling factor depends on the particular permeability assay/paracellular model used, and could be treated as an adjustable parameter, with its optimum value found from the best fit of the effective permeability and fraction absorbed data in the plug-flow model, as described in Section 3.4.2.

3.4.2. Absorption model

From the literature on absolute oral bioavailability data, and assuming that gut wall metabolism is negligible (i.e. $F_g \approx 1$), the fraction absorbed F_a can be calculated as follows (see Equation 3.2):

$$F_a \approx \frac{F}{F_h} \quad (3.39)$$

where $F_h = 1 - CL_h/Q_h$. The symbols CL_h and Q_h represent, respectively, first pass hepatic clearance [63] and hepatic blood flow (set at 23 $\text{mL min}^{-1} \text{kg}^{-1}$ body weight [2]). To evaluate the correlation between the effective permeability (P_e^{INT}) and the fraction absorbed, the $\%F_a$ data are fitted against the plug-flow absorption model [57-62,64]:

$$\%F_a = H(1 - \exp(-GP_e^{\text{INT}})) \quad (3.40)$$

where G represents the Graetz number (a dimensionless number which describes laminar flow in the intestine, treated as a tube, in the plug flow model). In the present study, G and H are regarded as fitting constants.

3.4.3. Correlation between *in vitro* permeability and the absorbed fraction of drugs in humans

Figure 3.14 shows the absorbed fraction of the 26 drug molecules as a function of the effective permeability ($\log P_e^{\text{INT}}$), with the solid curve representing the best fit to Equation 3.40 [65]. The optimised parameters are: $H = 100$, $G = 2.14 \times 10^6 \text{ cm}^{-1} \text{ s}$ and $S = 0.014$. The dotted curves are derived from estimated errors in the $\log P_e^{\text{INT}}$ values with an average value of 0.35, which is estimated based on the lab-to-lab variability of the Caco-2 paracellular parameters [58]. For the upper dashed curve, the H parameter is 100, while a value of 85 is used for the lower dashed curve (in part to reflect the estimated errors in the $\%F$ data). The estimated errors in the $\%F_a$ data were determined as a range of values spread over different published $\%F$ values. For those molecules where errors in the published $\%F$ values were not available, the average error value of 9 % was used. As shown in Figure 3.14, the absorbed fraction data of these molecules were generally well predicted, within experimental uncertainty, by the approach described in this chapter.

Figure 3.14. Correlation between fraction absorbed ($\%F_a$) and effective permeability (corrected for paracellular transport and unstirred water layer permeability (UWL thickness = 200 μm)). The solid curve is the best fit of the function represented by Equation 3.40. The dashed curves are based on estimated errors in the $\log P_e^{\text{INT}}$ values (0.35). Unfilled circle – acid; grey circle – base; black circle – neutral.

The 26 drugs used are: acetaminophen, antipyrine, atenolol, betamethasone, cefixime, cephalothin, chlorpheniramine, chlorthalidone, colchicine, diclofenac, eprosartan, metolazone, midazolam, nafcillin, naproxen, oxybutynin, pindolol, propranolol, quinine, risperidone, salicylic acid, theophylline, tolbutamide, verapamil, warfarin and zopiclone.

The average absolute bioavailability data were taken from several sources [68-72] and corrected for hepatic clearance [63,72] to generate the $\%F_a$ data [65]

3.4.4. Prediction of the absorbed fraction of amphoteric drugs in humans

Given the charge/polar nature of zwitterionic drugs, the permeability across an artificial membrane with low lipid content could be low. It was felt that the 20 % phospholipid content in the DS-DAMPA method would facilitate the permeation of charged species by offering a compensating source of hydrogen bonding within the membrane, which may provide a more sensitive measure of the permeation rates. Table 3.5 lists the P_e^{INT} values for selected amphoteric drugs deduced at donor pH 6.5 using the DS-DAMPA model, in conjunction with individual well stirring [39]. The effective permeability values ranges from 0.2×10^{-6} (ceftazidime) to $84 \times 10^{-6} \text{ cm s}^{-1}$ (melphalan), with a mean value of $14 \times 10^{-6} \text{ cm s}^{-1}$. As shown in Table 3.5, about a third of the drugs were predicted to permeate the intestinal barrier predominantly by the paracellular route, with acyclovir, ceftazidime, daunorubicin, famotidine, ganciclovir, levocarnithine and terbutaline almost entirely transported via the paracellular route, but with a low average absorbed fraction of less than 20 %.

Table 3.5. Effective permeability, P_e^{INT} , percent contribution of the paracellular component, Para, and the fraction absorbed data of the 33 selected amphoteric drug molecules, F_a

Drug	P_e^{INT} (pH 6.5) $10^{-6} \text{ cm s}^{-1}$	Para, %	MW	Fraction absorbed in human (F_a / %) ^a
Acyclovir	0.8	99	225.20	26 ± 9
Amdinocillin	0.9	46	325.40	14 ± 7
Benazepril	17	1	424.49	37 ± 7
Ceftazidime	0.2	96	546.60	0 ± 7
Cerivastatin	15	1	459.55	68 ± 16
Cetirizine	42	0	388.89	93 ± 7
Chlortetracycline	3.7	5	478.88	65 ± 9
Ciprofloxacin	2.8	18	331.34	100 ± 7
Daunorubicin	0.3	99	527.50	0 ± 7
Demeclocycline	3.8	4	464.85	66 ± 7
Doxycycline	20	1	444.43	95 ± 7
Enoxacin	7.7	6	320.32	100 ± 7
Famotidine	0.6	98	337.45	58 ± 7
Fexofenadine	2.8	6	501.65	30 ± 7
Ganciclovir	0.7	99	255.20	9 ± 7
Gatifloxacin	20	1	375.39	100 ± 7
Levocarnitine	1.3	98	161.20	10 ± 7
Lisinopril	0.6	45	405.49	26 ± 7
Lomefloxacin	15	2	351.35	97 ± 7
Meloxicam	13	1	351.40	97 ± 7
Melphalan	84	0	305.20	100 ± 12
Mesalamine	0.9	90	153.14	34 ± 7
Minocycline	50	0	457.48	100 ± 7
Norfloxacin	1.0	56	319.33	35 ± 7
Ofloxacin	4.7	8	361.37	100 ± 7
Pefloxacin	23	1	333.36	100 ± 8
Piroxicam	20	1	331.35	100 ± 7
Sparfloxacin	42	0	392.40	100 ± 7
Sulfadiazine	1.2	45	246.20	100 ± 7
Terbutaline	1.4	99	225.28	16 ± 7
Tetracycline	3.8	6	444.43	83 ± 7
Torsemide	22	1	348.42	82 ± 7
Trovafloxacin	57	0	416.35	97 ± 15

^a Average absolute bioavailability taken from several sources [68, 70-71, 73-81] and corrected for hepatic clearance [63,72] to generate the F_a data. Errors are determined as a range of absolute bioavailability values spread over different literature sources. For those molecules where errors in the published F values are not available, an average error value of 7 % was used.

This high paracellular fraction is somewhat surprising, given the discussions surrounding the human jejunal permeability measurements by Lennernäs and coworkers, who have suggested that the paracellular route may not be as important as that of the passive transcellular route in intestinal absorption for the 42 compounds (mostly drugs) tested in humans so far [66]. However, it could be that most of the compounds whose jejunal permeability has been determined are not ampholytes such as those considered here, although some molecules are included in both studies.

Figure 3.15 shows the fraction absorbed of the 33 ampholytes as a function of the $\log P_e^{\text{INT}}$ values deduced at the gradient pH 6.5, with the solid curve representing the best fit to Equation 3.40. In the previous study, the analysis was based on the absolute bioavailability data [39]. Despite most of the molecules exhibiting low hepatic clearance, the bioavailability data have been converted to the absorbed fraction in this study. Re-analysis of the absorbed fraction data revealed a consistent picture [39]. The best-fitted parameters were: $H = 100$, $G = 0.29 \times 10^6 \text{ cm}^{-1} \text{ s}$ and $S = 1.0$. The dotted curves were derived in the same way as

Figure 3.15. Correlation between fraction absorbed ($\%F_a$) and the effective permeability (corrected for paracellular transport and unstirred water layer permeability (UWL thickness = 500 μm)) for the 33 ampholytes studied. The solid curve is the best fit of the function represented by Equation 3.40. The dashed curves are based on estimated errors in the $\log P_e^{\text{INT}}$ values (0.35). The outliers are labelled with the drug names

in Figure 3.14. For most of the ampholytes studied in this work, the estimated errors in the F data could be up to $\pm 16\%$. This is based on the assumption of a 95 % confidence interval from different published % F values, where available. In instances where errors in the published % F values were not available, an average value of 7 % was assumed, based on known errors from the rest of the molecules. Based on this model, the P_e^{INT} values for the 50 % predicted absorbed fraction in humans could be estimated to be $2.3 \times 10^{-6} \text{ cm s}^{-1}$. It was noted that the values of S were about 70-fold greater than in the model using a rotating permeation cell (Section 3.4.3). This may be due to the different approaches used in calculating P_m values from the *in vitro* permeability data. As shown in Figure 3.15, the absorbed fraction data of most of the ampholytes was generally well-predicted by the approach described here. However, famotidine, sulfadiazine and ciprofloxacin were predicted as false negatives. This might have occurred due to the presence of facilitated transport mechanism(s) which the passive permeability model failed to account for. Benazepril was the only false positive. This molecule is an ester prodrug of benazeprilat. Following oral absorption, the prodrug is completely hydrolysed (primarily in the liver) to benazeprilat [67]. It is plausible that these data are under-estimated to some extent because of the fast hydrolysis reaction *in vivo*.

3.5. CONCLUDING REMARKS

PAMPA provides useful information to assess the passive permeability of research compounds across an artificial membrane. The method can be run with relatively low costs, compared to other cellular permeability assays. It was developed with the aim of enhanced throughput in mind, and can potentially be utilised in screening. Undoubtedly, this has been shown to be a success, evident by the fact that an increasing number of research laboratories are adopting PAMPA as part of their screening toolkit. In particular, PAMPA has an important role to play in supporting the development of chemical series and lead compounds in early drug discovery. For instance, in combination with other *in vitro* tests, PAMPA could form a part of a testing cascade in lead generation and/or lead optimisation campaigns. However, it is not sustainable to test every research compound using PAMPA. Instead, it would be more cost effective to apply this method in scenarios where passive permeability is likely to be an issue for the chemical series of interest.

The PAMPA method has improved considerably over the last decade. New lipid models have been developed to better mimic various cellular barriers, including intestinal epithelial layers and the blood-brain barrier. Pioneering PAMPA researchers soon realised the impact of unstirred water layers on the measured data, and subsequently developed the individual well stirring technique to control the thickness of the unstirred water layers. More recently, a novel rotating permeation cell, which offers precise control of the hydrodynamics in the donor and acceptor compartments, has been utilised to investigate the effects

of unstirred water layers on the accuracy of PAMPA data. This has provided new insights into the initial rate of transport and loading of the molecules into the artificial membrane. While the rotating permeation cell can only study one compound at a time, there is the potential to further develop this technique into a miniaturised version, for example, which would offer throughput that is comparable to the current PAMPA-based microtitre plate.

Mathematical models have been developed to predict the paracellular transport of test compounds. PAMPA permeability data, corrected for paracellular transport, were generated on two sets of marketed drugs, one of which included amphoteric drugs predominately charged at physiological pH. It was shown that these effective permeability data correlated well with data on the absorbed fraction in humans.

As the PAMPA technique evolves, it is expected that more practitioners will realise the existence and the effects of unstirred water layers, and carefully design their experiments to eliminate any artefacts that might be obtained from a static permeation study. To differentiate the change in permeation rates on analogues (with small structural changes) within a chemical series of interest will call for sensitive PAMPA data. It is envisaged that the research and optimisation of lipid models will continue to enhance the resolution of these data.

Acknowledgements

We gratefully acknowledge the inspiring discussion with Dr. Alex Avdeef on PAMPA methods. His advice on the paracellular transport model was invaluable. Thanks are especially due to Oksana Tsinman and Na Sun of pION Inc. for their contribution to the DS-PAMPA study on the amphoteric drugs described in this chapter.

REFERENCES

1. M.A. Navia, P.R. Chaturvedi. Design principles for orally bioavailable drugs. *Drug Discovery Today* **1**(5) (1996) 179-189.
2. M.V.S. Varma, R.S. Obach, C. Rotter, H.R. Miller, G. Chang, S.J. Steyn, A. El-Kattan, M.D. Troutman. Physicochemical space for optimum oral bioavailability: contribution of human intestinal absorption and first-pass elimination. *Journal of Medicinal Chemistry* **53**(3) (2010) 1098-1108.
3. P.D. Dobson, D.B. Kell. Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nature Reviews Drug Discovery* **7**(3) (2008) 205-220
4. K. Sugano, M. Kansy, P. Artursson, A. Avdeef, S. Bendels, L. Di, G.F. Ecker, B. Faller, H. Fischer, G. Gerebtzoff, H. Lennernäs, F. Senner. Coexistence of passive and carrier-mediated processes in drug transport. *Nature Reviews Drug Discovery* **9**(8) (2010) 597-614.
5. G.L. Amidon, H. Lennernäs, V.P. Shah, J.R. Crison. A theoretical basis for a biopharmaceutic drug classification: The correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharmaceutical Research* **12**(3) (1995) 413-420.

6. Center for Drug Evaluation and Research. Food and Drug Administration. Guidance for Industry: Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System 2000. <http://www.fda.gov/downloads/Drugs/Guidance-ComplianceRegulatoryInformation/Guidances/UCM070246.pdf>, accessed 12 August 2011.
7. M. Kansy, F. Senner, K. Gubernator. Physicochemical high throughput screening: Parallel artificial membrane permeation assay in the description of passive absorption processes. *Journal of Medicinal Chemistry* **41**(7) (1998) 1007-1010.
8. P. Artursson, J. Karlsson. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochemical and Biophysical Research Communications* **175**(3) (1991) 880-885.
9. V. Fade, S. Stavchansky. Link between drug absorption solubility and permeability measurements in Caco-2 cells. *Journal of Pharmaceutical Sciences* **87**(12) (1998) 1604-1607.
10. J.D. Irvine, L. Takahashi, K. Lockhart, J. Cheong, J.W. Tolan, H.E. Selick, J.R. Grove. MDCK (Madin-Darby canine kidney) cells: A tool for membrane permeability screening. *Journal of Pharmaceutical Sciences* **88**(1) (1999) 28-33.
11. M.H. Jacobs. Some aspects of cell permeability to weak electrolytes. *Cold Spring Harbor Symposia on Quantitative Biology* **8** (1940) 30-39.
12. P.A. Shore, B.B. Brodie, C.A. Hogben. The gastric secretion of drugs: a pH partition hypothesis. *The Journal of Pharmacology and Experimental Therapeutics* **119**(3) (1957) 361-369.
13. C.A. Hogben, D.J. Tocco, B.B. Brodie, L.S. Schanker. On the mechanism of intestinal absorption of drugs. *The Journal of Pharmacology and Experimental Therapeutics* **125**(4) (1959) 275-282.
14. E.R. Garrett, P.B. Chemburkar. Evaluation, control, and prediction of drug diffusion through polymeric membranes. II. Diffusion of aminophenones through silastic membranes: a test of the pH-partition hypothesis. *Journal of Pharmaceutical Sciences* **57**(6) (1968) 949-959.
15. V.F. Smolen. Misconceptions and thermodynamic untenability of deviations from pH-partition hypothesis. *Journal of Pharmaceutical Sciences* **62**(1) (1973) 77-79.
16. A. Avdeef, K.J. Box, J.E.A. Comer, C. Hibbert, K.Y. Tam. pH-Metric log P 10. Determination of liposomal membrane-water partition coefficients of ionizable drugs. *Pharmaceutical Research* **15**(2) (1998) 209-215.
17. K. Iseki, T. Hirano, Y. Fukushi, Y. Kitamura, S. Miyazaki, M. Takada, M. Sugawara, H. Saitoh, K. Miyazaki. The pH dependent uptake of enoxacin by rat intestinal brush-border membrane vesicles. *Journal of Pharmacy and Pharmacology* **44**(9) (1992) 722-726.
18. C. Ottiger, H. Wunderli-Allenspach. Partition behaviour of acids and bases in a phosphatidylcholine liposome-buffer equilibrium dialysis system. *European Journal of Pharmaceutical Sciences* **5**(4) (1997) 223-231.
19. A. Adson, T.J. Raub, P.S. Burton, C.L. Barsuhn, A.R. Hilgers, N.F.H. Ho, K.L. Audus. Quantitative approaches to delineate paracellular diffusion in cultured epithelial cell monolayers. *Journal of Pharmaceutical Sciences* **83**(11) (1994) 1529-1536.

20. K. Palm, K. Luthman, J. Ros, J. Gråsjö, P. Artursson. Effect of molecular charge on intestinal epithelial drug transport: pH- dependent transport of cationic drugs. *Journal of Pharmacology and Experimental Therapeutics* **291**(2) (1999) 435-443.
21. H. Lennernäs. Human jejuna effective permeability and its correlation with preclinical drug absorption models. *Journal of Pharmacy and Pharmacology* **49**(7) (1997) 627-638.
22. H. Lennernäs. Animal data: The contributions of the Ussing chamber and perfusion systems to predicting human oral drug delivery in vivo. *Advanced Drug Delivery Reviews* **59**(11) (2007) 1103–1120.
23. A. Avdeef. Absorption and Drug Development. Solubility, Permeability, and Charge State, *Wiley-Interscience*, 2003.
24. A. Avdeef, M. Strafford, E. Block, M. P. Balogh, W. Chambliss, I. Khan. Drug absorption in vitro model: filter-immobilized artificial membranes 2. Studies of the permeability properties of lactones in Piper methysticum Forst. *European Journal of Pharmaceutical Sciences* **14**(4) (2001) 271–280.
25. M. Velický, D.F. Bradley, K.Y. Tam, R.A.W. Dryfe. *In situ* artificial membrane permeation assay under hydrodynamic control: permeability-pH profiles of warfarin and verapamil. *Pharmaceutical Research* **27**(8) (2010) 1644–1658.
26. J.C. Dearden, G.M. Bresnen. The measurement of partition coefficients. *Quantitative Structure-Activity Relationships* **7**(3) (1988) 133-144.
27. A. Avdeef, P.E. Nielsen, O. Tsinman. PAMPA - A drug absorption in vitro model: 11. Matching the in vivo unstirred water layer thickness by individual-well stirring in microtitre plates. *European Journal of Pharmaceutical Sciences* **22**(5) (2004) 365-374.
28. A. Adson, P.S. Burton, T.J. Raub, C.L. Barsuhn, K.L. Audus, N.F.H. Ho. Passive diffusion of weak organic electrolytes across Caco-2 cell monolayers: Uncoupling the contributions of hydrodynamic, transcellular, and paracellular barriers. *Journal of Pharmaceutical Sciences* **84**(10) (1995) 1197-1204.
29. V.G. Levich. Physicochemical Hydrodynamics. *Englewood Cliffs*, London, 1962.
30. J. Karlsson, P. Artursson. A method for the determination of cellular permeability coefficients and aqueous boundary layer thickness in monolayers of intestinal epithelial (Caco-2) cells grown in permeable filter chambers. *International Journal of Pharmaceutics* **71**(1) (1991) 55-64.
31. G.E. Amidon, W.I. Higuchi, N.F.H. Ho. Theoretical and experimental studies of transport of micelle-solubilized solutes. *Journal of Pharmaceutical Sciences* **71**(1) (1982) 77-84.
32. W.J. Albery, J.F. Burke, E.B. Leffler, J. Hadgraft. Interfacial Transfer Studied with a Rotating Diffusion Cell. *Journal of the Chemical Society-Faraday Transactions I* **72** (1976) 1618-1626.
33. R.H. Guy, D.H. Honda. Solute transport resistance at the octanol - water interface. *International Journal of Pharmaceutics* **19**(2) (1994) 129-137.
34. D.E. Leahy, A.R. Wait. Solute transport resistance at water-oil interfaces. *Journal of Pharmaceutical Sciences* **75**(12) (1986) 1157-1161.
35. F. Wohnsland, B. Faller. High-throughput permeability pH profile and high-throughput alkane/water log P with artificial membranes. *Journal of Medicinal Chemistry* **44**(6) (2001) 923-930.

36. M. Bermejo, A. Avdeef, A. Ruiz, R. Nalda, J.A. Ruell, O. Tsinman, I. Gonzalez, C. Fernandez, G. Sanchez, T.M. Garrigues, V. Merino. PAMPA - a drug absorption in vitro model 7. Comparing rat in situ, Caco-2, and PAMPA permeability of fluoroquinolones. *European Journal of Pharmaceutical Sciences* **21**(4) (2004) 429-441.
37. A. Avdeef. High-throughput Measurement of Permeability Profiles. Drug Bioavailability, *Wiley-VCH* Weinheim, 2003.
38. C. Dagenais, A. Avdeef, O. Tsinman, A. Dudley, R. Beliveau. P-glycoprotein deficient mouse in situ blood-brain barrier permeability and its prediction using an in combo PAMPA model. *European Journal of Pharmaceutical Sciences* **38**(2) (2009) 121-137.
39. K.Y. Tam, A. Avdeef, O. Tsinman, N. Sun. The permeation of amphoteric drugs through artificial membranes - an in combo absorption model based on paracellular and transmembrane permeability. *Journal of Medicinal Chemistry* **53**(1) (2010) 392-401.
40. K. Sugano, H. Hamada, M. Machida, H. Ushio, K. Saitoh, K. Terada. Optimized conditions of bio-mimetic artificial membrane permeation assay. *International Journal of Pharmaceutics* **228**(1) (2001) 181-188.
41. K. Sugano, N. Takata, M. Machida, K. Saitoh, K. Terada. Prediction of passive intestinal absorption using bio-mimetic artificial membrane permeation assay and the paracellular pathway model. *International Journal of Pharmaceutics* **241**(2) (2002) 241-251.
42. E.M. Renkin. Filtration, diffusion, and molecular sieving through porous cellulose membranes. *The Journal of General Physiology* **38**(2) (1954) 225-243.
43. X. Chen, A. Murawski, K. Patel, C.L. Crespi, P.V. Balimane. A novel design of artificial membrane for improving the PAMPA model. *Pharmaceutical Research* **25**(7) (2008) 1511-1520.
44. A. Avdeef, O. Tsinman, K. Tsinman. Very thin PAMPA membranes indicate higher antipyrine permeability but are fragile and contain leaky water pores. *AAPS meeting* 2010. Poster presentation.
45. G.E. Flaten, A.B. Dhanikulaa, K. Luthman, M. Brandl. Drug permeability across a phospholipid vesicle based barrier: A novel approach for studying passive diffusion. *European Journal of Pharmaceutical Sciences* **27**(1) (2006) 80-90.
46. G. Corti, F. Maestrelli, M. Cirri, S. Furlanetto, P. Mura. Development and evaluation of an in vitro method for prediction of human drug absorption I. Assessment of artificial membrane composition. *European Journal of Pharmaceutical Sciences* **27**(4) (2006) 346-353.
47. G. Corti, F. Maestrelli, M. Cirri, N. Zerroukb, P. Mura. Development and evaluation of an in vitro method for prediction of human drug absorption II. Demonstration of the method suitability. *European Journal of Pharmaceutical Sciences* **27**(4) (2006) 354-362.
48. Z.S. Teksin, P.R. Seo, J.E. Polli. Comparison of drug permeabilities and BCS classification: Three lipid-component PAMPA system method versus caco-2 monolayers. *The American Association of Pharmaceutical Scientists Journal* **12** (2006) 238-241.

49. L. Di, E.H. Kerns, K. Fan, O.J. McConnell, G.T. Carter. High throughput artificial membrane permeability assay for blood-brain barrier. *European Journal of Medicinal Chemistry* **38**(3) (2003) 223-232.
50. A. Avdeef, O. Tsinman. PAMPA - A drug absorption in vitro model: 13. Chemical selectivity due to membrane hydrogen bonding: In combo comparisons of HDM-, DOPC-, and DS-PAMPA models. *European Journal of Pharmaceutical Sciences* **28**(1) (2006) 43-50.
51. H. Lennernäs. Human intestinal permeability. *Journal of Pharmaceutical Sciences* **87**(4) (1998) 403-410.
52. A.A. Cools, L.H.M. Janssen. Influence of sodium ion-pair formation on transport kinetics of warfarin through octanol-impregnated membranes. *Journal of Pharmacy and Pharmacology* **35**(10) (1983) 689-691.
53. A. Walter, J. Gutknecht. Monocarboxylic acid permeation through lipid bilayer membranes. *Journal of Membrane Biology* **77**(3) (1984) 255-264.
54. J.B. Dressman, G.L. Amidon, C. Reppas, V.P. Shah. Dissolution testing as a prognostic tool for oral drug absorption: Immediate release dosage forms. *Pharmaceutical Research* **15**(1) (1998) 11-22.
55. K. Kontturi, L. Murtomäki, J.A. Manzanares. Ionic Transport Processes in Electrochemistry and Membrane Science. *Oxford University Press*, 2008.
56. G.L. Flynn, S.H. Yalkowsky, T.J. Roseman. Mass transport phenomena and models: theoretical concepts. *Journal of Pharmaceutical Sciences* **63**(4) (1974) 479-510.
57. G.L. Amidon, P.J. Sinko, D. Fleisher. Estimating human oral fraction dose absorbed: A correlation using rat intestinal membrane permeability for passive and carrier-mediated compounds. *Pharmaceutical Research* **5**(10) (1988) 651-654.
58. A. Avdeef. Leakiness and size exclusion of paracellular channels in cultured epithelial cell monolayers-interlaboratory comparison. *Pharmaceutical Research* **27**(3) (2010) 480-489.
59. A. Avdeef. The rise of PAMPA. *Expert Opinion on Drug Metabolism & Toxicology* **1**(2) (2005) 325-342.
60. A. Avdeef, P. Artursson, S. Neuhoff, L. Lazorova, J. Gråsjö, S. Tavelin. Caco-2 permeability of weakly basic drugs predicted with the double-sink PAMPA pKa flux method. *European Journal of Pharmaceutical Sciences* **24**(4) (2005) 333-349.
61. D. Winne. Dependence of intestinal absorption in vivo on the unstirred layer. *Naunyn-Schmiedeberg's Archives of Pharmacology* **304** (1978) 175-181.
62. U. Fagerholm, H. Lennernäs. Experimental estimation of the effective unstirred water layer thickness in the human jejunum, and its importance in oral drug absorption. *European Journal of Pharmaceutical Sciences* **3**(5) (1995) 247-253.
63. R.S. Obach, F. Lombardo, N.J. Waters. Trend analysis of a database of intravenous pharmacokinetic parameters in human for 670 drug compounds. *Drug Metabolism and Disposition* **36**(7) (2008) 1385-1405.
64. L.X. Yu, G.L. Amidon. A compartmental absorption and transit model for estimating oral drug absorption. *International Journal of Pharmaceutics* **186**(2) (1999) 119-125.
65. M. Velický, K.Y. Tam, R.A.W. Dryfe. In situ artificial membrane permeation assay under hydrodynamic control: Correlation between drug in vitro permeability and

- fraction absorbed in humans. *European Journal of Pharmaceutical Sciences* **44**(3) (2011) 299-309.
66. H. Lennernäs. Intestinal permeability and its relevance for absorption and elimination. *Xenobiotica* **37**(10) (2007) 1015-1051.
 67. G. Kaiser, R. Ackermann, S. Brechbühler, W. Dieterle. Pharmacokinetics of the angiotensin converting enzyme inhibitor benazepril.HCl (CGS 14 824 A) in healthy volunteers after single and repeated administration. *Biopharmaceutics and Drug Disposition* **10**(4) (1989) 365-376.
 68. W.K. Sietsema. The absolute oral bioavailability of selected drugs. *International Journal of Clinical Pharmacology Therapy and Toxicology* **27**(10) (1989) 179-211.
 69. D. Tenero, D. Martin, B. Ilson, J. Jushchysyn, S. Boike, D. Lundberg, N. Zariffa, D. Boyle, D. Jorkasky. Pharmacokinetics of intravenously and orally administered eprosartan in healthy males: Absolute bioavailability and effect of food. *Biopharmaceutics and Drug Disposition* **19**(6) (1998) 351-356.
 70. C.T. Dollery. Therapeutic Drugs. *Churchill Livingstone*, 1999.
 71. FDA Drug Information (<http://www.fda.gov/Drugs/default.htm>).
 72. L.S. Goodman, A.G. Gilman, J.G. Hardman, L.E. Limbird. Goodman & Gilman's the pharmacological basis of therapeutics. *McGraw-Hill*, 2006.
 73. G. Lappin, Y. Shishikura, R. Jochimsen, R.J. Weaver, C. Gesson, B. Houston, B. Oosterhuis, O.J. Bjerrum, M. Rowland, C. Garner. Pharmacokinetics of fexofenadine: Evaluation of a microdose and assessment of absolute oral bioavailability. *European Journal of Pharmaceutical Sciences* **40**(2) (2010) 125-131.
 74. F.P. LaCreta, S. Kaul, G.D. Kollia, G. Duncan, D.M. Randall, D.M. Grasela. Interchangeability of 400-mg intravenous and oral gatifloxacin in healthy adults. *Pharmacotherapy* **20**(6) (2000) 59S-66S.
 75. C. Chen. Physicochemical, pharmacological and pharmacokinetic properties of the Zwitterionic antihistamines cetirizine and levocetirizine. *Current Medicinal Chemistry* **15**(21) (2008) 2173-2191.
 76. R. Whomsley, M.S. Benedetti. Development of new H1 antihistamines: the importance of pharmacokinetics in the evaluation of safe and therapeutically effective agents. *Current Medicinal Chemistry – Anti-Inflammatory & Anti-Allergy Agents* **4**(5) (2005) 451-464.
 77. K.N. Agwuh, A. MacGowan. A. Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines. *Journal of Antimicrobial Chemotherapy* **58**(2) (2006) 256-265.
 78. P.H. Hinderling. Evaluation of a novel method to estimate absolute bioavailability of drugs from oral data. *Biopharmaceutics & Drug Disposition* **24**(1) (2003) 1-16
 79. Physician's Desk Reference, PDR Network LLC, 2009.
 80. A.M. Frydman, Y. Leroux, M.A. Lefebvre, F. Djebbar, J.B. Fourtillan, J. Gaillot. Pharmacokinetics of pefloxacin after repeated intravenous and oral-administration (400-Mg Bid) in young healthy-volunteers. *Journal of Antimicrobial Chemotherapy* **17** Suppl B (1986) 65-79.
 81. R. Teng, L.C. Dogolo, S.A. Willavize, H.L. Friedman, J. Vincent. Oral bioavailability of trovafloxacin with and without food in healthy volunteers. *Journal of Antimicrobial Chemotherapy* **39** Suppl B (1997) 87-92.

PROTEIN-LIGAND INTERACTIONS

Geoff Holdgate

Contents

4.1. INTRODUCTION	167
4.2. OVERVIEW OF DRUG DISCOVERY	168
4.3. PROTEIN-LIGAND BINDING EQUILIBRIA, THERMODYNAMICS AND KINETICS	169
4.4. NON-COVALENT INTERACTIONS IN PROTEIN-LIGAND BINDING	172
4.4.1. Hydrogen bonding	173
4.4.2. Ionic or electrostatic interactions	173
4.4.3. Van der Waals interactions	173
4.4.4. Hydrophobic interactions.....	173
4.5. BIOPHYSICAL METHODS FOR CHARACTERISING PROTEIN-LIGAND INTERACTIONS	174
4.5.1. Isothermal titration calorimetry (ITC)	174
4.5.2. Surface Plasmon Resonance (SPR)	175
4.5.3. Optical waveguide grating (OWG)	178
4.5.4. Spectroscopic methods – Nuclear Magnetic Resonance (NMR)	178
4.5.4.1. 2D NMR chemical shift mapping	179
4.5.4.2. 1D NMR direct binding and reference displacement	179

4.5.5. Spectrometric methods – Mass Spectrometry (MS)	180
4.5.5.1. Direct detection of the ligand	180
4.5.5.2. Detection of the protein-ligand complex	181
4.6. BRIEF CASE STUDIES EXEMPLIFYING THE USE OF BIOPHYSICAL METHODS	181
4.6.1. DNA gyrase	181
4.6.2. Beta-site APP cleaving enzyme 1 (BACE-1)	183
4.6.3. MEK protein kinase	184
4.7. FUTURE TRENDS FOR BIOPHYSICAL APPROACHES	185
4.8. SUMMARY	186
REFERENCES	186

4.1. INTRODUCTION

The quest to discover or develop new medicines to facilitate healthcare management for a growing and ageing global population is a long and difficult task. Although knowledge of the human genome and the proteins encoded by it has helped to identify new drug targets, our understanding of proteins, ligands and their complexes is still relatively superficial. Proteins are the most common molecular target of drugs, since they play an essential role in cellular activity. Understanding protein-ligand interactions, whether the protein is an enzyme, transporter, ion channel, or G-protein coupled receptor (GPCR), or whether its ligand is a natural ligand, substrate or drug molecule is therefore of fundamental importance across many biological, biochemical, and biophysical disciplines.

As recognised in the late 19th [1] and early 20th [2,3] centuries, in order for the effective occurrence of cellular processes and drug-mediated interventions, these processes rely upon ligand binding to the protein target to form a complex. These cellular proteins, which are the targets for drug molecules are termed receptors. The term receptors is now more commonly used for a subset of proteins that act as intracellular binding partners for chemical messengers, and for clarity within this chapter we will continue to refer to protein-ligand interactions (although these may be interchanged with terminology such as receptor-ligand, receptor-drug, etc.).

The process termed molecular recognition by which the protein and ligand interact is dependent upon factors such as surface complementarity, hydrophobicity, and electrostatics. A detailed understanding of these principles at a microscopic and macroscopic level is required in order to begin to understand how to rationally design new drug molecules.

Thus, to fully understand protein–ligand interactions and to utilise this information to make an impact on drug discovery and healthcare requires that the biophysical properties of the protein, ligand and the protein–ligand complex be investigated.

4.2. OVERVIEW OF DRUG DISCOVERY

The goal of drug discovery is to utilise the knowledge of and interplay between chemistry, biology and pharmacology in order to design and develop new medicines. Understanding and modifying interactions between ligands, designed during the drug discovery process, and both target and other proteins are critically important steps that must take place in order to progress projects.

Historically, the approach to discovering drug molecules targeting proteins involved in disease mechanisms was to investigate compounds isolated from various natural sources. Overall, these compounds are involved in the treatment of many human diseases [4]. This important starting point has provided a rich source of drugs including chemotherapy agents (*e.g.* paclitaxel), immuno-suppressive drugs (*e.g.* cyclosporins) and cholesterol lowering drugs (*e.g.* the statins). Modern drug discovery, however, utilises both the skill of the medicinal chemist to provide creative solutions to improve compound properties, along with genomics and proteomics approaches (5) to identify proteins involved in relevant biological pathways.

Target selection is achieved from a combination of the understanding of biological processes and molecular approaches aimed at identifying protein target modulation which could provide clinical benefits in the relevant disease setting [6].

Most targets are proteins, with most drugs being small molecular compounds which interact physically with the target and alter its biological function. Biological understanding was traditionally the main target discovery strategy, and it remains important for diseases in which the relevant phenotype can only be detected at the whole body level, such as atherosclerosis, obesity, heart failure, hypertension and neurodegenerative disorders. Molecular approaches are driven by technologies that attempt to correlate molecular changes with human disease, such as changes in gene expression (genomics), protein expression (proteomics) or genetic variation. The absence or mutation of a particular gene can result in serious disease or the risk of contracting a disease, examples include the link between apolipoprotein A and cardiovascular disease, or BRCA 1 and 2 and breast and ovarian cancer. Following the selection of the protein target, several approaches are taken for hit and lead identification. These include *in silico* methods, combinatorial chemistry and high-throughput screening utilising the resultant corporate compound libraries. Hits are identified as those compounds showing activity in the assay. The lead identification process is designed to identify several structurally distinct chemical series that produce the desired pharmacological effects, have acceptable drug like properties or the potential for optimisation and are patentable. Another valuable source of leads is from competitor compounds, where the aim is to identify groups from those compounds, which may be modified to improve physicochemical properties, whilst avoiding competitor patent restrictions. This approach can lead to dramatic improvements in biological and pharmacokinetic profiles, and is reflected in the

quote from Nobel Prize winner Sir James Black (1924 – 2010): “The most fruitful basis for the discovery of a new drug is to start with an old drug”.

It is during this lead optimisation stage that structure-activity relationships (SARs) are developed with respect to potency for the target protein. Selectivity considerations, detailing which other proteins the ligand may bind to, and their relative affinities, also are investigated. It is also at this stage that drug metabolism and pharmacokinetics (DMPK) investigations begin.

During lead optimisation, 2-4 compounds that meet the candidate drug target profile are identified by applying medicinal chemistry to produce analogues of a lead series [7]. It is thus here, that these lead compounds are optimised with respect to potency, selectivity, DMPK and chemistry scale up is begun.

At the same time, the clinical strategy is defined, and markers of drug action and disease effect are characterised.

Once a suitable drug candidate is found preclinical testing begins, where the aim is to evaluate safety, toxicology, metabolism and pharmacokinetics of the developed compound. This will involve *in vitro*, *in vivo* and *ex vivo* testing as required by the regulatory authorities.

Clinical trials are then required, but before use a drug needs to be approved by either the Food and Drug Administration (FDA) or the European Agency for the Evaluation of medicinal Products (EMA). During the first part of these trials, the candidate drug is administered to healthy volunteers (Phase I), in order to assess drug safety, possible side effects and to gather basic pharmacokinetic information. During the next stage (Phase II), the drug is given to patients with the disorder for which the candidate drug is a proposed treatment. It is here that the dosage will be evaluated and preliminary data on efficacy in patients collected. Subsequently, the drug will be given to a larger number of patients (Phase III), allowing the drug to be compared to existing therapies and enabling statistics to be collected on any adverse reactions [8].

4.3. PROTEIN-LIGAND BINDING EQUILIBRIA, THERMODYNAMICS AND KINETICS

Assuming that the protein, P, has a single binding site for the ligand, L, and the binding interaction is a reversible bimolecular reaction:

We can describe the total protein, $[P]_t$, and total ligand, $[L]_t$, concentrations in terms of a pair of mass conservation equations:

$$[P]_t = [PL] + [P]_f$$

$$[L]_t = [PL] + [L]_f$$

where, $[P]_f$ and $[L]_f$ are the concentrations of free protein and ligand, respectively.

We also can then describe the change in Gibbs free energy (ΔG) for the binding reaction by the equation:

$$\Delta G = \Delta G^\circ + RT \ln \frac{[PL]}{[P]_f [L]_f}$$

where ΔG° is the standard free energy change, R is the gas constant ($8.31 \text{ J mol}^{-1} \text{ K}^{-1}$), and T is the temperature in Kelvin. At equilibrium, where $\Delta G = 0$, then the equation becomes:

$$\Delta G^\circ = -RT \ln \frac{[PL]}{[P]_f [L]_f}$$

If we represent the ratio of the reactants and product concentrations as the equilibrium dissociation constant $K_d = [P]_f [L]_f / [PL]$, then we obtain:

$$\Delta G^\circ = RT \ln K_d$$

The equilibrium dissociation constant is most often used in biochemical studies because it has units of molarity, and so may be related to the ligand concentration leading to half maximal saturation of the protein. It describes the strength of the interaction between protein and ligand, with lower values representing tighter binding.

Because the ΔG° may be described by the change in standard enthalpy (ΔH°) and standard entropy (ΔS°), these parameters can also be related to K_d :

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ = RT \ln K_d$$

The enthalpy represents the energy reduced by so called volume work of a reaction at constant pressure, and the entropy represents the degree of disorder introduced by the energy being distributed over the number of accessible degrees of freedom. We can see that for a spontaneous binding interaction, where ΔG° is negative, that a decrease in enthalpy and an increase in entropy are favourable contributions to an increase in binding affinity.

Thus, it can be seen that the equilibrium dissociation constant is derived from thermodynamic principles and can be related to fundamental thermodynamic parameters. A K_d value of 1 nM, which is a value commonly observed for optimised leads or even candidate drugs, corresponds to a Gibbs free energy of binding of $-53.4 \text{ kJ mol}^{-1}$ at body temperature, with a ten fold change in affinity representing a change of 5.9 kJ mol^{-1} .

The binding interaction may also be described in kinetic terms, because the equilibrium between free partners and the protein-ligand complex will be governed by the rate of complex formation and rate of its dissociation. The

second order rate constant for association is termed k_{on} , and the first order rate constant for dissociation is k_{off} , as shown above.

The K_d is given by the ratio of the rate constants:

$$K_d = \frac{k_{\text{off}}}{k_{\text{on}}}$$

For diffusion controlled binding, where the association rate constant (at least for enzyme–substrate interactions) can be up to $10^9 \text{ M}^{-1}\text{s}^{-1}$ [9] – $10^{10} \text{ M}^{-1}\text{s}^{-1}$ [10], while the dissociation rate constant for a K_d value of 1 nM will be $1 - 10 \text{ s}^{-1}$.

In the situation where a large excess of ligand is required to produce a significant degree of binding to the protein, then binding occurs with little change in the free ligand concentration, and so the association reaction occurs with pseudo-first order kinetics, so that:

$$[\text{PL}]_t = [\text{PL}]_{\text{eq}}(1 - \exp(-k_{\text{obs}}t))$$

where $[\text{PL}]_t$ is the concentration of the protein-ligand complex at time t , $[\text{PL}]_{\text{eq}}$ is the concentration of the complex at equilibrium and k_{obs} is the pseudo-first order rate constant.

The value of k_{obs} for a reversible interaction is given by:

$$k_{\text{obs}} = k_{\text{off}} + k_{\text{on}}[\text{L}]_f$$

Hence, a plot of k_{obs} versus $[\text{L}]_f$ will allow the rate constants and hence the K_d to be evaluated.

For this single step reversible equilibrium the residence time, τ , (the length of time the ligand occupies the protein binding site) is given by $1/k_{\text{off}}$.

Although it is possible to determine the K_d value from kinetic measurements, as shown above, these values can often be difficult to measure, because they occur on a short timescale, and so K_d values are often measured at equilibrium.

Many protein–inhibitor binding interactions of importance in drug discovery proceed more slowly than the diffusion controlled limit, and often exhibit slow binding inhibition.

This typically occurs via a two step mechanism,

where the enzyme encounters the ligand and subsequently a tightening of the initial interaction occurs, for example via a conformational change, allowing a tighter binding steady state complex PL^* to form.

This situation is described by two dissociation (or, more correctly, inhibition) constants, K_i and K_i^* .

The second inhibition constant is given by:

$$K_i^* = \frac{K_i k_4}{k_3 + k_4}$$

For this type of interaction the k_{obs} is given by a more complicated combination of rate constants:

$$k_{\text{obs}} = k_4 + \frac{k_3}{1 + \frac{K_i'}{[L]}}$$

where K_i' is the apparent K_i which will depend upon the mechanism of inhibition and the substrate concentration, and may be related to the true K_i value according to the Cheng-Prusoff equation [11]:

$$K_i' = \frac{K_{\text{is}} K_{\text{ii}} (K_m + [S])}{K_m K_{\text{ii}} + [S] K_m}$$

where K_{is} and K_{ii} are the inhibition constants at $[S] \ll K_m$ and $[S] \gg K_m$.

The residence time for the two step binding mechanism is given by:

$$\tau = \frac{k_2 + k_3 + k_4}{k_3 k_4}$$

Two step mechanisms such as this appear to be common in drug discovery, with many inhibitors displaying slow kinetics according to this mechanism. The second step is slow which is often attributed to conformational rearrangements or even irreversible inhibition such as in the case where k_2 or k_4 tend to zero.

Investigation into the kinetics and thermodynamics of the binding interaction is an invaluable tool in drug discovery as it may help to identify compounds which act by preferred or alternative mechanisms.

4.4. NON-COVALENT INTERACTIONS IN PROTEIN-LIGAND BINDING

Molecules can interact with each other via a number of non-covalent interactions that are mediated by surface complementarity between the protein binding site and the ligand. These weak attractive forces are important in molecular recognition occurring in biochemical binding reactions because they occur reversibly.

As these interactions are weak in nature, multiple interactions are usually required for the formation of a stable binding complex, and the interacting groups tend to be in close proximity.

The four types of non-covalent interactions important in protein-ligand binding are hydrogen bonding, ionic or electrostatic interactions, van der Waals forces and hydrophobic interactions.

4.4.1. Hydrogen bonding

Hydrogen bonds are electrostatic attractions between two dipoles and form when a hydrogen atom is shared by two electronegative atoms, referred to as the donor (to which the hydrogen is covalently bonded), and the acceptor. In proteins the electronegative heteroatoms are predominantly oxygen or nitrogen.

Hydrogen bonds are weak, contributing around $12.5 - 21 \text{ kJ mol}^{-1}$, with the actual strength being dependent upon several factors including the local medium, but in particular the distance and direction between the hydrogen and the acceptor [12].

Although individually weak, hydrogen bonding is the largest contributor to the binding energy of ligands, as well as to the structural elements within a protein structure.

4.4.2. Ionic or electrostatic interactions

These interactions occur when two oppositely charged groups are attracted to one another through a Coloumbic force. It has been suggested that these interactions are likely to be responsible for the initial recognition of protein and ligand.

The strength of the interaction is dependent upon the charges on the two atoms, as well as the distance between them and the dielectric constant of the medium.

Many ligands rely on ionic interactions for specific binding, with the greatest attractive force occurring in the low dielectric constant environment of the hydrophobic interior of the protein.

4.4.3. Van der Waals interactions

Van der Waals interactions occur as a result of the attraction between temporary dipoles that arise due to the uneven distribution of electrons between neighbouring atoms.

This attractive force is much weaker than ionic or hydrogen bonds, with an energy of no more than around 4 kJ mol^{-1} . However, due to high surface complementarity between protein and ligand, the number of van der Waals interactions can be large, with the collective force contributing significantly to complex stability.

4.4.4. Hydrophobic interactions

Hydrophobic interactions occur between two non-polar groups, due to the reduction in the unfavourable organisation of water around the non-polar groups, when the groups associate with one another [13].

For ligand binding, the hydrophobic region of the binding site tends to stabilise the binding of hydrophobic ligands. This hydrophobic portioning of ligands from solution to the protein active site can be a strong contributor to the binding energy.

4.5. BIOPHYSICAL METHODS FOR CHARACTERISING PROTEIN-LIGAND INTERACTIONS

4.5.1. Isothermal titration calorimetry (ITC)

The ITC experiment involves the monitoring of the heat change during the binding reaction (for a comprehensive protocol see [14]). The ligand solution is usually titrated from the injection syringe into the protein solution contained within the calorimeter cell. The instrument measures the heat change using a power compensation process whereby the difference in the variable power, proportional to the binding heat, applied to the sample cell and the constant power applied to the thermal reference cell is monitored by the instrument.

Depending upon the binding affinity and the amounts of available reagents, it is often possible to arrange the experimental conditions so that a single experiment can provide precise estimates of the affinity (K_d), the enthalpy (ΔH) and the stoichiometry (n) of the binding interaction. This also allows calculation of the entropy (ΔS) from the Gibbs-Helmholtz equation, above.

During the titration, in which small aliquots of the ligand solution are added, the first injections generate the largest heat change as most of the added ligand becomes bound to the protein. As the titration progresses the protein becomes increasingly saturated with ligand, and the heat differential heat change becomes smaller. Finally, following complete saturation, no further heat change is detected. However, sometimes significant, non-zero heats following saturation are observed, which are often attributable to the heat associated with dilution of the ligand, and can be corrected for by control titrations. Figure 4.1 shows a typical ITC thermogram.

Understanding the thermodynamic components of molecular interaction is important in drug discovery, as it allows optimisation of test compounds in a more meaningful way. The thermodynamic measurements that are made accessible through the ITC experiment are fundamental in trying to understand molecular interaction, and in applying that learning in the pursuit of compounds, not only with higher affinity, but with the appropriate thermodynamic and kinetic profiles for their biological function [15].

As shown above, the binding affinity of a test compound is related to the free energy of the interaction, which itself is dependent upon the enthalpic and entropic components. The situation is complicated by factors such as the influence of solvent water on the binding thermodynamics and the change in dynamics and conformation of the ligand and protein between the free and bound states. This makes the individual thermodynamic parameters incredibly difficult to predict.

Because ITC allows measurement of affinity and enthalpy in a single experiment, it can be useful in determining discontinuities in SAR which may be missed from K_d values alone.

Figure 4.1. Representative ITC thermogram for a compound binding to a target protein

Changes in enthalpy may provide a valuable approach for the selection of compounds during lead identification and in helping to guide the lead optimisation process towards compounds of higher quality. Enthalpic optimisation, where the enthalpy of interaction is increased moving from early to later marketed drugs, has been suggested as a useful approach to obtain advantages in the clinic [16,17]. The approach suggests that more efficient optimisation can be achieved if the contributions of both enthalpy and entropy are improved simultaneously [18].

By proactively using ITC to help guide medicinal chemistry in this way, we will inevitably initiate the exploration of the relationship between thermodynamics and structure in a more coherent manner that will enable us to increase and exploit our knowledge of molecular interaction.

4.5.2. Surface Plasmon Resonance (SPR)

Optical biosensors measure changes in some characteristic of light, coupled to changes (often in mass) at the sensor surface by making use of the electromagnetic evanescent-wave formed at the gold interface of the sensor chip surface. Biosensors employing SPR, such as BIAcore (GE Healthcare), are the

best-known optical biosensors, utilising this phenomenon to enable detection of protein-ligand interactions in real time.

At total internal reflection, the electromagnetic field component of the incident light (the so-called evanescent wave) penetrates into the gold film on the sensor chip and interacts with free electrons or plasmons. Photons of incident light are absorbed, the evanescent field strength amplified, and the light is no longer reflected. This decrease in the reflected intensity occurs at an angle which depends on the refractive index of the material in the flow cell, and is called the SPR angle. The refractive index of the flow cell changes concomitantly with mass on the surface and thus allows detection of ligand binding events, Figure 4.2 (for a review of the use of SPR in drug discovery see [19]). This potentially allows the kinetic and equilibrium constants for a given interaction to be determined.

Figure 4.2. Representative SPR sensorgram for a compound binding to an immobilised target protein

In order to monitor protein-ligand binding, one of the partners has to be immobilised onto the sensor surface. In direct binding assays (DBA) the target protein is coupled, without compromising the activity or functionality of the protein or the accessibility to the binding site. Several different methods are available in order to achieve this, with the most common being direct immobilisation via accessible primary amines exposed on the protein surface. This is achieved by activating the carboxymethyl-dextran matrix surface with a mixture of 1-ethyl -3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to introduce reactive succinimide esters that can react spontaneously with protein primary amines. Other immobilisation methods are available employing either covalent attachment using different chemistry strategies, or by employing non-covalent

capture using affinity methods, employing tags specifically incorporated within the protein.

The SPR signal is dependent on several factors associated with the target protein, including the molecular weight (MW) of the attached protein, the amount captured on the surface and the binding capacity of the protein, or the stoichiometry of the binding reaction. Factors associated with the ligand are also the MW, the total concentration, $[L]_t$ and the equilibrium dissociation K_d . The dependence upon MW and K_d for the small molecule ligand represents a particular challenge when working with fragments, where both size and affinity are low.

Issues that arise due to ligand molecular size may be circumvented by the use of alternative assay formats such as the surface competition assay (SCA) or inhibition in solution assay (ISA). The ISA overcomes these issues by monitoring binding of the macromolecule, whereby increasing test compound concentration results in a decrease in observed signal as the test compound competes for binding to the target protein, and thus makes systems amenable for small molecule work that may be out of the scope of direct binding.

The ISA format requires coupling of a tool compound, or so called target definition compound (TDC), to the biosensor surface which then serves as a probe for the binding site. The binding interaction between the TDC and the protein generates a control signal which will be lowered in the presence of competing test compounds. Only compounds showing kinetic competition with the TDC for binding to the target protein will reduce the amount of free protein available at equilibrium to bind to the TDC. This approach thus allows affinity information for the test compound to be derived [20].

An essential part of the initial development of an ISA is the identification of a suitable tool compound to be used as a TDC. Ideal properties for a suitable TDC would be binding which blocks the whole binding site, with rapid association kinetics and high potency (slow dissociation). This situation serves to ensure mass transport limited kinetics, so that the observed signal is proportional to the concentration of free target protein, with the biosensor essentially measuring the free protein concentration resulting from incubation with different concentrations of test compound. Attachment of the TDC via primary amines without compromising binding to the target protein is also the preferred attachment approach.

In addition to an absence of MW-limitations for proteins or ligands in the ISA, another major advantage is that the interaction of interest occurs in solution, alleviating any issues with potential changes in affinity caused by immobilisation. The protocols for immobilisation and regeneration are usually straightforward, as conditions usually too harsh to be used with immobilised protein can be routinely used with the small molecule attached to the surface. However, the main disadvantage compared to the DBA is that determination of the kinetic parameters is precluded by the ISA method.

4.5.3. Optical waveguide grating (OWG)

Biosensor instruments using optical waveguide-based systems have been growing in use recently. Both Corning and SRU Biosystems, manufacture instruments (EPIC and BIND, respectively) that allow increased sample throughput by using plate-based platforms.

These instruments make use of the evanescent waves formed when light travelling through a boundary between two media of different refractive indices undergoes total internal reflection. Wave coupling is then achieved by placing electromagnetic elements, such as optical waveguides, close together enabling propagation of a wave from one element to the next. Changes in the refractive index at the interface between the sensor surface and the solution phase, occurring as a consequence of ligand binding, modifies the wave coupling and triggers a change in the reflected or transmitted light. This allows changes in mass at the sensor surface, caused by binding to proteins attached to sensor surface, to be monitored (for a review of waveguide-based biosensors see [21]).

The main practical difference between the EPIC and BIND OWG systems to SPR-based systems is the absence of solution flow. Thus, the OWG systems are unable to measure accurate kinetics. However, this apparent disadvantage is compensated for by (1) increased throughput and (2) the ability to add several components either simultaneously or sequentially into the same well of the plate in order to study the effect of addition.

As with SPR-based platforms, ISA formats can be useful on OWG platforms. Many of the limitations described above for SPR are also problematic for OWG platforms. Limitations in sensitivity for small molecule binding represent a major challenge. Often, to overcome these issues, proteins are coupled at high density, resulting in high local concentrations. This leads to an increased risk of non-specific binding due to the potential increase in aggregated, unfolded, or functionally compromised proteins coupled to the sensor surface.

Thus, the immobilisation of a tool compound or TDC and making use of the ISA approach will circumvent these issues.

The use of these technologies is expected to expand as issues with protein immobilisation are overcome, perhaps via expansion of the available immobilisation chemistries and/or the sensitivity of detection improves.

4.5.4. Spectroscopic methods – Nuclear Magnetic Resonance (NMR)

Following the introduction of SAR by NMR by Fesik in 1996 [22], NMR has been an important tool for detecting protein-ligand binding in the lead generation phase of drug discovery. In fact, this ligand screening application has now almost completely replaced the use of NMR for structure solutions, which is predominantly achieved using X-ray crystallography.

Protein-ligand interactions for most soluble target proteins can be studied by NMR, and the assays are simple and robust generating few false negatives or false positives.

The main strength of NMR is that binding of low affinity ligands with K_d values in the μM to mM range can be readily detected, allowing NMR to be used as a front-line fragment screening method. In fact, NMR is often the method of choice for biophysical fragment screening.

Three main methods to monitor ligand binding by NMR are used: 2D NMR chemical shift mapping, 1D NMR direct binding and 1D NMR reference displacement.

4.5.4.1. 2D NMR chemical shift mapping

Protein-observe 2D NMR chemical shift mapping experiments can be conducted either using ^{15}N - ^1H or ^{13}C - ^1H (requiring uniform or amino acid specific ^{15}N or ^{13}C isotope labelling) correlations in Heteronuclear Single Quantum Coherence (HSQC) experiments [23], to provide a fingerprint of amide or methyl groups in the protein, respectively. Specific changes in individual residues are then monitored upon the addition of binding ligands. Residue peak assignments also can be obtained through a series of additional 3D NMR experiments, allowing analysis of the binding mode.

The approach has a wide dynamic range, identifying binders from mM to nM range, but quantitative K_d measurements are most reliable in the low affinity (μM to mM) range, under fast ligand exchange. Non-specific binding is generally not problematic in 2D NMR experiments since such binders are either not detected or tend to produce non-specific line broadening.

The requirement for an isotopically labelled protein and the size restriction up to around 40 kDa (80 kDa if uniform deuteration of the protein can be achieved) places some limitation on the application of 2D NMR.

4.5.4.2. 1D NMR direct binding and reference displacement

In 1D NMR ligand signals are observed directly. This can be advantageous in offering a built-in quality control and solubility measures for the test compounds studied. There is no limit on protein size, with larger proteins giving increased relaxation effects, resulting in magnified ligand binding effects. Generally, ^1H detection is used for 1D ligand-observe NMR, with the most common techniques being waterLOGSY, saturation transfer difference (STD), and $\text{T1}\rho$ and T2 filtered experiments [24,25].

1D NMR screening can be run either in direct binding mode where ligand binding is observed directly, or by monitoring the competition with an established reference ligand, often termed a spy molecule or reporter ligand.

The direct binding method is extremely sensitive, with binding effects observed even at ligand concentrations well below K_d , although competition with a ligand that is known to bind to the site of interest is required to ensure that the binding interaction is specific.

A major limitation of the 1D NMR direct binding approach is that the magnitude of the binding effect decreases when the binding approaches high affinity (nM) or

there is a slow ligand exchange regime, although this is not problematic for the competition method.

Typically, NMR approaches use compound mixtures, in order to increase the throughput of the techniques. Mixtures can be screened in direct 1D NMR without subsequent deconvolution, if reference spectra for the ligands in the mixture have been previously measured. Reference displacement methods require deconvolution to identify hits present in the mixture. Thus, typical screens comprising up to several thousand compounds are often completed in a timescale covering a few days to several weeks depending upon the screening mode.

4.5.5. Spectrometric methods – Mass Spectrometry (MS)

MS approaches for assessing protein-ligand binding may be grouped into 2 main types: (1) direct detection of the ligand and (2) detection of the protein-ligand complex.

4.5.5.1. Direct detection of the ligand

MS analysis of small molecules is a routine technology. Its strength is that hits can be identified by mass, allowing mixtures of compounds (with different MW) to be utilised during study. This, however, requires the separation of binders from non-binding compounds directly before the MS analysis step. This separation of bound from unbound compounds can be achieved in a variety of ways including size exclusion chromatography, ultrafiltration, equilibrium dialysis, or frontal affinity chromatography. These approaches are suited to liquid chromatography electrospray ionisation MS (LC-ESI-MS) which facilitates protein denaturation and the release of the bound ligand.

For size exclusion based separations, the target protein is incubated with one or a mixture of potential ligands before chromatography. The chromatographic step rapidly separates unbound compounds from those bound to the protein before analysis by LC-ESI-MS. This approach is amenable to high throughput screening (HTS).

Using the ultrafiltration approach, the target protein is incubated with potential ligands before being concentrated in a centrifugal device with a semi-permeable membrane. Unbound compounds pass through the membrane and become significantly diluted, beyond MS detection limits. Bound ligands are retained in the protein-containing sample, and are identified by LC-ESI-MS.

In equilibrium dialysis (ED) compound mixtures are allowed to fully equilibrate between two chambers separated by a membrane permeable to only the compounds, with one chamber containing the target protein. The contents of the two chambers are analyzed quantitatively by LC-ESI-MS; with bound ligands identified as the total concentration in the protein-containing chamber will be higher than that on the protein free side [26].

A fundamentally different approach is frontal affinity chromatography, in which the target protein is immobilized onto a liquid chromatography column. Com-

pounds are introduced and detected after the column step by ESI-MS. Changes in retention volume, indicating binding, allows bound ligands to be identified [27]. With the introduction of tool ligands with known affinity, competition experiments can be performed to estimate binding affinity for test compounds.

4.5.5.2. Detection of the protein-ligand complex

Native mass spectrometry [28] has been shown to identify non-covalent protein-ligand complexes, as the conditions allow the protein to retain a native fold and retain bound ligands in the gas phase [29]. This screening approach has been employed for standard libraries [30] and also has been used for screening fragments [31].

4.6. BRIEF CASE STUDIES EXEMPLIFYING THE USE OF BIOPHYSICAL METHODS

The most powerful use of biophysical methods is in the combination of approaches, either across the biophysical methodology range, or coupled with biochemical data aimed at addressing specific problems during the drug discovery process. The examples below refer to drug discovery projects carried out at AstraZeneca, where the use of biophysical methods was integral to project progression.

4.6.1. DNA gyrase

DNA gyrase is a bacterial topoisomerase, essential for DNA replication and cell viability. Topoisomerases are the only DNA replication targets validated by drugs in the clinical setting, and DNA gyrase is well conserved across all bacterial pathogens of interest.

The DNA gyrase enzyme relieves the supercoiling of DNA generated during replication and transcription, and uses the energy from ATP to catalyse the DNA topology changes. It is a tetrameric enzyme composed of a dimer of dimers, with the A subunits involved with DNA cleavage and resealing and the B subunit (GyrB) responsible for the ATPase activity.

24 kDa and 43 kDa fragments of the DNA gyrase B subunit were used for structural biology efforts and with both ITC and NMR methods in order to help identify and characterise gyrase binding compounds. ITC was used to investigate the binding affinity and thermodynamics of compounds binding to these protein fragments. The utility of the ITC method can be emphasised in this project, as these fragments of the B subunit lack the topoisomerase-linked ATPase activity of the intact tetramer, and so traditional biochemical methods following catalytic activity could not be used to measure test compound affinity.

In a subsequent fragment based approach, 2D NMR was used to screen a library of low molecular weight fragment compounds containing both generic fragments and fragments of known gyrase inhibitors. The 2D NMR method allowed both affinity and binding site information to be obtained. The fragment hits were

found to have high ligand efficiency ($\Delta G/\text{heavy atom}$) of up to 0.46, and shared a hydrogen bond donor/acceptor motif similar to the adenine moiety of ATP.

Having identified a naphthol fragment as an efficient binder to the primary fragment site, a secondary screen was undertaken using the GyrB:naphthol complex. Following identification of a weak (5 mM) binding quinoline, docking methods suggested likely orientations of the 2 fragments and allowed a linking strategy to be followed to improve potency, Figure 4.3a.

Figure 4.3. (a) Docking suggesting likely binding poses of pyrrole and quinoline fragments in the 24 kDa fragment of DNA Gyrase B subunit; (b) Crystal structure demonstrating how pyrrole substitutions were optimised to fill site 1, whilst additional H-bonds were formed with the amide occupying site 2

Improved potency was driven by optimisation of the GyrB interactions, making substitutions on a pyrrole moiety to fill site 1. Additional hydrogen bonding interactions were incorporated into site 2, which extended into the novobiocin binding pocket, whilst maintaining the ligand efficiency at 0.39, Figure 4.3b.

The pyrrolamide series [32] of established compounds maintain potency against a range of drug resistant bacterial strains, are bactericidal with low resistance frequency and demonstrate potency against a broad spectrum of bacteria.

4.6.2. Beta-site APP cleaving enzyme 1 (BACE-1)

Combinations of NMR and SPR methods have been applied in the search for inhibitors of BACE-1.

BACE-1, also known as beta-secretase 1, is an aspartic acid protease which cleaves amyloid precursor protein (APP) extracellularly. This cleavage step is a prerequisite for the subsequent cleavage by gamma-secretase to the 40 or 42 amino acid amyloid β peptides. The aggregation of these peptides in the brain leads to Alzheimer's disease.

Figure 4.4. Discovery of isocytosines and dihydroisocytosines from initial fragment screening hits versus BACE-1.

1D NMR screening was undertaken with BACE-1 protein expressed as a C-terminal fusion of the Fc part of human IgG1. Due to the large size of this fusion protein, which causes increased relaxation effects, enhanced ligand binding effects occur and low micromolar concentrations of protein could be used to carry out the screening. A fragment library of 2000 generic fragments was screened using waterLOGSY competition experiments. The fragment library was screened in compound mixtures containing 6 compounds, and resulted in a hit rate of around 0.5 %. Following the initial fragment screen, an inhibition in solution assay was established on the BIAcore platform, using a TDC peptide. The P1 (S)-statin substituted substrate analogue (KTEEISEVNstatin-DAEF) was captured using amine coupling, and displays nM affinity to BACE-1. A reference surface

was also prepared with a scrambled version of the peptide (KFES-statin-ETIA-EVENV). Initial binding of the fragment hits from the NMR screen were tested at 1 mM, with subsequent concentration responses carried out over the 0.1 – 5 mM range. This fragment based NMR screening approach coupled with BIAcore follow up confirmation and analogue testing resulted in the identification of a range of 6-substituted isocytosines as novel warheads for BACE-1 inhibition, with ligand efficiencies of around 0.3, that were subsequently developed into inhibitors with nanomolar potency and cellular activity, Figure 4.4 [33].

4.6.3. MEK protein kinase

MEK protein kinase is also known as MKK and MAPKK. It exists as two isoforms, MEK1 and MEK2, which have a 79 % sequence identity and each has a similar ability to phosphorylate ERK1 and ERK2. MEK1 is regulated by activating phosphorylations at Ser217 and Ser221. Compounds that affect MEK may act by 2 different mechanisms: (1) prevention of activation (PoA), where binding to the non-activated enzyme interferes with its phosphorylation by RAF isoforms such as B-RAF, or (2) by inhibition of catalysis (IoC), where binding to activated MEK prevents the phosphorylation of substrates such as ERK. Targeting non-activated kinases via the PoA mechanism offers some advantages over the traditional approach of IoC, such as greater selectivity due to a more diverse range of protein structures as the catalytic machinery is not required to be in place, and reduced competition by the ATP substrate as the non-activated forms tend to have a lower intrinsic affinity for ATP.

Biophysical methods have a useful application in identifying potential PoA compounds, since they offer simpler assay set-up than the biochemical PoA assays which involve upstream, activated kinase phosphorylation of the non-activated target kinase. BIAcore inhibition in solution assays allows the detection of binding to both the enzyme and substrate kinases individually without the need for substrate turnover. ISAs were established for both B-RAF and MEK1 by immobilising ureidoquinazoline and cyanoquinoline as target definition compounds, respectively. These assays allowed comparisons between K_d values for the individual proteins from ISA and IC_{50} values at $[ATP] = K_m$ in both IoC and PoA assays. Kinase drug discovery often involves the ranking of compound potency in assays that follow IoC when ATP is present around its K_m concentration, and it is often used to show selectivity. However, it is clear from the biochemical and biophysical studies here, that a single compound gives different IC_{50} values against the same target protein in different assays, which introduces challenges into the evaluation of potency and selectivity. ITC was therefore employed to investigate the dependence of compound K_d values upon $[ATP]$, and showed that the MEK-1 inhibitor U-0126 demonstrates noncompetitive kinetics, whereas an alkyl amide compound showed uncompetitive kinetics. This work illustrates that the determination of the mechanism of action and measurement of equilibrium dissociation and inhibition constants helps in the understanding of the structure–activity relationships of MEK inhibitors [34].

4.7. FUTURE TRENDS FOR BIOPHYSICAL APPROACHES

The future use of biophysical methods to characterise protein-ligand interactions will undoubtedly be facilitated by improvements in 2 key areas: (1) increased throughput and/or sensitivity of current instrumentation and (2) increased application to currently intractable targets by emerging target preparation methods or new emerging technology, or both.

Successful improvements in throughput have been exemplified over the last 20 years or so by the transformation of SPR from a single molecule characterisation tool to a method capable of driving fragment based lead generation projects, where thousands of potential ligands are tested in a single screen.

The throughput of OWG instruments has also progressed from early instruments to 384-well plate based (EPIC) and even 1536-well plates (BIND), and the Advion nanomate holds a 96 or 384 rack of conductive tips as well as a 96 or 384-well sample plate, allowing high throughput MS.

These methods will be increasingly used as orthogonal methods to verify the validity of hits derived from traditional HTS.

The possibility of increased throughput for these biophysical technologies to 1536-well based may be technically feasible, allowing an even greater impact on drug discovery in the future.

Recent efforts in building enthalpy arrays offer the potential to apply thermodynamic profiling to both a wider number and range of protein-ligand interactions and systems [35]. These instruments are arrays of nanocalorimeters that enable label-free detection of molecular interactions using small sample volumes and short measurement times compared to traditional instruments.

In terms of a newly emerging technology, an approach that has gained in interest recently is back-scattering interferometry (BSI), which is able to provide a label-free, homogenous and mass-independent detection of ligand binding in solution with much lower requirements on sample quantity and purity than OWG and SPR methods [36]. BSI uses light interaction with a microfluidic channel to measure temporal changes in refractive index (RI), brought about upon ligand binding. Studies of unlabeled proteins with test compounds can be carried out, both in solution and following target immobilisation to the surface. Laser illumination of the microfluidic channel produces a highly modulated fringe pattern perpendicular to the channel. The bright and dark features shift position with changes in the RI of the sample and monitoring this shift forms the basis of the BSI measurement.

New technology such as this, alongside novel methods to prepare stabilised membrane proteins, such as GPCRs, using a small number of point mutations, for example the STaR technology from Heptares [37] will allow the application of biophysical methods to membrane protein systems that have been difficult to tackle previously.

Extending measurements to whole cells is also something that has been beginning to occur, particularly with OWG instruments, owing to the ability of the instrument to detect the dynamic mass redistribution that occurs with changes in cell morphology upon addition of active ligands.

The use of in-cell NMR makes it possible to study biological macromolecules while they remain in living cells. The first in-cell protein NMR experiments were performed in yeast by labelling with ^{19}F , although in-cell ^{15}N -NMR and ^{13}C -NMR experiments have since been conducted in *Escherichia coli* [38].

The future for in-cell NMR will be the routine application of these methods to eukaryotic cells with the highly desirable opportunity to study large protein complexes and membranes.

4.8. SUMMARY

Detecting, characterising and modifying ligand binding interactions is of fundamental importance to drug discovery. Recently, a wider range of lead generation approaches, employed alongside traditional HTS, have been adopted by the pharmaceutical industry. The most important of these methods have been the biophysical techniques used either as frontline screening methods, especially in relation to fragment based lead generation, or as orthogonal methods to validate hits from high throughput or high concentration screens.

These biophysical approaches include techniques operating via a number of different physical processes that together offer the possibility of measuring not only affinity, but also the thermodynamic and kinetic contributions to that observed affinity.

This powerful combination of kinetic and thermodynamic data, coupled with structural information, allows a more detailed understanding of the binding interaction and facilitates decision making during the hit to lead and lead optimisation processes.

Further refinement, both in terms of new methodology that will allow the study of systems currently not amenable to biophysical methods and in the application of increased throughput or increased sensitivity extensions to existing methods, or both, will ensure that biophysical methods will remain embedded in high quality lead generation programmes for the foreseeable future.

REFERENCES

1. E. Fischer. Einfluss der configuration auf die wirkung der enzyme. *Berichte Deutschen Chemischen Gese* **27** (1894) 2985–2993.
2. J.N. Langley. On the reaction of cells and of nerve endings in certain poisons, chiefly as regards the reaction of striated muscle to nicotine and curare. *Journal of Physiology* **33**(4-5) (1905) 374–413.

3. P. Ehrlich. Address in pathology on chemotherapeutics: scientific principles, methods and results. *The Lancet* **182**(4694) (1913) 445–451.
4. M.-Q. Zhang, B. Wilkinson. Drug discovery beyond the 'rule of five'. *Current Opinion on Biotechnology* **18**(6) (2007) 478–488.
5. N.G. Ahn, A.H.-J. Wang. Proteomics and genomics: perspectives on drug and target discovery. *Current Opinion on Chemical Biology* **12**(1) (2008) 1–3.
6. K.K. Kopec, D. Bozyczko-Coyne, M. Williams. Target identification and validation in drug discovery: the role of proteomics. *Biochemical Pharmacology* **69**(8) (2005) 1133–1139.
7. J.G. Lombardino, J.A. Lowe. A guide to drug discovery: The role of the medicinal chemist in drug discovery – then and now. *Nature Reviews Drug Discovery* **3**(10) (2004) 853–862.
8. G. Thomas. Medicinal Chemistry – An Introduction. Wiley, 2000.
9. R.A. Alberty, G.G. Hammes. Application of the theory of diffusion-controlled reactions to enzyme kinetics. *Journal of Physical Chemistry* **62**(2) (1958) 154–159.
10. K.C. Chou, S.P. Jiang. Studies on the rate of diffusion-controlled reactions of enzymes. *Scientia Sinica* **17** (1974) 664–680.
11. Y.C. Cheng, W.H. Prusoff. Relationship between the inhibition constant (K_1) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochemical Pharmacology* **22**(23) (1973) 3099–3018.
12. A. Warshel, A. Papazyan, P.A. Kollman, W.W. Cleland, M.M. Kreevay, P.A. Frey. On low-barrier hydrogen bonds and enzyme catalysis. *Science* **269** (1995) 102–106.
13. W. Blokzijl, J. Engberts. Hydrophobic effects. Opinion and facts. *Angewandte Chemie International Edition in English* **32**(11) (1993) 1545–1579.
14. G.A. Holdgate. Isothermal titration calorimetry and differential scanning calorimetry, in: *Ligand-macromolecular Interactions in Drug Discovery*. Ed. A.C.A. Roque. Humana Press Inc., 2010.
15. G.A. Holdgate. Thermodynamics of binding interactions in the rational drug design process. *Expert Opinion on Drug Discovery* **2**(8) (2007) 1103–1114.
16. E. Freire. Do enthalpy and entropy distinguish first in class from best in class? *Drug Discovery Today* **13**(19) (2008) 869–874.
17. J.E. Ladbury, G. Klebe, E. Freire. Adding calorimetric data to decision making in lead discovery: a hot tip. *Nature Reviews Drug Discovery* **9**(1) (2010) 23–27.
18. E. Freire. A thermodynamic approach to the affinity optimization of drug candidates. *Chemical Biology & Drug Design* **74**(5)(2009) 468–472.
19. W. Huber, F. Mueller. Biomolecular interaction analysis in drug discovery using surface plasmon resonance technology. *Current Pharmaceutical Design* **12**(31) (2006) 3999–4021.
20. R. Karlsson. Real-time competitive kinetic analysis of interactions between low-molecular-weight ligands in solution and surface-immobilized receptors. *Analytical Biochemistry* **221**(1) (1994) 142–151.
21. H. Mukundan, A.S. Anderson, W.K. Grace, K.M. Grace, N. Hartman, J.S. Martinez and B.I. Swanson. Waveguide-based biosensors for pathogen detection. *Sensors* **9**(7) (2009) 5783–5809.
22. S.B. Shuker, P.J. Hajduk, R.P. Meadows, S.W. Fesik. Discovering high-affinity ligands for proteins: SAR by NMR. *Science* **274** (1996) 1531–1534.
23. P.J. Hajduk, D.J. Augeri, J. Mack, R. Mendoza, J. Yang, S.F. Betz, S.W. Fesik. NMR-based screening of proteins containing ^{13}C -labeled methyl groups. *Journal of the American Chemical Society* **122**(33) (2000) 7898–7904.

24. C. Dalvit, P. Pevarello, M. Tato, M. Veronesi, A. Vulpetti, M. Sundström. Identification of compounds with binding affinity to proteins via magnetization transfer from bulk water. *Journal of Biomolecular NMR* **18**(1) (2000) 65-68.
25. M. Mayer, B. Meyer. Characterization of ligand binding by saturation transfer difference NMR spectroscopy. *Angewandte Chemie International Edition* **38**(17) (1999) 1784-1788.
26. H. Wan, M. Rehngren. High-throughput screening of protein binding by equilibrium dialysis combined with liquid chromatography and mass spectrometry. *Journal of Chromatography A* **1102**(1) (2006) 125-134.
27. N.W. Chan, D.F. Lewis, P.J. Rosner, M.A. Kelly, D.C. Schriemer. Frontal affinity chromatography-mass spectrometry assay technology for multiple stages of drug discovery: applications of a chromatographic biosensor. *Analytical Biochemistry* **319**(1) (2003) 1-12.
28. R.H. van den Heuvel, A.J. Heck. Native protein mass spectrometry: from intact oligomers to functional machineries. *Current Opinion in Chemical Biology* **8**(5) (2004) 519-526.
29. J.M. Daniel, S.D. Friess, S. Rajagopalan, S. Wendt, R. Zenobi. Quantitative determination of noncovalent binding interactions using soft ionization mass spectrometry. *International Journal of Mass Spectrometry* **216**(1) (2002) 1-27.
30. S.A. Hofstadler, K.A. Sannes-Lowery. Interrogation of noncovalent complexes by ESI-MS: A powerful platform for high throughput drug discovery. in: *Mass Spectrometry in Medicinal Chemistry (First Edition)* Eds: K. Wanner, G. Höfner, Wiley-VCH Verlag. 2007, p. 321-338.
31. D.A. Erlanson, R.S. McDowell, T. O'Brien. Fragment-based drug discovery. *Journal of Medicinal Chemistry* **47**(14) (2004) 3463-3482.
32. G.S. Basarab, M.B. Gravestock, S.I. Hauck. Pyrrole derivatives as DNA gyrase and topoisomerase inhibitors. *US Patent Application* US2008/0269214 A1, 2008.
33. P.D. Edwards, J.S. Albert, M. Sylvester, D. Aharony, D. Andisik, O. Callaghan, J.B. Campbell, R.A. Carr, G. Chessari, M. Congreve, M. Frederickson, R.H.A. Folmer, S. Geschwindner, G. Koether, K. Kolmodin, J. Krumrine, R.C. Mauger, C.W. Murray, L.-L. Olsson, S. Patel, N. Spear, G. Tian. Application of fragment-based lead generation to the discovery of novel, cyclic amidine beta-secretase inhibitors with nanomolar potency, cellular activity and high ligand efficiency. *Journal of Medical Chemistry* **50**(24) (2007) 5912-5925.
34. W.S. VanScyoc, G.A. Holdgate, J.E. Sullivan, W.H. Ward. Enzyme kinetics and binding studies on inhibitors of MEK protein kinase. *Biochemistry* **47**(17) (2008) 5017-5027.
35. M.I. Recht, D. De Bruyker, A.G. Bell, M.V. Wolkin, E. Peeters, G.B. Anderson, A.R. Kolatkar, M.W. Bern, P. Kuhn, R.H. Bruce, F.E. Torres. Enthalpy array analysis of enzymatic and binding reactions. *Analytical Biochemistry* **377**(1) (2008) 33-39.
36. D. J. Bornhop, J.C. Latham, A. Kussrow, D.A. Markov, R.D. Jones, H.S. Sørensen. Free-resolution, label-free molecular interactions studied by back-scattering interferometry. *Science* **317** (2007) 1732-1736.
37. N. Robertson, A. Jazayeri, J. Errey, A. Baig, E. Hurrell, A. Zhukov, C. J. Langmead, M. Weir, F.H. Marshall. The properties of thermostabilised G protein-coupled receptors (StaRs) and their use in drug discovery. *Neuropharmacology* **60**(1) (2011) 36-44.
38. S. Reckel, F. Lohr, V. Dotsch. In-cell NMR spectroscopy. *ChemBioChem* **6**(9) (2005) 1601-1606.

NMR SPECTROSCOPY FOR STUDYING INTERACTIONS OF BIOACTIVE MOLECULES

Predrag Novak and Tomislav Jednačak

Contents

5. 1. INTRODUCTION	191
5.2. LIGAND-PROTEIN INTERACTIONS	203
5.3. LIGAND-DNA, LIGAND-RNA AND LIGAND MEMBRANE INTERACTIONS	209
5.4. STRUCTURAL ANALYSIS OF LIGANDS	214
5.5. STRUCTURAL ANALYSIS OF PEPTIDES AND PROTEINS	215
5.6. MACROLIDE-RIBOSOME INTERACTIONS	217
Acknowledgement.....	220
REFERENCES	220

5.1. INTRODUCTION

One of the main prerequisites for the successful design of bioactive molecules and drugs is the elucidation of the three-dimensional structure of small molecular ligands, receptors and their complexes. Key steps in this process involve the identification of structural elements and groups responsible for bioactivity. On the other hand, our ability to design novel drug candidates simply from high-resolution structures of biomolecules is still limited. A deeper understanding of molecular mechanisms and dynamics involved in the interaction of ligands with macromolecules is of crucial importance. There are many techniques for probing ligand-receptor interactions, such as equilibrium dialysis, fluorescence spectroscopy, capillary electrophoresis, ultrafiltration, etc. Generally, these techniques require time-consuming separation steps which might influence binding equilibria or analyte derivatisation steps which may change ligand activity. NMR spectroscopy is a very useful method for the characterisation of ligand-receptor interactions since it can provide a wealth of information without destroying the sample. In this review, a description of the main NMR techniques to study the interactions of bioactive molecules with their biological receptors is given and the principal advantages and disadvantages are discussed.

Today, NMR spectroscopy is one of the most powerful and valuable methods in modern drug discovery and development. There is a plethora of one- and multi-dimensional techniques developed in recent years to study the structures of biologically important molecules such as proteins and nucleic acids and their interactions with other macromolecules or small molecules, for example. Many sequences have been proposed to screen for small molecular binders in the early stages of the drug discovery process. Drug discovery usually includes several critical stages, *i.e.* hit and lead generation, lead optimisation and preclinical development. Although knowledge of the 3D structures of ligand-receptor complexes might accelerate to a certain extent the search for molecules with higher activity, optimisation of physicochemical properties which dictate *in vivo* potency and efficacy play an important role as well. These properties include solubility, absorption, distribution, metabolism, excretion and toxicity, known as ADMET.

Initially, the main contribution of NMR spectroscopy to drug design included the determination of the three-dimensional structure of biological targets, mostly

proteins, and many efforts have been made by biological NMR groups to develop different two- and multi-dimensional techniques for that purpose. The first protein structures determined by NMR were published more than 25 years ago [1,2]. Although knowledge of protein structure is certainly an important factor for the drug discovery process, the predictions at that time regarding the impact of NMR were overly optimistic, primarily due to the fact that the size and solubility of proteins are still the biggest limiting factor for successful NMR analysis. Today, protein structure determination by NMR spectroscopy usually includes isotopic labelling of the protein over-expressed in genetically modified bacteria [3]. Hence, the availability of ^{13}C , ^{15}N and ^2H isotopically enriched proteins combined with numerous multidimensional NMR experiments enable sequential resonance assignment and solution state structure determination of proteins or protein-ligand complexes up to 82 kDa in size [4]. Recently developed sequences which reduce the transverse relaxation rates such as transverse relaxation optimized spectroscopy (TROSY) [5-7] have enabled the recording of high-resolution NMR spectra of macromolecules with masses up to 100 kDa and beyond. However, even for smaller proteins with good resonance dispersion and narrow line-widths, the process of structure determination can be very time consuming. Recent developments in membrane protein NMR studies have revived the role of structural NMR in pharmaceutical research [8]. More recently, new solid-state NMR techniques have emerged as a powerful tool to study proteins that were not accessible to solution state measurements. This has made possible studies of integral membrane proteins, protein aggregates and fibrous proteins, regardless of the solid state characteristics of the studied system, which is one of the main prerequisites for X-ray diffraction analysis where crystalline samples are needed. More recent information on protein structure analysis can be found in the related literature [9-13]. In modern drug discovery, NMR provides much more than macromolecular 3D structure determination since it has found widespread use in hit and lead generation, metabonomics and complex mixture analysis. Excellent discussions of the principles and applications of NMR techniques in metabonomics and mixture analysis can be found in a number of books and articles [14-21].

Apart from the structural information on the macromolecular target in the process of drug design, it is crucial to understand the principles of how a particular biological receptor interacts with a drug-like molecule. This is one of the prerequisites for the discovery of high affinity ligands for biologically important protein targets. Ligand-receptor interactions by using NMR might also include the elucidation of free and bound ligand conformations, the mobility of the ligand, receptor and their complex, determination of binding constants, determination of the reactive groups responsible for binding and also provide information on the orientation or immersion depth of the ligand in its target.

The binding of a ligand molecule to its receptor in its simplest form follows the bimolecular association reaction with second-order kinetics according to Equation 5.1:

where k_{on} and k_{off} are the on (association) and off (dissociation) rate constants.

The dissociation constant K_D can then be expressed by Equation 5.2:

$$K_d = \frac{k_{\text{off}}}{k_{\text{on}}} = \frac{[R][L]}{[RL]} \quad (5.2)$$

As already mentioned, there have been many NMR sequences developed in the last two decades that can provide useful information on the interactions between biological targets and small molecules which can be used to screen for potential binders [22-28]. These can be divided into two main groups: ligand-based and receptor-based techniques. Theoretically, all NMR parameters are sensitive to binding processes and can be used to study ligand-receptor interactions. Most frequently used are those that are easily obtained and with highest sensitivity and include chemical shifts, translational diffusion coefficients, relaxation times and the nuclear Overhauser effect (NOE). Some of these parameters are simply used to indicate if binding occurs, while others provide information on the location of ligand binding sites and surface mapping features and determine binding constants and changes in the mobility of ligand-receptor complexes. NMR screening for biologically active molecules includes the identification of small molecule ligands for macromolecular targets by the observation of a change in an NMR parameter upon interaction. The parameters that are usually used to study interactions between ligand and their biological receptors are given in Figure 5.1.

Figure 5.1. NMR parameters used for studying ligand-receptor interactions

Both approaches have advantages and disadvantages. The receptor-based methods do not rely on fast exchange and therefore allow for the characterisation of both high and low affinity binders. They can also easily distinguish between specific and non-specific binding. The main prerequisite for this approach is *a priori* knowledge of the 3D structure of the protein obtained either from NMR or X-ray diffraction analysis. Monitoring changes in NMR parameters (usually chemical shifts) identifies not only binding of molecules, but also localises their binding sites. Some major drawbacks of this approach include the size of the target protein which prevents complete resonance assignment and structure elucidation as well as isotopic enrichments (^{13}C , ^{15}N , ^2H etc.) necessary for structural analysis. One of the main disadvantages of receptor-based techniques is the over-expression and purification of milligram quantities of a soluble protein, since in drug discovery, targets are selected on the basis of their therapeutic potential and not their compliance with NMR. For ligand-based methods, the target size is irrelevant and there is no need for isotopic labelling. The receptor concentration is much smaller, usually in the micromolar range. This approach mostly utilises 1D techniques, which are faster than 2D or higher dimensional techniques needed to solve protein structures. There are also some drawbacks connected to these techniques, such as the inability to *a priori* localise the binders on the receptor and the requirement for fast exchange between free and bound states. Also, the large excess of ligand molecules used in such experiments might increase the risk of non-specific binding. However, the ligand-based approach has broader applicability today and is frequently used for primary and secondary screening.

An excellent receptor-based method called structure-activity relationship (SAR) by NMR was developed by researchers at Abbott [29-31]. The method is based on perturbations in protein hetero-nuclear single quantum coherence NMR spectra (^1H - ^{15}N or ^1H - ^{13}C -HSQC) upon the addition of a compound mixture. It requires ^{15}N or ^{13}C isotopic enrichment of proteins. In the first step, a library of small molecules is screened to identify those that bind to the target protein. Binding is detected by comparing two-dimensional ^1H - ^{15}N or ^1H - ^{13}C -HSQC spectra of the protein prior to and after the addition of a ligand or ligand mixture to detect changes in chemical shifts upon binding. The ligands that bind to one or more pockets in spatial proximity are identified, optimised and linked together to produce high-affinity ligands. Since the first application of this method on the FKBP protein, it has been used to discover many high-affinity ligands for other therapeutically relevant proteins.

For experiments that rely on the observation of ligand molecules, the choice of NMR parameters is more diverse (Figure 5.1). The high sensitivity of ligand parameters to binding have enabled a number of valuable techniques to be developed to better understand the binding process at a molecular level and to identify new bioactive compounds [22-28]. Upon binding of a small molecule ligand to its target macromolecule, the chemical shifts of both the ligand and the target resonances are perturbed. Hence, for the detection of whether a ligand binds or not, in the case of well-separated signals it is sufficient to perform simple titration expe-

riments and observe changes in proton chemical shifts and line-widths. This is shown in Figure 5.2 for binding of the antibiotic erythromycin to the ribosome.

The dissociation constant may also be estimated by nonlinear curve fitting of the chemical shifts vs. concentration. Other methods to identify and characterise ligand-receptor interactions can generally be divided into NOE-based, relaxation-based and diffusion-based methods.

Figure 5.2. Proton spectrum of erythromycin (a) and proton spectra of erythromycin after the incremental addition of *E. coli* ribosome (lines b, c and d) recorded at 700 MHz

A number of NMR techniques based on the NOE effect have been developed recently. It is well known that NOEs are invaluable tools for determining 3D structures of small molecules and biomolecules. Upon binding of the ligand molecule to its biological receptor, the NOEs undergo significant changes leading to the observation of transferred NOEs or transferred nuclear Overhauser enhancements (trNOEs) which stem from different correlation times, τ_c , of free and bound molecules. Small molecular ligands have short correlation times and usually give rise to positive NOEs or no NOEs. On the other hand, large macromolecules exhibit strong negative NOEs. This is depicted in Figure 5.3. If a small molecule binds, it behaves like a macromolecule and shows strong negative trNOEs, providing a fast exchange regime between the bound and free states.

This is demonstrated in Figure 5.4 for binding of the macrolide antibiotic azithromycin to ribosomes isolated from *E. coli*. It can be seen that the 2D NOESY spectrum of azithromycin in buffered solution displays only a few positive NOE

Figure 5.3. Properties of the ligand, receptor and their complex

cross-peaks (all others vanish since $\tau_c \omega_0 \approx 1$, $M_w = 774$). However, after the addition of 70S *E. coli* ribosomes, the observed trNOEs are all negative with higher intensities, indicating interactions between azithromycin and ribosomes and the formation of a complex. In practise, these experiments are performed with high excess of the ligand (from ten to hundred-fold excess) so that signals of the receptor molecules are not visible in the spectrum and no filtering techniques are necessary. Furthermore, the analysis of trNOEs provides information on the bound conformation. In addition, one can also observe intermolecular trNOEs between the ligand and receptor protein which allows for the determination of the orientation of bound ligands in protein binding pockets [32,33].

Another very popular and fast method that relies on the NOE effect is saturation transfer difference NMR spectroscopy (STD-NMR), proposed by B. Mayer and M. Meyer [34,35]. The method has become one of the most frequently used NMR methods to characterise interactions between ligands and receptors. It is based on the transfer of saturation from the receptor to the bound ligand which in turn is moved into solution by exchange and detected. As depicted in Figure 5.5, STD takes the difference of the two experiments. In the first experiment (on-resonance), only receptor resonances are selectively irradiated or saturated using selective pulses. The saturation propagates to all receptor protons *via* the network of an efficient intra-molecular proton-proton cross-relaxation process known as spin diffusion. As seen in Figure 5.5, compounds that bind to the receptor become saturated as well via inter-molecular proton-proton cross-relaxation. The saturation is then carried into solution by the exchange process and detected. Subsequently, a reference spectrum is recorded with the irradiation frequency set far from any signal giving rise to an off-resonance spectrum. The subtraction of the on-resonance spectrum from the off-resonance spectrum gives rise to a difference spectrum, showing only resonances that have experienced saturation.

Figure 5.4. (a) NOESY spectrum of azithromycin in TRIS buffer and (b) TrNOESY spectrum of azithromycin in TRIS buffer after the addition of 70S *E. coli* ribosomes

The degree of ligand saturation depends on the residence time of the ligand in the receptor binding pocket. When it dissociates from the receptor, the saturation is transferred to the solution where the free ligand gives rise to signals with narrow line widths. There are several advantages of the STD method.

First, STD has no limitation with respect to the size of the receptor and is ideal for large receptor proteins and even whole ribosomes (see Section 5.6). Second, it

Figure 5.5. Schematic representation of an STD experiment: (a) the off-resonance or reference experiment involves rf-irradiation off-resonance from ligand and receptor protons giving intensity I_0 , (b) the on-resonance experiment applies rf-irradiation for selective saturation of receptor resonances producing attenuated signal intensity I_{SAT} and (c) STD spectrum which gives only resonances of the binding compounds (receptor resonances are usually invisible due to either low concentrations or relaxation filtering)

requires low amounts of the receptor (usually in the micromolar range) and it benefits from the fact that only bound ligands show signals in the spectrum and therefore is very suitable for screening purposes. A typical one-dimensional STD pulse sequence is shown in Figure 5.6.

Figure 5.6. STD pulse sequence with a $T_{1\rho}$ relaxation filter to suppress unwanted receptor resonances

Furthermore, STD may be combined with other pulse sequences, thus generating experiments such as STD - total correlation spectroscopy (STD-TOCSY) or STD-HSQC, for example [36]. STD-NMR spectroscopy can be successfully used to identify groups of atoms in direct contact with the receptor since these experience the highest degree of saturation; this allows for epitope mapping or group epitope mapping (GEM) [35]. Protons closest to the target molecule are saturated to a higher degree, giving stronger STD enhancements. This information can be used to identify structural moieties that are important for binding. The binding constants can also be assessed from STD-NMR spectra by performing competition titration experiments. A variation of STD-NMR spectroscopy is the water ligand-observed gradient spectroscopy (waterLOGSY) method which uses the fact that intermolecular water-ligand NOE will be negative only when the ligand is bound to the protein [37,38]. The transfer of magnetisation is from water to receptor to binding ligand. The method utilises either the steady-state NOE experiment with on-resonance saturation applied to the water signal or selective inversion of the water resonance signal in the enhanced protein hydration observed through gradient spectroscopy nuclear Overhauser effect (e-PHOGSY NOE) experiment [39]. Protein-ligand interactions can also be studied by monitoring inter-molecular NOEs using NOE pumping or reverse NOE pumping (RNP) techniques [40]. RNP uses a relaxation filter followed by the NOE mixing element where polarisation is transferred from a binding ligand to the protein, resulting in a spectrum containing only the signals of the binding ligands. Recently, a method called INPHARMA (Interligand Noes for PHARmacophore MAPPING) methodology was introduced [41]. It is based on the observation of spin-diffusion mediated, trNOE data between two ligands (L_1 and L_2) that bind weakly and competitively to a macromolecular receptor (R). As L_1 binds to the receptor, the magnetisation is transferred from the ligand to the

receptor protons during the mixing time of the NOESY experiment. During the same mixing time, L_1 dissociates from the receptor and L_2 binds, which allows the transfer of magnetisation from the receptor to L_2 protons.

The chemical exchange during INPHARMA experiment can be explained by two different models:

- a) A three-step model, which includes the state where both ligands and the receptor are found also in free form. The three-step model can be described by:

- b) A two-step model, where the receptor is never in free form, due to the presence of the two ligands in large excess. This model can be described by:

In contrast to STD and waterLOGSY experiments, the information obtained in the INPHARMA spectrum is highly site-specific. INPHARMA provides rapid determination of the relative orientation of different chemical structures in the macromolecule binding pocket. INPHARMA methodology was successfully applied to a mixture of baccatin III (L_1) and epothilone A (L_2) in the presence of tubulin (R). The results showed that the intermolecular NOEs between epothilone A and baccatin III were mediated by the protons in tubulin.

Binding of the ligand to the target will have a dramatic effect to its relaxation properties. Free small molecule ligands are characterised by small longitudinal and transverse relaxation rates, ($R_1=1/T_1$ and $R_2=1/T_2$). When bound, ligands share relaxation properties of the receptors with large relaxation rates. Therefore, binding will cause significant line-broadening of ligand resonances. A comparison of transverse relaxation times in the presence and absence of the receptor is very sensitive probe of binding due to its almost direct dependence on the overall molecular correlation time. T_2 can be determined by using a simple Carr–Purcell–Meiboom–Gill (CPMG) experiment [42,43] or, alternatively, one can use $T_{1\rho}$ determined from rotating frame Overhauser effect spectroscopy (ROESY)-type experiments to detect binding. A variation of the relaxation-based approach is the method called SLAPSTIC (Spin Labels Attached to Protein Side chains as a Tool to identify Interacting Compounds) [44,45] which allows for the screening of primary binding sites by covalently attaching spin labels to protein targets. The use of paramagnetic relaxation enhancement (PRE) experiments could provide further structural information on the orientation or immersion depth of the ligand in its target. Upon the addition of an inert paramagnetic agent to the solvent, relaxation enhancements of ligand nuclei depend on the insertion depth in the target. If the ligand is in fast exchange between the free and bound state,

solvent PREs are partly transferred to the free ligand where they can be observed with high resolution and without any size limitation of the receptor [46-49]. Information regarding the orientation of the ligand with respect to the receptor can be determined and further used for guiding docking calculations, provided that the three-dimensional atomic resolution structure of the receptor is available. Identifying parts of a drug which are buried deepest in the receptor can help with directing chemical modifications of the ligand to design better inhibitors.

Experiments based on translational diffusion can also be used to probe interactions of ligands with a target. The diffusion-editing techniques are based on the fact that, upon binding, the hydrodynamic radius r_H of the ligand increases substantially, leading to a decrease in the diffusion rate. The translational diffusion coefficients can be obtained according to Stokes-Einstein equation:

$$D = kT/6\eta\pi r_H \quad (5.5)$$

where k represents the Boltzmann constant, T is the temperature, η is the solvent viscosity and r_H is hydrodynamic radius. From Equation 5.5, it is clear that a small ligand molecule with a small hydrodynamic radius will have larger diffusion coefficient with respect to larger molecules such as proteins. Hence, if a ligand binds to the receptor, its diffusion coefficient will decrease. A variety of NMR pulse sequences have been developed to investigate diffusion coefficients in solution [50]. Diffusion-editing has been successfully used for the characterisation of interactions between small and intermediate sizes proteins. With the development of reliable gradient technology, many NMR pulse schemes using gradients have been proposed to determine diffusion coefficients such as bipolar pulsed field gradient longitudinal eddy-current delay (bpPFGLED), depicted in Figure 5.7.

Figure 5.7. The bpPFGLED pulse sequence.

The signal intensity during the diffusion experiment will decay exponentially according to Equation 5.6:

$$I = I_0 \exp[-D(\gamma\delta G)^2(\Delta - \delta/3)] \quad (5.6)$$

where I_0 is the signal intensity in the absence of field gradients, D is the diffusion coefficient, γ is the gyromagnetic ratio, δ is gradient length, G is gradient strength and Δ is the diffusion time. Diffusion coefficients can be determined from the slope of the linear dependence of the natural logarithm of both sides of the Equation 5.6. The experimental parameters can be optimised to suppress the signals of non-binding compounds and to detect the resonances of bound ligands.

Figure 5.8 shows the results from an experiment performed on a mixture of three compounds in the presence of porcine pancreatic elastase. It can be seen that only the signals of the compound that binds, *e.g.* phenylphosphonic acid, appears in the spectrum of the mixture, while signals of the other non-binding compounds are not present.

Figure 5.8. The bpPFGLED spectra of a mixture of phenylphosphonic acid and two small organic compounds at (a) low gradient strength, (b) high gradient strength and (c) after the addition of porcine pancreatic elastase at high gradient strength.

Diffusion-based methods can further be used to provide knowledge on which parts of the ligand are involved in the binding surface and to determine the binding epitopes. A key advantage of diffusion epitope mapping over the aforementioned GEM approach is that it bypasses problems of longitudinal relaxation, which in some cases might lead to erroneous STD enhancements in GEM experiments. The main disadvantage of diffusion experiments is their lower sensitivity compared to relaxation-based or NOE-based approaches and some limitations exist regarding the size of the receptor.

Although very powerful and efficient, ligand-based NMR methods also have some limitations. Most of these methods report cases of weak to medium affinity binding. Usually, these experiments are performed with a large excess of ligands which may lead to non-specific binding. Other limitations include molecules with low water solubility, those that bind with high affinity to the receptor and molecules that have small off-rate constants and hence are not detected. In such cases, competition experiments are very useful to monitor ligand-receptor

interactions, thus avoiding many drawbacks connected with the direct ligand-based methodologies [51-54]. Competition ligand-based methods are robust and reliable tools for providing valuable information on specific binding and could serve as quantitative measures of the ligand affinity for the receptor. A competition method that combines waterLOGSY and transverse relaxation has recently been proposed as a sensitive method for fragment screening [55].

The NMR methods discussed above will continue to play significant roles in the process of modern drug discovery, including hit generation, lead generation and optimisation phases, thus increasing the chances of success to produce drug candidates with high potency, reduced toxic effects and desired physicochemical properties.

In the next section, recent applications of the NMR techniques discussed above to study ligand-receptor interactions are reviewed.

5.2. LIGAND-PROTEIN INTERACTIONS

Ligand-protein interactions can be monitored by various experimental methods. As mentioned above, ligand-based NMR techniques are useful tools for investigations of such interactions.

STD-NMR spectroscopy was used to investigate the binding properties of the caged nucleotides guanosine 5'-*O*-(3-thiotriphosphate), *P* 3(*S*)-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl) ester (DMNPE-GTP- γ -S) and adenosine 5'-diphosphate, *P*²-(1-(2-nitrophenyl)ethyl) ester (NPE-ADP) to rabbit muscle creatine kinase (RMCK) and human annexin A6 (hAnxA6) [56]. The obtained results indicated the strong binding of caged nucleotides to the investigated proteins. Similarly, Blume *et al.* [57] used the same technique to study the interaction of the epimerase site of the bifunctional enzyme UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase with its natural substrate, UDP-*N*-acetylglucosamine (UDP-GlcNAc) and derivatives. STD-NMR allowed for ligand epitope mapping and the determination of ligand binding affinity. The binding epitopes of the uridine moieties of UMP, UDP, UDP-GalNAc and UDP-GlcNAc were similar, suggesting that the binding mode of the UDP moiety was the same in all cases. However, small differences in the binding epitopes of hexopyranose units of UDP-GlcNAc and UDP-GalNAc reflected the inability of the enzyme to process UDP-GalNAc. The results of STD titrations showed that UDP had the greatest binding affinity to the epimerase site of the enzyme.

Furthermore, a combination of STD-NMR and ¹H-¹⁵N HSQC experiments was used to investigate the binding of 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR) and guanosine monophosphate (GMP) to histidine triad nucleotide binding protein (HINT1) [58]. The presence of ligand binding was checked using STD-NMR spectroscopy. The ligand binding site was established from ¹H and ¹⁵N chemical shift perturbations. The binding of AICAR to HINT1 revealed

considerable chemical shift perturbation similarity to GMP, indicating that the two ligands probably bind in the same basic location.

Ji *et al.* [59] applied STD-NMR spectroscopy to map a group epitopes and to measure the dissociation constant of specific interactions between L-tryptophan (Trp) and human serum albumin (HSA). STD spectra recorded in ligand-protein solutions with and without the inhibitor naproxen pointed towards the interaction between Trp and HSA. The STD spectrum in the absence of the inhibitor reflected specific as well as non-specific binding of Trp. However, in the presence of naproxen, the specific sites were occupied by the inhibitor. Hence, the resulting STD spectrum reflected only non-specific contributions to the interaction between Trp and HSA.

Several groups have studied competitive ligand binding using STD-NMR spectroscopy. Sadeghi-Khomami *et al.* [60] studied the binding of pNP- β -D-glucose to hydrolytically inactive nucleophile mutants of the family-3 retaining β -glucosidase DesR in the presence of erythromycin A. The results of competitive STD-NMR experiments indicated that erythromycin A binds to the active site and acts as a competitive glycosidase inhibitor. Similarly, STD-NMR spectroscopy was used to study the interaction between bifunctional galactofuranosyltransferase GlfT2 and two trisaccharide acceptor substrates [61]. The bifunctionality of the enzyme was explored by competition STD experiments and saturation transfer double difference experiments (STDD). The results showed that both acceptor substrates bind competitively at the same site.

STD-NMR spectroscopy provided insight into enzyme-ligand interactions in yeast hexokinase isoform PII under physiological conditions [62]. Competitive STD titration experiments allowed for ranking the ligands according to their binding affinities.

Goncalves *et al.* [63] investigated the binding of a synthetic Toll-like receptor (TLR4) antagonist to cluster differentiation antigen CD14 by STD-NMR. The results indicated that the binding was principally mediated through the lipid chains of the synthetic compound. These findings strongly suggest that TLR4 activation is inhibited by competitively occupying CD14.

Furthermore, the competition STD method was successfully applied to detect the presence of a competing high-affinity ligand, diazepam, in a compound mixture by the reduction of the STD signals of a low-affinity ligand, β -site amyloid precursor protein cleaving enzyme 1 [64].

Plesniak *et al.* [65] characterised the conformation of novobiocin in the pocket of histidine protein kinase EnvZ using STD and trNOE experiments. Thus, STD-NMR spectroscopy identified the novobiocin moieties that interacted with EnvZ. The authors suggested that novobiocin binds to EnvZ in a conformation and orientation similar to its binding to DNA gyrase B.

Similarly, Lee *et al.* [66] characterised binding inhibitors of *Escherichia coli* β -ketoacyl-acyl carrier protein synthase III (ecKAS III) using STD-NMR

spectroscopy. The results suggested that the antimicrobial compound, 4-cyclohexyliminomethyl-benzene-1,3-diol (YKAs3003), was a potent inhibitor of pathogenic KAS III. It was shown that the binding models of YKAs3003 and eCKAS III determined from *in silico* screening correlated well with the STD results.

According to Houliston *et al.* [67] STD experiments enable the investigation of antibody-ligand interaction in target ganglio-oligosaccharides added directly to patient sera, without antibody fractionation. Very strong saturation transfer was observed in one-third of glycan resonances upon the addition of ganglio-oligosaccharides to patient sera; this delineated the region of contact with serum antibodies.

Murata *et al.* [68] have shown how STD-NMR spectroscopy can be applied to identify binding epitopes and to study the binding of gibberelin A₃ to the monoclonal antibody 4-B8(8)/E9. STD experiments provided useful information on the hapten-antibody interaction in the solution state. The results obtained by STD-NMR were in excellent agreement with the results of crystallographic analysis.

Assadi-Porter *et al.* [69] demonstrated the use of STD and STDD-NMR experiments for monitoring specific ligand binding to the human taste type 1 receptors 2 and 3 (T1R2 + T1R3). Subtraction of STD spectra recorded in the presence and the absence of sweet receptors yielded two-dimensional ¹H-¹⁵N HSQC STDD spectra containing signals only from specific binding. The results indicated that the mutant receptor (T1R2 + T1R3(D535Q)) binds the ligand brazzein much less tightly than does the wild-type receptor (T1R2+T1R3).

Streiff *et al.* [70] proposed the application of STD-NMR spectroscopy for anaesthetic binding proteins. The measurements were conducted on a series of protein/anaesthetic solutions. STD spectra provided evidence for Ca²⁺-dependent halothane binding to calmodulin. Those findings demonstrated the specificity of anaesthetics for a particular protein conformation.

Macnaughtan *et al.* [71] combined STD-NMR and trNOE experiments to study the conformation of UDP-GlcNAc and β-D-GlcpNAc-(1→2)-α-D-Manp(1→6)-β-D-Glcp-OOctyl, as well as their interaction with *N*-acetylglucosaminyltransferase V. In addition, the relative orientation of the two bound ligands was determined in a paramagnetic relaxation enhancement experiment using a spin-labelled ligand analogue, 5'-diphospho-4-*O*-2,2,6,6-tetramethylpiperidine-1-oxyl (UDP-TEMPO).

Furthermore, Wen *et al.* [72] used STD-NMR and molecular modelling protocols to probe the binding modes of the glycosidase inhibitors kifunensine and salacinol to *Drosophila melanogaster* Golgi α-mannosidase 11. A comparison of the experimental and theoretical effects of ligand protons in these complexes allowed for the selection of likely ligand binding modes. The results indicated that reliable binding modes of a ligand to a protein in aqueous solution can be obtained with the combined use of STD-NMR spectroscopy and a computational approach.

Becker *et al.* [73] used ligand-detected ¹H NMR experiments, T_{1ρ}-NOESY, diffusion and STD-NMR to distinguish the binding epitopes of propranolol enantiomers

with alpha(1)-acid glycoprotein. STD epitope mapping provided insights into different orientations of the enantiomers with respect to the alpha(1)-acid glycoprotein binding pocket. It was concluded that ligand-protein interactions can be elucidated by using the combination of several NMR epitope mapping experiments.

Several research groups have proposed the implementation of STD-NMR spectroscopy in chromatographic optimisation studies [74,75]. STD-NMR in combination with molecular dynamics simulations can be used to screen chiral stationary phases for evidence of molecular interactions as a predictor of chiral column selection and to study the mechanism of chemically selective displacement chromatography.

Recently, several improvements have been made in STD-NMR [76,77]. Novel competition STD experiments based on the combination of filtering and isotope labelling schemes have been demonstrated using a mixture containing either unlabelled or ^{13}C labelled D-maltose in the presence of malectin. The signals of the unlabelled ligand are present only in ^{13}C -filtered STD spectrum, while the edited experiment shows no signals. However, when using ^{13}C labelled maltose, only the ^{13}C -edited experiment produces STD signals. The combination of ^{13}C -filtered and ^{13}C -edited experiments allows for the separation of the STD signals of the labelled reference ligand from the signals corresponding to the unlabelled hit compounds. Similarly, a novel STD experiment was proposed for group selective saturation of amide protons in ^{15}N labelled hosts. The pulse sequence of the experiment is based on a train of bilinear rotation decoupling (BIRD) pulses that inverts only protons attached to ^{15}N and results in saturation of the amide protons. The ^{15}N GS STD experiment produces clean and artifact-free STD spectra. The feasibility study indicated that ^{15}N GS STD experiments can be used as the basis for studying intermolecular interactions where the standard STD experiment is difficult to optimise.

TrNOE and molecular dynamics simulations (MD) were used to study the interaction between *Serratia marcescens* chitinase B (*SmChiB*) and the dipeptide *N*-acetyl-Arg- $\{N^{\omega}$ -(*N*-methylcarbamoyl)}-*N*-methyl-Phe [78]. The dipeptide was derived from the naturally produced cyclopentapeptide chitinase inhibitor argifin. Molecular dynamics simulations and trNOE experiments indicated that both argifin and the dipeptide bind to (*SmChiB*) with similar affinities. The less favourable van der Waals and non-polar solvation energies of the dipeptide compared with argifin were compensated for by the improved total electrostatic component.

Several authors have demonstrated the application of NMR methodology based on relaxation rate analysis. Spin-lattice relaxation rate enhancements of ligand protons were used in order to investigate the affinity of epinephrine and isoproterenol for two different systems: fibrinogen and platelets [79]. The affinity index for epinephrine-fibrinogen was three times greater than the isoproterenol-fibrinogen affinity index. On the contrary, the affinity indexes for epinephrine-

platelet and isoproterenol-platelet interactions were similar, indicating that the ligands interact with the receptor with the same strength.

Furthermore, Lerche *et al.* [80] applied hyperpolarised ^{13}C NMR screening as a competitive assay to characterise the binding of salicylate and ascorbate to human serum albumin. Signal enhancements were achieved using dynamic nuclear polarisation (DNP) ^{13}C NMR spectroscopy. Dynamic nuclear polarisation creates a non-equilibrium state and the enhanced spin polarisation returns to its equilibrium with the relaxation time T_1 . As enhanced polarisation is much faster in macromolecule-bound ligands than in free ligands, relaxation time T_1 can be used to distinguish free and bound states.

TROSY was applied to investigate ternary systems composed of liver bile acid binding protein, bile acids and membrane mimetic systems, such as anionic liposomes [81]. The absence of the folded protein resonances in the TROSY spectrum of the liposome-protein complex indicated the formation of partially unfolded membrane-bound species. NMR titration of the liposome-protein complex was performed with increasing amounts of bile acids. The signals of the folded protein became more intense at higher bile acid/protein ratios. Hence, the presence of bile acid produced a shift in equilibrium towards the ligand-bound folded protein.

Relaxation rate analysis can be combined with NMR techniques based on monitoring chemical shift perturbations. Shi *et al.* [82] combined the aforementioned method to investigate the human vinexin SH3 domain. Site-specific backbone amide (^{15}N) ^1H and side chain ^{19}F chemical shifts as well as relaxation analysis of SH3 domains in the absence or presence of a peptide ligand indicated different internal motions due to ligand binding at the three different sites. Similarly, Fenwick *et al.* [83] performed solution NMR experiments to gain insight into the structure and dynamics of the bilobed ligand-binding core of glutamate receptor 2 in complexes with a set of willardine partial agonists. The regions of the receptor experiencing structural and/or dynamic changes due to interactions with the ligand were identified by main chain amide $^1\text{H}/^{15}\text{N}$ chemical shift deviations. Backbone dynamics on the chemical shift timescale were detected using measurements of ^{15}N relaxation exchange rates (R_{ex}). The NMR results showed that conformational exchange dynamics vary largely with efficacy and the extent of desensitisation.

Bai *et al.* [84] used global ^1H - ^{15}N HSQC chemical shift perturbations to elucidate the structure of P2-ibuprofen complexes. Two-dimensional ^1H - ^{15}N HSQC spectra showed that the chemical shift information was communicated from one β -strand to another, in a “molecular wave” pattern. It was proposed that the ligand induced adjustments in hydrogen bonds stabilising the β -sheet structure to overcome unfavourable entropic contributions to binding. The information transfer across β -sheets could be explained by the correlation between the amide nitrogen temperature coefficient and the nitrogen chemical shift perturbation. The correlation between *R*- and *S*- ibuprofen temperature coefficients indicated

that there is little enantiomeric selection for these compounds. The same method was used for the structure determination of 19 distinct ligand-protein complexes in high-throughput ligand affinity screens [85]. The chemical shift perturbation data was used to guide and filter AutoDock calculations using the AutoDockFilter program. The results obtained using chemical shift perturbation experiments were in accordance with the original X-ray structures. Martín-Pastor *et al.* [86] applied chemical shift perturbation methods to study the interaction of the GH secretagogue receptor (GHS-R1a) with ghrelin and its des-acyl analogue in living cells. The results showed that ghrelin possesses higher binding affinity than des-acylghrelin and that the *n*-octanoic group is involved in the interaction with the (GHS-R1a) receptor. Furthermore, the differences between proton chemical shifts of bound and free ligand were used to elucidate the interaction of the isotopically labelled form of the fully active and chemically defined form of endotoxin, Kdo₂-Lipid A, with serum glycoprotein sCD14 [87].

Ligand-protein interactions have been recently investigated by solid-state NMR spectroscopy. Krabben *et al.* [88] determined the contact area of neurotoxin II from Asian cobra *Naja naja oxiana* when interacting with membrane-bound nicotinic acetylcholine receptors from *Torpedo californica*. The comparison of the chemical shift values for the free and receptor-bound toxin indicated that more than 75% of the assigned ¹³C resonances did not change within ± 0.5 ppm. Hence, it was concluded that the observed chemical shift changes were related to large conformational modifications. The strongest chemical shift changes were associated with the residues at the ligand binding interface. Furthermore, Xu *et al.* [89] assigned uniformly microcrystalline ¹³C and ¹⁵N enriched yeast triosephosphate isomerase (TIM) using high-resolution solid-state NMR spectroscopy and compared the results with solution-state NMR assignments. An overall agreement between solid-state and solution-state NMR spectra was observed. Some of the observed chemical shift perturbations, as compared to the solution NMR assignment, were related to crystal packing interactions. Furthermore, the binding of a substrate analogue D-glycerol-3-phosphate (D-G3P) to TIM was studied by solid-state NMR for the first time. Site-specific perturbations of the enzyme's chemical shifts upon ligand binding indicated protein conformational changes. Similarly, solid-state ²H NMR spectroscopy was used to investigate the orientation and conformation of retinal within the rhodopsin binding pocket [90]. This approach elucidated the mechanism of rhodopsin activation as a guide for ligand-based drug design.

There are several articles concerning the combined use of NMR spectroscopy and isothermal titration calorimetry. This approach provides a complete thermodynamic characterisation of the investigated system, as was demonstrated with the aminoglycoside antibiotic paromomycin [91]. Furthermore, it was shown that isothermal titration calorimetry in combination with NMR methods was able to elucidate the structure of different enzyme-inhibitor complexes and the dynamics of their interaction [92-94].

NMR experiments based on the paramagnetic effects of lanthanide ions can provide unique insight to ligand-protein complex structure. This technique has been used for the structural characterisation of the ternary 30 kDa complex between the lanthanide-labelled N-terminal domain of the epsilon exonuclease subunit from *E. coli* DNA polymerase III, the theta subunit and thymidine [95].

Although modern ligand-based NMR methods are most widely used to monitor ligand-protein interactions, standard one- and two- dimensional NMR methods are able to structurally characterise the investigated ligand-protein complexes [96-98]. Alcaraz *et al.* [99] used ^{13}C NOESY-HSQC spectra to characterise the adducts of the catalytic domain of matrix metalloprotease-3 (MMP3) with three different non-peptide inhibitors. Similarly, Murail *et al.* [100] determined the structure of the mouse translocator protein (mTSPO) and the stability of its tertiary folding regarding the presence of (2-chlorophenyl)-*N*-methyl-*N*-(1-methyl-propyl)-3-isoquinoline carboxamide (PK 11195) ligand using two-dimensional NMR methods. The proton chemical shifts and ^{15}N - ^1H HSQC correlations indicated that PK 11195 binding enhanced the stability of the mTSPO tertiary fold.

As mentioned in the Introduction, ligand-protein interactions can be studied using protein-detected techniques, such as SAR by NMR. Tsantrizos *et al.* [101] used SAR by NMR to optimise small-molecule hepatitis C virus NS3 protease inhibitors. The observed results have shown that the binding affinity and potency were highly dependent on the ring size, the stereochemistry of each chiral centre and the electrostatic potential of the aromatic substituents.

5.3. LIGAND-DNA, LIGAND-RNA AND LIGAND MEMBRANE INTERACTIONS

NMR spectroscopy has also been used to study various interactions of small molecules (ligands) with DNA, RNA and membrane receptors.

Di Micco *et al.* [102] demonstrated an STD approach based on two parallel sets of STD experiments, in which saturation was elicited by irradiating at a suitable frequency chosen from specific DNA resonances for the characterisation of different DNA ligands. The signal-to-noise ratios of all the protons showing the STD effects were compared with the reference STD spectrum. The results suggested that the ligands making proximate contacts with aromatic base protons received more saturation upon the irradiation of DNA aromatic protons, whereas the external ligands were more affected by irradiation in the deoxyribose region.

Furthermore, ligand-DNA complexes have been studied by diffusion methods. Ramalho *et al.* [103] utilised a combination of, pulsed field gradient NMR (PFG NMR) spectroscopy, spin-lattice relaxation rate analysis and docking methods to investigate the interaction of 5-nitroimidazole radiosensitisers with duplex DNA. The intensity of radiosensitiser-macromolecule interactions was successfully evaluated using PFG NMR and docking data. Furthermore, the measurements of

spin-lattice selective relaxation rates represented a powerful tool to investigate the binding affinity of the ligand towards the receptor.

Moreover, novel methods have been proposed for studying ligand-RNA binding.

Kreutz *et al.* [104] applied ^{19}F NMR spectroscopy to identify site-specific ligand-RNA binding. The approach relied on site-specific labelling of RNA with 2'-deoxy-2'-fluoro(2'-F) nucleosides, thereby replacing the 2'-hydroxy group with a fluorine atom. The downfield shift of fluorine resonance upon ligand binding and a clear decrease of linewidth indicated that RNA has a more rigid structure in the bound state. Furthermore, Foloppe *et al.* combined NMR spectroscopy and a fluorescence resonance energy transfer (FRET) binding assay to identify aminoacyl-tRNA acceptor site (A-site) ligands [105]. The compounds that showed affinity for the A-site in a FRET-based binding assay were further investigated using NMR spectroscopy. Intermolecular NOEs confirmed the binding of these compounds to RNA.

The study of ligand-membrane interactions using a combination of NMR spectroscopy and other experimental or computational techniques have recently gained particular attention in order to understand metabolic pathways and develop drugs with the desired properties.

Melo *et al.* [106] studied the interaction between the dengue virus (DV) fusion peptide and different model membranes by fluorescence and NMR spectroscopy. The interaction was strongest in dodecylphosphocholine and anionic phosphatidylcholine/phosphatidylglycerol vesicles, which were the only vesicles fused by the DV fusion peptide. The three-dimensional structure of the DV fusion peptide bound to dodecylphosphocholine micelles and anionic phosphatidylcholine/phosphatidylglycerol vesicles was solved by transferred NOESY (trNOESY) and solution homonuclear NMR spectroscopy. The majority of the trNOE cross-peaks were from the hydrophobic triad, corroborating the dodecylphosphocholine-bound structure. Follet *et al.* [107] used a combination of electron spin resonance (ESR) and NMR spectroscopy in solution as well as solid-state NMR spectroscopy to investigate the physicochemical properties and membrane interactions of anti-apoptotic 2-(4-fluorophenyl)-3-(pyridine-4-yl)imidazo [1,2-*a*] pyridine derivatives with different side chain lengths and conformations. The results pointed towards the fact that improved solubility in biological media could be achieved by choosing a six-atom side chain length and that enhancement of membrane interactions/penetration could be achieved by hydroxyl substitutions on the C6 lateral chain. Cabeça *et al.* [108] applied molecular dynamics (MD) simulations and NMR spectroscopy to examine the interaction of the neutral and protonated species of the local anaesthetic prilocaine (PLC) with phosphatidylcholine (PC) bilayers. The diffusion-ordered 2D NMR spectroscopy (DOSY) experiments showed that neutral PLC has a higher affinity for the liposome environment. Furthermore, STD results revealed that the interaction of prilocaine molecules with the bilayer is pH-dependent. The findings were in accord with MD simulations.

The progress in NMR spectroscopy for studying interactions of bioactive molecules has been demonstrated in several reviews.

Fielding [109] recently reviewed NMR methods for the determination of protein-ligand dissociation constants. A protein and a ligand in thermodynamic equilibrium are characterised by the dissociation constant, K_D . In the simplest case of a protein with a single binding site, K_D is defined as:

$$K_D = \frac{[P][L]}{[PL]} \quad (5.7)$$

where $[P]$, $[L]$ and $[PL]$ are the equilibrium concentrations of the protein, ligand and complexed state, respectively. The object of the NMR observation might be the ligand or the protein.

Hence, when viewing the ligand, the NMR experiment has to be able to distinguish between the ligand in the free and bound states, so that $[L]$ and $[PL]$ can be quantified. For a system in fast exchange, the observed NMR response (M_{obs}) of a ligand is a molar fraction weighted average of the NMR parameters of the free and bound states:

$$M_{\text{obs}} = X_{L(\text{free})}M_{L(\text{free})} + X_{L(\text{bound})}M_{L(\text{bound})} \quad (5.8)$$

where $X_{L(\text{free})}$ and $X_{L(\text{bound})}$ are the molar fractions of the free and bound ligand, and $M_{L(\text{free})}$ and $M_{L(\text{bound})}$ are the NMR parameters of the ligand in its free and bound states, respectively.

Alternatively, when observing the protein, the NMR experiment has to distinguish between and quantify $[P]$ and $[PL]$. For the observation of a protein, the observed NMR response is defined as:

$$M_{\text{obs}} = X_{P(\text{free})}M_{P(\text{free})} + X_{P(\text{bound})}M_{P(\text{bound})} \quad (5.9)$$

where X_P and M_P are now the molar fraction and the NMR characteristic of the non-bound and occupied protein, respectively.

Kleckner and Foster [110] provided an overview of eight distinct NMR-based methods for studying protein dynamics. The methods are arranged by increasing experimental complexity and the observable timescale of the dynamic exchange τ_{ex} :

- a) real-time (RT) NMR ($\tau_{\text{ex}} > 1$ s; $k_{\text{ex}} < 1$ s⁻¹),
- b) exchange spectroscopy (EXSY) ($\tau_{\text{ex}} \approx 10$ -5000 ms; $k_{\text{ex}} \approx 0.2$ -100 s⁻¹; slow exchange ; $k_{\text{ex}} \ll \Delta\nu$),
- c) lineshape analysis ($\tau_{\text{ex}} \approx 10$ -100 ms; $k_{\text{ex}} \approx 10$ -100 s⁻¹; slow-intermediate exchange; $k_{\text{ex}} \leq \Delta\nu$),
- d) Carr-Purcell Meiboom-Gill relaxation dispersion (CPMG RD) ($\tau_{\text{ex}} \approx 0.3$ -10 ms; $k_{\text{ex}} \approx 100$ -3000 s⁻¹; intermediate-fast exchange $k_{\text{ex}} \approx \Delta\nu$),
- e) Rotating frame relaxation dispersion (RFRD) ($\tau_{\text{ex}} \approx 20$ -100 μ s; $k_{\text{ex}} \approx 10000$ -50000 s⁻¹; intermediate-fast exchange $k_{\text{ex}} \geq \Delta\nu$),
- f) Nuclear spin relaxation (NSR) (ps-ns dynamics indirectly probed),
- g) Residual dipolar coupling (RDC) (ps-ms dynamics indirectly probed),

- h) Paramagnetic relaxation enhancement (PRE) ($\tau_{\text{ex}} \approx 10 \mu\text{s}$; $k_{\text{ex}} \approx 100000 \text{ s}^{-1}$; fast exchange $k_{\text{ex}} \gg \Delta I$).

Madl *et al.* [111] discussed the utility and computational approaches of combining solution-state NMR with small-angle X-ray and neutron scattering (SAXS/SANS) experiments for the structural analysis of large protein complexes. The combination of NMR and SAXS/SANS in particular is a valuable approach that complements crystallographic studies for structure determination and the investigation of dynamic properties of multi-domain proteins and their complexes in solution.

Reckel *et al.* [112] discussed the aim of in-cell NMR spectroscopy. The non-invasive character of in-cell NMR spectroscopy makes it the only biophysical method that enables the direct in-cell observation of conformational changes, post-translational modifications and binding events with high structural resolution in living cells. This unique ability is based on the sensitivity of the chemical shift of an NMR-active nucleus to changes in its chemical environment.

Grzesiek and Sass [113] have reviewed recent advances and applications of solution NMR methods for the characterisation of macromolecular structure and dynamics as well as molecular interactions. The combination of enhanced computational approaches and the in-cell detection of molecules enable a detailed characterisation of folding transitions and intracellular interaction studies.

Blobel *et al.* [114] have discussed different approaches to monitoring protein interactions by NMR at concentrations close to physiological values in unlabelled species. This review emphasises the role of small molecules and NMR active ^{129}Xe nuclei as reporters in the detection of protein-protein interactions.

Von Itzstein [115] reviewed the latest developments in structure-based inhibitor design for microbial-assisted and mammalian-assisted carbohydrate-recognising proteins. The inhibitors were studied using STD-NMR, crystallography and computational techniques.

Krishna and Jayalakshmi have provided a comprehensive description of complete relaxation and conformational exchange theory adapted for saturation transfer measurements (CORCEMA-ST) [116]. The CORCEMA-ST protocol can be applied to obtain quantitative data from STD-NMR spectra.

CORCEMA-ST is based on the two-state model depicted in Scheme 1. The two-state model involves the ligand and the receptor in their free (L_f , R_f) and bound (R_b , L_b) states. The exchange rate is determined by on- and off-rate constants ($k_{L,\text{on}}$, $k_{R,\text{on}}$ and $k_{L,\text{off}}$, $k_{R,\text{off}}$, respectively).

According to the two-state model, the expression of the observable magnetisation experiment is given by:

$$\mathbf{I}(t) = \mathbf{I}_0 + [1 - e^{-(\mathbf{R}+\mathbf{K})t}](\mathbf{R}+\mathbf{K})^{-1} \mathbf{Q} \quad (5.10)$$

Scheme 1.

where t is the time period in which the protons remain unsaturated. $\mathbf{I}(t)$ is the intensity matrix composed of column matrices $\mathbf{I}_f$ and $\mathbf{I}_b$ representing free and bound protons, respectively. $\mathbf{I}_0$ is the thermal equilibrium matrix, whereas the term $\mathbf{R} + \mathbf{K}$ is the dynamic matrix composed of relaxation rate matrix $\mathbf{R}$ and kinetic matrix $\mathbf{K}$. The matrix $\mathbf{Q}$ is composed of $\mathbf{Q}_f$ and $\mathbf{Q}_b$ submatrices corresponding to the free and bound states, respectively.

A feasibility study was performed utilising a known ligand-protein complex that demonstrated the ability of an STD-NMR intensity-restrained CORCEMA optimisation procedure (SICO) to identify the global minimum conformation for the bound ligand. Furthermore, it was shown that CORCEMA-ST can be successfully utilised in obtaining quantitative structural information on the conformation of ligands within the binding pockets of large proteins.

Goncalves *et al.* [117] reviewed a recent development in NMR methods for the structural characterisation of G protein-coupled receptors. This review outlines three key NMR interactions that yield structural information on G protein-coupled receptors:

- chemical shifts that provide information on the local environment, conformational changes and hydrogen bonding,
- dipolar couplings used to measure internuclear distances and orientations,
- quadrupolar couplings that are sensitive to molecular motion.

The investigations of structural changes occurring upon the activation of rhodopsin by a molecular switch mechanism demonstrated the ability of NMR spectroscopy to correlate the structure and function of G protein-coupled receptors.

Law *et al.* [118] discussed the role of computational chemistry, NMR spectroscopy and X-ray crystallography in fragment-based drug design (FBDD). The ability of FBDD to optimise ligand fragment properties is illustrated by several ligand-receptor complexes. It was outlined that computational chemistry, in combination with experimental techniques, could have a major impact on the success of FBDD programmes in the future and that it is ready to respond to the challenges that exist in the FBDD field.

Schanda [119] has provided an excellent review focused on the existing methods for fast multidimensional NMR spectroscopy used for biomolecular studies. The long data acquisition times required for the recording of conventional nD Fourier transform (FT) NMR spectra of biomolecules is very restricting for several reasons: limited stability of biological samples, instrumental costs and the inability to monitor many biologically relevant reactions in real time. These issues can be resolved using different fast multidimensional NMR techniques based on two approaches. The first replaces the regular $(n - 1)$ -dimensional sampling grid by some other time domain sampling that requires fewer sampling points. Several methods, such as sparse sampling, spectral aliasing and projection NMR, represent this approach. In the second approach, time-domain sampling is replaced by frequency-domain sampling (Hadamard spectroscopy) or a spatial encoding of evolution frequencies (ultrafast NMR spectroscopy).

Fast acquisition methods make NMR ready for use as a powerful tool in the analysis of the structure and dynamics of biological molecules. Hence, fast multidimensional NMR spectroscopy is capable of elucidating phenomena that are out of reach of many other techniques.

Donald and Martin [120] presented an overview of novel algorithms for protein structure determination, automated assignment, characterisation of membrane proteins and protein complexes as well as fold recognition using only unassigned NMR data. The methods presented in this review demonstrate how sparse dipolar couplings can be exploited to resolve the assignment problem, which is a key bottleneck in NMR structural biology.

Schwalbe *et al.* [25] emphasised the importance of NMR spectroscopy in understanding the structure and dynamics of biomacromolecules in drug discovery. The implementation of novel techniques improved the speed and accuracy of solution- and solid-state NMR spectroscopy. Hence, it was recognised that NMR spectroscopy was capable of determining the structure of medium-sized proteins in native and non-native states as well as characterising ligand-receptor interactions.

5.4. STRUCTURAL ANALYSIS OF LIGANDS

The structure of ligands is crucial for their function and interactions with the receptor. Some applications of NMR techniques for ligand structure elucidation are demonstrated by several examples in the following paragraph.

Modarresi-Alam *et al.* [121] used variable temperature 1H NMR spectroscopy to study restricted S–N rotation in aryl-*N*-(arylsulphonyl)-*N*-(triphenylphosphoranylidene) imidocarbamates. The doublets of the aryl substituents were employed to determine the barriers to rotation at the coalescence temperature. It was shown that a change in the substituents on the oxygen, sulphonyl and nitrogen of the imidoyl moiety had no appreciable influence on the observed barrier to rotation.

Seneci *et al.* [122] synthesised and characterised proapoptotic second mitochondria-derived activator of caspases (Smac) mimics and inhibitor of apoptosis protein (IAP) inhibitors. The SAR of this class of inhibitors was elucidated by computational methods and structural studies (NMR spectroscopy, X-ray diffraction). One lead molecule for further *in vitro* and *in vivo* evaluation was identified.

Fuchs *et al.* [123] prepared several heterocyclic analogues of thioflavones based on a Sonogashira-type coupling of *o*-halo(hetero)aryl chlorides with phenyl-acetylene. NMR spectroscopic studies provided the structure determination of the prepared compounds.

Zhuang *et al.* [124] used paramagnetism-based NMR constraints, including field-induced RDCs and pseudo-contact shifts (PCSs) for the structure determination of a galectin-3-carbohydrate. The paramagnetic Dy³⁺ ion was complexed to the peptide, making it possible to observe RDCs as well as PCSs for the protein and the ligand. The determined structure was in agreement with the crystal structure of a galectin-3-*N*-acetyllactosamine complex.

Tiziani *et al.* [125] applied DOSY to assess possible interactions between daidzein and daidzein soy protein and carotenoids. The results showed that the affinity between daidzein and the soy protein isolate was higher compared to the daidzein dissolved in the same model system. The shifting of the particular proton NMR signals pointed towards two main contributions to the interactions between the phytochemicals and the protein: hydrogen bonding from hydroxyl groups and hydrophobic interactions.

5.5. STRUCTURAL ANALYSIS OF PEPTIDES AND PROTEINS

A large number of proteins and peptides serve as ligand receptors. Hence, the elucidation of macromolecular structure and dynamics is crucial for the characterisation of ligand-protein complexes. The recent applications of NMR methods for peptide and protein structure determination are presented in the following section.

Arroyo and Mayo [126] elucidated the structure of angiostatic peptide anginex using NMR structural studies in dodecylphosphocholine micellar environment. Conformational modelling was performed using NOESY data. The results showed that anginex forms a three-stranded anti-parallel β -sheet conformation in a dodecylphosphocholine micellar solution.

Daly *et al.* [127] characterised the structure of several cyclotides (*cyclo peptides*), containing a head-to-tail cyclised peptide backbone and a knotted arrangement of three conserved disulphide bonds. Excellent signal dispersion in NMR spectra makes NMR spectroscopy the most commonly used technique for studying cyclotide structures. The NOESY spectrum of the cyclotide kalata B1 revealed a continuous cycle of sequential cross-peaks, which spanned all 29 amino acids in the sequence and provided evidence for its circular backbone. Furthermore, the

NMR analysis of kalata B1 and kalata B7 in the presence of dodecylphosphocholine micelles illustrated their binding to the membrane in different orientations.

Furthermore, NMR screening methods were applied to study the structural aspects of allosteric regulation by deuterated functionally active thermostable dimeric Lac repressor proteins [128]. The optimisation of conditions most suitable for lengthy NMR recordings was performed using a high-throughput thermofluor method based on the measurement of the fluorescence intensity of a protein solution at different pH values, temperatures and salt concentrations.

Yao *et al.* [129] applied PFG NMR for evaluating the estimates of protein rotational correlation times. The rotational correlation time, τ_c , can be expressed as a function of the translation diffusion coefficient, D_t :

$$\tau_c = \frac{2}{9} \frac{(R_h^{\text{ref}} D_t^{\text{ref}})^2}{D_t^3} \quad (5.11)$$

where R_h^{ref} and D_t^{ref} are the hydrodynamic radius and diffusion coefficient of the reference molecule, respectively.

The estimates of protein rotational correlation times can be useful if:

- a) isotopically labelled material is not available,
- b) relaxation rates are difficult to interpret,
- c) a full relaxation analysis is difficult because of limited sensitivity.

Kodama *et al.* [130] used time-sharing NMR measurements as a rapid, effective and unbiased approach for the identification of a protein-protein interface without resonance assignments. Only a single protein ^{15}N - and ^{13}C -labelled sample was required for the analysis. The measurements were performed using a new pulse sequence that allowed simultaneous aromatic ^1H - ^{13}C and ω_1 -TROSY-type backbone ^1H - ^{15}N correlations, together with single quantum methyl ^1H - ^{13}C correlations.

Sakurai *et al.* [131] investigated the structure, dynamics and folding of β -lactoglobulin as well as its interaction with ligands by NMR spectroscopy. The results indicated that β -lactoglobulin undergoes a conformational change during pH-dependent Tanford transition and ligand binding. The folding mechanism and perturbations due to the introduction of a mutation were explained by a schematic representation using folding funnels.

Buchko *et al.* [132] studied leucine-rich amelogenin protein (LRAP), a splice-variant isoform of amelogenin using NMR spectroscopy. The main difference in the ^1H - ^{15}N NMR spectrum of LRAP compared to the full-length amelogenin protein was an additional set of amide resonances for seven non-proline residues, which indicated the presence of two different conformations. The specific changes in molecular dynamics of LRAP upon NaCl addition, manifested

by the reduction in intensity and disappearance of ^1H - ^{15}N HSQC cross-peaks, pointed towards the formation of protein nanospheres.

Moriya *et al.* [133] determined the protein-protein interface and solvent accessibility upon complex formation between ubiquitin and a C90S mutant of yeast ubiquitin hydrolase 1 (YUH 1) using ^{13}C chemical shift perturbations induced by freely diffusing 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPOL).

5.6. MACROLIDE-RIBOSOME INTERACTIONS

Macrolide antibiotics, such as erythromycin, azithromycin and telithromycin (Figure 5.9) are effective therapeutic agents for treating infectious diseases [134,135]. They are still the centre of interest of many research groups and pharmaceutical companies and much effort is directed toward the discovery of new macrolide antibiotics by chemical modification of the existing classes of natural derivatives. The goal is to obtain novel therapeutic agents having an improved overall biological profile with a special emphasis on resistant bacterial strains. The continuing emergence of multidrug-resistant bacterial strains imposes a serious threat to the health-care community and intensifies the search for new and more effective agents in order to overcome this problem. Of particular interest are azalides, semisynthetic derivatives of erythromycin A [134,136], which have been found to display a wide antimicrobial spectrum and the ability to concentrate within host cells with high accumulation ratios.

There are two major mechanisms of resistance to macrolides, *i.e.* target site modifications by methylation that prevent the binding of the antibiotic to the ribosome (encoded by the *erm* gene) and efflux mechanisms, mediated by the *mef* (*streptococci*) and *msr* (*staphylococci*) genes. The methylation of ribosomal rRNA bases leads to cross-resistance to macrolides (M), lincosamides (L) and streptogramin B (S_B), the so-called MLS_B phenotype [137].

Figure 5.9. Chemical structures of (a) erythromycin, (b) azithromycin and (c) telithromycin

Macrolides exert their activity by interacting with the bacterial 50S ribosomal subunit at, or close to, the peptidyl-transferase centre and thus inhibit the growth

of the nascent peptide chain. As mentioned above, resistance to antibiotics has become a global problem and much effort is now directed toward new and more potent classes of drugs. An effective approach to overcoming this problem is to understand the principles of how these drugs interact with the ribosome [138]. Recently, crystal structures of some ribosome-macrolide complexes [139-141] have shed new light on the binding mechanisms of macrolides to ribosomes and hence provide a good basis for the rational design of new ligands and inhibitors. However, when analysing solid state structures of ribosome-macrolide complexes, one should keep in mind some discrepancies between the structures obtained for the halophilic archeon *H. Marismortui* [139,141,142] and *D. radiodurans* [140,143]. The proposed models differ even though ribosomal 50S subunits of the two bacteria have drug binding sites whose sequences are highly conserved. Furthermore, the crystal structure data obtained so far on complexes of macrolides with ribosomes isolated from clinically non-relevant bacteria do not explain all the effects of macrolides on different pathogenic strains. We believe that steps taken in the process of drug design should also include the elucidation of the solution-state structures of free and bound ligand molecules since the structural features of the complex may not be exactly the same in solution as in the solid state [142,145].

Novak *et al.* [146-150] have shown that a systematic approach combining NMR and molecular modelling calculations could be applicable to conformational studies of free and bound macrolides and their interactions with ribosomes. Their results [146-150], and those of other groups [151-155], have shown that macrolides adopt two major conformational families, folded-out and folded-in, referring to the outward and inward folding of the ring fragment 3C-5C (Figure 5.10).

Figure 5.10. Superposition of the two major conformations of azithromycin: folded-in (black) and folded-out (grey)

The authors concluded that the vicinal coupling constants $^3J_{\text{H2H3}}$ and NOE proton-proton contacts such as H3-H11 and H4-H11 are good indicators of aglycone fold-

ing. Furthermore, $^3J_{\text{CH}}$ coupling constants over the glycosidic bonds might provide information about the position and mobility of sugar units, with respect to the lactone ring. Longitudinal relaxation of methyl protons could also be useful to probe motions of methyl groups which reflect aglycone ring folding. Additionally, by applying trNOESY and STD-NMR experiments, it was possible to characterise the interactions of macrolides and ribosomes [146,148].

STD experiments have indicated three common regions closest to the ribosome surface: desosamine sugar, cladinose sugar and the methyl group at position 13 (Figure 5.11).

Figure 5.11. STD binding epitopes of a macrolide derivative.

These were also found by X-ray diffraction to be the reactive groups responsible for binding to the ribosome. The bound conformations of the studied macrolides were found to be very similar to those observed in the free state, which were also in agreement with the results obtained by crystallography [142,144]. Furthermore, it was found that the absence of cladinose sugar was the main cause of the inability of decladinosyl macrolides to bind to the ribosome. Recently, more detailed studies using a combination of STD-NMR and *in vitro* protein inhibition on a series of macrolide compounds have shown that hydrophobic interactions involving cladinose, methyl groups of the lactone ring and the 13-alkyl moiety are a prerequisite for optimal positioning of the desosamine sugar unit. A synergy between the desosamine 3'-dimethylamino and 2'-hydroxyl interactions with the ribosome has been proposed as crucial for successful protein synthesis inhibition. Recently, NMR self-diffusion and solvent paramagnetic relaxation enhancements have been used to determine the interaction strength and localization of a series of macrolide antibiotics with membrane-mimetic micelles. It has been proven that protonated nitrogen atoms play a crucial role in binding to membrane-mimetics [156].

STD-NMR spectroscopy has also been employed to characterise the epitopes of azithromycin, oleandomycin and telithromycin macrolide antibiotic binding to

bovine serum albumin (BSA) [157]. The structural parts of azithromycin and oleandomycin in intimate contact with bovine serum albumin were found to be similar, while those of telithromycin showed similarities but also some differences. The authors explained these findings by different structural elements of antibiotics that interacted with the protein, especially the alkyl-heteroaryl side chain in telithromycin and cladinose and desosamine sugars in azithromycin and oleandomycin.

Acknowledgement

This study was supported by the Ministry of Science, Education and Sports of the Republic of Croatia (Project No. 119-1191342-1083).

REFERENCES

1. M.P. Williamson, T.F.D. Havel, K. Wüthrich. Solution Conformation of Proteinase Inhibitor IIA from Bull Seminal Plasma by ^1H Nuclear Magnetic Resonance and Distance Geometry. *Journal of Molecular Biology* **182**(2) (1985) 295-315.
2. K. Wutrich. NMR of Proteins and Nucleic Acid, Wiley, 1986.
3. H. Patzelt, N. Goto, H. Iwai, K. Lundstrom, E. Fernholz. Modern Methods for the Expression of Proteins in Isotopically Enriched Form. In O. Zerbe (Ed.), *BioNMR in Drug Research*, Wiley-VCH, 2003 pp. 1-38.
4. V. Tugarinov, W.Y. Choy, V.Y. Orekhov, L.E. Ka. Solution NMR-derived Global Fold of a Monomeric 82-kDa Enzyme. *Proceedings of the National Academy of Science of the United States of America* **102**(3) (2005) 622-627.
5. K. Pervushin, R. Riek, G. Wider, K. Wüthrich. Attenuated T2 Relaxation by Mutual Cancellation of Dipole-dipole Coupling and Chemical Shift Anisotropy Indicates an Avenue to NMR Structures of Very Large Biological Macromolecules in Solution. *Proceedings of the National Academy of Science of the United States of America* **94**(23) (1997) 12366-12371.
6. K. Pervushin, R. Riek, G. Wider, K. Wütrich. Transverse Relaxation-Optimized Spectroscopy (TROSY) for NMR Studies of Aromatic Spin Systems in ^{13}C -Labeled Proteins. *Journal of American Chemical Society* **120**(25) (1998) 6394-6400.
7. M. Pellechia, P. Sebbel, U. Herrmanns, K. Wüthrich, R. Glockshuber. Pilus chaperone FimC-adhesin FimH interactions mapped by TROSY-NMR. *Nature Structural & Molecular Biology* **6**(4) (1999) 336-339.
8. R. Bader, M. Lerch, O. Zerbe, NMR of Membrane-Associated Peptides and Proteins. In *BioNMR in Drug Research*, (Ed: O. Zerbe) Wiley-VCH, 2003 pp. 95-120.
9. C. Fernandez, G. Wider. TROSY in NMR studies of the Structure and Function of Large Biological Macromolecules. *Current Opinion in Structural Biology* **13**(5) (2003) 570-580.
10. A.E. McDermott. Structural and Dynamic Studies of Proteins by Solid-state NMR Spectroscopy: Rapid Movement Forward. *Current Opinion in Structural Biology* **14**(5) (2004) 554-561.
11. J.E. Kay. NMR Studies of Protein Structure and Dynamics. *Journal of Magnetic Resonance* **173**(2) (2005) 193-207.

12. C.D. Schwieters, J.J. Kuszewski, G. Marius Clore. Using Xplor-NIH for NMR Molecular Structure Determination. *Progress in Nuclear Magnetic Resonance Spectroscopy* **48**(1) (2006) 47-62.
13. M.R. Jensen, P.R.L. Markwick, S. Meier, C. Griesinger, M. Zweckstetter, S. Grzesiek, P. Bernado, M. Blackledge. Quantitative Determination of the Conformational Properties of Partially Folded and Intrinsically Disordered Proteins Using NMR Dipolar Couplings. *Structure* **17**(9) (2009) 1169-1185.
14. M. Coen. A Metabonomic Approach for Mechanistic Exploration of Pre-clinical Toxicology. *Toxicology* **278**(3) (2010) 326-340.
15. J. Griffin. Metabonomics: NMR Spectroscopy and Pattern Recognition Analysis of Body Fluids and Tissues for Characterisation of Xenobiotic Toxicity and Disease Diagnosis. *Current Opinion in Chemical Biology* **7**(5) (2003) 648-654.
16. D. G. Robertson, J. Lindon. Metabonomics in Toxicity Assessment. *Taylor & Francis Group*, Boca Raton, 2005.
17. D. Bartos, S. Görög. Recent Advances in the Impurity Profiling of Drugs. *Current Pharmaceutical Analysis* **4**(4) (2008) 215-230.
18. P. Novak, P. Tepeš, M. Cindrić, M. Ilijaš, S. Dragojevic, K. Mihaljevic. Combined Use of Liquid Chromatography-nuclear Magnetic Resonance Spectroscopy and Liquid Chromatography-mass Spectrometry for the Characterization of an Acarbose Degradation Product. *Journal of Chromatography A* **1033**(2) (2004) 299-303.
19. P. Novak, M. Cindrić, P. Tepeš, S. Dragojević, M. Ilijaš, K. Mihaljević. Identification of Impurities in Acarbose by Using an Integrated Liquid Chromatography-nuclear Magnetic Resonance and Liquid Chromatography-Mass Spectrometry Approach. *Journal of Separation Science* **28**(13) (2005) 1442-1447.
20. P. Novak, P. Tepeš, I. Fistrić, I. Bratoš, V. Gabelica. The Application of LC-NMR and LC-MS for the Separation and Rapid Structure Elucidation of an Unknown in 5-aminosalicylic Acid. *Journal of Pharmaceutical and Biomedical Analysis* **40**(5) (2006) 1268-1272.
21. P. Novak, P. Tepeš, M. Ilijaš, I. Fistrić, I. Bratoš, A. Avdagić, V. Gabelica Marković, M. Dumić. Rapid LC-NMR and LC-MS Characterization of an Unknown Component in a Novel Antifungal Drug Icofungipen. *Journal of Pharmaceutical and Biomedical Analysis* **50**(1) (2009) 68-72.
22. B.J. Stockman, C. Dalvit. NMR Screening Techniques in Drug Discovery and Drug Design. *Progress in Nuclear Magnetic Resonance Spectroscopy* **41** (2002) 187-231.
23. J.W. Peng, J. Moore, N. Abdul-Manan. NMR Experiments for Lead Generation in Drug Discovery. *Progress in Nuclear Magnetic Resonance Spectroscopy* **44**(3) (2004) 225-256.
24. B. Meyer, T. Peters. NMR Spectroscopy Techniques for Screening and Identifying Ligand Binding to Protein Receptors. *Angewandte Chemie International Edition* **42**(8) (2003) 864-890.
25. H. Scwalbe, H.U. Stiltz, H. Kessle. NMR Spectroscopy of Biomacromolecules in Drug Discovery and Beyond. *ChemBioChem* **6**(9) (2005) 1475-1478.
26. P.J. Hajduk, J.R. Huth, C. Tse. Predicting Protein Druggability. *Drug Discovery Today* **10**(23) (2005) 1675-1682.
27. P. Tepeš, P. Novak. Interakcije receptora i liganda pod lupom spektroskopije NMR. *Kemija u Industriji* **57**(4) (2008) 165-173.

28. L. Fielding. NMR Methods for the Determination of Protein-ligand Dissociation Constants. *Progress in Nuclear Magnetic Resonance Spectroscopy* **51**(4) (2007) 219-242.
29. P.J. Hajduk, R.P. Meadows, S.W. Fesik. One-dimensional Relaxation- and Diffusion-edited NMR Methods for Screening Compounds that Bind to Macromolecules. *Journal of American Chemical Society* **119**(50) (1997) 12257-12261.
30. S.B. Shuker, P.J. Hajduk, R.P. Meadows, S.W. Fesik. Discovering High-Affinity Ligands for Proteins: SAR by NMR. *Science* **274**(5292) (1996) 1531-1534.
31. P.J. Hajduk, D.J. Augeri, J. Mack, R. Mendoza, J. Yang, S.F. Betz, S.W. Fesik. NMR-Based Screening of Proteins Containing ^{13}C -Labeled Methyl Groups. *Journal of American Chemical Society* **122**(33) (2000) 7898-7904.
32. D. Li, L.A. Levy, S.A. Gabel, M.S. Lebetkin, E.F. Derosé, M.J. Wall, E.E. Howell, R.E. London. Interligand Overhauser Effects in Type II Dihydrofolate Reductase. *Biochemistry* **40**(14) (2001) 4242-4252.
33. H. N. Moseley, W. Lee, C.H. Arrowsmith, N. Rama Krishna. Quantitative Determination of Conformational, Dynamic, and Kinetic Parameters of a Ligand-protein/DNA Complex from a Complete Relaxation and Conformational Exchange Matrix Analysis of Intermolecular Transferred NOESY. *Biochemistry* **36**(18) (1997) 5293-5299.
34. M. Mayer, B. Meyer. Characterization of Ligand Binding by Saturation Transfer Difference NMR Spectroscopy. *Angewandte Chemie International Edition* **38**(12) (1999) 1784-1788.
35. M. Mayer, B. Meyer. Group Epitope Mapping by Saturation Transfer Difference NMR To Identify Segments of a Ligand in Direct Contact with a Protein Receptor. *Journal of American Chemical Society* **123**(25) (2001) 6108-6617.
36. M. Vogtherr, T. Peters. Application of NMR Based Binding Assays to Identify Key Hydroxy Groups for Intermolecular Recognition. *Journal of American Chemical Society* **122**(25) (2000) 6093-6099.
37. C. Dalvit, P. Floersheim, M. Zurini, A. Widmer. Use of Organic Solvents and Small Molecules for Locating Binding Sites on Proteins in Solutions. *Journal of Biomolecular NMR* **14**(1) (1999) 23-32.
38. C. Dalvit, P. Pevarello, M. Tatò, M. Veronesi, A. Vulpetti, M. Sundström. Identification of Compounds with Binding Affinity to Proteins via Magnetization Transfer from Bulk Water. *Journal of Biomolecular NMR* **18**(1) (2000) 65-68.
39. I. Bertini, C. Dalvit, J. Gaspard Huber, M. Piccidi. ePHOGSY Experiments on a Paramagnetic Protein: Location of the Catalytic Water Molecule in the Heme Crevice of the Oxidized Form of Horse Heart Cytochrome *c*. *Federation of European Biochemical Societies Letters* **415**(1) (1997) 45-48.
40. A. Chen, M.J. Shapiro. NOE Pumping as High-Throughput Method To Determine Compounds with Binding Affinity to Macromolecules by NMR. *Journal of American Chemical Society* **122**(2) (2000) 414-415.
41. J. Orts, C. Griesinger, T. Carolmagno. The INPHARMA Technique for Pharmacophore Mapping: A Theoretical Guide to the Method. *Journal of Magnetic Resonance* **200** (2009) 64-73.
42. S. Meiboom, D. Gill. Modified Spin-Echo Method for Measuring Nuclear Relaxation Times. *Review of Scientific Instruments* **29**(8) (1958) 688-691.

43. H.Y. Carr, E.M. Purcell. Effects of Diffusion on Free Precession in Nuclear Magnetic Resonance Experiments. *Physical Review* **94**(3) (1954) 630-638.
44. W. Jahnke, L.B. Perez, C.G. Paris, A. Strauss, G. Feudrich, C.M. Nalin. Second-site NMR Screening with a Spin-Labeled First Ligand. *Journal of American Chemical Society* **122**(30) (2000) 7394-7395.
45. W. Jahnke, S.Rüdissler, M. Eurini. Spin Label Enhanced NMR Screening. *Journal of American Chemical Society* **123**(13) (2001) 3149-3150.
46. G.M. Clore. Exploring Sparsely Populated States of Macromolecules by Diamagnetic and Paramagnetic NMR Relaxation. *Protein Science* **20**(2) (2011) 229-246.
47. P.H.J. Keizers, M. Ubbink. Paramagnetic Tagging for Protein Structure and Dynamics Analysis. *Progress in Nuclear Magnetic Resonance Spectroscopy* **58**(1) (2011) 88-96.
48. T. Madl, W. Bermel, K. Zangger. Use of Relaxation Enhancements in a Paramagnetic Environment for the Structure Determination of Proteins Using NMR Spectroscopy. *Angewandte Chemie International Edition* **48**(44) (2009) 8259-8262.
49. K. Zangger, M. Respondek, C. Göbl, W. Hohlweg, K. Rasmussen, G. Gramp, T. Madl. Positioning of Micelle-bound Peptides by Paramagnetic Relaxation Enhancements. *Journal of Physical Chemistry B* **113** (2009) 4400-4406.
50. L.H. Lucas, C.K. Larive. Measuring Ligand-protein Binding Using NMR Diffusion Experiments. *Concepts of Magnetic Resonance; Part: A* **20A**(1) (2004) 24-41.
51. C. Dalvit, M. Fasolini, M. Flocco, S. Knapp, P. Pevarello, M. Veronesi. NMR-Based Screening with Competition Water-ligand Observed via Gradient Spectroscopy Experiments: Detection of High-affinity Ligands. *Journal of Medicinal Chemistry* **45**(12) (2002) 2610-2614.
52. C. Dalvit, M. Flocco, S. Knapp, M. Mostardini, R. Perego, B.J. Stockman, M. Veronesi, M. Varasi. High-throughput NMR-based Screening with Competition Binding Experiments. *Journal of American Chemical Society* **124**(26) (2002) 7702-7709.
53. W. Jahnke, P. Floersheim, C. Ostermeier, X. Zhang, R. Hemmig, K. Hurth, D.P. Uzunov. NMR Reporter Screening for the Detection of High-Affinity Ligands. *Angewandte Chemie International Edition* **41**(18) (2002) 3420-3423.
54. A. H. Siriwardena, F. Tian, S. Noble, J.H. Prestegard. A Straightforward NMR-Spectroscopy-Based Method for Rapid Library Screening. *Angewandte Chemie International Edition* **41**(18) (2002) 3454-3457.
55. C. Dalvit. Theoretical Analysis of the Competition Ligand-Based NMR Experiments and Selected Applications to Fragment Screening and Binding Constant Measurements. *Concepts of Magnetic Resonance; Part: A* **32** (5) (2008) 341-372.
56. J. Bendorowicz-Pikula, R. Buchet, F.J. Cañada, M. Clémancey, P. Groves, J. Jiménez-Barbero, J.M. Lancelin, O. Marcillat, S. Pikula, A. Sekrecka-Belniak, A. Strzelecka-Kiliszek. Characterization of Caged Compounds Binding to Proteins by NMR Spectroscopy. *Biochemical Biophysical Research Communications* **400**(3) (2010) 447-451.
57. A. Blume, A.J. Benie, F. Stolz, R.R. Schmidt, W. Reutter, S. Hinderlich, T. Peters. Characterization of Ligand Binding to the Bifunctional Key Enzyme in the Sialic Acid Biosynthesis by NMR. *Journal of Biological Chemistry* **279**(53) (2004) 55715-55721.

58. G. Bai, B. Feng, J.B. Wang, E. Pozharski, M. Shapiro. Studies on Ligand Binding to Histidine Triad Nucleotide Binding Protein 1. *Bioorganic & Medicinal Chemistry* **18**(18) (2010) 6756-6762.
59. Z. Ji, Z. Yao, M. Liu. Saturation Transfer Difference Nuclear Magnetic Resonance Study on the Specific Binding of Ligand to Protein. *Analytical Biochemistry* **385**(2) (2009) 380-382.
60. A. Sadeghi-Khomami, M.D. Lumsden, D.L. Jakeman. Glycosidase Inhibition by Macrolide Antibiotics Elucidated by STD-NMR Spectroscopy. *Chemistry & Biology* **15**(7) (2008) 739-749.
61. M.G. Szczepina, R.B. Zheng, G.C. Completo, T.L. Lowary, B.M. Pinto. STD-NMR Studies Suggest that two Acceptor Substrates for GlfT2, a Bifunctional Galactofuranosyltransferase Required for the Biosynthesis of *Mycobacterium tuberculosis* Arabinogalactan, Compete for the Same Binding Site. *ChemBioChem* **10**(12) (2009) 2052-2059.
62. A. Blume, M. Fitzen, A.J. Benie, T. Peters. Specificity of Ligand Binding to Yeast Hexokinase PII Studied by STD-NMR. *Carbohydrate Research* **344**(12) (2009) 1567-1574.
63. M. Piazza, L. Yu, A. Teghanemt, T. Gioannini, J. Weiss, F. Peri. Evidence of a Specific Interaction between New Synthetic Antisepsis Agents and CD14. *Biochemistry* **48**(51) (2009) 12337-12344.
64. Y.S. Wang, D. Liu, D.F. Wyss. Competition STD-NMR for the Detection of High-affinity Ligands and NMR-based Screening. *Magnetic Resonance in Chemistry* **42**(6) (2004) 485-489.
65. L.A. Plesniak, K. Botsch, M. Leibrand, M. Kelly, D. Sem, J.A. Adams, P. Jennings. Transferred NOE and Saturation Transfer Difference NMR Studies of Novobiocin Binding to EnvZ Suggest Binding Mode Similar to DNA Gyrase. *Chemical Biology & Drug Design* **71**(1) (2008) 28-35.
66. J.Y. Lee, K.W. Jeong, J.U. Lee, D.I. Kang, Y. Kim. Novel *E. coli* Beta-ketoacyl-acyl Carrier Protein Synthase III Inhibitors as Targeted Antibiotics. *Bioorganic & Medicinal Chemistry* **17**(4) (2009) 1506-1513.
67. R.S. Houlston, B.C. Jacobs, A.P. Tio-Gillen, J.J. Verschuuren, N.H. Khieu, M. Gilbert, H.C. Jarrel. STD-NMR Used to Elucidate the Fine Binding Specificity of Pathogenic Anti-ganglioside Antibodies Directly in Patient Serum. *Biochemistry* **48**(2) (2009) 220-222.
68. T. Murata, H. Hemmi, M. Nakajima, M. Yoshida, I. Yamaguchi. Epitope Mapping of Gibberellin to the Anti-gibberellin A(4) Monoclonal Antibody by Saturation Transfer Difference NMR spectroscopy. *Biochemical Biophysical Research Communications* **307**(3) (2003) 498-502.
69. F.M. Assadi-Porter, M. Tonelli, E.L. Maillet, J.L. Markley, M. Max. Interactions Between the Human Sweet-sensing T1R2-T1R3 Receptor and Sweeteners Detected by Saturation Transfer Difference NMR Spectroscopy. *Biochimica et Biophysica Acta - Biomembranes* **1798**(2) (2010) 82-86.
70. J.H. Streiff, N. O. Juranic, S.I. Macura, D.O. Warner, K.A. Jones, W.J. Perkins. Saturation Transfer Difference Nuclear Magnetic Resonance Spectroscopy as a Method for Screening Proteins for Anesthetic Binding. *Molecular Pharmacology* **66**(4) (2004) 929-935.

71. M.A. Macnaughtan, M. Kamar, G. Alvarez-Manilla, A. Venot, J. Glushka, J.M. Pierce, J.H. Prestegard. NMR Structural Characterization of Substrates Bound to N-acetylglucosaminyltransferase V. *Journal of Molecular Biology* **366**(4) (2007) 1266-1281.
72. X. Wen, Y. Yuan, D.A. Kuntz, D.R. Rose, B.M. Pinto. A Combined STD-NMR/molecular Modeling Protocol for Predicting the Binding Modes of the Glycosidase Inhibitors Kifunensine and Salacinol to Golgi Alpha-mannosidase II. *Biochemistry* **44**(18) (2005) 6729-673.
73. B.A. Becker, C.K. Larive. Probing the Binding of Propranolol Enantiomers to Alpha1-acid Glycoprotein with Ligand-detected NMR Experiments. *The Journal of Physical Chemistry B* **112**(43) (2008) 13581-13587.
74. M.R. Thompson, D.R. McKenzie, J.L. Likos, J.K. Gard. Protein-free Ligand Screening: Simplification of Chiral Chromatographic Development via Novel Adaptation of NMR Screening Methodologies. *Magnetic Resonance in Chemistry* **47**(7) (2009) 541-550.
75. C.J. Morrison, R. Godawat, S.A. McCallum, S. Garde, S.M. Cramer. Mechanistic Studies of Displacer-protein Binding in Chemically Selective Displacement Systems Using NMR and MD Simulations. *Biotechnology and Bioengineering* **102**(5) (2009) 1428-1437.
76. K. Fehér, P. Groves, G. Batta, J. Jiménez-Barbero, C. Muhle-Goll, K.E. Kővér. Competition Saturation Transfer Difference Experiments Improved with Isotope Editing and Filtering Schemes in NMR-based Screening. *Journal of American Chemical Society* **130**(50) (2008) 17148-17153.
77. K.E. Kővér, P. Groves, J. Jiménez-Barbero, G. Batta. Molecular Recognition and Screening Using a ^{15}N group Selective STD-NMR Method. *Journal of American Chemical Society* **129**(37) (2007) 11579-11582.
78. H. Gouda, T. Sunazuka, T. Hirose, K. Iguchi, N. Yamaotsu, A. Sugawara, Y. Noguchi, Y. Saito, T. Yamamoto, T. Watanabe, K. Shiomi, S. Ōmura, S. Hirono. NMR Spectroscopy and Computational Analysis of Interaction Between *Serratia marcescens* Chitinase B and a Dipeptide Derived from Natural-product Cyclopentapeptide Chitinase Inhibitor Argifin. *Bioorganic & Medicinal Chemistry* **18**(16) (2010) 5835-5844.
79. C. Bonechi, S. Martini, C. Rossi. Interaction Study of Bioactive Molecules with Fibrinogen and Human Platelets Determined by ^1H NMR Relaxation Experiments. *Bioorganic & Medicinal Chemistry* **17**(4) (2009) 1630-1635.
80. M.H. Lerche, S. Meier, P.R. Jensen, H. Baumann, B.O. Petersen, M. Karlsson, J.Ø. Duus, J.H. Ardenkjær-Larsen. Study of Molecular Interactions with ^{13}C DNP-NMR. *Journal of Magnetic Resonance* **203**(1) (2010) 52-56.
81. M. Pedò, F. Löhr, M. D'Onofrio, M. Assfalg, V. Dötsch, H. Molinari. The Interplay of Ligand Binding and Quaternary Structure in the Diverse Interactions of Dynein Light Chain LC8. *Journal of Molecular Biology* **394**(5) (2009) 852-863.
82. P. Shi, Z. Xi, H. Wang, C. Shi, Y. Xiong, C. Tian. Site-specific Protein Backbone and Side-chain NMR Chemical Shift and Relaxation Analysis of Human Vinexin SH3 Domain Using a Genetically Encoded $^{15}\text{N}/^{19}\text{F}$ -labeled Unnatural Amino Acid. *Biochemical Biophysical Research Communications* **402**(3) (2010) 461-466.

83. M K. Fenwick, R.E. Oswald. NMR Spectroscopy of the Ligand-binding Core of Ionotropic Glutamate Receptor 2 Bound to 5-substituted Willardiine Partial Agonists. *Journal of Molecular Biology* **378**(3) (2008) 673-685.
84. G. Bai, H. Mo, M. Shapiro. NMR Evaluation of Adipocyte Fatty Acid Binding Protein (aP2) with *R*- and *S*-ibuprofen. *Bioorganic & Medicinal Chemistry* **16**(8) (2008) 4323-4330.
85. J. Stark, R. Powers. Rapid Protein-ligand Costructures Using Chemical Shift Perturbations. *Journal of American Chemical Society* **130**(2) (2008) 535-545.
86. M. Martín-Pastor, A. De Capua, C.J.P. Álvarez, M.D. Díaz-Hernández, J. Jiménez-Barbero, F.F. Casanueva, Y. Pazos. Interaction between Ghrelin and the Ghrelin Receptor (GHS-R1a), a NMR Study Using Living Cells. *Bioorganic & Medicinal Chemistry* **18**(4) (2010) 1583-1590.
87. S. Albright, P. Agrawal, N.U. Jain. NMR Spectral Mapping of Lipid A Molecular Patterns Affected by Interaction with the Innate Immune Receptor CD14. *Biochemical Biophysical Research Communications* **378**(4) (2009) 721-726.
88. L. Krabben, B.J. van Rossum, S. Jehle, E. Bocharov, E.N. Lyukmanova, A.A. Schulga, A. Arseniev, F. Hucho, H. Oschkinat. Loop 3 of Short Neurotoxin II is an Additional Interaction Site with Membrane-bound Nicotinic Acetylcholine Receptor as Detected by Solid-state NMR Spectroscopy. *Journal of Molecular Biology* **390**(4) (2009) 662-671.
89. Y. Xu, J. Lorieau, A. E. McDermott. Triosephosphate Isomerase: ^{15}N and ^{13}C Chemical Shift Assignments and Conformational Change upon Ligand Binding by Magic-angle Spinning Solid-state NMR Spectroscopy. *Journal of Molecular Biology* **397**(1) (2010) 233-248.
90. K. Tanaka, A.V. Struts, S. Krane, N. Fujioka, G.F.J. Salgado, K. Martinez-Mayorga, M.F. Brown, K. Nakanishi. Synthesis of CD_3 -Labeled 11-*cis*-Retinals and Application to Solid-State Deuterium NMR Spectroscopy of Rhodopsin. *Bulletin of the Chemical Society of Japan* **80**(11) (2007) 2177-2184.
91. C.M. Barbieri, D.S. Pilch. Complete Thermodynamic Characterization of the Multiple Protonation Equilibria of the Aminoglycoside Antibiotic Paromomycin: a Calorimetric and Natural Abundance ^{15}N NMR Study. *Biophysical Journal* **90**(4) (2006) 1338-1349.
92. H.W. Huang, S.K. Mohan, C. Yu. The NMR Solution Structure of Human Epidermal Growth Factor (hEGF) at Physiological pH and its Interactions with Suramin. *Biochemical Biophysical Research Communications* **402**(4) (2010) 705-710.
93. C.D. Amero, D.W. Byerly, C.A. McElroy, A. Simmons, M.P. Foster. Ligand-induced Changes in the Structure and Dynamics of *Escherichia coli* Peptide Deformylase. *Biochemistry* **48**(32) (2009) 7595-7607.
94. K.T. Welch, K.G. Virga, N.A. Whittemore, C. Özen, E. Wright, C.L. Brown, R.E. Lee, E.H. Serpersu. Discovery of Non-carbohydrate Inhibitors of Aminoglycoside-modifying Enzymes. *Bioorganic & Medicinal Chemistry* **13**(22) (2005) 6252-6263.
95. M. John, G. Pintacuda, A.Y. Park, N.E. Dixon, G. Otting. Structure Determination of Protein-ligand Complexes by Transferred Paramagnetic Shifts. *Journal of American Chemical Society* **128**(39) (2006) 12910-12916.
96. U. Rothweiler, A. Czarna, M. Krajewski, J. Ciombor, C. Kalinski, V. Khazak, G. Ross, N. Skobeleva, L. Weber, T.A. Hodak. Isoquinolin-1-one Inhibitors of the MDM2-p53 Interaction. *ChemMedChem* **3**(7) (2008) 1118-1128.

97. S. Jin, M.Y. Li, C.Y. Zhu, V. Tran, B.H. Wang. Computer-based *de novo* Design, Synthesis, and Evaluation of Boronic Acid-based Artificial Receptors for Selective Recognition of Dopamine. *ChemBioChem* **9**(9) (2008) 1431-1438.
98. M. Hohwy, L. Spadola, B. Lundquist, P. Hawtin, J. Dahmen, I. Groth-Clausen, E. Nilsson, S. Persdotter, K. von Wachenfeldt, R.H.A. Folmer, K. Edman. Novel Prostaglandin D Synthase Inhibitors Generated by Fragment-based Drug Design. *Journal of Medicinal Chemistry* **51**(7) (2008) 2178-2186.
99. L.A. Alcaraz, L. Banci, I. Bertini, F. Cantini, A. Donaire, L. Gonnelli. Matrix Metalloproteinase-inhibitor Interaction: the Solution Structure of the Catalytic Domain of Human Matrix Metalloproteinase-3 with Different Inhibitors. *Journal of Biological Inorganic Chemistry* **12**(8) (2007) 1197-1206.
100. S. Murail, J.C. Robert, Y.M. Coïc, J.M. Neumann, M.A. Ostuni, Z.X. Yao, V. Papadopoulos, N. Jamin, J.J. Lacapère. Secondary and Tertiary Structures of the Transmembrane Domains of the Translocator Protein TSPO Determined by NMR. Stabilization of the TSPO Tertiary Fold Upon Ligand Binding. *Biochimica et Biophysica Acta - Biomembranes* **1778**(6) (2008) 1375-1381.
101. Y.S. Tsantrizos. The Design of a Potent Inhibitor of the Hepatitis C Virus NS3 Protease: BILN 2061 - From the NMR Tube to the Clinic. *Biopolymers* **76**(4) (2004) 309-323.
102. S. Di Micco, C. Bassarello, G. Bifulco, R. Riccio, L. Gomez-Paloma. Differential-Frequency Saturation Transfer Difference NMR Spectroscopy Allows the Detection of Different Ligand-DNA Binding Modes. *Angewandte Chemie International Edition* **45**(2) (2006) 224-228.
103. T.C. Ramalho, T.C.C. França, W.A. Cortopassi, A.S. Gonçalves, A.W.S. da Silva, E.F.F. da Cunha. Topology and Dynamics of the Interaction Between 5-nitroimidazole Radiosensitizers and Duplex DNA Studied by a Combination of Docking, Molecular Dynamic Simulations and NMR Spectroscopy. *Journal of Molecular Structure* **992**(1) (2011) 65-71.
104. C. Kreutz, H. Kählig, R. Konrat, R. Micura. A General Approach for the Identification of Site-specific RNA Binders by ¹⁹F NMR Spectroscopy: Proof of Concept. *Angewandte Chemie International Edition* **45**(21) (2006) 3450-3453.
105. N. Follpe, I.J. Chen, B. Davis, A. Hold, D. Morley, R. Howes. A Structure-Based Strategy to Identify New Molecular Scaffolds Targeting the Bacterial Ribosomal A-site. *Bioorganic & Medicinal Chemistry* **12**(5) (2004) 935-947.
106. M.N. Melo, F.J.R. Sousa, F.A. Carneiro, M.A.R.B. Castanho, A.P. Valente, F.C.L. Almeida, A.T. Da Poian, R. Mohana-Borges. Interaction of the Dengue Virus Fusion Peptide with Membranes Assessed by NMR: The Essential Role of the Envelope Protein Trp101 for Membrane Fusion. *Journal of Molecular Biology* **392**(3) (2009) 736-746.
107. S. Follot, J.C. Debouzy, D. Crouzier, C. Enguehard-Gueiffier, A. Gueiffier, F. Nachon, B. Lefebvre, F. Fauvelle. Physicochemical Properties and Membrane Interactions of Anti-apoptotic Derivatives 2-(4-fluorophenyl)-3-(pyridin-4-yl)imidazo[1,2-a]pyridine Depending on the Hydroxyalkylamino Side Chain Length and Conformation: an NMR and ESR Study. *European Journal of Medicinal Chemistry* **44**(9) (2009) 3509-3518.

108. L.F. Cabeça, M. Pickholz, E. De Paula, A.J. Marsaioli. Liposome-prilocaine Interaction Mapping Evaluated Through STD-NMR and Molecular Dynamics Simulations. *The Journal of Physical Chemistry B* **113**(8) (2009) 2365-2370.
109. L. Fielding. NMR methods for the Determination of Protein–ligand Dissociation Constants. *Progress in Nuclear Magnetic Resonance Spectroscopy* **51**(4) (2007) 219-242.
110. I.R. Kleckner, M.P. Foster. An Introduction to NMR-based Approaches for Measuring Protein Dynamics. *Biochimica et Biophysica Acta –Proteins & Proteomics* **1814**(8) (2011) 942-968.
111. T. Madl, F. Gabel, M. Sattler. NMR and Small-angle Scattering-based Structural Analysis of Protein Complexes in Solution. *Journal of Structural Biology* **173**(3) (2011) 472-482.
112. S. Reckel, R. Hänsel, F. Löhr, V. Dötsch. In-cell NMR Spectroscopy. *Progress in Nuclear Magnetic Resonance Spectroscopy* **51**(2) (2007) 91-101.
113. S. Grzesiek, H.J. Sass. From Biomolecular Structure to Functional Understanding: New NMR Developments Narrow the Gap. *Current Opinion in Structural Biology* **19**(5) (2009) 585-595.
114. J. Blobel, R. Fayos, J. García, O. Marimon, Y. Pérez, M. Pons. Low-molecular-weight Spies of Protein–protein Interactions. *Comptes Rendus Chimie* **11**(2008) 499-505.
115. M. von Itzstein. Disease-associated Carbohydrate-recognising Proteins and Structure-based Inhibitor Design. *Current Opinion in Structural Biology* **18**(5) (2008) 558-566.
116. M.R. Krishna, V. Jayalakshmi. Complete Relaxation and Conformational Exchange Matrix Analysis of STD-NMR Spectra of Ligand-receptor Complexes. *Progress in Nuclear Magnetic Resonance Spectroscopy* **49**(1) (2006) 1-25.
117. J.A. Goncalves, S. Ahuja, S. Erfani, M. Eilers, S.O. Smith. Structure and Function of G Protein-coupled Receptors Using NMR Spectroscopy. *Progress in Nuclear Magnetic Resonance Spectroscopy* **57**(2) (2010) 159-180.
118. R. Law, O. Barker, J.J. Barker, T. Hestekamp, R. Godemann, O. Andersen, T. Fryatt, S. Courtney, D. Hallett, M. Whittaker. The Multiple Roles of Computational Chemistry in Fragment-based Drug Design. *Journal of Computer-Aided Molecular Design* **23**(8) (2009) 459-473.
119. P. Schanda. Fast-pulsing Longitudinal Relaxation Optimized Techniques: Enriching the Toolbox of Fast Biomolecular NMR Spectroscopy. *Progress in Nuclear Magnetic Resonance Spectroscopy* **55**(3) (2009) 238-265.
120. B.R. Donald, J. Martin. Automated NMR Assignment and Protein Structure Determination using Sparse Dipolar Coupling Constraints. *Progress in Nuclear Magnetic Resonance Spectroscopy* **55**(2) (2009) 101-127.
121. A.R. Modarresi-Alam, F. Khamooshi, M. Rostamizadeh, H. Keykha, M. Nasrollahzadeh, H.R. Bijanzadeh, E. Kleinpeter. Dynamic ^1H NMR Spectroscopic Study of the Restricted SN Rotation in Aryl-N-(arylsulfonyl)-N-(triphenylphosphoranylidene)imidocarbamates. *Journal of Molecular Structure* **841**(1) (2007) 61-66.
122. P. Seneci, A. Bianchi, C. Battaglia, L. Belvisi, M. Bolognesi, A. Caprini, F. Cossu, E. de Franco, M. de Matteo, D. Delia, C. Drago, A. Khaled, D. Lecis, L. Manzoni, M. Marizzoni, E. Mastrangelo, M. Milani, I. Motto, E. Moroni, D. Potenza, V. Rizzo, F.

- Servida, E. Turlizzi, M. Varrone, F. Vasile, C. Scolastico. Rational Design, Synthesis and Characterization of Potent, Non-peptidic Smac Mimics/XIAP Inhibitors as Proapoptotic Agents for Cancer Therapy. *Bioorganic & Medicinal Chemistry* **17**(16) (2009) 5834-5856.
123. F.C. Fuchs, G.A. Eller, W. Holzer. Heterocyclic Analogs of Thioflavones: Synthesis and NMR Spectroscopic Investigations. *Molecules* **14**(9) (2009) 3814-3832
 124. T. Zhuang, H.S. Lee, B. Imperiali, J.H. Prestegard. Structure Determination of a Galectin-3-carbohydrate Complex Using Paramagnetism-based NMR Constraints. *Protein Science* **17**(7) (2008) 1220-1231.
 125. S. Tiziani, S.J. Schwartz, Y. Vodovotz. Intermolecular Interactions in Phytochemical Model Systems Studied by NMR Diffusion Measurements. *Food Chemistry* **107**(2) (2008) 962-969.
 126. M.H. Arroyo, K.H. Mayo. NMR Solution Structure of the Angiostatic Peptide Angi-nex. *Biochimica et Biophysica Acta –Proteins & Proteomics* **1774** (2007) 645-651.
 127. N.L. Daly, K.J. Rosengren, D.J. Craik. Discovery, Structure and Biological Activities of Cyclotides. *Advanced Drug Delivery Reviews* **61**(11) (2009) 918-930.
 128. J. Romanuka, H. van den Bulke, R. Kaptein, R. Boelens, G.E. Folkers. Novel Strategies to Overcome Expression Problems Encountered with Toxic Proteins: Application to the Production of Lac Repressor Proteins for NMR Studies. *Protein Expression Purification* **67**(2) (2009) 104-112.
 129. S. Yao, J.J. Babon, R.S. Norton. Protein Effective Rotational Correlation Times from Translational Self-diffusion Coefficients Measured by PFG-NMR. *Biophysical Chemistry* **136**(2) (2008) 145-151.
 130. Y. Kodama, M.L. Reese, N. Shimba, K. Ono, E. Kanamori, V. Dötsch, S. Noguchi, Y. Fukunishi, E. Suzuki, I. Shimada, H. Takahashi. Rapid Identification of Protein-protein Interfaces for the Construction of a Complex Model Based on Multiple Unassigned Signals by Using Time-sharing NMR Measurements. *Journal of Structural Biology* **174**(3) (2011) 434-442.
 131. K. Sakurai, T. Konuma, M. Yagi, Y. Goto. Structural Dynamics and Folding of β -lactoglobulin Probed by Heteronuclear NMR. *Biochimica et Biophysica Acta – General Subjects* **1790**(6) (2009) 527-537.
 132. G.W. Buchko, B.J. Tarasevich, J. Roberts, M.L. Snead, W.J. Shaw. A Solution NMR Investigation into the Murine Amelogenin Splice-variant LRAP (Leucine-Rich Amelogenin Protein). *Biochimica et Biophysica Acta –Proteins & Proteomics* **1804**(9) (2010) 1768-1774.
 133. J. Moriya, M. Sakakura, Y. Tokunaga, R.S. Prosser, I. Shimada. An NMR Method for the Determination of Protein Binding Interfaces Using TEMPOL-Induced Chemical Shift Perturbations. *Biochimica et Biophysica Acta –General Subjects* **1790**(10) (2009) 1368-1376.
 134. W. Schönfeld, S. Mutak. Azithromycin and novel azalides. In: W. Schönfeld and H. A. Kirst (Eds.), *Macrolide Antibiotics*, Birkhäuser Verlag, Basel, 2002, pp. 73-95.
 135. S. Pal. A Journey Across the Sequential Development of Macrolides and Ketolides Related to Erythromycin. *Tetrahedron* **62**(14) (2006) 3171-3200.
 136. S. Mutak. Azalides from Azithromycin to New Azalide Derivatives. *Journal of Antibiotics* **60**(2) (2007) 85-122.

137. J. Poehlsgaard, S. Douthwaite. Macrolide Antibiotic Interaction and Resistance on the Bacterial Ribosome. *Current Opinion in Investigational Drugs* **4**(2) (2003) 140-148.
138. A.S. Mankin. Macrolide Myths. *Current Opinion in Microbiology* **11** (2008) 414-421.
139. N. Ban, P. Nissen, J. Hansen, P.B. Moore, T.A. Steitz. The Complete Atomic Structure of the Large Ribosomal Subunit at 2.4 Å Resolution. *Science* **289**(5481) (2000) 905-920.
140. F. Schlünzen, R. Zarivach, J. Harms, A. Bashan, A. Tocilj, R. Albrecht, A. Yonath, F. Franceschi. Structural Basis for the Interaction of Antibiotics with the Peptidyl Transferase Centre in Eubacteria. *Nature* **413**(6858) (2001) 814-821.
141. J.L. Hansen, J.A. Ippolito, N. Ban, P. Nissen, P.B. Moore, T.A. Steitz. The Structures of Four Macrolide Antibiotics Bound to the Large Ribosomal Subunit. *Molecular Cell* **10**(1) (2002) 117-128.
142. D. Tu, G. Blaha, P.B. Moore, T.A. Steitz. Structures of MLS_BK Antibiotics Bound to Mutated Large Ribosomal Subunits Provide a Structural Explanation for Resistance. *Cell* **121**(2) (2005) 257-270.
143. F. Schlünzen, J.M. Harms, F. Franceschi, H.A.S. Hansen, H. Bartels, R. Zarivach, A. Yonath. Structural Basis for the Antibiotic Activity of Ketolides and Azalides. *Structure* **11**(3) (2003) 329-338.
144. J.A. Dunkle, L. Xiong, A.S. Mankin, J.H.D. Cate. Structures of the *Escherichia coli* Ribosome with Antibiotics Bound near the Peptidyl Transferase Center Explain Spectra of Drug Action. *Proceedings of the National Academy of Science of the United States of America* **107**(40) (2010) 17152-17157.
145. M.A. Johnson, B.M. Pinto. Saturation-transfer Difference NMR Studies for the Epitope Mapping of a Carbohydrate-mimetic Peptide Recognized by an Anti-carbohydrate Antibody. *Bioorganic & Medicinal Chemistry* **12**(1) (2004) 295-300.
146. P. Novak, I. Tatić, P. Tepeš, S. Koštrun, J. Barber. Systematic Approach to Understanding Macrolide-ribosome Interactions: NMR and Modeling Studies of Oleandomycin and its Derivatives. *The Journal of Physical Chemistry A* **110**(2) (2006) 572-579.
147. P. Novak, Z. Banić Tomišić, P. Tepeš, G. Lazarevski, J. Plavec, G. Turkalj. Conformational Analysis of Oleandomycin and its 8-methylene-9-oxime Derivative by NMR and Molecular Modeling. *Organic & Biomolecular Chemistry* **3**(1) (2005) 39-47.
148. P. Novak, J. Barber, A. Čikoš, B. Arsić, J. Plavec, G. Lazarevski, P. Tepeš, N. Košutić-Hulita. Free and Bound state Structures of 6-*O*-methyl Homoerythromycins and Epitope Mapping of their Interactions with Ribosomes. *Bioorganic & Medicinal Chemistry* **17**(16) (2009) 5857-5867.
149. N. Košutić-Hulita, D. Matak-Vinković, M. Vinković, P. Novak, G. Kobrehel, G. Lazarevski. Conformational Behaviour of 11-*O*-methylazithromycin in the Solid and Solution State. *Croatica Chemica Acta* **74**(2) (2001) 327-341.
150. M.B. Krajačić, M. Dumić, P. Novak, M. Cindrić, S. Koštrun, A. Fajdetić, S. Alihodžić, K. Brajša, N. Kujundžić. Discovery of Novel Ureas and Thioureas of 3-decladinosyl-3-hydroxy 15-membered Azalides Active Against Efflux-Mediated Resistant *Streptococcus pneumoniae*. *Bioorganic Medicinal Chemistry Letters* **21**(2) (2011) 853-856.
151. J.R. Everett, J.W. Tyler. The Conformational Analysis of Erythromycin A. *Journal of Chemical Society Perkin Transactions* **2**(11) (1987) 1659-1667.

152. W.E. Steinmetz, B.L. Shapiro, J.J. Roberts. The Structure of Erythromycin Enol Ether as a Model for its Activity as a Motilide. *Journal of Medicinal Chemistry* **45**(22) (2002) 4899-4902.
153. G. Lazarevski, M. Vinković, G. Kobrehel, S. Đokić, B. Metelko, D. Vikić-Topić. Conformational Analysis of Azithromycin by Nuclear Magnetic Resonance Spectroscopy and Molecular Modeling. *Tetrahedron* **49**(3) (1993) 721-730.
154. A. Awan, R.J. Brennan, A.C. Regan, J. Barber. The Conformations of the Macrolide Antibiotics Erythromycin A, Azithromycin and Clarithromycin in Aqueous Solution: a ^1H NMR Study. *Journal of Chemical Society Perkin Transactions* **2**(8) (2000) 1645-1652.
155. G. Bertho, J. Gharby-Benarous, M. Delaforge, C. Lang, A. Parent, J.P. Girault. Conformational Analysis of Ketolide, Conformations of RU 004 in Solution and Bound to Bacterial Ribosomes. *Journal of Medicinal Chemistry* **41**(18) (1998) 3373-3386.
156. S. Kosol, E. Schrank, M. Bukvić-Krajačić, G. Wagner, H. Meyer, C. Göbl, K. Zangger, P. Novak. Probing the Interactions of Macrolide Antibiotics with Membrane-Mimetics by NMR Spectroscopy. *Journal of Medicinal Chemistry*, doi:10.1021/jm300647f
157. P. Novak, P. Tepeš, V. Lazić. Epitope Mapping of Macrolide Antibiotics to Bovine Serum Albumin by Saturation Transfer Difference NMR Spectroscopy. *Croatica Chemica Acta* **80**(2) (2007) 211-216.

CHIRAL SEPARATION FOR ENANTIOMERIC DETERMINATION IN THE PHARMACEUTICAL INDUSTRY

Nelu Grinberg, Su Pan

Contents

6.1. INTRODUCTION	235
6.2. ENANTIOMERS, DIASTEREOMERS, RACEMATES	236
6.3. REQUIREMENTS FOR CHIRAL SEPARATION	237
6.4. THE TYPES OF MOLECULAR INTERACTIONS.....	237
6.4.1. Chiral separation through hydrogen bonding.....	237
6.4.2. Chiral separation through inclusion compounds.....	243
6.4.2.1. Cyclodextrins	243
6.4.2.2. Crown ethers.....	245
6.4.3. Charge transfer.....	259
6.4.4. Chiral separation through a combination of charge transfer, hydrogen bonding and electrostatic interactions.....	260

6.4.5. Ligand exchange	270
6.4.6. Mixed types of interactions	273
6.4.6.1. Polysaccharide phases	273
6.4.6.2. Antibiotic Phases	309
6.4.6.3. Protein Phases	314
6.5. CONCLUSION	319
REFERENCES.....	319

6.1. INTRODUCTION

Chirality plays a major role in biological processes. Enantiomers of a bioactive molecule often have different biological effects, as their pharmacological activity may reside in only one enantiomer of a racemic mixture. In some cases, enantiomers may have qualitatively similar pharmacological activity, but different quantitative potencies, while in others one enantiomer can be potentially toxic. Since drugs that are produced by chemical synthesis are usually a mixture of enantiomers, there is a need to quantify the level of enantiomeric impurity in an active pharmaceutical ingredient (API) [1].

The history of enantiomeric separation starts with the work of Pasteur. He discovered in 1848 that the spontaneous resolution of racemic ammonium sodium tartrate yielded two enantiomorphous crystals. Individual solutions of these enantiomorphous crystals led to *levo* and *dextro* rotation of polarised light. Because the difference in optical rotation was observed in solution, Pasteur suggested that, like two sets of crystals, the molecules are mirror images of each other and the phenomenon is due to molecular asymmetry [2].

While Pasteur made the historical discovery, subsequent advances in the resolution of enantiomers by crystallisation were based on empirical results. Several attempts to separate enantiomers using paper chromatography were met with unreliable results. In 1952, Dalgliesh postulated that three points of simultaneous interaction between the enantiomeric analyte and the stationary phase are required for the separation of enantiomers [3].

Developments in the field of life sciences and in the pharmaceutical industry brought enantiomeric separation to a new level. In the late 1950s/early 1960s, many drugs were synthesised and used in a racemic form. An example with tragic consequences is the use of thalidomide, a sedative and sleep-promoting drug used in the early 1960s which produced severe malformations in the newborn babies of women who took it in the early stage of pregnancy. Later, it was demonstrated that only the (*S*)-enantiomer possesses teratogenic properties [4].

The introduction of gas chromatography provided a stimulus to the field of enantiomeric separation. In 1966, a group from the Weizmann Institute of Science in Israel reported the first successful separation of enantiomers using gas chromatography (GC).

In a letter addressed to Emanuel Gil-Av after the publication of the first separation of enantiomers by a chiral GC stationary phase [5], A. J. P. Martin wrote: *"As you no doubt know, I had not expected such attempts to lead to much success, believing that the substrate-solvent association would normally be too loose to distinguish between the enantiomers."* At the time, there were only a few reports on the separation of enantiomers using chromatographic methods. Later developments in high performance liquid chromatography (HPLC) gave an additional boost to the field. Today, there are over 60 types of rugged, well-characterised columns capable of separating enantiomers. Unfortunately, there is a great deal of trial and error involved in choosing a particular column for chiral separation.

Chiral separation plays an important role in the determination of enantiomeric excess of API. The present chapter will present some of the rationale behind the interactions between enantiomeric solutes and a particular stationary phase along with some applications pertinent to each kind of interaction.

6.2. ENANTIOMERS, DIASTEREOMERS, RACEMATES

Chirality is due to the fact that the stereogenic centre, also called the chiral centre, has four different substitutions. These molecules are called asymmetrical. When a chiral compound is synthesised in an achiral environment, the compound is generated as a 50:50 equimolar mixture of the two enantiomers and is called a racemic mixture. This is because, in an achiral environment, enantiomers are energetically degenerate and interact in an identical way with the environment. Enantiomers can be differentiated from each other only in a chiral environment. Such conditions are provided during chromatographic enantiomeric separation offered by the asymmetric environment of the chiral column [6].

There are two approaches for achieving enantiomeric separation. The first approach involves a chiral mobile phase along with an achiral stationary phase, when a chiral additive is dissolved in the mobile phase and is constantly pumped through an achiral stationary phase. The second approach consists of chiral stationary phases where a chiral compound is immobilised to a solid matrix (*i.e.* silica gel, polymers, *etc.*). The separation of enantiomers using either chiral stationary or mobile phases involves the formation of transient diastereomeric complexes between the enantiomeric analytes and the chiral moiety present in the chromatographic column.

Diastereomers are chiral molecules containing two or more chiral centres with the same chemical composition and connectivity. They differ in stereochemistry about one or more chiral centres. If two stereoisomers are not enantiomers of one another, they can in principle be separated in an achiral environment *i.e.* using a non-chiral stationary phase [6].

6.3. REQUIREMENTS FOR CHIRAL SEPARATION

The chirality of the selector (chiral stationary phase) or the selectand (enantiomeric analytes) can arise from an asymmetric carbon, molecular asymmetry or the helicity of a polymer. Also, the bonds between substituents of the selectand and the selector can involve a single bond, but could also involve multiple bonds or surfaces. Such bonds represent the leading interactions between the selectand and selector. In chromatographic chiral separation, they control the retention on a certain column. Only when the leading interactions take place and the asymmetric moiety of the two bodies are brought in close proximity do the secondary interactions (*e.g.* van der Waals, steric hindrance, dipole-dipole) become effectively involved. Secondary interactions can affect the conformation and the formation energy of diastereomeric associates [7].

The interaction energy of one enantiomer in the active chiral phase can be described as the contribution of the interaction energies of all possible forms of chiral phase/solute associates. These associates are in equilibrium with fast interconversion rates. Each form contributes to the total free energy according to its particular formation energy and its particular molar fraction [7,8]. These complexes between the selector and selectand should also be as mutually exclusive as possible, to prevent a given interaction from occurring at multiple sites in the diastereomeric complex [6].

6.4. THE TYPES OF MOLECULAR INTERACTIONS

Chiral separations generally rely on the formation of transient diastereomeric complexes with differing stabilities. Complexes are defined as two or more compounds bound to one another in a definite structural relationship by forces such as hydrogen bonding, ion pairing, metal ion-to-ligand attraction, π -acid/ π -base interactions, van der Waals attraction and entropic component of desolvation. In the following sections, the most important types of molecular interactions in chiral separation are discussed.

6.4.1. Chiral separation through hydrogen bonding

Hydrogen bonding is a donor-acceptor interaction specifically involving hydrogen atoms [9]. When a covalently bonded hydrogen atom forms a second bond to another atom, the second bond is referred to as a hydrogen bond.

A hydrogen bond is formed by the interaction between the partners R-X-H and :Y-R' according to:

where R-X-H is the proton donor and :Y-R' makes an electron pair available for the bridging bond. The strength of hydrogen bonds depends on the solvent

conditions in which the complex occurs. For instance, in the presence of an ionic medium (which generates an electrical field), the H-bonds of the solvate become polarised and, consequently, their symmetry can change from a symmetrical to an asymmetrical H-bond. The change in symmetry leads to weakening of the H-bonds between solvate molecules [10].

Amide groups and peptide bonds are the most important functional groups involved in designing chiral phases that involve hydrogen bonding. Two configurations of the planar peptide bond are possible; they can be in either in a *trans* or *cis* configuration, which are in equilibrium (Figure 6.1). The *trans* form is energetically favoured, due to less repulsion between non-bonded atoms [11].

Figure 6.1. Configurational equilibrium of amide group

The amide group is also an integral functional group of the backbone of proteins and determines the secondary structure of α -helix and β -sheet. Preservation of the energy balance in the system necessitates that the backbone polypeptide groups form hydrogen bonded interactions between themselves in the protein folded state. Such requirement has important consequences for protein folding because the peptide carbonyl oxygen and N-H groups are regularly arranged along the polypeptide backbone. Extended arrangements having optimum hydrogen-bonded interactions among residues either within or between polypeptide chains reflect the periodic regularity and result in the formation of protein secondary structures [12]. In α -helix, the polypeptide backbone follows a path of a rigid right handed helical spring to form an arrangement in which each carbonyl group forms a hydrogen bond with the amide NH group.

β -sheets are formed when two or more almost fully extended polypeptide chains are brought together side by side, so that regular hydrogen bonds can form between the peptide amide NH and carbonyl oxygen groups of the adjacent chains. β -sheets can occur in two different arrangements: parallel β -sheet and antiparallel β -sheet (Figure 6.2).

In the parallel arrangement, the two adjacent polypeptide chains can close through hydrogen bonds a C5:C7 ring while in the antiparallel they can close both C5:C5 and a C7:C7 ring (*vide infra*).

Figure 6.2. Parallel (a) and antiparallel (b) β -sheets

The first successful chiral phases used under GC conditions were N-trifluoroacetyl (TFA)-L- α -amino acid esters. These phases separated racemates of the more volatile members of the same compounds [12]. The diamide stationary phase contained two hydrogen bonding sites, a C5 and a C7 site, where hydrogen bonding selector/selectand-associations could take place [7]:

The structure of the diamide phase appeared to be similar to an anti-parallel β -sheet of poly-L-alanine. X-ray diffraction of the D,L-leucyl derivative showed the C₅:C₅ association, while the C₇ site involved three molecules in the anti-parallel arrangement, closing a C₅:C₅ associate of the L-diamide selector with L- and D- α -amino acid derivatives [5].

Derivatised amino acids such as N-TFA- α -amino acid esters, which can form hydrogen bonds at these sites, can be separated successfully on such types of chiral stationary phases (CSP). Such a chiral phase is known under the trade name Chirasil-Val® and is used for the separation of enantiomers by gas chromatography [13,14]. Table 6.1 lists some chiral compounds successfully separated on Chirasil-Val® column.

Table 6.1. Names and structures of compounds separated on Chiralsil-Val® chiral stationary phase (CSP) consisting of embedded poly(dimethylsiloxane) with valine-*tert*-butylamide

Name	Structure	Conditions	Ref.
N,O-HFB-tranylcypromine		GC - 140°C	[18]
3-Dechloroethyl ifosfamide		GC - 195°C	[19]
N-Acetyl-4-chlorophenyl-alanine methyl ester		GC - 160°C	[20]
Di-N,O-ethoxycarbonyl serine ethyl ester		GC - 150°C	[21]
Di-N,O-PFP- <i>allo</i> -threonine isopropyl ester		GC - Temperature programming: 65°C, 4°C/min, 210°C.	[22]
N-TFA-2-Aminoheptane		GC - 100°C.	[23]

Name	Structure	Conditions	Ref.
N-TFA-Aspartic acid di-n-propyl ester		GC - Temperature program 80°C for 3 min, followed by 4°C/min to 200°C	[24]

Similar interactions occur in HPLC under normal phase chromatography conditions using hexane with polar modifiers such as alcohols [15]. Another type of chiral phase based on hydrogen bonding interactions is the poly(acrylamide)-type phase. Developed by Blaschke, the phase is comprised of poly(acrylamide) that incorporates phenylalanine ethyl ester. The phase has a helical structure, and the interactions are based on hydrogen bonding between the polar groups of the enantiomer and the CO-NH groups of the polymer [16,17]. The compounds separated on poly(acrylamide) type HPLC columns are listed in Table 6.2.

Table 6.2. The HPLC conditions and mobile phase used for the separation of listed compounds on poly(acrylamide) chiral stationary phase: Chiraspher®

Name	Structure	Mobile Phase	Ref.
2-Phenyl-2,3-dihydro-benzofuran-3-carboxylic acid methyl ester		Chiraspher-NT 100 % Heptane	[25]
[1,1']Binaphthalenyl-2,2'-diol		90:10 CO ₂ / 2-propanol (SFC*)	[26]
4-Hydroxy-2,5-dimethyl-furan-3-one		7:3 pentane / (C ₂ H ₅) ₂ O	[27]

Name	Structure	Mobile Phase	Ref.
8-Chloro-2-(2,6-difluoro-benzyl)-3-methyl-1,1-dioxo-1,2,3,5-tetrahydro-1lambda*6*-benzo[f][1,2,5]thiadiazepin-4-one		50:50:2 hexane / CHCl ₃ / 2-propanol	[28]
8-Chloro-3-methyl-2-(2-methyl-allyl)-1,1-dioxo-1,2,3,5-tetrahydro-1lambda*6*-benzo[f][1,2,5]thiadiazepin-4-thione		80:20 hexane / 2-propanol	[29]
5-(2-Dimethylamino-ethoxy)-3,9-dimethoxy-7H-benzo[c]fluoren-7-ol		75:25 hexane / ethanol	[30]
Bunitrolol		60:20:20:0.05 hexane / ethanol / methanol / (25%aq) NH ₃	[31]
Cytosoxone		95:5 hexane / 2-propanol	[32]
3-Methyl-3,4-dihydro-2H-benzo[1,2,4]thiadiazine 1,1-dioxide		70:30 hexane / tetrahydrofuran	[33]
2,3,3a,4-Tetrahydro-1H-pyrrolo[2,1-c][1,2,4]benzo-thiadiazine 5,5-dioxide		70:30 hexane / tetrahydrofuran	[34]
3-Methyl-3,4-dihydro-2H-1,2,4-benzothiadiazine 1,1-dioxide		60:40 hexane / tetrahydrofuran	[35]
2,3,3a,4-Tetrahydro-1H-pyrrolo[2,1-c][1,2,4]benzo-thiadiazine 5,5-dioxide		70:30 hexane / tetrahydrofuran	[35]

*SFC – supercritical fluid chromatography;

6.4.2. Chiral separation through inclusion compounds

Inclusion complexing partners are classified as hosts and guests. Complexes are structured by contacts at multiple binding sites between hosts and guests. The binding energy at a single contact site is at most a few kilocalories per mole. Contacts at several sites between hosts and guests depends on the complementary placement of the binding sites; specifically, hosts must have binding sites which cooperatively contact and attract the binding sites of guests without generating non-bonded repulsion [36]. There are two types of hosts which have been successfully employed in the chromatographic separation of enantiomers: hosts which have a hydrophobic interior and hosts with a hydrophilic interior. The hydrophilic interior means that the cavity contains heteroatoms such as oxygen, where lone pair electrons are able to participate in bonding to electron acceptors such as an organic cation (e.g., chiral crown ethers). In contrast, a host with a hydrophobic interior cavity is able to include hydrocarbon-rich parts of a molecule [37]. This type of host is found in the cyclodextrins.

6.4.2.1. Cyclodextrins

The most popular are α -, β - and γ -cyclodextrin (α -CD, β -CD and γ -CD, respectively) with six, seven and eight glucose rings respectively, also called cyclohexa-, cyclohepta- and cyclooctaamylose (or CA6, CA7 and CA8). Structures, such as CA6, CA7 and CA8 have a doughnut shape and are able to host small molecules inside the cavity. Similar to amylose, the glucose units in the CAs are linked by $\alpha(1\rightarrow4)$ bonds that adopt a 4C_1 chair conformation (Figure 6.3). They may be considered as rigid building blocks giving fairly limited conformational freedom of the macrocycle in rotation of the C6-O6 groups and limited rotational movements about the glycosidic link C1(n)-O4(n-1)-C4(n-1). All glucose groups are aligned in *cis* configuration with the secondary O2 and O3 hydroxyls on one side and the primary O6 hydroxyls on the other side. They have the overall shape of a hollow, truncated cone with the wide side occupied by O2 and O3 and the narrow side by O6 [38].

Empirical rules for successful chiral recognition candidates using cyclodextrin selectors have evolved based on extensive chromatographic data. For instance, in the guest molecule, the presence of at least one aromatic ring enhances chiral recognition with β -CD, although two rings appear to be more beneficial, particularly if the chiral centre is positioned between the two rings or between a single aromatic ring and a carbonyl [39]. The enhanced chiral recognition is attributed to increased molecular rigidity [40].

The chromatographic separation of enantiomers using CDs is usually performed using aqueous-organic mobile phases. The apparent pH of these mobile phases must be carefully controlled in order to handle the charge of the enantiomeric analytes. For example, separation of basic enantiomeric analytes [41] cannot be achieved at a pH lower than 5. This is a consequence of the protonation of

nitrogens in the analyte molecules. At higher pH, complete separation can be achieved, indicating that enantiomeric separation requires the nitrogens to be partially deprotonated. Simultaneously, the hydrogen bonding between the β -CD and the analytes occurs through O-H to N.

Figure 6.3. Chemical structure of CA7 (β -CD) where the numbering of glucose unit (1-7) is performed counterclockwise (left). Atom numbering scheme for a glucose unit (right) (Adapted from [38])

The concentration of an organic modifier in a hydroorganic mobile phase also influences retention. For instance, the retention of analytes decreases as the amount of acetonitrile in the hydroorganic mobile phase increases up to a point, after which the retention starts increasing again. Such behaviour may indicate a change in retention interactions with an increased amount of acetonitrile in the mobile phase. No reversal of elution order was observed, indicating that no change in the enantioselective interactions occurred [41].

Polar organic mobile phases, such as mixtures of methanol and acetonitrile with small amounts of acetic acid, as well as triethylamine, can also be effective for the separation of enantiomers mediated by CDs. Under these conditions, the interior of the CD cavity is occupied by acetonitrile. The overwhelming concentration of acetonitrile renders its displacement by enantiomeric analytes basically impossible. Acetonitrile is a polar aprotic solvent, with limited capacity for hydrogen bond formation. As a consequence, analytes are thought to undergo hydrogen bonding under these conditions, with the secondary hydroxyl groups located at the rim of the CDs. The addition of methanol and traces of acetic acid and triethylamine allow solute retention to be modulated through solvent mediation of the hydrogen bond strength [42].

Derivatised CDs have also been used successfully in HPLC. Armstrong et al. [43, 44] synthesised several derivatised β -CDs and used them as chiral stationary phases under normal phase conditions. Under these conditions, inclusion is unlikely. A number of substituted derivatives were prepared including acetic anhydride, (*R*) and (*S*)-1-(1-naphthyl)ethyl isocyanate, 2,6-dimethylphenyl iso-

cyanate and *p*-toluoyl chloride. The presence of aromatic substitution provides possibilities for π - π interaction with the aromatic substituents of the enantiomeric analytes [43,44]. For example, in (*R*)-(-)- or (*S*)-(+)-1-(1-naphthyl)ethyl carbamate of β -CD, the naphthyl ethyl moiety has some π donor character. Incorporation of 3,5-dinitro phenyl substituents on chiral analytes promotes the formation of a π - π complex. At the same time, the carbamate functionality which links the aromatic group to the β -CD produces sites which are able to undergo hydrogen bonding as well as dipole stacking with the enantiomeric analytes.

Table 6.3 gives HPLC conditions for the enantiometric separation of selected substances using cyclodextrins CSP.

6.4.2.2. Crown ethers

Crown ethers can be described as heteroatomic macrocycles with repeating units of $(-X-C_2H_4-)$ where the heteroatom X is usually oxygen, but may also be sulphur or nitrogen. They can also incorporate aromatic moieties which enhance their lipophilicity. Each oxygen atom possesses two unshared electron pairs. All six oxygens of the cyclic ether are turned inward to provide dipole-to-ion attractive interactions between the host and guest. The main source of interaction is ion-dipole attraction between $^+NH\cdots O$ and $^+N\cdots O$. Three hydrogen bonds can be formed between the protonated amine hydrogens and the crown ether oxygens. The ethylene units of the crown ether are turned outward and form a lipophilic barrier around the hydrogens of the hydrophilic protonated amine ion. While the host molecule is roughly planar, and the nitrogen of the guest is situated slightly out of the plane at the apex of a shallow tripod, it may be argued that the association between the crown ether and the ammonium is not really an inclusion complex. The alkyl group attached to the nitrogen extends along the axis perpendicular to the plane of the cyclic ether. The protonated amine can complex at either of the two faces of the cyclic polyether. The counterion, X^- , in a non-polar environment, ion pairs with positive nitrogen atom from the face opposite that occupied by the protonated amine [45]. Cram used this principle to design chiral crown ethers. To achieve enantiomeric separation, Cram introduced additional units such as naphthalene rings into the crown ether structure, which provided additional interactions capable of discriminating between enantiomers. The host containing two chiral elements provided the highest chiral selectivity [46]. Shimbo *et al.* used a chiral 18-crown-6 dynamically coated on a reversed phase stationary phase [47,48]. This crown ether is commercially available under the trade name Crownpak®. This crown ether is able to resolve a large number of enantiomeric amines, amino alcohols and amino acids using reversed phase conditions.

It was found that additives such as perchlorate ion play an important role in chiral separation. In a series of anions such as ClO_4^- , CF_3COO^- , NO_3^- and $H_2PO_4^-$, the retention factor of amino alcohols such as *cis* and *trans* amino indanol (at a constant pH of 2) increases in the order $ClO_4^- > CF_3COO^- > NO_3^- > H_2PO_4^-$. The selectivity factor, however, is not influenced by the nature of the anion [49]. For

more hydrophobic analytes, retention can be modulated by the addition of organic modifiers such as methanol in the mobile phase. However, there is not a linear relationship between the amount of the organic modifier in the mobile phase and the retention factor of the enantiomeric analytes, indicating multiple types of retention interactions [50]. A different type of crown ether used to separate enantiomers is derived from 18-crown-6 tetracarboxylic acid, covalently immobilised on silica gel via the reaction between 18-crown-6 tetracarboxylic acid and amino propyl silica gel [51,52]. NMR spectroscopy of the complex between the 18-crown-6 tetracarboxylic acid and phenylglycine or phenylglycine methyl ester showed for the chiral recognition of the more stable complex the following interactions: 1) three $\text{-NH}\cdots\text{O}$ hydrogen bonds in a tripod arrangement between the polyether oxygens of 18-crown-6-tetra carboxylic acid and the ammonium moiety of the enantiomer; 2) a hydrophobic interaction between the polyether ring of 18-crown-6-crown ether and the phenyl ring of the enantiomer; 3) hydrogen bonding between the carboxylic acid of the crown ether and the carbonyl oxygen of the enantiomer [53]; 4) if the analyte contains an aromatic moiety, in some instances $\text{CH} - \pi$ interactions are possible from the carbon adjacent to the carboxyl group and the aromatic moiety.

Table 6.4 gives the HPLC mobile phase composition for the enantiomeric separation of selected compounds on the CSP containing (+/-)-18-crown-6 ether.

Table 6.3. Compositions of mobile and stationary phases for cyclodextrins-based HPLC enantiomeric separations of selected substances

Name/Structure	Stationary phase	Mobile Phase	Ref.
Isoproterenol	Perphenylcarbamate- β -cyclodextrin covalently bonded to aminopropyl silica gel (Kieselgel 100) via a mono-6-urea linkage (Staudinger reaction)	80:20 1% triethylammonium acetate (pH 4.65) / CH_3CN	[54]

Name/Structure	Stationary phase	Mobile Phase	Ref.
<p>OPA-2-ME-tyrosine</p>	Amino- β -cyclodextrin bonded to silica (unknown spacer)	20:80 methanol / aqueous 1% triethylammonium acetate buffer pH 7.0	[55]
<p>Taxifolin</p>	Permethylated- β -cyclodextrin cross-linked and bonded to aminopropyl silicagel via urea	40:60 methanol / triethylammonium acetate buffer pH 5.0 (prepared with Et ₃ N 1%, pH adjusted with glacial acetic acid)	[56]
<p>Mandelic acid</p>	β -cyclodextrin bonded to silica (Nucleodex beta-OH)	86.4:9.1:4.5 H ₂ O / CH ₃ COOH / CH ₃ CN 0.05 M ammonium acetate (pH 3.0)	[57]
<p>2-(1H-Indol-3-yl)-succinic acid</p>	β -cyclodextrin (<i>S</i>) and (<i>R,S</i>)-hydroxypropyl derivative bonded to silica (Cyclobond RSP)	40:60:2 methanol / H ₂ O / acetic acid	[58]
<p>Oxazepam</p>	β -cyclodextrin (<i>S</i>) and (<i>R,S</i>)-hydroxypropyl derivative bonded to silica (Beta-RSP-2000)	19:8:73 CH ₃ CN / 1% triethylamine acetate buffer (pH 4.5) / H ₂ O	[59]

Name/Structure	Stationary phase	Mobile Phase	Ref.
Propranolol	Perphenylcarbamate-heptakis(6-azido-6-deoxy) β -cyclodextrin cross-linked and immobilised to amino-propyl silicagel via multiple urea linkages (CHIDEX-MKP)	80:20 hexane / 2-propanol	[60]
(2-Nonyloxy-phenyl)-carbamic acid 1-methyl-2-piperidin-1-yl-ethyl ester	γ -cyclodextrin bonded to LiChrospher (ChiraDex-Gamma)	95:5 100 mM (pH 5) triethylammonium acetate / acetonitrile	[61]
Ephedrine	β -cyclodextrin phenylcarbamate bonded silica (undisclosed spacer) (Ultron ES-PhCD)	4:1 20 mM KH_2PO_4 (pH 4.6) / acetonitrile	[62]
N-[2-(7-Fluoro-1,2,3,4-tetrahydro-naphthalen-1-yl)-ethyl]-acetamide	β -cyclodextrin covalently bonded to glycidoxypropyl silica (Cyclobond I 2000)	methanol	[63]

Name/Structure	Stationary phase	Mobile Phase	Ref.
1-(3-Amino-propyl)-1-(4-fluoro-phenyl)-1,3-dihydro-isobenzofuran-5-carbonitrile	Acetylated cyclodextrin bonded to glycidoxypropyl silica (Cyclobond I 2000 Ac)	55:45 methanol / 10mM citrate triethylamine buffer (pH 6.3)	[64]
Acetoxy-phenyl-acetic acid 2,2,2-trifluoro-ethyl ester	Permethylated- β -cyclodextrin covalently bonded to silicagel undisclosed spacer (Sumichiral OA 7500)	80:20 methanol / H ₂ O	[65]
3-Hydroxy-1-[4-(7-nitro-benzo[1,2,5]oxadiazol-4-yl)-piperazin-1-yl]-butan-1-one	β -cyclodextrin phenylcarbamate bonded silica (undisclosed spacer) (Ultron ES-PhCD)	80:20 methanol / H ₂ O	[66]
Tyrosine phenylisocyanate	β -cyclodextrin covalently bonded to glycidoxypropyl silica (Cyclobond I)	490:10:1:3 acetonitrile / methanol / acetic acid / triethylamine	[67]

Name/Structure	Stationary phase	Mobile Phase	Ref.
<p>4-Bromo-phenylethanol</p>	Perphenylcarbamate-heptakis(6-azido-6-deoxy) β -cyclodextrin cross-linked and immobilised to aminopropyl silicagel via multiple urea linkages (CHIDEX-MKP)	2-propanol	[68]
<p>Methadone</p>	β -cyclodextrin (<i>S</i>) and (<i>R,S</i>)-hydroxypropyl derivative bonded to silica (β -RSP-2000)	90:10 1% triethylamine / acetonitrile (pH 3.2 with H_3PO_4)	[69]
<p>N-(3-Chloro-benzoyl)-alanylglycine</p>	(<i>R</i>)-Naphthylethyl-isocyanate derivatised β -cyclodextrin bonded to silica (Cyclobond I 2000 RN)	490:10:1:2 acetonitrile / methanol / acetic acid / triethylamine	[70]
<p>Phenylalanine phenylisocyanate</p>	(<i>R</i>)-Naphthyl-ethylisocyanate derivatised β -cyclodextrin bonded to silica (480:20:1:2 CH_3CN / methanol / $\text{CH}_3\text{CO}_2\text{H}$ / Et_3N)	480:20:1:2 acetonitrile / methanol / acetic acid / triethylamine	[67]
<p>Ala-Phe phenylisocyanate</p>	β -cyclodextrin-(<i>S</i>)-naphthylethyl carbamate derivative (Cyclobond I SN)	475:25:1:3 acetonitrile / methanol / acetic acid / triethylamine	[67]
<p>Baclofen</p>	γ -cyclodextrin bonded to silica (CYCLOBOND II)	90:10 10 mM Ammonium acetate (pH 6.8)	[71]

Name/Structure	Stationary phase	Mobile Phase	Ref.
Benzoic acid 2-(6-amino-purin-9-ylmethoxy)-2-phenyl-ethyl ester	β -cyclodextrin (<i>S</i>) and (<i>R,S</i>)-hydroxypropyl derivative bonded to silica (Cyclobond RSP)	98:2 CH ₃ CN / methanol	[72]
3-Methoxy-6a,11a-dihydro-6H-benzo[4,5]furo[3,2-c]chromene	α -cyclodextrin bonded to silica (CYCLOBOND III)	80:20 H ₂ O / methanol	[73]
Dimethyl-[4-(2-phenyl-4,5,6,7-tetrahydro-benzofuran-4-yl)-phenyl]-amine	β -cyclodextrin (<i>S</i>) and (<i>R,S</i>)-hydroxypropyl derivative bonded to silica (Cyclobond RSP)	60:40:0.1:0.1 CH ₃ OH / H ₂ O / triethylamine / CH ₃ CO ₂ H (pH 8)	[74]
2-Styryl-2,3-dihydro-benzofuran-5-carboxylic acid methyl ester	Heptakis-2,3-O-dimethyl β -Cyclodextrin covalently bonded to glycidox isopropyl silica (Cyclobond I DM)	40:60 methanol / H ₂ O	[75]
2-Ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine	β -cyclodextrin (<i>S</i>) and (<i>R,S</i>)-hydroxypropyl derivative bonded to silica (β -RSP-2000)	19:9:72 CH ₃ CN / 1% triethylammonium acetate (pH 4.5) in H ₂ O / H ₂ O	[76]

Name/Structure	Stationary phase	Mobile Phase	Ref.
8-Iodo-5-methoxy-7-(4-methoxy-phenyl)-5H-pyrano[4,3-b]pyridine	β -cyclodextrin covalently bonded to glycidoxypropyl silica (Cyclobond I)	Not reported	[77]
4-[4-(2-Hydroxy-cyclohexylmethyl)-phenoxy]-3-methyl-but-2-enoic acid ethyl ester	β -cyclodextrin bonded to silica (Nucleodex beta-OH)	4:1 methanol / H ₂ O	[78]
2'-Hydroxyflavanone	Perphenylcarbamate- β -cyclodextrin covalently bonded to aminopropyl silicagel (Kromasil silica) via a mono-6-urea linkage (CHIDEX-SKP)	50:50 H ₂ O / methanol	[79]
Thioridazine	β -cyclodextrin bonded to LiChrospher (ChiraDex)	50:50 Phosphate buffer 0.05M, pH 6.5 / acetonitrile	[80]
Bis(alpha-methylbenzyl)amine	Acetylated cyclodextrin bonded to glycidoxypropyl silica (Cyclobond I 2000 Ac)	100:0.1 H ₂ O / triethylammonium acetate (pH 4.1)	[81]

Name/Structure	Stationary phase	Mobile Phase	Ref.
1,2,5,6,9,10-Hexabromo- cyclododecane	Permethylated-beta- cyclodextrin covalently bonded to silicagel (Nucleodex beta-PM)	Gradient acetonitrile / H ₂ O	[82]
4-[(2-Hydroxymethyl-4- oxo-4,6,7,8-tetrahydro-3H- cyclopenta[g]quinazolin-6- yl)-prop-2-ynyl-amino]- benzoic acid	β -cyclodextrin covalently bonded to glycidoxypropyl silica (Cyclobond I)	25 mM Na ₂ HPO ₄ / 25mM NaH ₂ PO ₄ / acetonitrile	[83]
6-(Azetidin-1-ylcarbonyl)- 5-[(3R)-3-hydroxy-3-(2-me- thylphenyl)propyl]-1,2-di- methyl-1H-benzimidazol-4- ol	β -cyclodextrin bonded to LiChrospher (ChiraDex)	20:80 methanol / H ₂ O	[84]

Name/Structure	Stationary phase	Mobile Phase	Ref.
2-Hydroxy-2-phenyl-acetamide	β -cyclodextrin bonded to silica (Nucleodex beta-OH)	50:50 methanol / H ₂ O	[85]
6-phenyl-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol	2,3-O-((4-Trifluoromethyl-2,6-Dinitrophenyl)- β -cyclodextrin covalently bonded to glycidoxypopyl silica (Cyclobond I 2000 DNP)	90:10 hexane / 2-propanol / triethylamine 0.2%	[86]
8-hydroxy-6-phenyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocine-3(2H)-carboxamide	β -cyclodextrin-3,5-dimethylphenylcarbamate derivative (Cyclobond I 2000 DMP)	90:10 hexane / 2-propanol / triethylamine 0.2%	[86]
3-methyl-6-phenyl-1,2,3,4,5,6-hexahydro-1,5-methano-3-benzazocine	β -cyclodextrin covalently bonded to glycidoxypopyl silica (Cyclobond I 2000)	65:35 ammonium acetate buffer pH 7 / acetonitrile	[86]

Name/Structure	Stationary phase	Mobile Phase	Ref.
1,3-Dibenzyl-tetrahydrofuro[3,4-d]imidazole-2,4-dione	β -cyclodextrin phenyl-carbamate bonded silica (carbamate spacer) (Chiral CD-Ph)	70:30 acetonitrile / H ₂ O	[87]
N-(2,4-Dinitrophenyl)tyrosinol	β -Cyclodextrin bonded to LiChrospher (ChiraDex)	40:60 CH ₃ CN / triethylammonium phosphate buffer 20 mM, pH 7	[88]
Clopidogrel	β -Cyclodextrin bonded to LiChrospher (ChiraDex)	15:5:80 CH ₃ CN / methanol / 0.01 M aqueous KH ₂ PO ₄	[89]
trans-Permethrin	β -Cyclodextrin bonded to LiChrospher (ChiraDex)	Gradient methanol / H ₂ O (56:44 0-16 min, then a linear gradient to 70:30 in 30 min, then 10 min at 56:44)	[90]

Table 6.4. Mobile phase compositions for the enantiomeric separation of selected substances on (+/-)-18-crown-6 ether coated on octadecylsilyl silica(ODS) stationary phase

Name	Structure	Mobile Phase	Ref.
Amino-phenyl-acetic acid methyl ester		Aqueous HClO ₄ (pH 2.0)	[91]
2-Amino-4-phenyl-butamide		HClO ₄ Solution (pH 2)	[92]
3-Amino-3-cyclohex-3-enyl-propionic acid		Aqueous HClO ₄ (pH 2.0)	[93]
Amino-phenyl-acetic acid		Aqueous HClO ₄ (pH 1.5-2.0)	[94]
2-Amino-pentanedioic acid		Aqueous HClO ₄ (pH 2.0)	[95]
2-Amino-3-(4-hydroxy-[1,2,5]thiadiazol-3-yl)-propionic acid		Aqueous trifluoro acetic acid (pH 2)	[96]
2-Amino-cyclohexanol		95:5:2 H ₂ O / methanol / CF ₃ CO ₂ H	[97]

Name	Structure	Mobile Phase	Ref.
Alanine		Aqueous HClO ₄ (pH 1.5)	[98]
Leucine		Aqueous HClO ₄ (pH 2)	[99]
2-Amino-butyric acid methyl ester		Aqueous HClO ₄ (pH 1.3)	[100]
Baclofen		90:10 10 mM ammonium acetate (pH 6.8)	[71]
2-Amino-3-phenyl-propionitrile		90:10 10 mM H ₂ O HClO ₄ / methanol	[101]
(RR,SS)-1-(4-Hydroxy-phenyl)-2-amino-1-propanol		Aqueous HClO ₄ (pH 1.0)	[102]
7-Ethyl-1,2,3,4-tetrahydro-1-naphthylamine hydrochloride		90:10 H ₂ O + 0.1% trifluoroacetic acid / methanol	[103]
2-Amino-propionamide		60 mM HClO ₄	[104]

Name	Structure	Mobile Phase	Ref.
Phenylglycine		90:10 HClO ₄ H ₂ O (pH 1.2) / methanol	[105]
2-Amino-3-phenyl-propionic acid ethyl ester		90:10 HClO ₄ (pH 2) / methanol	[106]
2-Amino-succinamic acid		100 mM HClO ₄ (pH 1)	[107]
7-Methoxy-2-aminotetralin		15:85 methanol / aqueous 1% HClO ₄	[108]
2-Amino-3-[3-(2-fluoro-ethyl)-4-methoxy-phenyl]-propionic acid		95:5 Aqueous HClO ₄ (pH 2) / methanol	[109]
<i>p</i> -Borono-phenylalanine		0.4% Aqueous HClO ₄	[110]
Amino-(2-hydroxy-4-propyl-2H-pyrazol-3-yl)-acetic acid		HClO ₄	[111]

Name	Structure	Mobile Phase	Ref.
2-Amino-2-phenyl-acetamide		HClO ₄ (pH 1.3)	[85]
Phenylalanine		Aqueous HClO ₄ (pH 1.5)	[112]
2-Amino-cyclobutane-carboxylic acid		0.17 M HClO ₄ (pH 1)	[113]
2-Amino-propionic acid		30 mM HClO ₄ (pH 2)	[114]
2-Amino-4-[[[(E)-2-carboxyethenyl](hydroxy)phosphoryl]butanoic acid		Aqueous HClO ₄ pH 1.0	[115]
2-Phenylpropan-1-amine		Aqueous 0.03 N HClO ₄ (pH 6) buffer	[116]

6.4.3. Charge transfer

Charge transfer complexes are an electron donor/electron acceptor associations for which an intermolecular electronic charge transfer is observed [117]. Sometimes, it is very difficult to discriminate between electronic charge transfer and donor-acceptor complexes. A donor-acceptor complex may also involve adduct formation by proton transfer. At the same time, there is no definite boundary between coordination complexes and charge transfer complexes. In charge transfer complexes, an electron may be transferred from a non-bonding

orbital of the donor to an anti-bonding orbital of the acceptor. A coordination complex involves the donation of a lone pair of electrons from the donor to a vacant orbital of the acceptor molecule. In general, the acceptor is a metal atom of the transition series. The adducts are usually stabilised by other bonding contributions [118].

Charge transfer complexes can be classified according to the type of orbitals interacting:

1. π donors and π acceptors in which it is a π orbital which donates or accepts the bonding electron, which is delocalized over the orbital of the adduct. This type of charge transfer complexes are usually strong complexes.
2. σ donors and σ acceptors in which the bonding involves the s orbital of the reactants.
3. n donors where the bonding orbital is localized within the reactant molecules. An example of this effect is of tertiary nitrogen [118].

In the following we will focus on the aromatic interactions which occur between a π donor molecule and a π acceptor counterpart. They provide stability to duplex DNA and are believed to contribute to the unique properties of thermophilic proteins. They may play a role in amyloid aggregation in Alzheimer's disease, being a common motif in biomolecular recognition. They are integral part in enantiomeric recognition when the stationary phase consists of a π donor/acceptor and the analyte contains a π donor/acceptore moiety

Aromatic interactions have been suggested to consist of van der Waals, hydrophobic and electrostatic forces. The relative contribution and magnitude of each of these components is still a matter of debate. The controversy arises from the fact that aromatic groups interact in one of several geometries, depending on the nature of the rings involved [119]. The electrostatic component has been suggested to arise from interactions of the quadrupole moments of the aromatic rings. The edge-face geometry can be considered as a CH- π interaction found in benzene in the solid and liquid state, and is commonly observed between aromatic residues in proteins [120]. Aromatic rings can also act as hydrogen bond acceptors. The presence of a solvent affects the complexation constant describing the equilibrium between the individual components of the complex. This is due to the competition of solvent molecules toward each component of the complex. The solvent does not have to be a charge-transfer competitor. Competitive interactions such as hydrogen bonds can also affect the equilibrium. When the equilibrium constant of complexation is quite low, the influence of the solvent is very significant, due to its overwhelming concentration compared to the concentration of the complex.

6.4.4. Chiral separation through a combination of charge transfer, hydrogen bonding and electrostatic interactions

In 1966, Pirckle first reported that (*S*)-1-phenylmethylaniline caused ^{19}F -NMR non-equivalency of 2,2,2-trifluoro-1-phenylethanol in a carbon tetrachloride

solution [121]. In later studies, 2,2,2-trifluoro-1-(9-anthryl)ethanol, an NMR shift reagent, was used as a mobile phase additive to separate racemates of 2,4-dinitrophenyl methyl sulphoxide on a silica gel column [122]. Later, one enantiomer of this fluoroalcohol was covalently attached to silica gel and used for the resolution of a large number of solutes, including sulphoxides, lactones, derivatives of alcohols, amines, amino acids, hydroxy acids and mercaptans [123]. The model used to describe complex formation between the selectand and the selector consists of three simultaneous points of interaction first described by Dalglish [3,124]. The Pirkle-type phases are based on amino acids bound on one side to the silica gel matrix and on the other side derivatised with an aromatic moiety which can be either a π donor or a π acceptor. This chiral stationary phase (CSP) undergoes π - π interactions with enantiomeric analytes which have an aromatic moiety. The complex is stabilised through additional interactions such as hydrogen bonding, dipole-dipole interactions or steric repulsion [125]. An improved chiral stationary phase synthesised by Pirkle's group possesses both dinitrobenzoyl and naphthyl moieties, allowing for simultaneous face-to-face π - π interactions and phase-to-edge interactions [126]. The elution order is controlled through the configuration of the CSP. These phases were used under normal phase conditions, with mixtures of solvents such as hexane-isopropyl alcohol. They were also used under supercritical and reversed phase conditions [125]. Table 6.5 lists some enantiomeric compounds and the compositions of CSP and mobile phase for the separation of their racemic mixture based on simultaneous charge transfer and hydrogen bonding interactions.

Another type of CSP capable of undergoing charge transfer interactions is the one developed by Lindner's group [127], based on cinchona alkaloids. They can operate under both normal phase and reversed phase. Under reversed phase conditions, they operate through an ion pairing interaction with acidic enantiomeric analytes. Under normal phase conditions, they operate through π - π interactions as well as ion pairing [128,129].

The basis of the interactions in the complex consists of electrostatic interactions between the quinuclidine of the amine ion and the carboxylate of the selectand, the π - π interactions between the quinoline ring of the selector and the dinitrobenzoyl of the selectand and the steric repulsion between the leucine side chain and the carbamate moiety [129]. Table 6.6 lists some successful separation on the commercial Chiral AX[®] column based on quinine CSP.

Table 6.5. Selected compounds and the conditions for the separation of their racemic mixture on CSP working through charge transfer and H-bonding

Name/Structure	Stationary phase	Mobile Phase	Ref.
3- <i>tert</i> -Butyl-3H-naphtho[1,2-c]furan-1-one	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenz-amido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>S,S</i>)-Whelk-O1)	10:90 hexane / 2-propanol	[130]

Name/Structure	Stationary phase	Mobile Phase	Ref.

3-Methyl-2-phenyl-2,3-dihydro-benzofuran	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenzamido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>S,S</i>)-Whelk-O1)	Heptane	[25]

1-Trimethylsilanyl-2-azabicyclo[3.1.0]hexane-2-carboxylic acid <i>tert</i> -butyl ester	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenzamido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>S,S</i>)-Whelk-O1)	95:5 hexane / 2-propanol	[131]

4-Bromo-benzoic acid 1-(6-oxocyclohex-1-enyl)- <i>hept</i> -4-enyl ester	(3 <i>S</i> ,4 <i>R</i>)-4-(3,5-Dinitrobenzamido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>R,R</i>) Whelk-O1)	95:5 hexane / 2-propanol	[132]

4-Cyano-3-(4-fluoro-phenyl)-butyric acid methyl ester	(3 <i>S</i> ,4 <i>R</i>)-4-(3,5-Dinitrobenzamido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>R,R</i>) Whelk-O1)	90:10 hexanes / ethanol	[133]

Name/Structure	Stationary phase	Mobile Phase	Ref.
<p>Butyric acid 1-(4-methoxyphenyl)-ethyl ester</p>
<p>((E)-1-Hydroxy-5-methyl-hex-2-enyl)-phosphonic acid dimethyl ester</p>	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenzamido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>S,S</i>)-Whelk-O1)	90:10 hexane / 2-propanol	[134]
<p>Tiropamide</p>	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenzamido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>S,S</i>)-Whelk-O1)	80:20 hexane / ethanol	[135]
<p>N-[2-Methoxymethoxy-1-(2-methoxy-5-methyl-phenyl)-ethyl]-acetamide</p>	(3 <i>S</i> ,4 <i>R</i>)-4-(3,5-Dinitrobenzamido)-3-[3-(trioxysilyl)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>R,R</i>) Whelk-O2)	700:300:2:1 methanol / H ₂ O / acetic acid / triethylamine	[136]
	4-(3,5-Dinitrobenzamido)-3-tetrahydrophenanthrene derivative incorporated into polymethylhydrosiloxane (coated on silicagel 300A) ((<i>S,S</i>)-polyWhelk-O1)	30:20 hexanes / 2-propanol	[137]

Name/Structure	Stationary phase	Mobile Phase	Ref.

2-(Benzhydrylidene-amino)-4-(2,2-dimethyl-propylidene)-pentanedioic acid 1- <i>tert</i> -butyl ester 5-methyl ester	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenz-amido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>S,S</i>)-Whelk-01)	99:1 hexane / 2-propanol	[138]

2-Phenylcyclopropanecarboxylic acid	(3 <i>S</i> ,4 <i>R</i>)-4-(3,5-Dinitrobenz-amido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>R,R</i>) Whelk-01)	98:1.5:0.5 phexane / ethyl acetate / acetic acid	[139]

6,6'-Dibromo-1,1'-bi-2-naphthol	(3 <i>S</i> ,4 <i>R</i>)-4-(3,5-Dinitrobenz-amido)-3-[3-(trioxysilyl)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>R,R</i>) Whelk-02)	98:2 hexane / 2-propanol	[140]
[(<i>S</i>)-(<i>S</i>)-(1-Acetyl-2-oxocyclopentyl)- <i>m</i> -tolyl-methyl]-carbamic acid allyl ester	(3 <i>S</i> ,4 <i>R</i>)-4-(3,5-Dinitrobenz-amido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>R,R</i>) Whelk-01)	90:10 hexane / 2-propanol	[141]

Name/Structure	Stationary phase	Mobile Phase	Ref.

3-[(Indan-1-ylamino)-indan-1-one	(3 <i>S</i> ,4 <i>R</i>)-4-(3,5-Dinitrobenz-amido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>R,R</i>)-Whelk-O1)	0.95:0.95:8:3:87.1 triethylamine / H ₃ PO ₄ / 2-propanol / acetonitrile / H ₂ O	[142]

1-[2-(3,3-Dimethyl-but-1-ynyl)-pyrimidin-5-yl]-2-methylpropan-1-ol	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenz-amido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>S,S</i>)-Whelk-O1)	Gradient 4-40 % methanol (SFC)*	[143]

4a,7-Dimethyl-1,4,4a,8a-tetrahydro-1,4-methanonaphthalene-5,8-dione	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenz-amido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>S,S</i>)-Whelk-O1)	99:1 hexane / 2-propanol	[144]

2-[(4-Methoxy-phenylamino)-phenyl-methyl]-hexanoic acid ethyl ester	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenz-amido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>S,S</i>)-Whelk-O1)	99:1 hexane / 2-propanol	[145]

Name/Structure	Stationary phase	Mobile Phase	Ref.
<p>(3-Oxo-1-phenyl-butyl)- carbamic acid tert-butyl ester</p>
<p>(3<i>R</i>,4<i>S</i>)-4-(3,5-Dinitrobenz- amido)-3-[3-(dimethylsily- loxy)propyl]-1,2,3,4-tetra- hydrophenanthrene ((<i>S,S</i>)-Whelk-01)</p>	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenz- amido)-3-[3-(dimethylsily- loxy)propyl]-1,2,3,4-tetra- hydrophenanthrene ((<i>S,S</i>)-Whelk-01)	85:15 hexane / 2- propanol	[146]
<p>2,2,6,6-Tetramethyl-1-[2,4,4- trimethyl-1-(toluene-4- sulphonyl)-pyrrolidin-2- ylmethoxy]-piperidine</p>	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenz- amido)-3-[3-(dimethylsily- loxy)propyl]-1,2,3,4-tetra- hydrophenanthrene ((<i>S,S</i>)-Whelk-01)	97:3 hexane / 2- propanol	[147]
<p>2-(Hydroxy-phenyl-methyl)-5- oxo-2,5-dihydro-pyrrole-1- carboxylic acid tert-butyl ester</p>	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenz- amido)-3-[3-(dimethylsily- loxy)propyl]-1,2,3,4-tetra- hydrophenanthrene ((<i>S,S</i>)-Whelk-01)	85:15 hexane / ethanol	[148]

Name/Structure	Stationary phase	Mobile Phase	Ref.
<p>4-Oxo-3-phenyl-3-((<i>E</i>)-3-phenyl-allyl)-3,4-dihydro-2H-quinoline-1-carboxylic acid allyl ester</p>
<p>1-Phenyl-2-propanol</p>	(3 <i>R</i> ,4 <i>S</i>)-4-(3,5-Dinitrobenzamido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>S,S</i>)-Whelk-O1)	80:20 hexanes / 2-propanol	[149]
<p>Methyl-phenyl(prop-2-en-1-yloxy)ethanoate</p>	(3 <i>S</i> ,4 <i>R</i>)-4-(3,5-Dinitrobenzamido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>R,R</i>)-Whelk-O1)	90:10 hexane / 2-propanol + acetic acid (apparent pH: 3.6)	[150]
(3 <i>S</i> ,4 <i>R</i>)-4-(3,5-Dinitrobenzamido)-3-[3-(dimethylsilyloxy)propyl]-1,2,3,4-tetrahydrophenanthrene ((<i>R,R</i>)-Whelk-O1)	99:1 hexane / 2-propanol	[151]

*SFC – supercritical fluid chromatography

Table 6.6. Selected compounds and the conditions for their HPLC separation on Chiral AX® CSP containing quinine

Name/Structure	Stationary phase	Mobile Phase	Ref.
<p>N-(2,4-Dinitro-phenyl)-alpha-naphthalen-1-ylmethyl-proline</p>	(2,6-Diisopropyl-phenyl)-carbamoyl-Quinine bonded to mercaptopropylsilica (Chiral AX QN-2)	30:70 Aqueous ammonium acetate 0.05 M / methanol (pH 6.5)	[152]
<p>2-Benzoyloxycarbonylamino-3-phenyl-propionic acid</p>	<i>tert</i> -Butylcarbamoyl-Quinine bonded to mercaptopropyl silica (Chiral AX QN-1)	80:20 methanol / 50 mM sodium phosphate buffer (pH 5.6)	[153]
<p><i>cis</i>-Chrysanthemic acid</p>	<i>tert</i> -Butylcarbamoyl-quinine bonded to mercaptopropyl silica (Chiral AX QN-1)	80:20 methanol / H2O, 10 mM ammonium acetate buffer (pH 6.0)	[154]

Name/Structure	Stationary phase	Mobile Phase	Ref.
1-Furan-2-yl-1,2-dihydro-imidazo[5,1-b]quinazoline-3,9-dione	<i>tert</i> -Butylcarbamoyl-quinine bonded to mercaptopropyl silica (Chiral AX QN-1)	90:10 0.1 M Ammonium acetate buffer (pH 6.0) / acetonitrile	[155]
1-(4-Chloro-phenyl)-1,2-dihydro-imidazo[5,1-b]quinazoline-3,9-dione	(2,6-Diisopropyl-phenyl)-carbamoyl-quinine bonded to mercaptopropyl silica (Chiral AX QN-2)	60:40 0.1 M Ammonium acetate buffer (pH 6.0) / methanol	[155]
Ala-OPA (Alanine ortho-phthalaldehyde derivative)	<i>tert</i> -Butylcarbamoyl-quinine bonded to mercaptopropyl silica (Chiral AX QN-1)	80:20 methanol / aqueous 0.1 M ammonium acetate buffer (pH 6.0)	[156]
2-Methoxy-2-(1-naphthyl)propionic acid	<i>tert</i> -Butylcarbamoyl-quinine bonded to mercaptopropyl silica (Chiral AX QN-1)	96:4 methanol / acetic acid	[157]

Name/Structure	Stationary phase	Mobile Phase	Ref.
Fmoc-Glu	<i>tert</i> -Butylcarbamoyl-quinine bonded to mercaptopropyl silica (Chiral AX-QN)	methanol / 0.25% acetic acid (44 mM)	[158]
NBD- <i>trans</i> -Hydroxyproline*	<i>tert</i> -Butylcarbamoyl-quinine bonded to mercaptopropyl silica (Chiral AX QN-1)	50:50 methanol / acetonitrile / 10 mM citric acid	[159]
NBD-Proline*	(2,6-Diisopropyl-phenyl)-carbamoyl-quinine bonded to mercaptopropyl silica (Chiral AX QN-2)	50:50 methanol / acetonitrile / 10 mM citric acid	[159]

* NBD - 4-nitrobenzo-2-oxa-1,3-diazole

6.4.5. Ligand exchange

Ligand exchange chromatography (LEC) is a typical example of complexation chromatography. Complexes formed during LEC encompass a metal cation associated with ligands (anions or neutral molecules) able to donate electron pairs to a vacant orbital of the metal [160]. The term ligand exchange was introduced Helfferich in 1961 when he described the substitution of organic diamine molecules with metal ion-coordinated molecules containing amine groups in a polymeric phase [161]. The technique was further developed by Davankov and Rogozin for the separation of enantiomers [162]. This technique is applicable to those enantiomers which are able to form metal complexes with the moiety bound onto the stationary phase. Enantiomeric analytes such as amino acids and hydroxy acids were successfully separated into enantiomers using LEC.

In their paper, the authors described the model of interaction during the enantiomeric separation with LEC [162]:

"In such a process, one ligand (the optically active one) should be rigidly attached to a stationary phase, while the other (racemic) ligand should be able to move with the mobile phase. The metal atom forming the complex may be combined with either ligand. The important point is that the complex generated should be kinetically labile, i.e. readily decomposed and reformed."

Based on the model, the chiral phase is usually an amino acid anchored to a silica matrix through a spacer. Table 6.7 shows several examples of enantiomeric separations through LEC. The mobile phase used in conjunction with these chiral phases is a buffer containing millimolar amounts of copper. At a constant pH, the retention increases with an increase in the length of the spacer. The pH of the mobile phase influences the stability constant of the complexes and, consequently, the retention as well as the enantioselectivity. The retention of hydrophobic analytes can be controlled by the amount of an organic modifier such as acetonitrile or methanol. The flow rate of the mobile phase has major role on the resolution; the slower the flow rate, the higher the resolution [163].

Table 6.7. Conditions for LEC enantiomeric separation of selected compounds

Name/Structure	Stationary Phase	Mobile Phase	Ref.
5,5-Dimethylthiazolidine-4-carboxylic acid	(S)-Leucinol monosodium salt covalently bonded to aminoundecyl silica	80:20 aqueous CuSO ₄ 0.2 mM / methanol	[164]
2R,3R-2S,3S-2-Hydroxy-3-methyl-pentanoic acid	N,N-Dioctyl-L-alanine coated octadecyl silica (Chiralpak MA(+))	85:15 2 mM CuSO ₄ / acetonitrile	[165]
N-Methyl glutamic acid	N,S-Dioctyl-(D)-penicillamine coated on octadecylsilanised silica (Chirex 3126)	90:10 2 mM CuSO ₄ / acetonitrile	[166]

Name/Structure	Stationary Phase	Mobile Phase	Ref.
Proline	N,S-Dioctyl-(<i>D</i>)-penicillamine coated on octadecylsilanised silica (Chirex D-penicillamine)	95:5 2 mM CuSO ₄ / acetonitrile	[167]
2-(1-Amino-1-carboxy-ethyl)-cyclopropanecarboxylic acid	N,S-Dioctyl-(<i>D</i>)-penicillamine coated on octadecylsilanised silica (Chirex D-penicillamine)	1 mM CuSO ₄	[168]
2-Amino-4-methylselanylbutyric acid	Isoleucine reacted on (3,4-epoxycyclohexyl)ethyltrimethoxy-silane reacted on silica	50 mM KH ₂ PO ₄ , 0.1 mM CuSO ₄ (pH 5.5)	[169]
3-(4-Hydroxyphenyl)lactic acid	Chitosan (95% deacetylated) bonded to aminopropylsilica with glutaraldehyde	Aqueous 3 mM CuSO ₄	[170]
Alanine	S-Trityl-(<i>R</i>)-cysteine coated on Lichrospher 100 RP18 silica.	0.5 mM CuSO ₄ (pH 4.5)	[171]
Amino-(3-hydroxyadamantan-1-yl)-acetic Acid	(<i>L</i>)-Proline bonded to silica gel (Chiralpak (WH))	Aqueous 0.3 mM CuSO ₄	[172]

6.4.6. Mixed types of interactions

The previous sections described the main types of interactions occurring between the enantiomeric analytes and the stationary phase (hydrogen bonding, inclusion complexes, charge transfer and ligand exchange). In the following section, we will present enantiomeric separation based on combinations of these interactions. These types of stationary phases include polysaccharides, antibiotic phases (macrocyclic antibiotics) and protein phases.

6.4.6.1. Polysaccharide phases

Many polysaccharides serve as extracellular elements in the cell walls of unicellular microorganisms and higher plants and on the outer surfaces of animal cells. There are many different structural polysaccharides. Cellulose is the most abundant extracellular structural polysaccharide of all biomolecules, plant or animal. Structurally, cellulose is a polysaccharide composed of several thousand molecules of D-glucose joined by $\beta(1\rightarrow4)$ glycosidic linkages. Complete hydrolysis of all glycosidic bonds of cellulose yields D-glucose.

Starch is a mixture of a water-dispersible fraction called amylose and a second component, amylopectin. Amylose is a polysaccharide made up of about 100 to several thousand D-glucose units joined together by $\alpha(1\rightarrow4)$ glycosidic bonds [173]. This difference in the structure of cellulose and amylose results in polymeric structures with very different properties. Because of their β linkages, the D-glucose chains in cellulose assume an extended conformation and undergo side-by-side aggregation due to hydrogen bonding between the hydroxyl groups of adjacent cellulose chains. Due to the geometry of their $\alpha(1\rightarrow4)$ linkages, the main chains of D-glucose units in amylose tend to assume a coiled, helical conformation [174].

Cellulose has been used in thin layer chromatography to separate enantiomers of tryptophan and tryptophan derivatives using aqueous mobile phases [175-177], while amylose has been used to separate biphenyl atropisomers and other optical isomers [178]. The interactions between the enantiomeric analytes and these chiral stationary phases occur only through H-bonding as well as inclusion between the polymeric chains of these biomolecules. The chiral discrimination of these native biopolymers is thereby reduced. Modification of hydroxy groups through esters or carbamate formation greatly enhances the enantioselective properties of these polysaccharides due to multiple possibilities of H-bonds and π - π interactions as well as through inclusion complex formations. Phenyl esters and phenyl carbamates of amylose and cellulose are the most popular CSPs today. For example, the major chiral adsorption site in cellulose tris-methylbenzoate is considered to be the polar carbonyl groups of the esters, which can interact with the racemate through hydrogen bonding and dipole-dipole interactions for discrimination between enantiomers [179]. The cellulose and amylose carbamate assume a left-handed three-fold helical conformation,

while the corresponding amylose has a left-handed four-fold helix. A chiral helical channel is formed along the backbone. Aromatic moieties are located at the surface of the chain while the polar groups are located in the interior [180]. The shape of the chiral cavities varies depending on the nature of the substitution on the polysaccharide [125]. Chiral recognition is attributed to shape-selective inclusion along with π - π interactions, hydrogen bonding and van der Waals interactions [181]. These stationary phases operate under normal phase as well as under reversed phase mobile phase conditions. Under normal phase conditions, combinations of hexane/alcohols are used. Under reversed phase conditions, it is assumed that the interaction between the CSP and the enantiomeric analytes occurs through shape-specific interactions into the chiral cavities [182].

Tables 6.8 – 6.19 give some selected examples of enantiomeric separation using cellulose derivatised and amylose derivatised CSPs.

Table 6.8. Conditions for chiral separations of selected compounds using cellulose tris(3,5-dimethylphenylcarbamate)/macroporous silica gel (Chiralcel OD®) stationary phase

Name	Structure	Mobile Phase	Ref.
1-[[4-(4-Chloro-phenyl)-1-methyl-1H-pyrrol-3-yl]-phenyl-methyl]-1H-imidazole		90:10 hexane / ethanol / 0.1% diethylamine	[183]
Indapamide		50:50 hexane / ethanol	[184]
Dia-(1 <i>S</i> 3 <i>R</i> ,1 <i>R</i> 3 <i>R</i>)-3-benzyl-oxymethyl-1,3-dihydro-isobenzofuran-1-thymine		80:20 hexane / ethanol	[185]

Name	Structure	Mobile Phase	Ref.
Dia-(1 <i>R</i> 3 <i>R</i> ,1 <i>S</i> 3 <i>R</i>)-3-benzyl-oxymethyl-1,3-dihydro-isobenzofuran-1-uracil		80:20 hexane / ethanol	[185]
5,7-Dimethyl-1,4-dioxo-1,5,8a-tetrahydro-4H-naphthalene-4a-carboxylic acid methyl ester		95:5 hexane / 2-propanol	[186]
5-(<i>N,N'</i> -Bisbenzyloxy-carbonyl-hydrazino)-4-hydroxy-heptan-2-one		60:40:0.1 H ₂ O / acetonitrile / tri-fluoro acetic acid	[187]
3-Methyl-1,3,4-triphenyl-butan-1-one		85:15 hexane / methyl- <i>t</i> -butyl ether	[188]
5-(3,5-Dinitro-benzoyloxy)-3-aza-tricyclo[2.2.1.0*2,6*]heptane-3-carboxylic acid <i>tert</i> -butyl ester		50:50 hexane / ethanol	[189]
Diniconazole		85:15 hexane / ethyl acetate	[190]

Name	Structure	Mobile Phase	Ref.
Phosphinamide derivative		90:10 hexane / 2-propanol	[191]
(5-Methyl-4,5-dihydro-pyrazol-1-yl)-pyridin-3-yl-methanone		90:10 hexane / 2-propanol	[192]
1-(4-Chloro-phenyl)-3-phenyl-propane-1,3-diol		95:5 hexane / 2-propanol	[193]
6-Chloro-2,2-dimethyl-1a,7b-dihydro-2H-1,3-dioxa-cyclopropa[a]naphthalene		99:1 hexane / 2-propanol	[194]
1-(1-Methyl-1H-imidazol-2-yl)-2-(2,3,4,9-tetrahydro-1H-carbazol-1-yl)-ethanone		90:10 hexanes / 2-propanol	[195]
Nadolol		100% acetonitrile	[196]
(1-Isobutyl-pyrrolidin-2-yl)-phenyl-methanol		99.5:0.5 hexane / 2-propanol	[197]

Name	Structure	Mobile Phase	Ref.
Acetic acid 2-benzyl-3-(<i>tert</i> -butyl-dimethyl-silanyloxy)-propyl ester		95:5 hexane / 2-propanol	[198]
3-Furan-2-yl-2,3-dihydro-benzofuran-3-ol		90:10 hexane / 2-propanol	[199]
4-Acetyl-5-ethyl-3-hydroxy-2,3-dihydro-furan-3-carboxylic acid ethyl ester		95:5 hexane / 2-propanol	[200]
Indan-1-yl-carbamic acid 2,2,2-trichloro-ethyl ester		9:1 hexane / 2-propanol	[201]
3-Phenethyl-decanoic acid methyl ester		99.2:0.8 hexane / 2-propanol	[202]
(3,5-Dimethyl-phenyl)-(1-phenyl-ethyl)-amine		99:1 hexane / 2-propanol	[203]
3-[4-Methyl-3-[methyl-(7H-pyrrolo[2,3-d]pyrimidin-4-yl)-amino]-piperidin-1-yl]-3-oxo-propionitrile		methanol	[204]

Name	Structure	Mobile Phase	Ref.
2,2,4-Trimethyl-4-(2-phenyl-allyl)-[1,3]dioxan-5-one		99:1 hexanes / ethanol	[205]
2-(Benzoyl-methyl-amino)-3-methyl-butyric acid methyl ester		50:1 hexane / 2-propanol	[206]
3-Ethyl-4-oxo-3-phenyl-[1,2]diazetidine-1,2-dicarboxylic acid dimethyl ester		95:5 hexane / 2-propanol	[207]
4-(4-Fluorophenylsulphonyl)-2-phenylmorpholine		95:5 hexane / 2-propanol	[208]
[1-(4-Fluoro-phenyl)-ethyl]-(3-methoxy-phenyl)-amine		95:5 hexane / 2-propanol	[209]
Methyl 4-bromo-alpha-methyl-N-[(propan-2-yloxy)carbonyl]-L-phenylalaninate		80:20 hexane / ethanol	[210]

Name	Structure	Mobile Phase	Ref.
2-Amino-4-(4-methylphenyl)-8-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitrile		15:85 2-propanol / hexane	[211]
5-Bromo-N-hydroxy-6-methyl-N-[3-methylbut-3-en-2-yl]pyridin-2-amine		95:5 hexanes / 2-propanol	[212]
1-Chloro-2-but-3-enyl benzoate		1000:1 hexane / 2-propanol	[213]

Table 6.9. Conditions for chiral separations of selected compounds using amylose tris(3,5-dimethylphenylcarbamate) coated on silica (Chiralpak AD®) stationary phase

Name	Structure	Mobile Phase	Ref.
1-[[4-(4-Chloro-phenyl)-1-methyl-1H-pyrrol-3-yl]-phenyl-methyl]-1H-imidazole		90:10 hexane / ethanol / 0.1% diethylamine	[183]
5-[2-[1-(3,5-Bis-trifluoromethyl-phenyl)-ethoxy]-3-phenyl-morpholin-4-ylmethyl]-2,4-dihydro-[1,2,4]triazol-3-one		93:7 hexane / propanol	[214]
2-Amino-3-[4-((E)-styryl)-phenyl]-propionic acid methyl ester		90:10 hexane / 2-propanol	[215]
Omeprazole		20 mM phosphate buffer (pH 4.65) / acetonitrile (Chiralpak AD-RH)	[216]

Name	Structure	Mobile Phase	Ref.
2-Phenyl-2-(N-phenyl-aminoxy)-ethanol		90:10 hexane / ethanol	[217]
5-Methyl-2-thioxo-oxazolidine-4-carboxylic acid tert-butyl ester		10:1 hexane / 2-propanol	[218]
3-Phenyl-propane-1,2-diol		98:2 hexane / 2-propanol	[219]
1,2-Di-furan-2-yl-2-hydroxy-ethanone		90:10 hexane / 2-propanol	[220]
4-Ethoxy-6-(4-nitro-phenyl)-5,6-dihydro-pyran-2-one		85:15 hexane / 2-propanol	[221]
3-Naphthalen-1-yl-pentan-1-ol		50:1 hexane / 2-propanol (Chiralpak AD-H)	[222]
N-(1-Phenyl-ethyl)-acetamide		95:5 hexane / 2-propanol	[223]

Name	Structure	Mobile Phase	Ref.
3-Methyl-2-phenyl-pent-4-en-2-ol		999:1 hexane / 2-propanol (Chiralpak AD-H)	[224]
[2-(2-Bromo-phenyl)-3-((E)-styryl)-cyclopropyl]-phenyl-methanone		100:4 hexane / 2-propanol	[225]
(6- <i>tert</i> -Butoxycarbonyl-amino-5-oxo-cyclohex-3-enyl)-carbamic acid <i>tert</i> -butyl ester		20:1 hexane / 2-propanol	[226]
2-(Hydroxy-pyridin-4-yl-methyl)-cyclohexanone		90:10 hexane / 2-propanol	[227]
5-(N-Phenyl-aminooxy)-hept-1-en-4-ol		97:3 hexanes / 2-propanol	[228]
Efaroxan 2-(2-Ethyl-2,3-dihydro-benzofuran-2-yl)-4,5-dihydro-1H-imidazole		98:2:0.05 hexane / 2-propanol / diethylamine	[229]
Diarylphosphinamide derivative		50:1 hexane / 2-propanol	[230]

Name	Structure	Mobile Phase	Ref.
1-Methyl-2,4,5-triphenyl-4,5-dihydro-1H-imidazole		90:10 heptane / 2-propanol	[231]
2-[(1H-Indol-3-yl)-phenyl-methyl]-3-methyl-butyr-aldehyde		85:15 hexane / 2-propanol	[232]
2-[Hydroxy-(4-nitro-phenyl)-methyl]-cyclopentanone		95:5 hexane / 2-propanol	[233]
2-Ethyl-3-methyl-5-phenyl-pyrrolidine-2,4-dicarboxylic acid dimethyl ester		40:1 hexane / 2-propanol	[234]
2-[1-(4-Chloro-phenyl)-2-nitro-ethyl]-malonic acid diethyl ester		65:35 hexane / 2-propanol	[235]
4-Hydroxy-4-m-tolyl-butan-2-one		90:10 hexane / 2-propanol	[236]
Methyl 4-bromo-alpha-methyl-N-[(propan-2-yloxy)carbonyl]-L-phenyl-alaninate		80:20 hexanes / ethanol (AD-H)	[210]

Name	Structure	Mobile Phase	Ref.
Ethyl 3-(4-methoxy-1H-indol-3-yl)butanoate		95:5 hexane / 2-propanol	[237]
Koga base		95:5 Isobutanol / methanol / 0.03% triethylamine	[238]

Table 6.10. Conditions for chiral separations of selected compounds using cellulose tris(3,5-dimethylphenylcarbamate) immobilised on silica (Chiralpak IB®) stationary phase

Name	Structure	Mobile Phase	Ref.
5-Ethyl-1-methyl-5-propyl-pyrimidine-2,4,6-trione		90:10 hexane / 2-propanol	[239]
N- <i>p</i> -Methoxy-1,α-dihydroaristoyagonine		80:20 hexane / ethanol + 0.5% trifluoroacetic acid	[240]
Monastrol		60:40 ethylacetate / hexane	[241]
7b-Phenyl-1a,2,3,7b-tetrahydro-1-oxa-cyclopropa[a]naphthalene		95:5 hexane / 2-propanol	[242]
2-(3-Ethyl-2,6-dioxo-piperidin-3-yl)-isoindole-1,3-dione		80:20 methyl- <i>t</i> -butyl ether / tetrahydrofuran	[243]
N-Phthaloyl-valine		95:5 hexane / 2-propanol / 0.1% CF ₃ CO ₂ H	[244]
3-(4-Methoxy-phenylamino)-2-methyl-3-phenyl-propan-1-ol		90:10 hexane / 2-propanol	[145]

Name	Structure	Mobile Phase	Ref.
4-[[Carboxy-(3,4-dimethoxy-phenyl)-methyl]-carbamoyl]-butyric acid		66:30:4:0.1 hexane / CHCl ₃ / ethanol / trifluoroacetic acid	[245]
Metanephrine		65:35:0.1 hexane / ethanol / diethylamine	[246]
BOC-Phg-Oet		40:60 methyl-t-butyl ether / hexane / 0.1% trifluoroacetic acid	[247]
3-(4-Methoxy-phenyl)-1,4-diphenyl-azetidin-2-one		hexane / 2-propanol	[248]
(Cyclobutyl-phenyl-methyl)-(4-methoxy-phenyl)-amine		99:1 hexane / 2-propanol	[249]
Flurbiprofen <i>n</i> -butyl ester		85:15:0.4:0.1 hexane / <i>tert</i> -butyl methyl ether / 2-propanol / trifluoroacetic acid	[250]
(7 <i>E</i>)-6-Hydroxytetradec-7-ene-2,4-diyne-1-yl acetate		90:10 heptane / ethyl acetate	[251]

Table 6.11. Conditions for chiral separations of selected compounds using amylose tris(3,5-dimethylphenylcarbamate) immobilised on silica (Chiralpak IA®) stationary phase

Name	Structure	Mobile Phase	Ref.
3,5-Dinitro-benzoic acid 2-phenyl-1-trifluoromethyl-cyclopropylmethyl ester		90:10 hexane / 2-propanol	[252]
Pirprofen		80:20:0.1 hexane / 2-propanol / trifluoroacetic acid	[253]
Propafenone		60:40:0.1 hexane / tetrahydrofuran / diethylamine	[254]
[1]Benzothieno[5',4':2,3][1]benzothieno[5,4-b]thieno[3,2-e][1]benzothiophene		19:1 hexane / dichloromethane	[255]
3-[[[7-Ethyl-5-methoxy-9-oxo-6,7,8,9-tetrahydro-5H-5,8-epoxybenzo[7]annulen-6-yl]carbonyl]-1,3-oxazolidin-2-one		80:20 hexane / 2-propanol	[256]
3-p-Tolyl-cyclohexanone		98:2 hexane / 2-propanol	[257]

Name	Structure	Mobile Phase	Ref.
Rabeprazole		80:20:0.1 heptane / 2-propanol / triethylamine	[258]
2-(3-Ethyl-2,6-dioxo-piperidin-3-yl)-isoindole-1,3-dione		80:20 CH ₂ Cl ₂ / tetrahydrofuran	[259]
[(4-Isopropoxy-phenylamino)-phenyl-methyl]-phosphonic acid dimethyl ester		9:1 hexane / 2-propanol	[260]
Flavanone		90:10 methanol / 10 mM sodium phosphate (Na ₂ HPO ₄ + NaH ₂ PO ₄) buffer pH 3.0 (pH adjusted with 0.1 N NaOH or 10% H ₃ PO ₄)	[261]
1-Phenylethanol		95:5 hexane / 2-propanol	[262]
4-Cyclohexyl-4-hydroxy-butan-2-one		90:10 heptane / 2-propanol	[263]
2,4,5-Triphenyl-1-(toluene-4-sulphonyl)-4,5-dihydro-1H-imidazole		75:25 heptane / 2-propanol	[231]
BOC-Val-Oet		40:60 methyl-t-butyl ether / hexane / 0.1% trifluoroacetic acid	[247]

Name	Structure	Mobile Phase	Ref.
3-(Phenylthio)cyclopentanone		90:10 hexane / 2-propanol	[264]
2-(N-Phenyl-aminooxy)-hexan-1-ol		95:5 hexane / 2-propanol	[265]
Protohypericin		90:10:0.25 methanol / acetonitrile / N,N-diisopropylammonium acetate	[266]
(E)-1-(Triisopropylsilyl)tetradeca-6-en-1,3-diyn-5-ol		99:1 heptane / 2-propanol	[251]
[(endo)-1-(Hydroxymethyl)-1,5,6,10b-tetrahydropyrazolo[5,1-a]isoquinolin-3(2H)-yl](phenyl)methanone		4:1 hexane / ethanol	[267]

Table 6.12. Conditions for chiral separations of selected compounds using cellulose tris(4-methyl-benzoate) coated on silica gel (Chiralcel OJ®) stationary phase

Name	Structure	Mobile Phase	Ref.
cis-1,2-Indanediol		Various mobile phases (OJ-R)	[268]
Dia-(1S3R,1R3R)-3-benzyl-oxyethyl-1,3-dihydro-isobenzofuran-1-thymine		80:20 hexane / ethanol	[185]
2-Benzyl-indan-1-one		90:10 hexane / 2-propanol	[269]

Name	Structure	Mobile Phase	Ref.
Disopyramide		73:27 CO ₂ / ethanol (SFC*)	[270]
Mepivacaine		85:15:0.1 isohexane / ethanol or 2-propanol / diethylamine	[271]
2,2-Dimethyl-3-(2-methylpropenyl)-cyclopropane-carboxylic acid		90:10 hexane / 2-propanol	[272]
4-Hydroxy-4-(3-nitrophenyl)-butan-2-one		80:20 hexane / 2-propanol	[273]
2-Hydroxy-4-phenyl-pent-4-enoic acid ethyl ester		97:3 hexane / 2-propanol	[274]
3-(tert-Butyl-dimethylsilanyloxy)-2-(3-trimethylsilyl-prop-2-ynylidene)-cyclopentanol		75:25 methanol / H ₂ O (OJ-R)	[275]
6-Biphenyl-4-yl-4-ethoxy-5,6-dihydro-pyran-2-one		80:20 hexane / 2-propanol	[276]
1-(4-Chloro-phenyl)-3-methyl-but-3-en-1-ol		50:1 hexane / 2-propanol	[277]
3-Benzylsulphanyl-pentan-1-ol		96:4 hexane / 2-propanol	[278]

Name	Structure	Mobile Phase	Ref.
4-Methyl-N-(1-phenyl-ethyl)-benzenesulphonamide		70:30 hexane / 2-propanol	[279]
3-[Hydroxy-(4-nitro-phenyl)-methyl]-but-3-en-2-one		96:4 hexane / 2-propanol	[280]
2-(3-Oxo-1,3-diphenyl-propyl)-malonic acid dibenzyl ester		90:10 hexane / 2-propanol	[281]
1-(4-Chloro-phenyl)-3-methyl-but-3-en-1-ol		50:1 hexane / 2-propanol	[282]
1-Ethynyl-4-methyl-2-oxo-cyclohex-3-enecarboxylic acid tert-butyl ester		90:10 hexane / 2-propanol	[283]
1,2,3,4-Tetrahydro-2,2,4,7-tetramethylquinoline		70:30 CO ₂ / ethanol (SFC*)	[284]
3-(4-Nitro-phenyl)-5-phenyl-4,5-dihydro-isoxazole		100 methanol	[285]
10-Hydroxycamptothecin		100 methanol	[286]

Name	Structure	Mobile Phase	Ref.
Acetic acid 3-tert-butoxycarbonylamino-7,9-dichloro-8-oxo-1-oxa-spiro[4.5]deca-6,9-dien-2-yl ester		90:10 hexane / ethanol	[287]
Anti-bicyclo[3.3.1]nonane-3,7-dione dioxime		99:1 hexane / 2-propanol	[288]
3-(4-Chloro-phenyl)-1-phenyl-pent-4-yn-1-one		85:15 hexane / 2-propanol	[289]
2-Ethylhexyl 3-[(2-[[2-phenylpropanoyl]amino]phenyl)sulphanyl]propanoate		H ₂ O / acetonitrile (gradient 40:60 to 0:90 in 30min)	[290]
2-[2-Hydroxypropyl]-1H-isindole-1,3(2H)-dione		Not reported	[291]
(RR,SS)-2-(4-Benzylpiperazin-1-yl)-1-(5-chloro-6-methoxynaphthalen-2-yl)propan-1-ol		80:20:0.1 hexane / 2-propanol / diethylamine	[292]

* SFC – supercritical fluid chromatography

Table 6.13. Conditions for chiral separations of selected compounds using cellulose triphenylcarbamate coated on silica (Chiralcel OC®) stationary phase

Name	Structure	Mobile Phase	Ref.
3-(3-Hydroxy-1-isopropyl-2,2-dimethyl-4-oxo-azetidin-3-yl)-benzoic acid methyl ester		90:10 hexane / 2-propanol	[293]
4-(3-Methyl-2-oxo-tetrahydro-furan-3-yl)-benzoic acid ethyl ester		90:10 hexane / ethanol	[294]
3-Hydroxy-1,3,4,5-tetrahydro-benzo[b]azepin-2-one		97:3 hexane / 2-propanol	[295]
4-Methyl-3,6-dioxabicyclo[3.1.0]hexan-2-one		10:1 hexane / 2-propanol	[296]
3-Allyl-2-butyl-4-methyl-1-[(4-methylphenyl)sulphonyl]-2,5-dihydro-1H-pyrrole		97:3 hexane / 2-propanol	[297]
2-tert-Butoxycarbonylamino-3-thiazol-4-yl-propionic acid		65:35 hexane / 2-propanol	[298]
5-[2-[2-(2-Ethoxy-phenoxy)-ethylamino]-propyl]-2-methoxy-benzenesulphonamide, Tamsulosin		Not reported	[299]
3-Methyl-4-oxo-4-pyridin-2-yl-butylaldehyde		90:10 heptane / 2-propanol	[300]
Metaproterenol		90:10 hexane / ethanol / 0.2% butylamine	[301]

Name	Structure	Mobile Phase	Ref.
di-O,O-(p-Toluyyl)-D-tartaric acid		90:10 hexane / 2-propanol	[302]
[3a,4-Dimethyl-hexahydro-benzofuran-(2 <i>E</i>)-ylidene]-acetic acid isopropyl ester		30:1 hexane / ethanol	[303]
2,6-Diethoxy-1,5-dimethyl-2,6-diphenyl-3,7-dioxa-bicyclo[3.3.1]nonane		100 % hexane	[304]
2-(<i>tert</i> -Butyl-dimethyl-silanyloxy)-2-(2-nitro-phenyl)-pent-4-enal		99:1 heptane / 2-propanol	[305]
1-Cyclohexenyl-1-oxopent-4-en-2-yl acetate		99:1 heptane / 2-propanol	[306]
Pilocarpine, 3-Ethyl-4-(3-methyl-3H-imidazol-4-ylmethyl)-dihydro-furan-2-one		30:70 hexanes / ethanol	[307]

Name	Structure	Mobile Phase	Ref.
Imino thiazoline derivative		90:10 hexane / 2-propanol	[308]
2,2,2-Trifluoro-N-[2-hydroxy-1-phenylethyl]acetamide		70:30 hexane / 2-propanol	[309]
Table 6.14. Conditions for chiral separations of selected compounds using cellulose tris(4-Methyl-phenylcarbamate)coated on silica (Chiralcel OG®) stationary phase			
Name	Structure	Mobile Phase	Ref.
N-(2-Hydroxy-1-phenylethyl)-4-methylbenzenesulphonamide		70:30 hexane / 2-propanol	[310]
3-Phenyl-cyclopentanol		90:10 hexane / 2-propanol	[311]
2-Acetylamino-2-[5-trimethylsilanyl-penta-2,3-dienyl]-malonic acid diethyl ester		98:2 hexane / 2-propanol	[312]
2-Methyl-1,2,3,4,9,13b-hexahydro-2,4a,5-triazatribenzo[a,c,e]cycloheptene		98:2:0.1 hexane / ethanol / diethylamine	[313]
Z-ketoxime derivative		50:1 hexane / 2-propanol	[314]

Name	Structure	Mobile Phase	Ref.
Ethaboxam		70:30 hexane / ethanol	[315]
4-Phenyl-tetrahydro-pyran-2-one		90:10 hexane / 2-propanol	[316]
Metaproterenol		90:10 hexane / ethanol / 0.2% butylamine	[301]
Butyric acid 2'-hydroxy-[1,1']binaphthalenyl-2-yl ester		15:1 hexane / 2-propanol	[317]
19-Methyl-6,7,9,10-tetrahydro-dibenzo[b,g][1,3]thiazolo[3,2-d][1,9,12,4,6]trioxadiazacyclo tetradecine		60:40 hexane / 2-propanol	[308]
Diisopropyl 1-(3-benzyl-5-chloro-2-oxindolin-3-yl)hydrazine-1,2-dicarboxylate		90:10 hexane / 2-propanol	[318]
2,6-Dimethylphenyl 3-(1,3-benzodioxol-5-yl)-3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propanoate		90:10 hexane / 2-propanol	[319]
2,6-Dimethylphenyl (3R)-3-(3,5-dimethylphenyl)-3-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)propanoate		90:10 hexane / 2-propanol	[319]

Table 6.15. Conditions for chiral separations of selected compounds using cellulose tris(4-Chloro-phenylcarbamate) coated on silica (Chiralcel OF®) stationary phase

Name	Structure	Mobile Phase	Ref.
Econazole		425:74:1 hexane / 2-propanol / diethylamine	[320]
3-Methyl-1-(2-oxo-3-phenyl-tetrahydro-pyran-4-ylmethyl)-piperidine-3-carboxylic acid ethyl ester		Gradient 96:4 CO ₂ / methanol to 60:40 CO ₂ / methanol (SFC*)	[321]
2-Acetyl-4,4-dimethyl-2-(3-phenyl-allyl)-cyclohexanone		96:4 hexane / 2-propanol	[322]
6-Bromo-7,8-dihydrobenzo[def]chrysene-7,8-diol		50:50 hexane / 2-propanol	[323]
3-Benzyloxymethyl-5-hydroxy-2-phenyl-1-pyrrolidin-1-yl-pentan-1-one		Gradient 96:4 CO ₂ / methanol to 60:40 CO ₂ / methanol (SFC*)	[324]
2-(1-Methyl-1H-indol-3-yl)-1,2-diphenyl-ethanol		91:9 hexane / 2-propanol	[325]
3-(2-Nitro-1-p-tolyl-ethyl)-2-phenyl-1H-indole		85:15 hexane / 2-propanol	[326]

Name	Structure	Mobile Phase	Ref.
3-Hydroxy-pyrrolidine-1-carboxylic acid tert-butyl ester		4:1 hexane / 2-propanol	[327]
3,5-Dinitro-benzoic acid 4-(tert-butyl-dimethyl-silanyloxy)-1,3,3-trimethyl-butyl ester		Gradient 99:1 to 97:3 hexane : 2-propanol	[328]
1-[2-(3,3-Dimethyl-but-1-ynyl)-pyrimidin-5-yl]-2-methyl-propan-1-ol		70:30 CO ₂ / methanol	[143]
<i>sn</i> -1/3-Monocaproylglycerol (<i>sn</i> -1/3-6:0)		95:5 hexane / 2-propanol	[329]
anti-1-(2- <i>tert</i> -Butylphenyl)-3-(<i>tert</i> -butylthiomethyl)pyrrolidine-2,5-dione		90:10 hexane / 2-propanol	[330]
<i>tert</i> -Butyl 3-(4-aminophenylthio)-2-phthalimidopropionate-2-d1		50:50 hexane / 2-propanol	[330]

* SFC - supercritical fluid chromatography

Table 6.16. Conditions for chiral separations of selected compounds using cellulose tribenzoate coated on macroporous silica(Chiralcel OB/OB-H®) stationary phase

Name	Structure	Mobile Phase	Ref.
5-Methyl-3-phenyl-cyclopent-2-enone		90:10 hexane / 2-propanol	[331]
2-Furan-2-yl-3-(2-trimethyl-silanyl-vinyl)-cyclopropane-carboxylic acid methyl ester		200:1 hexane / 2-propanol	[332]
4-Dimethylamino-2-(6-methoxy-naphthalen-2-yl)-butan-2-ol		98:2:0.1 hexane / ethanol / diethylamine	[333]
Acetic acid 1-methyl-3-phenyl-allyl ester		80:20 hexane / 2-propanol	[334]
3-(1-Methyl-1-nitro-ethyl)-cyclohexanone		80:20 hexane / ethanol	[335]
Phenyl-o-tolyl-methanol		9:1 hexane / 2-propanol	[336]

Name	Structure	Mobile Phase	Ref.
2-Methanesulphinyl-pyridine		85:15 hexane / 2-propanol	[337]
1,2-Di-thiophen-2-yl-ethane-1,2-diol		85:15 hexane / 2-propanol	[338]
2-Methyl-1-phenyl-propan-1-ol		95:5 hexane / 2-propanol	[339]
Benzoic acid 1-methyl-butyl ester		49:1 hexane / 2-propanol	[327]
(4-Methoxy-phenyl)-(1-phenyl-ethyl)-amine		85:15 CO ₂ / methanol (SFC*)	[340]
<i>E</i> -1,3-Di(2-methylphenyl)-but-1-ene		98:2 heptane / 2-propanol	[341]
(2-Chloro-phenyl)-thiophen-2-yl-methanol		98:2 hexane / 2-propanol	[342]

Name	Structure	Mobile Phase	Ref.
<i>tert</i> -Butyl(dimethylamino)phenyl phosphine-borane complex		99:1 hexane / 2-propanol	[343]
(3-Chloro-phenyl)-trimethylsilanyloxy-acetonitrile		99.75:0.25 hexane / 2-propanol	[344]
1-(4-Chloro-phenyl)-propan-1-ol		98:2 hexanes / ethanol	[345]
2-Hydroxy-1,4-diphenyl-pent-4-en-1-one		97:3 hexane / 2-propanol	[346]
1-[2-(3,3-Dimethyl-but-1-ynyl)-pyrimidin-5-yl]-2-methyl-propan-1-ol		Gradient 96-60:4-40 CO ₂ / methanol	[143]
1-(Methanesulphonyl)-2-methyl-benzene		80:20 hexane / 2-propanol	[347]
4-(2-Methyl-1-oxo-1,2,3,4-tetrahydro-naphthalen-2-yl)-benzonitrile		80:20 hexane / 2-propanol	[348]
2-(Cyclopentyl-hydroxy-methyl)-cyclohex-2-enone		99:1 hexane / 2-propanol	[349]

Name	Structure	Mobile Phase	Ref.
3-Phenylcyclopentanone		99.75:0.25 hexane / 2-propanol	[350]
4-[1-Hydroxypropyl]phenol		90:10 hexane / 2-propanol	[351]
1-(Pyridin-2-yl)ethyl acetate		95:5 hexane / 2-propanol	[352]
2-But-3-enyl benzoate		1000:1 hexane / 2-propanol	[213]
(<i>SR,RS</i>)-2-Ethenyl-1-phenylpropane-1,3-diol	(<i>SR,RS</i>)-2-ethenyl-1-phenylpropane-1,3-diol	93:7 hexane / 2-propanol	[353]
5-(Benzyloxymethyl)-2,2,5-trimethylheptan-3-one		0.05:99.95 2-propanol / hexane	[354]

* SFC – supercritical fluid chromatography

Table 6.17. Conditions for chiral separations of selected compounds using cellulose tricinamate coated on silica gel (Chiralcel OK®) stationary phase

Name	Structure	Mobile Phase	Ref.
Econazole		425:74:1 hexane / 2-propanol / diethylamine	[320]
Miconazole		425:74:1 hexane / 2-propanol / diethylamine	[320]
Sulconazole		425:74:1 hexane / 2-propanol / diethylamine	[320]
2-Phenyl-4,5-dihydro-oxazole-4-carboxylic acid methyl ester		90:10 hexane / 2-propanol	[355]
2-Phenoxy-propionic acid butyl ester		Not reported	[356]
Phosphine borane derivative		80:20 hexane / ethanol	[357]

Name	Structure	Mobile Phase	Ref.
2-[(3-Chloro-phenyl)-hydroxy-methyl]-acrylic acid		90:10 hexane / 2-propanol	[358]
2-(4-Ethyl-phenoxy)-propionic acid		98:2:0.1 hexane / 2-propanol / trifluoroacetic acid	[359]
2-Phenoxy-propionic acid		98:2:0.1 hexane / 2-propanol / trifluoroacetic acid	[359]

Table 6.18. Conditions for chiral separations of selected compounds using cellulose tris(3,5-dichlorophenylcarbamate) photochemically immobilised on silica (Chiralpak IC®) stationary phase

Name	Structure	Mobile Phase	Ref.
Benzoin		60:20:20 hexane / dimethoxymethane / tetrahydrofuran	[360]
1-Naphthalen-2-yl-propane-1,3-diol		10:1 hexane / 2-propanol	[361]
3-(Dimethoxymethyl)-6-methoxy-2-(4-nitrophenyl)tetrahydro-2H-pyran		50:1 hexane / 2-propanol	[362]

Name	Structure	Mobile Phase	Ref.
7a-Benzyl-2,3,7,7a-tetrahydro-6H-indene-1,5-dione		60:40 hexane / 2-propanol	[363]
3-Formyl-2-nitromethylhexanoic acid methyl ester		90:10 hexane / ethanol	[364]
3-(4-Chloro-phenyl)-4-nitro-butan-1-ol		10:1 hexane / 2-propanol	[361]
2-Phenylglutaric anhydride		90:10 CO ₂ / tetrahydrofuran (SFC)	[284]
Terazosin		90:10:0.1 methanol / H ₂ O / diethylamine	[365]
[4-(4-Amino-6-hydroxy-7-methoxyquinazolin-2-yl)pi-perazin-1-yl][(5S)-5-methyltetrahydrofuran-2-yl]methanone		95:5:0.1 methanol / H ₂ O / diethylamine	[365]
1,1,1,2,3,3,3,4,4,4,5,5,5,6,6,7,7,8,8-Hepta-decafluoro-N-(4-methoxybenzyl)octane-2-sulphonamide		40:60 0.1% Aqueous acetic acid / methanol + 0.1% acetic acid	[366]

Name	Structure	Mobile Phase	Ref.
[3-(6-Benzyloxy-hexyl)-oxiranyl]-methanol		95:5 hexane / 2-propanol	[367]
5,5-Diphenyl-4-methyl-2-oxazolidinone		40:60 acetonitrile / H ₂ O	[368]
(<i>SS,RR</i>)-2-[1-(2-Chlorophenyl)-2-nitroethyl]pentanal		85:15 hexane / 2-propanol	[369]
Methyl 2-(2-formylphenyl)-2-phenylpropanoate		5:95 2-propanol / hhexane	[370]
<i>tert</i> -Butyl 2-methylidene-3-[[[(4-methylphenyl)sulphonyl]amino]hexanoate		80:20 hexane / 2-propanol	[371]
<i>tert</i> -Butyl (<i>RR</i>),(<i>SS</i>)-3-methyl-5-nitro-2-oxo-4-phenylpentanoate		98:2 hexane / ethanol	[372]

Table 6.19. Conditions for chiral separations of selected compounds using amylose tris[(S)alpha-phenethyl]carbamate coated on silica gel (Chiralpak AS®) stationary phase

Name	Structure	Mobile Phase	Ref.
Dia-(1 <i>S</i> 3 <i>R</i> ,1 <i>R</i> 3 <i>R</i>)-3-benzyl-oxymethyl-1,3-dihydro-iso-benzofuran-1-thymine		80:20 hexane / ethanol	[185]
Dia-(1 <i>R</i> 3 <i>R</i> ,1 <i>S</i> 3 <i>R</i>)-3-benzyl-oxymethyl-1,3-dihydro-iso-benzofuran-1-uracil		80:20 hexane / 2-propanol	[185]
Bromo- <i>p</i> -tolyl-acetic acid benzyl ester		99.8:0.2 hexane / 2-propanol	[373]
2-(3-Bromo-phenyl)-oxirane		90:10 hexane / 2-propanol	[374]
6-Methyl-5-(<i>N</i> -phenyl-aminooxy)-hept-3-en-2-one		98:2 hexane / 2-propanol	[375]
3-(4-Cyanophenyl)-2,3-epoxy-1-phenylpropanone		9:1 hexane / 2-propanol	[376]
2-(1-Hydroxy-propyl)-cyclohex-2-enone		Not reported	[377]
Ethaboxam		80:20 hexane / 2-propanol	[315]

Name	Structure	Mobile Phase	Ref.
1-(2'-Hydroxymethyl-[1,1']binaphthalenyl-2-ylmethyl)-cyclohexanol		hexane / 2-propanol	[378]
Atenolol		90:10 hexane / ethanol / 0.2% butylamine	[301]
Chlorthalidone		100 methanol	[196]
5-Benzyl-3-(4-chlorophenyl)-4,6-dioxo-octahydro-pyrrolo[3,4-c]pyrrole-1-carboxylic acid methyl ester		50:50 hexane / 2-propanol	[379]
Pantoprazole		50:50:0.1 Heptane / ethanol / triethylamine	[258]

Name	Structure	Mobile Phase	Ref.
N-Allyl-N-[1-(2-chloro-phenyl)-ethyl]-2,2,2-trifluoroacetamide		99:1 hexane / 2-propanol	[380]
<i>tert</i> -Butyl (benzyloxy)[6-oxodecan-4-yl]carbamate		99.5:0.5 hexane / 2-propanol	[146]
6-[2-[4-(3-Fluorophenyl)piperazin-1-yl]propanoyl]-1,3-benzoxazol-2(3H)-one		90:10 hexane / ethanol	[381]
Methanesulphonic acid 1-(6-chloro-pyridin-3-yl)-ethyl ester		70:30 Isohexane / 2-propanol	[382]
3-(2-Nitro-1-p-tolyl-ethyl)-pentane-2,4-dione		85:15 hexane / 2-propanol	[383]
Triadimenol-(1 <i>RS</i> ,2 <i>SR</i>)-1-(4-chlorophenoxy)-3,3-dimethyl-1-(1 <i>H</i> -1,2,4-triazol-1-yl)butan-2-ol		2:98 ethanol / hexane	[384]
2-Ethylhexyl 3-[(2-[[2-phenylpropanoyl]amino]phenyl)sulphonyl]propanoate		H ₂ O / acetonitrile (gradient 40:60 (0 min), 10:90 (30 min)) to (gradient 40:60 to 10:90 in 30 min)	[290]

Name	Structure	Mobile Phase	Ref.
(Z)-5-Hydroxy-5-phenyl-oct-2-enitrile		20:1 hexane / 2-propanol	[385]

6.4.6.2. Antibiotic Phases

The glycopeptide macrocycle antibiotics are classes of compounds with highly enantioselective properties. They include vancomycin, teicoplanin and ristocetin A [386]. Such high enantioselective properties are due to their amphoteric character, their molecular structure (which in solution accentuates enantioselective interactions) and their hydrophilic and hydrophobic functional groups (which makes them soluble in aqueous and organic solvents) [387]. Such properties make them amenable for use as chiral selectors in either capillary electrophoresis or HPLC (Tables 6.20-6.22). There have been over a hundred different glycopeptide antibiotics identified in the fermentation broth of various bacteria. All have a heptapeptide core of seven amino acid residues with a covalently joined side chain and sugar substituents at various positions. In this classification scheme, vancomycin and eremomycin are assigned to group I. Groups II and III include compounds such as avoparcin, ristocetin and teicoplanin, which have aromatic residues at different positions in the molecule [388]. They possess multiple stereogenic centres and can interact with a solute through hydrogen bonding, dipole-dipole interactions, π - π interactions, hydrophobic interactions, electrostatic interactions and sterically-based interactions. Due to the multiple interactions that they can exhibit, these macrocyclic antibiotics have been immobilised on a silica matrix and used as chiral stationary phases. These columns show very good selectivity toward a large class of compounds [389]. They operate in different modes: normal phase, polar organic and sub/supercritical modes [125,389]. In reversed phase mode, the mobile phase should be buffered in the range of pH between 4 and 7. Electrostatic and hydrophobic interactions are the main types of interactions responsible for the enantiomeric separation on these phases.

Table 6.20. Mobile phase compositions for enantiomeric separation of selected compounds on Vancomycin bonded to silica (Chirobiotic V®) stationary phase

Name	Structure	Mobile Phase	Ref.
N-CBZ-Phenylalanine		90:10 1% triethylammonium acetate, pH 4.1 / acetonitrile	[184]

Name	Structure	Mobile Phase	Ref.
Nisoldipine		50:50 triethylammonium acetate buffer / methanol	[390]
Norephedrine		30-90% methanol or acetonitrile / aqueous 0.1% triethylammonium acetate (pH 4.0)	[391]
6-Hydroxybuspirone		98.8:0.8:0.4 methanol / acetic acid / triethylamine	[392]
5-Amino-6,7,8,9-tetrahydro-5H-benzocycloheptene-6-carboxylic acid		10:90 0.1% triethylammonium acetate pH 6.5 / methanol	[393]
1-[2-(3,3-Dimethyl-but-1-ynyl)-pyrimidin-5-yl]-2-methyl-propan-1-ol		Gradient 4% to 40% / methanol (SFC*)	[143]
Pregabalin (and its <i>R</i> -enantiomer)		80:20 ethanol / H ₂ O	[394]

Name	Structure	Mobile Phase	Ref.
4-Biphenyl-4-yl-2,7,7-trimethyl-5-oxo-1,4,5,6,7,8-hexahydro-quinoline-3-carboxylic acid ethyl ester		80:20 hexane / 2-propanol	[395]

* SFC – supercritical fluid chromatography

Table 6.21. Mobile phase compositions for enantiomeric separation of selected compounds on Amphoteric teicoplanin covalently bonded to silica (Chirobiotic T[®]) stationary phase

Name	Structure	Mobile Phase	Ref.
N-CBZ-Phenylalanine		80:20 Triethylammonium acetate pH 4.1 / methanol	[184]
5,9-Dimethyl-2-(2-methyl-propenyl)-2,3-dihydro-furo[3,2-g]chromen-7-one		95:5 CO ₂ / methanol (SFC*)	[396]
2-Amino-3-(4-trifluoromethyl-phenyl)-propionic acid		70:30 methanol / H ₂ O	[397]
2-Aminomethyl-3-(3,4-dihydroxy-phenyl)-propionic acid		70:30 methanol / H ₂ O	[398]
Tyrosine		20:80 H ₂ O (0.2% triethylammounium acetate, pH 3.8) / methanol	[399]

Name	Structure	Mobile Phase	Ref.
2-[4-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-3-oxo-1,3,4,5-tetrahydro-benzo[c]azepin-2-yl]-propionic acid		100:0.1:0.1 methanol / acetic acid / triethylamine	[400]
3-Amino-4-methyl-pentanoic acid		70:30 methanol / 0.1% triethylammounium acetate (pH 4.1)	[401]
2-Aminomethylpentanoic acid		30:70 0.1% triethylammonium acetate (pH 4.1) / methanol	[402]
Warfarin		80:20 heptane / ethanol	[403]

* SFC – supercritical fluid chromatography

Table 6.22. Mobile phase compositions for enantiomeric separation of selected compounds on Ristocetin A covalently bonded to silica gel (Chirobiotic R®) stationary phase

Name	Structure	Mobile Phase	Ref.
1,2,3,6-Tetrahydro-pyridine-2-carboxylic acid (baikaine)		50:50 H ₂ O / methanol	[404]
γ -phenyl- γ -butyrolactone		95:5 hexane / ethanol	[405]
α -benzyl-proline		Various percentages of methanol / H ₂ O or triethylammonium acetate 0.1% / methanol pH 4.1	[152]

Name	Structure	Mobile Phase	Ref.
4-Benzoyloxymethyl-3-phenyl-tetrahydro-pyran-2-one		Gradient of 96:4 CO ₂ / methanol to 60:40 CO ₂ / methanol	[324]
6a,11a-Dihydro-6H-benzo[4,5]furo[3,2-c]chromene		60:40 H ₂ O / methanol	[73]
3,4-Dihydroxyphenylalanine		1:1 methanol / H ₂ O	[406]
[(<i>SS,RR</i>)-4-(1,3-Dioxo-1,3-dihydro-isoindol-2-yl)-5-methyl-3-oxo-1,3,4,5-tetrahydro-benzo[<i>c</i>]azepin-2-yl]-acetic acid		100:0.01:0.01 methanol / CH ₃ CO ₂ H / triethylamine	[400]
Camptothecin		100:0.1:0.1 methanol / CH ₃ CO ₂ H / triethylamine	[286]
(1 <i>RS</i> ,2 <i>SR</i> ,4 <i>SR</i> ,5 <i>RS</i>)-2-Amino-4,5-dihydroxy-cyclohexane-carboxylic acid		20:80 0.1% Aqueous triethylammonium acetate pH 4.1 / ethanol	[407]
(1 <i>SR</i> ,2 <i>RS</i> ,3 <i>SR</i>)-2-Amino-3-hydroxy-cyclopentane-carboxylic acid		20:80 0.1% Aqueous triethylammonium acetate pH 4.1 / ethanol	[407]

6.4.6.3. Protein Phases

Proteins are biopolymers consisting of amino acids linked together through peptide bonds arranged in a certain sequence. Molecular recognition in protein-ligand complexes is responsible for the selective binding of a low molecular weight ligand to a specific protein which can be characterised by a binding constant. All these interactions are usually non-covalent in nature. The experimentally determined binding constants, K_b , are typically in the range of 10^{-2} to 10^{-12} M $^{-1}$, which corresponds to a negative free energy of binding of 10 to 80 kJmol $^{-1}$ [408]. The interactions between ligands and proteins include a wide range of interactions including electrostatic, van der Waals, steric, hydrophobic and hydration forces [409] related to the active sites of the protein. The binding of a small molecule ligand to a protein requires shape and property complementarity. In the course of binding, the ligand and protein have to adapt to each other to achieve a successful recognition process. The small molecule ligand is usually the more flexible partner, and thus can adopt a large variety of different low energy conformations upon interaction with a protein [408]. The enantioselective recognition of molecules with one chiral centre requires a protein to interact with a minimum of three substrate locations, while stereoselectivity towards a substrate with two or three stereocentres requires interactions with a minimum of four or five substrate locations, respectively [410]. The inherent chirality of proteins makes them very good candidates for the separation of enantiomers. Proteins which can tolerate organic solvents, as well as high temperatures, and which can function over a wide pH range are useful as chiral stationary phases. Several proteins such as α_1 -acid glycoprotein (AGP) (Table 6.23), bovine serum albumin (BSA) (Table 6.24), human serum albumin (HSA), ovomucoid from chicken egg whites (OVM) (Table 6.25), avidin, cellobiohydrolase I (CBH I) (Table 6.26), pepsin, ovotransferrin and β -lactoglobulin have been immobilised on silica gel and successfully used as CSPs for enantiomeric separation [389]. Mobile phase optimisation with these CSPs is done through variations in pH, ionic strength, temperature and organic modifiers. Enantioselectivity can be changed as a function of pH. Increasing ionic strength increases hydrophobic interactions between the enantiomers and the CSP. The nature of the organic modifier also influences the enantioselectivity, as reversal of the elution order has been observed to be dependent upon the organic modifier used. Temperature also influences enantioselectivity; a decrease in temperature generally enhances enantioselectivity. Reversal of elution order has also been observed with a change in temperature. The protein CSPs have a broad range of applications and have been used for the separation of neutral, acidic and basic enantiomeric drugs [125].

Table 6.23. Mobile phase compositions for enantiomeric separation of selected compounds on α_1 -acid glycoprotein silica (Chiral-AGP®) stationary phase

Name/Structure	Structure	Mobile Phase	Ref.
----------------	-----------	--------------	------

Name/Structure	Structure	Mobile Phase	Ref.
1-(2,2-Dimethyl-8,8a-dihydro-3aH-indeno[1,2-d]oxazol-3-yl)-3-phenylpropan-1-one		88:12 10 mM potassium phosphate buffer (pH 7) / 2-propanol	[411]
(2-Phenyl-2,3-dihydro-benzofuran-3-yl)-methanol		90:10 0.01M phosphate buffer / 2-propanol	[25]
Bupivacaine		97:3 H ₂ O / 2-propanol ammonium acetate 5 mM (pH 7.0)	[412]
Bromoketamine		94:6 10 mM ammonium acetate buffer (pH 7.6) / 2-propanol	[413]
5-Ethyl-1,2-dimethyl-4,4-diphenyl-3,4-dihydro-2H-pyrrolium		Gradient of methanol / 20 mM ammonium formate (pH 5.7)	[414]
Reboxetine, 2-[(2-Ethoxyphenoxy)-phenyl-methyl]-morpholine		12:88 acetonitrile / phosphate buffer (pH 6)	[415]
EDDP D3		82:18 ammonium acetate buffer 10 mM (pH 7.0) / CH ₃ CN	[416]

Name/Structure	Structure	Mobile Phase	Ref.
Ibuprofen		990:10:0.2 phosphate buffer (pH 6.9) / acetonitrile / dimethyloctylamine	[417]
<i>tris</i> (1,2-Dimethyl-3-indolyl)phosphine oxide		30:70 CH ₃ CN / phosphate buffer (pH 4.6)	[418]
Pindolol		90:10 phosphate buffer / acetonitrile	[419]
2-(2-Fluoro-phenyl)-4,5-di-phenyl-4,5-dihydro-1H-imidazole		80:20 0.1% acetic acid, H ₂ O with NH ₄ OH (pH 7) / acetonitrile	[231]
N-(2,4-Dinitrophenyl)tyrosinol		98:2 sodium phosphate buffer 10 mM (pH 4) / acetonitrile	[88]
<i>trans</i> -2-(4-Methoxyphenyl)-3-phenyl-1-tosylaziridine		1:99 2-propanol / aqueous 10 mM sodium phosphate buffer (pH 7)	[420]

Table 6.24. Mobile phase compositions for enantiomeric separation of selected compounds on Bovine serum albumin (BSA) immobilised on silica gel (Chiral BSA-RP®) stationary phase

Name/Structure	Structure	Mobile Phase	Ref.
2-Phenyl-pentanenitrile		88:10:2 phosphate buffer (0.1 M, pH 7.4) / acetonitrile/ 1-butanol	[421]
N-Benzoylisoleucine		50 mM phosphate buffer (pH 7)	[422]
Ibuprofen		50 mM phosphate buffer (pH 7.8) / 1-propanol 4.5% / octanoic acid 4 mM	[423]
Leucovorin		98:2 50 mM Na ₂ HPO ₄ buffer (pH 5.2) / 1-propanol	[424]
N-Formyltetrahydrofolate		10 mM sodium phosphate buffer (pH 7.0)	[425]

Table 6.25. Mobile phase compositions for enantiomeric separation of selected compounds on Ovomucoid protein immobilised to silica (OVM®) stationary phase

Name/Structure	Structure	Mobile Phase	Ref.
----------------	-----------	--------------	------

Name/Structure	Structure	Mobile Phase	Ref.
Ibuprofen		90:10 20 mM phosphate buffer (pH 5.1) / ethanol	[426]
Benzoin		90:10 20 mM phosphate buffer (pH 5.1) / ethanol	[426]
NK-104 3-epimer and enantiomer		100:5:10 20 mM KH ₂ PO ₄ in H ₂ O / acetonitrile / methanol	[427]

Table 6.26. Mobile phase compositions for enantiomeric separation of selected compounds on Cellobiohydrolase (CBH I) immobilised onto silica (Chiral-CBH®) stationary phase

Name/Structure	Structure	Mobile Phase	Ref.
4-(2-Methanesulphonyl-4-methoxy-phenyl)-piperidine		2-propanol	[428]
O-Desethyl-reboxetine		6:9:85 acetonitrile / 2-propanol / 50 μM EDTA in 10 mM phosphate buffer (pH 6.1)	[429]
Synephrine		95:5 10 mM sodium phosphate buffer (pH 6.0) / 2-propanol	[430]

6.5. CONCLUSION

The separation of enantiomers is a technique which is driven by the development of enantiomerically pure drugs. A large number of chiral stationary phases are available on the market, providing the opportunity to choose the right column for the separation of enantiomeric drugs. Understanding the interactions between these CSPs and enantiomeric analytes can provide a rationale for the development of an enantiomeric separation method.

REFERENCES

1. N. Grinberg. Chiral Separation in Pharmaceutical Industry. *American Pharmaceutical Reviews* **65** (2006) 12.
2. B. Feibush, N. Grinberg. The history of enantiomeric resolution. In *Chromatographic Chiral separations*, (Editors M. Zief, L.J. Crane) *Marcel Dekker Inc.*, 1988, p. 1.
3. C.E. Dalglish. The optical resolution of aromatic amino acids on paper chromatograms, *Journal of the Chemical Society* (1952) 3940.
4. S. Allenmark. Separation of enantiomers by protein-based chiral phases. In *A practical approach to chiral separations by liquid chromatography* (Editor G. Subramanian) *VCH Weinheim* 1994, p. 183.
5. E. Gil-Av, B. Feibush, R. Charles-Sigler. Separation of enantiomers by gas chromatography with an optically active stationary phase. *Tetrahedron Letters* **7** (1966) 1009-1015.
6. W.H. Pirkle, T. Pochapsky. Theory and design of chiral stationary phases for direct chromatographic separation of enantiomers. In *Packings and stationary phases in chromatographic techniques*. (Editor K.K. Unger) *Marcel Dekker, Inc.*, New York, 1990, p. 783.
7. B. Feibush. Chiral separation of enantiomers via selector/selectand hydrogen bondings. *Chirality* **10** (1998) 382-395.
8. S. Topiol. A general criterion for molecular recognition: Implications for chiral interactions. *Chirality* **1** (1989) 69-79.
9. G.J. Jeffrey. An introduction to hydrogen bonding. *Oxford University Press*, New York, 1997.
10. G. Zundel. Easily polarizable hydrogen bonds - their interactions with the environment - ir continuum and anomalous large proton conductivity. In *The hydrogen bond. Recent developments in theory and experiments. II Structure and spectroscopy. Vol. II.* (Eds. P. Schuster, G. Zundel, C. Sandorfy) *North Holland Publishing Company*, Amsterdam, 1976, p. 683.
11. T.E. Creighton. Proteins Structure and molecular properties, *W. H. Freeman*, New York, 1993.
12. N. Grinberg, T. Burakowski, A. M. Stalcup. Chiral separation. In *HPLC for pharmaceutical scientists* (Eds. Y. Kazakevich, R. Lobrutto) *John Wiley & Sons*, Hoboken, New Jersey, 2007, p. 987,
13. B. Koppenhoefer, E. Bayer. Chiral recognition in the resolution of enantiomers by GLC. *Chromatographia* **19** (1984) 123-130.

14. B. Koppenhoefer, E. Bayer. Chiral recognition in gas chromatographic analysis of enantiomers on chiral polysiloxanes. *Journal of Chromatography Library* **32** (1985) 1-42.
15. Y. Dobashi, S. Hara. A chiral stationary phase derived from (R,R)-tartramide with broaden scope of application to the liquid chromatographic resolution of enantiomers. *The Journal of Organic Chemistry* **52** (1987) 2490-2496.
16. G. Blaschke. Chromatographic resolution of chiral drugs on polyamides and cellulose triacetate. *Journal of Liquid Chromatography* **9** (1986) 341-368.
17. G. Blaschke. Substituted polyacrylamides as chiral phases for the resolution of drugs. In *Chromatographic chiral separation*. (Eds. M. Zieff and L.J. Crane) Marcel Dekker, Inc., New York, 1988, p. 179.
18. I. Abe, K. Terada, T. Nakahara. Enantiomer separation of pharmaceuticals by capillary gas chromatography with novel chiral stationary phases. *Biomedical Chromatography* **14** (2000) 125-129.
19. G.P. Kaijser, J.H. Beijnen, A. Bult, H.J. Keizer, W.J.M. Underberg. Chromatographic analysis of the enantiomers of ifosfamide and some of its metabolites in plasma and urine. *Journal of Chromatography B: Biomedical Sciences and Applications* **690** (1997) 131-138.
20. A.S.C. Chan, W. Hu, C.-C. Pai, C.-P. Lau, Y. Jiang, A. Mi, M. Yan, J. Sun, R. Lou, J. Deng. Novel Spiro Phosphinite Ligands and Their Application in Homogeneous Catalytic Hydrogenation Reactions. *Journal of the American Chemical Society* **119** (1997) 9570-9571.
21. I. Abe, N. Fujimoto, T. Nishiyama, K. Terada, T. Nakahara. Rapid analysis of amino acid enantiomers by chiral-phase capillary gas chromatography. *Journal of Chromatography A* **722** (1996) 221-227.
22. C.A. Bewley, H. He, D.H. Williams, D.J. Faulkner. Aciculitins A-C: Cytotoxic and Antifungal Cyclic Peptides from the Lithistid Sponge *Aciculites oreintalis*. *Journal of the American Chemical Society* **118** (1996) 4314-4321.
23. A. Berthod, E.Y. Zhou, K. Le, D.W. Armstrong. Determination and use of Rohrschneider-McReynolds constants for chiral stationary phases used in capillary gas chromatography. *Analytical Chemistry* **67** (1995) 849-857.
24. B. Koppenhoefer, U. Muehleck, K. Lohmiller. Backbone modification of Chirasil-Val: Effect of nonpolar side chains on enantiomer separation in gas chromatography. *Chromatographia* **40** (1995) 718-723.
25. L. Juhasz, L. Szilagyi, S. Antus, J. Visy, F. Zsila, M. Simonyi. New insight into the mechanism of hypervalent iodine oxidation of flavanone. *Tetrahedron* **58** (2002) 4261-4265.
26. M. Johannsen, S. Peper, A. Depta. Simulated moving bed chromatography with supercritical fluids for the resolution of bi-naphthol enantiomers and phytol isomers. *Journal of Biochemical and Biophysical Methods* **54** (2002) 85-102.
27. T. Raab, T. Hauck, A. Knecht, U. Schmitt, U. Holzgrabe, W. Schwab. Tautomerism of 4-hydroxy-2,5-dimethyl-3(2H) furanone: Evidence for its enantioselective biosynthesis. *Chirality* **15** (2003) 573-578.
28. F. La Torre, R. Cirilli, R. Ferretti, B. Gallinella, R. Costi, R. Di Santo. Conversion of a racemic mixture of 8-chloro-2-(2,6-difluorophenylmethyl)-2,3-dihydro-3-methyl-

- 1,2,5-benzothiadiazepin-4(5h)-one 1,1-dioxide into a single enantiomer via a chromatographic resolution/racemization method. *Chirality* **15** (2003) 429-432.
29. R. Cirilli, R. Costi, R. Di Santo, M. Artico, A. Roux, B. Gallinella, L. Zanitti, F. La Torre. Enantioselective liquid chromatography of C₃-chiral 2,3-dihydro-1,2,5-benzothiadiazepin-4(5H)-one and thione 1,1-dioxides on polyacrylamide- and polysaccharide-based chiral stationary phases. *Journal of Chromatography A* **993** (2003) 17-28.
 30. R. Kučera, M. Nobilis, L. Skalova, B. Szotakova, P. Cisai, T. Jira, J. Klimes, V. Wsol. Use of chiral liquid chromatography for the evaluation of stereospecificity in the carbonyl reduction of potential benzo[c]fluorene antineoplastics benfluron and dimefluron in various species. *Journal of Pharmaceutical and Biomedical Analysis* **37** (2005) 1049-1057.
 31. I.A. Sayyed, V.V. Thakur, M.D. Nikalje, G.K. Dewkar, S.P. Kotkar, A. Sudalai. Asymmetric synthesis of aryloxypropanolamines via OsO₄-catalyzed asymmetric dihydroxylation. *Tetrahedron* **61** (2005) 2831-2838.
 32. A.S. Paraskar, A. Sudalai. Enantioselective synthesis of (-)-cytoxazone and (+)-*epi*-cytoxazone, novel cytokine modulators via Sharpless asymmetric epoxidation and L-proline catalyzed Mannich reaction. *Tetrahedron* **62** (2006) 5756-5762.
 33. G. Cannazza, D. Braghiroli, P. Juliani, C. Parenti. Energy barrier determination of enantiomerization of chiral 3,4-dihydro-1,2,4-benzothiadiazine 1,1-dioxide type compounds by enantioselective stopped-flow HPLC. *Tetrahedron: Asymmetry* **17** (2006) 3158-3162.
 34. G. Cannazza, M.M. Carrozzo, D. Braghiroli, C. Parenti. Enantiomerization of chiral 2,3,3a,4-tetrahydro-1H-pyrrolo[2,1-c][1,2,4]benzothiadiazine 5,5-dioxide by stopped-flow multidimensional HPLC. *Journal of Chromatography B* **875** (2008) 192-199.
 35. G. Cannazza, M.M. Carrozzo, U. Battisti, D. Braghiroli, C. Parenti. On-line racemization by high-performance liquid chromatography. *Journal of Chromatography A* **1216** (2009) 5655-5659.
 36. D.J. Cram, G.M. Lein. Host-guest complexation. 36. Spherand and lithium and sodium complexation rates equilibria. *Journal of the American Chemical Society* **107** (1985) 3657-3668.
 37. S.G. Allenmark. Chromatographic enantioseparation: methods and applications, *Ellis Horwood Limited*, New York, 1988.
 38. W. Saenger, J. Jacob, K. Gessler, T. Steiner, D. Hoffman, H. Sanbe, K. Koizumi, S.M. Smith, T. Takaha. Structure of the common cyclodextrins and their larger analogues - beyond the doughnut. *Chemical Reviews* **98** (1998) 1787-1802.
 39. D.W. Armstrong, T.J. Ward, R.D. Armstrong, T.E. Beesley. Separation of drug stereoisomers by formation of β -cyclodextrin inclusion complexes. *Science* **232** (1986) 1132-1134.
 40. S.M. Han, Y.I. Han, D.W. Armstrong. Structural factors affecting the chiral recognition and separation on β -cyclodextrin bonded phase. *Journal of Chromatography A* **441** (1988) 376-381.
 41. J.I. Seeman, H.V. Secor, D.W. Armstrong, K.D. Timmons, T.J. Ward. Enantiomeric resolution and chiral recognition of racemic nicotine and nicotine analogues by β -cyclodextrins complexation. Structure-enantiomeric resolution relationship in host guest interaction. *Analytical Chemistry* **60** (1988) 2120-2127.

42. A. Berthod, L. Berthod, D.W. Armstrong. Selectivity of a native β -cyclodextrin column in the separation of catechins. *Journal of Liquid Chromatography & Related Technologies* **28** (2005) 1669-1678.
43. A.M. Stalcup, S.-C. Chang, D.W. Armstrong. Effect of the configuration of the substituents of derivatized β -cyclodextrin bonded phases on enantioselectivity in normal phase liquid chromatography. *Journal of Chromatography A* **540** (1991) 113-128.
44. D.W. Armstrong, A.M. Stalcup, M.L. Hilton, J.D. Duncan, J. J. R. Faulkner, S.-C. Chang. Derivatized cyclodextrins for normal phase liquid chromatographic separation of enantiomers. *Analytical Chemistry* **62** (1990) 1610-1615.
45. D.J. Cram, J.M. Cram. Host-guest-chemistry. *Science* **183** (1974) 803-809.
46. D.S. Lingenfelter, R.G. Helgeson, D.J. Cram. Host-guest complexation. 23. High chiral recognition of amino acid and ester guests by hosts containing one chiral element. *The Journal of Organic Chemistry* **46** (1981) 393-406.
47. T. Shimbo, T. Yamaguchi, H. Yanagishita, D. Kitamoto, K. Sakaki, M. Sugiura. Improved crown ether-based stationary phase. *Journal of Chromatography A* **625** (1992) 101-108.
48. T. Shimbo, T. Yamaguchi, K. Nishimura, M. Sugiura. Chromatographic separation of racemic amino acids by use of chiral crown ether-coated reversed-phase packings. *Journal of Chromatography A* **405** (1987) 145-153.
49. R.A. Thompson, Z. Gee, N. Grinberg, D. Ellison, P. Tway. Mechanistic aspects of the stereospecific interaction for aminoindanol with crown ether column. *Analytical Chemistry* **67** (1995) 1580-1587.
50. S. Chen, H. Yuan, N. Grinberg, A. Dovletoglou, G. Bicker. Enantiomeric separation of trans-2-aminocyclohexanol on a crown ether stationary phase using evaporative light scattering detection. *Journal of Liquid Chromatography & Related Technologies* **26** (2003) 425-442.
51. M.H. Hyun, J.S. Jin, W. Lee. Liquid chromatographic resolution of racemic amino acids and their derivatives on a new chiral stationary phase based on crown ether. *Journal of Chromatography A* **822** (1998) 155-161.
52. H. J. Choi, M.H. Hyun. Liquid chromatographic chiral separation by Crown Ether-based chiral stationary phases. *Journal of Liquid Chromatography & Related Technologies* **30** (2007) 853-875.
53. E. Bang, J.-W. Jung, W. Lee, D.W. Lee, W. Lee. Chiral recognition of (18-crown-6)tetracarboxylic acid as a chiral selector determined by NMR spectroscopy. *Journal of the Chemical Society, Perkin Transactions II* (2001) 1685-1692.
54. S.C. Ng, L. Chen, L.F. Zhang, C.B. Ching. Facile preparative HPLC enantioseparation of racemic drugs using chiral stationary phases based on mono-6^A-azido-6^A-deoxy-perphenylcarbamoylated β -cyclodextrin immobilized on silica gel. *Tetrahedron Letters* **43** (2002) 677-681.
55. O.A. Shpigun, E.N. Shapovalova, I.A. Ananieva, A.V. Pirogov. Separation and enantioseparation of derivatized amino acids and biogenic amines by high-performance liquid chromatography with reversed and chiral stationary phases. *Journal of Chromatography A* **979** (2002) 191-199.
56. S.C. Ng, T.T. Ong, P. Fu, C.B. Ching. Enantiomer separation of flavour and fragrance compounds by liquid chromatography using novel urea-covalent bonded

- methyated β -cyclodextrins on silica. *Journal of Chromatography A* **968** (2002) 31-40.
57. M. Kaspereit, P. Jandera, M. Skavrada, A. Seidel-Morgenstern. Impact of adsorption isotherm parameters on the performance of enantioseparation using simulated moving bed chromatography. *Journal of Chromatography A* **944** (2002) 249-262.
 58. D.W. Armstrong, Y.S. Liu, L.F. He, K.H. Ekborg-Ott, C.L. Barnes, C.F. Hammer. Potent enantioselective auxin: indole-3-succinic acid. *Journal of Agricultural and Food Chemistry* **50** (2002) 473-476.
 59. C. Pham-Huy, G. Villain-Pautet, H. Hua, N. Chikhi-Chorfi, H. Galons, M. Thevenin, J.R. Claude, J.M. Warnet. Separation of oxazepam, lorazepam, and temazepam enantiomers by HPLC on a derivatized cyclodextrin-bonded phase: application to the determination of oxazepam in plasma. *Journal of Biochemical and Biophysical Methods* **54** (2002) 287-299.
 60. L. Chen, L.F. Zhang, C.B. Ching, S.C. Ng. Synthesis and chromatographic properties of a novel chiral stationary phase derived from heptakis(6-azido-6-deoxy-2,3-di-*O*-phenylcarbamoylated)- β -cyclodextrin immobilized onto amino-functionalized silica gel via multiple urea linkages. *Journal of Chromatography A* **950** (2002) 65-74.
 61. K. Hrobonova, J. Lehotay, J. Cizmarik, M. Rencova, D.W. Armstrong. Study of mechanism of enantioseparation. II. HPLC chiral analysis of alkoxysubstituted esters of phenylcarbamic acid. *Journal of Liquid Chromatography & Related Technologies* **25** (2002) 1711-1720.
 62. Y. Makino, Y. Urano, T. Nagano. Impurity profiling of ephedrine in methamphetamine by high-performance liquid chromatography. *Journal of Chromatography A* **947** (2002) 151-154.
 63. E. Lipka, M.P. Vaccher, E. Fourmaintraux, J.P. Bonte, C. Vaccher. Chiral Separation and Determination of the Enantiomeric Purity of Tetrahydronaphthalenic Derivatives, Melatoninergic Ligands, by HPLC using β -Cyclodextrins. *Chromatographia* **58** (2003) 665-670.
 64. F.C. Kugelberg, B. Carlsson, J. Ahlner, F. Bengtsson. Stereoselective single-dose kinetics of citalopram and its metabolites in rats. *Chirality* **15** (2003) 622-629.
 65. H. Matsuzawa, K. Mikami. Efficient enantiomeric resolution via introduction of a fluorine tag as a resolving reagent with β -cyclodextrin columns model study on fluorinated *O*-acetylmandelate and ibuprofen amide. *Tetrahedron Letters* **44** (2003) 6227-6230.
 66. Y.C. Tsai, T.-H. Liao, J.-A. Lee. Identification of L-3-hydroxybutyrate as an original ketone body in rat serum by column-switching high-performance liquid chromatography and fluorescence derivatization. *Analytical Biochemistry* **319** (2003) 34-41.
 67. S. Chen. The HPLC Enantioresolution of Phenyl Isocyanated Amino Acids, Peptides and Amino Alcohols on Cyclodextrin Bonded Phases Using the Acetonitrile-Based Mobile phase. *Chromatographia* **59** (2004) 697-703.
 68. S.C. Ng, C.B. Ching, L. Zhang, and L. Chen, US Patent, v. 6720285 (2004).
 69. J.G. Gerber, R.J. Rhodes, J. Gal. Stereoselective metabolism of methadone N-demethylation by cytochrome P4502B6 and 2C19. *Chirality* **16** (2004) 36-44.

70. S. Chen, T. Ward. Comparison of the chiral separation of amino-acid derivatives by a teicoplanin and RN- β -CD CSPs using waterless mobile phases: Factors that enhance resolution. *Chirality* **16** (2004) 318-330.
71. R. Goda, N. Murayama, Y. Fujimaki, K. Sudo. Simple and sensitive liquid chromatography–tandem mass spectrometry method for determination of the *S*(+)- and *R*(-)-enantiomers of baclofen in human plasma and cerebrospinal fluid. *Journal of Chromatography B* **801** (2004) 257-264.
72. E. Lipka, V. Glaçon, G. Mackenzie, D. Ewing, C. Len, D. Postel, M.-P. Vaccher, J.-P. Bonte, C. Vaccher. HPLC Separation and Determination of Enantiomeric Purity of Novel Nucleoside Analogs, on Cyclodextrin Chiral Stationary Phases, Using Reversed and Polar Organic Modes. *Analytical Letters* **37** (2005) 385-398.
73. M.M. Warnke, C.R. Mitchell, R.V. Rozhkov, D.E. Emrich, R.C. Larock, D.W. Armstrong. Use of native and derivatized cyclodextrins based and macrocyclic glycopeptides based chiral stationary phases for the enantioseparation of pterocarpanes by HPLC. *Journal of Liquid Chromatography & Related Technologies* **28** (2005) 823-834.
74. X. Han, T. Yao, Y. Liu, R.C. Larock, D.W. Armstrong. Separation of chiral furan derivatives by liquid chromatography using cyclodextrin-based chiral stationary phases. *Journal of Chromatography A* **1063** (2005) 111-120.
75. R.J. Soukup, R.V. Rozhkov, R.C. Larock, D.W. Armstrong. The Use of Cyclodextrin-Based LC Stationary Phases for the Separation of Chiral Dihydrobenzofuran Derivatives. *Chromatographia* **61** (2005) 219-224.
76. H. He, C. Sun, X.R. Wang, C. Pham-Huy, N. Chikhi-Chorfi, H. Galons, M. Thevenin, J.R. Claude, J.M. Warnet. Solid-phase extraction of methadone enantiomers and benzodiazepines in biological fluids by two polymeric cartridges for liquid chromatographic analysis. *Journal of Chromatography B* **814** (2005) 385-391.
77. X. Han, Q. Zhong, D. Yue, N. Della Ca, R.C. Larock, D.W. Armstrong. Separation of Enantiomers of Isochromene Derivatives by HPLC Using Cyclodextrin-Based Stationary Phases. *Chromatographia* **61** (2005) 205-211.
78. Z. Wimmer, D. Saman, M. Zarevucka, M. Wimmerova. Synthesis of the (1*S*,2*S*)- and (1*R*,2*S*)-stereoisomers of the respective *E*- and *Z*-isomers of ethyl 4-[(2-hydroxycyclohexyl)methyl]phenoxy-3-methyl-2-butenate using yeast whole cell bioreduction of the parent ketones. *Tetrahedron: Asymmetry* **16** (2005) 2810-2815.
79. Y.F. Poon, I.W. Muderawan, S.C. Ng. Synthesis and application of mono-2^A-azido-2^A-deoxyperphenylcarbamoylated β -cyclodextrin and mono-2^A-azido-2^A-deoxyperacetylated β -cyclodextrin as chiral stationary phases for high-performance liquid chromatography. *Journal of Chromatography A* **1101** (2006) 185-197.
80. R. Bhushan, D. Gupta. HPLC resolution of thioridazine enantiomers from pharmaceutical dosage form using cyclodextrin-based chiral stationary phase. *Journal of Chromatography B* **837** (2006) 133-137.
81. K. Huang, Z.S. Breitbach, D.W. Armstrong. Synthesis of the (1*S*,2*S*)- and (1*R*,2*S*)-stereoisomers of the respective *E*- and *Z*-isomers of ethyl 4-[(2-hydroxycyclohexyl)methyl]phenoxy-3-methyl-2-butenate using yeast whole cell bioreduction of the parent ketones. *Tetrahedron Asymmetry* **17** (2006) 2821-2832.
82. R. Koeppen, R. Becker, F. Emmerling, C. Jung, I. Nehls. Enantioselective preparative HPLC separation of the HBCD—Stereoisomers from the technical product and their

- absolute structure elucidation using X-ray crystallography. *Chirality* **19** (2007) 214-222.
83. V. Bavetsias, E.A. Henderson, E. McDonald. Cyclopenta[g]quinazolinone-based inhibitors of thymidylate synthase targeting α -folate receptor overexpressing tumours: synthetic approaches to 4- $\{N-[(6RS)-2$ -hydroxymethyl-4-oxo-3,4,7,8-tetrahydro-6H-cyclopenta[g]quinazolin-6-yl]-N-(prop-2-ynyl)amino}benzoic acid. *Tetrahedron* **63** (2007) 1537-1543.
 84. A.M. Palmer, V. Chiesa, H.C. Holst, J. Le Paih, A. Zanotti-Gerosa, U. Nettekoven. Synthesis of enantiopure 3,6,7,8-tetrahydrochromeno[7,8-d]imidazoles via asymmetric ketone hydrogenation in the presence of RuCl₂[Xyl-P-Phos][DAIPEN. *Tetrahedron: Asymmetry* **19** (2008) 2102-2110.
 85. C. Ewert, S. Lutz-Wahl, L. Fischer. Enantioselective conversion of α -arylnitriles by *Klebsiella oxytoca*. *Tetrahedron: Asymmetry* **19** (2008) 2573-2578.
 86. W.M. Barker, K. Worm, R.E. Dolle. High-performance liquid chromatographic enantioseparation of methanobenzazocines. *Journal of Chromatography A* **1216** (2009) 7708-7714.
 87. J.-Y. Zheng, Z. Wang, Q. Zhu, Y.-J. Zhang, H.-D. Yan. Resolution of biotin intermediate lactone by enzyme catalyzed stereoselective lactonization in organic solvent. *Journal of Molecular Catalysis B: Enzymatic*. **56** (2009) 20-23.
 88. R. Bhushan, R. Kumar. Comparative application of microwave, ultrasonication, ultracentrifugation and conventional heating for preparation of sample as dinitrophenyl derivative for direct enantioseparation of certain amino alcohols and 1-amino-2-propanol from vitamin B₁₂ hydrolysate on α_1 -acid glycoprotein and β -cyclodextrin columns. *Journal of Chromatography A* **1216** (2009) 7941-7945.
 89. K. Nikolić, K. Ivković, Ž. Bešović, S. Marković, D. Agbaba. A validated enantiospecific method for determination and purity assay of clopidogel. *Chirality* **21** (2009) 878-885.
 90. M. Shishovska, V. Trajkovska. HPLC-method for determination of permethrin enantiomers using chiral β -cyclodextrin-based stationary phase. *Chirality* **22** (2010) 527-533.
 91. M.I. Youshko, F. van Rantwijk, R.A. Sheldon. Enantioselective acylation of chiral amines catalysed by aminoacylase I. *Tetrahedron: Asymmetry* **12** (2001) 3267-3271.
 92. P. Lopez-Serrano, M.A. Wegman, F. van Rantwijk, R.A. Sheldon. Enantioselective enzyme catalysed ammoniolysis of amino acid derivatives. Effect of temperature. *Tetrahedron: Asymmetry* **12** (2001) 235-240.
 93. Peter, L. Lazar, F. Fulop, D.W. Armstrong. High-performance liquid chromatographic enantioseparation of β -amino acids. *Journal of Chromatography A* **926** (2001) 229-238.
 94. M.X. Wang, S.J. Lin. Practical and Convenient Enzymatic Synthesis of Enantiopure α -Amino Acids and Amides. *The Journal of Organic Chemistry* **67** (2002) 6542-6545.
 95. R. Watanabe, M. Kita, D. Uemura. A novel dipeptide, N- γ -glutamyl boletine, and a cyclic iminium toxin from the mushroom *Tylopilus* sp. (Boletaceae). *Tetrahedron Letters* **43** (2002) 6501-6504.

96. T.N. Johansen, Y.L. Janin, B. Nielsen, K. Frydenvang, H. Bräuner-Osborne, T.B. Stenbol, S.B. Vogensen, U. Madsen, P. Krogsgaard-Larsen. 2-Amino-3-(3-hydroxy-1,2,5-thiadiazol-4-yl)propionic acid: resolution, absolute stereochemistry and enantiopharmacology at glutamate receptors. *Bioorganic and Medicinal Chemistry* **10** (2002) 2259-2266.
97. S. Chen, H. Yuan, N. Grinberg, A. Dovletoglou, G. Bicker. Enantiomeric separation of *trans*-2-aminocyclohexanol on a crown ether stationary phase using evaporative light scattering detection. *Journal of Liquid Chromatography & Related Technologies* **26** (2003) 425-442.
98. R. Nonokawa, E. Yashima. Detection and Amplification of a Small Enantiomeric Imbalance in α -Amino Acids by a Helical Poly(phenylacetylene) with Crown Ether Pendants. *Journal of the American Chemical Society* **125** (2003) 1278-1283.
99. B.-F. Li, K. Yuan, M.-J. Zhang, H. Wu, L.-X. Dai, Q.R. Wang, X.-L. Hou. Highly Diastereoselective Strecker Reaction of Enolizable Aliphatic Sulfinimes. *The Journal of Organic Chemistry* **68** (2003) 6264-6267.
100. M.I. Youshko, L.M. van Langen, R.A. Sheldon, V.K. Svedas. Application of aminoacylase I to the enantioselective resolution of α -amino acid esters and amides. *Tetrahedron: Asymmetry* **15** (2004) 1933-1936.
101. T. Sonke, B. Kaptein, A.F.V. Wagner, P.J.L.M. Quaedflieg, S. Schultz, S. Ernste, A. Schepers, J.H.M. Mommers, Q.B. Broxterman. Peptide deformylase as biocatalysts for the synthesis of enantiomerically pure amino acid derivatives. *Journal of Molecular Catalysis B: Enzymatic*. **29** (2004) 265-277.
102. Nishiyama, A., N. Kishimoto, and N. Nagashima, US Patent Application; Patent Version Number 20050277791 (2005).
103. Hom, R., J. Tucker, V. John, and N. Shah, US Patent Application, v. 20050267199 (2005).
104. Y. Asano, S. Yamaguchi. Dynamic Kinetic Resolution of Amino Acid Amide Catalyzed by D-Aminopeptidase and α -Amino- ϵ -caprolactam Racemase. *Journal of the American Chemical Society* **127** (2005) 7696-7697.
105. U. Müller, F. van Assema, M. Gunsio, S. Orf, S. Kremer, D. Schipper, A. Wagemans, C.A. Townsend, T. Sonke, R. Bovenberg, M. Wubbolts. Metabolic engineering of the *E. coli* L-phenylalanine pathway for the production of D-phenylglycine (D-Phg). *Metabolic Engineering* **8** (2006) 196-208.
106. V. Zimmermann, M. Beller, U. Kragl. Modelling the reaction course of a dynamic kinetic resolution of amino acid derivatives: identifying and overcoming bottlenecks. *Organic Process Research and Development* **10** (2006) 622-627.
107. C. Carboni, H.G.T. Kierkels, L. Gardossi, K. Tamiola, D.B. Janssen, P.J.L.M. Quaedflieg. Preparation of D-amino acids by enzymatic kinetic resolution using a mutant of penicillin-G acylase from *E. coli*. *Tetrahedron: Asymmetry* **17** (2006) 245-251.
108. Pannuri, S., S. Kamat, and A.R. Martin-Garcia, US Patent Application, v. 20070134772, (2007).
109. A. Yoshida, S. Hikichi, N. Mizuno. Acid-base catalyses by dimeric disilicoicosatungstates and divacant γ -Keggin-type silicodecatungstate parent: Reactivity of the polyoxometalate compounds controlled by step-by-step protonation of lacunary W=O sites. *Journal of Organometallic Chemistry* **692** (2007) 455-459.

110. Y. Hattori, T. Asano, M. Kirihata, Y. Yamaguchi, T. Wakamiya. Development of the first and practical method for enantioselective synthesis of ^{10}B -enriched *p*-borono-L-phenylalanine. *Tetrahedron Letters* **49** (2008) 4977-4980.
111. R.P. Clausen, C. Christensen, K.B. Hansen, J.R. Greenwood, L. Jørgensen, N. Micale, J.C. Madsen, B. Nielsen, J. Egebjerg, H. Bräuner-Osborne, S.F. Traynelis, J.L. Kristensen. *N*-Hydroxypyrazolyl Glycine Derivatives as Selective *N*-Methyl-D-aspartic Acid Receptor Ligands. *Journal of Medicinal Chemistry* **51** (2008) 4179-4187.
112. J. Zheng, H. Zhu, K. Hong, Y. Wang, P. Liu, X. Wang, X. Peng, W. Zhu. Novel Cyclic Hexapeptides from Marine-Derived Fungus, *Aspergillus sclerotiorum* PT06-1. *Organic Letters* **11** (2009) 5262-5265.
113. C. Fernandes, E. Pereira, S. Faure, D.J. Aitken. Expedient preparation of all isomers of 2-aminocyclobutanecarboxylic acid in enantiomerically pure form. *The Journal of Organic Chemistry* **74** (2009) 3217-3220.
114. T. Nuijens, C. Cusan, J.A.W. Kruijtz, D.T.S. Rijkers, R.M.J. Liskamp, P.J.L.M. Quaedflieg. Enzymatic Synthesis of *C*-Terminal Arylamides of Amino Acids and Peptides. *The Journal of Organic Chemistry* **74** (2009) 5145-5150.
115. C. Selvam, N. Oueslati, I.A. Lemasson, I. Brabet, D. Rigault, T. Courtiol, S. Cesarini, N. Triballeau, H.-O. Bertrand, C. Goudet, J.-P. Pin, F.C. Acher. A virtual screening hit reveals new possibilities for developing group III metabotropic glutamate receptor agonists. *Journal of Medicinal Chemistry* **53** (2010) 2797-2813.
116. H. Yilmaz, G. Topal, R. Cakmak, H. Hosgoren. Resolution of ($\pm$)- β -methylphenylethylamine by a novel chiral stationary phase for Pirkle-type column chromatography. *Chirality* **22** (2010) 252-257.
117. C.J. Bender. Theoretical models of charge-transfer complexes. *Chemical Society Reviews* **15** (1986) 475-502.
118. F. Gutmann, C. Johnson, H. Keyzer, J. Molnar. Charge Transfer Complexes in Biological Systems, *Marcel Dekker, Inc.*, New York, 1997.
119. M.L. Waters. Aromatic interactions in model systems. *Current Opinion in Chemical Biology* **6** (2002) 736-741.
120. W.B. Jennings, F.M. Farrell, J.F. Malone. Attractive intermolecular edge-to-face aromatic interactions in flexible organic molecules. *Accounts of Chemical Research* **34** (2001) 885-894.
121. W.H. Pirkle. The nonequivalence of physical properties of enantiomers in optically active solvents. Differences in nuclear magnetic resonance spectra. I. *Journal of the American Chemical Society* **88** (1966) 1837-1837.
122. W.H. Pirkle, D.L. Sikkenga. Resolution of optical isomers by liquid chromatography. *Journal of Chromatography A* **123** (1976) 400-404.
123. W.H. Pirkle, D.W. House, J.M. Finn. Broad spectrum resolution of optical isomers using chiral high-performance liquid chromatographic bonded phases. *Journal of Chromatography A* **192** (1980) 143-158.
124. W.H. Pirkle, J. Finn. Separation of enantiomers by liquid chromatographic methods. In *Asymmetric synthesis. Analytical methods*. (Eds. J.D. Morrison, J.W. Scott) *Academic Press*, New York, 1983, p. 87.

125. R. Thompson. A Practical guide to HPLC enantioseparations for pharmaceutical compounds. *Journal of Liquid Chromatography & Related Technologies* **28** (2005) 1215-1231.
126. W.H. Pirkle, C.J. Welch, B. Lamm. Design, synthesis and evaluation of an improved enantioselective naproxen selector. *The Journal of Organic Chemistry* **57** (1992) 3854-3860.
127. M. Lammerhofer, W. Lindner. Quinine and quinidine derivatives as chiral selectors. Brush type chiral stationary phases for high performance liquid chromatography based on chincona carbamates and their applications as chiral anion exchanger. *Journal of Chromatography A* **741** (1996) 33-48.
128. M. Lammerhofer, W. Lindner. Liquid chromatographic enantiomer separation and chiral recognition by Cinchona Alcaloid-Derived Enantioselective Separation Materials. In *Advances in Chromatography* (Eds. E. Grushka, N. Grinberg) CRC Press, Boca Raton, 2008, pp. 1-109.
129. S. Schefzig, W. Lindner, K.B. Lipkowitz, M. Jalaie. Enantiomeric discrimination by a quinine-based chiral stationary phase. A computational study. *Chirality* **12** (2000) 7-15.
130. J. Clayden, D. Mitjans, L.H. Youssef. Lithium–Sulfoxide–Lithium Exchange for the Asymmetric Synthesis of Atropisomers under Thermodynamic Control. *Journal of the American Chemical Society* **124** (2002) 5266-5267.
131. W.F. Bailey, P. Beak, S.T. Kerrick, S. Ma, K.B. Wiberg. An Experimental and Computational Investigation of the Enantioselective Deprotonation of Boc-piperidine. *Journal of the American Chemical Society* **124** (2002) 1889-1896.
132. N.T. McDougal, S.E. Schaus. Asymmetric Morita–Baylis–Hillman Reactions Catalyzed by Chiral Brønsted Acids. *Journal of the American Chemical Society* **125** (2003) 12094-12095.
133. M.S. Taylor, E.N. Jacobsen. Enantioselective Michael Additions to α,β -Unsaturated Imides Catalyzed by a Salen–Al Complex. *Journal of the American Chemical Society* **125** (2003) 11204-11205.
134. M.-J. Kim, Y.I. Chung, Y.K. Choi, H.K. Lee, D. Kim, J. Park. (S)-Selective Dynamic Kinetic Resolution of Secondary Alcohols by the Combination of Subtilisin and an Aminocyclopentadienylruthenium Complex as the Catalysts. *Journal of the American Chemical Society* **125** (2003) 11494-11495.
135. A. He, B. Yan, A. Thanavaro, C.D. Spilling, N.P. Rath. Synthesis of Nonracemic Allylic Hydroxy Phosphonates via Alkene Cross Metathesis. *The Journal of Organic Chemistry* **69** (2004) 8643-8651.
136. J.J. Ryoo, K.S. Heo, E.S. Choi, J.H. Park, W. Lee. Enantioseparation of tiropamide by HPLC. *Chirality* **16** (2004) S51-S54.
137. J.C.D. Le, B.L. Pagenkopf. Asymmetric Hydrogenation of *o*-Alkoxy-Substituted Arylenamides. *The Journal of Organic Chemistry* **69** (2004) 4177-4180.
138. P.V. Ramachandran, S. Madhi, L. Bland-Berry, M.V.R. Reddy, M.J. O'Donnell. Catalytic Enantioselective Synthesis of Glutamic Acid Derivatives via Tandem Conjugate Addition–Elimination of Activated Allylic Acetates under Chiral PTC Conditions. *Journal of the American Chemical Society* **127** (2005) 13450-13451.
139. M.P. Doyle, J.P. Morgan, J.C. Fettingner, P.Y. Zavalij, J.T. Colyer, D.J. Timmons, M.D. Carducci. “Matched/Mismatched” Diastereomeric Dirhodium(II) Carboxamidate

- Catalyst Pairs. Structure–Selectivity Correlations in Diazo Decomposition and Hetero-Diels–Alder Reactions. *The Journal of Organic Chemistry* **70** (2005) 5291–5301.
140. J. Huang, P. Zhang, H. Chen, T. Li. Preparation and Evaluation of Proline-Based Chiral Columns. *Analytical Chemistry* **77** (2005) 3301–3308.
141. A. Ting, S. Lou, S.E. Schaus. Highly diastereoselective asymmetric Mannich reactions of 1,3-dicarbonyls with acyl imines. *Organic Letters* **8** (2006) 2003–2006.
142. A.L. Simplicio, P. Matias, J.F. Gilmer, J.M. Clancy. Chiral separation and identification of β -aminoketones of pharmacological interest by high performance liquid chromatography and capillary electrophoresis. *Journal of Chromatography A* **1120** (2006) 89–93.
143. C.J. Welch, M. Biba, P. Sajonz. Fast methods of enantiopurity determination for the Soai reaction: Towards a general enantioenrichment detector?. *Chirality* **19** (2007) 34–43.
144. D. Liu, E. Canales, E.J. Corey. Chiral Oxazaborolidine–Aluminum Bromide Complexes Are Unusually Powerful and Effective Catalysts for Enantioselective Diels–Alder Reactions. *Journal of the American Chemical Society* **129** (2007) 1498–1499.
145. A.V. Malkov, S. Stoncius, K. Vrankova, M. Arndt, P. Kocovsky. Dynamic Kinetic Resolution in the Asymmetric Synthesis of β -Amino Acids by Organocatalytic Reduction of Enamines with Trichlorosilane. *Chemistry - A European Journal* **14** (2008) 8082–8085.
146. X. Lu, L. Deng. Asymmetric Aza-Michael Reactions of α,β -Unsaturated Ketones with Bifunctional Organic Catalysts. *Angewandte Chemie International Edition (English)* **47** (2008) 7710–7713.
147. P.H. Fuller, J-W. Kim, S.R. Chemler. Copper Catalyzed Enantioselective Intramolecular Aminooxygenation of Alkenes. *Journal of the American Chemical Society* **130** (2008) 17638–17639.
148. C. Curti, A. Sartori, L. Battistini, G. Rassu, F. Zanardi, G. Casiraghi. Asymmetric, catalytic, vinylogous aldol reactions using pyrrole-based dienoxysilanes. Enantioselective synthesis of α,β -unsaturated γ -butyrolactam synthons. *Tetrahedron Letters* **50** (2009) 3428–3431.
149. A.E. Nibbs, A.-L. Baize, R.M. Herter, K.A. Scheidt. Catalytic asymmetric alkylation of substituted isoflavanones. *Organic Letters* **11** (2009) 4010–4013.
150. R. Aneja, P. Luthra, S. Ahuja. High-performance liquid chromatography separation of enantiomers of mandelic acid and its analogs on a chiral stationary phase. *Chirality* **22** (2010) 479–485.
151. Z. Li, H.M.L. Davies. Enantioselective C–C Bond Formation by Rhodium-Catalyzed Tandem Ylide Formation/[2,3]-Sigmatropic Rearrangement between Donor/Acceptor Carbenoids and Allylic Alcohols. *Journal of the American Chemical Society* **132** (2010) 396–401.
152. A. Peter, E. Vekes, A. Arki, D. Tourwe, W. Lindner. Direct high-performance liquid chromatographic enantioseparation of α -substituted proline analogues on a quinine-derived chiral anion-exchanger stationary phase. *Journal of Separation Science* **26** (2003) 1125–1132.

153. M. Lammerhofer, D. Hebenstreit, E. Gavioli, W. Lindner, A. Mucha, P. Kafarski, P. Wieczorek. High-performance liquid chromatographic enantiomer separation and determination of absolute configurations of phosphinic acid analogues of dipeptides and their α -aminophosphinic acid precursors. *Tetrahedron: Asymmetry* **14** (2003) 2557-2565.
154. W. Bicker, M. Lammerhofer, W. Lindner. Direct high-performance liquid chromatographic method for enantioselective and diastereoselective determination of selected pyrethroid acids. *Journal of Chromatography A* **1035** (2004) 37-46.
155. K. Gyimesi-Forras, J. Kokosi, G. Szasz, A. Gergely, W. Lindner. Liquid chromatographic enantiomer separations of novel quinazolinone derivatives on quinine carbamate based chiral stationary phases using hydro-organic mobile phases. *Journal of Chromatography A* **1047** (2004) 59-67.
156. K. Gyimesi-Forras, A. Leitner, A. Akasaka, W. Lindner. Comparative study on the use of *ortho*-phthalaldehyde, naphthalene-2,3-dicarboxaldehyde and anthracene-2,3-dicarboxaldehyde reagents for α -amino acids followed by the enantiomer separation of the formed isoindolin-1-one derivatives using quinine-type chiral stationary phases. *Journal of Chromatography A* **1083** (2005) 80-88.
157. K. Gyimesi-Forras, K. Akasaka, M. Lammerhofer, N.M. Maier, T. Fujita, M. Watanabe, N. Harada, W. Lindner. Enantiomer separation of a powerful chiral auxiliary, 2-methoxy-2-(1-naphthyl)propionic acid by liquid chromatography using chiral anion exchanger-type stationary phases in polar-organic mode; investigation of molecular recognition aspects. *Chirality* **17** (2005) S134-S142.
158. X. Xiong, W.R.G. Baeyens, H.Y. Aboul-Enein, J.R. Delanghe, T. Tu, J. Ouyang. Comparison of performance of Chirobiotic T, T2 and TAG columns in the separation of β^2 - and β^3 -homocysteine. *Talanta* **71** (2007) 573-581.
159. Y. Tojo, K. Hamase, M. Nakata, A. Morikawa, M. Mita, Y. Ashida, W. Lindner, K. Zaitsev. Automated and simultaneous two-dimensional micro-high-performance liquid chromatographic determination of proline and hydroxyproline enantiomers in mammals. *Journal of Chromatography B* **875** (2008) 174-179.
160. V.A. Davankov. Ligand exchange chromatography of chiral compounds. In: *Complexation chromatography* (Ed. D. Cagniant) Marcel Dekker, New York, 1992, p. 197.
161. F. Helfferich. Ligand exchange: a novel separation technique. *Nature* **189** (1961) 1001-1002.
162. S.V. Rogozhin, V.A. Davankov. Ligand chromatography on asymmetric complex-forming sorbent as a new method for resolution of racemates. *Journal of the Chemical Society D: Chemical Communications* (1971) 490.
163. A.M. Rizzi. Efficiency in chiral high performance ligand exchange chromatography. Influence of complexation process, flow rate and capacity factor. *Journal of Chromatography A* **542** (1991) 221-237.
164. M.H. Hyun, S.C. Han, C.W. Lee, Y.K. Lee. Preparation and application of a new ligand exchange chiral stationary phase for the liquid chromatographic resolution of α -amino acid enantiomers. *Journal of Chromatography A* **950** (2002) 55-63.
165. H. Luesch, P.G. Williams, W.Y. Yoshida, R.E. Moore, V.J. Paul. Ulongamides A-F, New β -Amino Acid-Containing Cyclodepsipeptides from Palauan Collections of the

- Marine Cyanobacterium *Lyngbya* sp. *Journal of Natural Products* **65** (2002) 996-1000.
166. P.G. Williams, W.Y. Yoshida, R.E. Moore, V.J. Paul. Tasiamide, a Cytotoxic Peptide from the Marine Cyanobacterium *Symploca* sp. *Journal of Natural Products* **65** (2002) 1336-1339.
 167. H. Luesch, D. Hoffmann, J.M. Hevel, J.E. Becker, T. Golakoti, R.E. Moore. Biosynthesis of 4-Methylproline in Cyanobacteria: Cloning of *nosE* and *nosF* Genes and Biochemical Characterization of the Encoded Dehydrogenase and Reductase Activities. *The Journal of Organic Chemistry* **68** (2003) 83-91.
 168. H. Pajouhesh, K. Curry, H. Palouhesh, M.H. Maresht, B. Patrick. Stereospecific synthesis and absolute configuration of the (2*S*,3*S*,4*S*)-isomer of 2-methyl-2-(carboxycyclopropyl)glycine (MCCG). *Tetrahedron: Asymmetry* **14** (2003) 593-596.
 169. X. Huang, J. Wang, Q. Wang, B. Huang. Chiral Speciation and Determination of DL-Selenomethionine Enantiomers on a Novel Chiral Ligand-Exchange Stationary Phase. *Analytical Sciences* **21** (2005) 253-257.
 170. Y. Liu, H. Zou, J. Haginaka. Preparation and evaluation of a novel chiral stationary phase based on covalently bonded chitosan for ligand-exchange chromatography. *Journal of Separation Science* **29** (2006) 1440-1446.
 171. B. Natalini, R. Sardella, A. Macchiarulo, R. Pellicciari. *S*-Trityl-(*R*)-cysteine, a powerful chiral selector for the analytical and preparative ligand-exchange chromatography of amino acids. *Journal of Separation Science* **31** (2008) 696-704.
 172. Vu, T.C., D.B. Brzozowski, R. Fox, J.D. Godfrey JR., R.L. Hanson, S.V. Kolotuchin, J.A. Mazzullo, R.N. Patel, J. Wang, K. Wong, J. Yu, J.J. Zhu, D.R. Magnin, D.J. Augeri, and L.G. Hamann, US Patent Application, v. 20090018311 (2009).
 173. F.A. Carey. Organic Chemistry. *The McGraw-Hill Companies Inc*, New York, 1996.
 174. A.L. Lehninger. Principles of biochemistry. *Worth Publisher, Inc*, New York, 1982.
 175. M. Lederer. Adsorption chromatography. VI. Further studies on the separation of D-and L-tryptophan on cellulose with aqueous solvents. *Journal of Chromatography A* **510** (1990) 367-371.
 176. M. Lederer. Adsorption chromatography. VII. Chiral separation on cellulose with aqueous solvents. *Journal of Chromatography A* **604** (1992) 55-62.
 177. H.T.K. Xuan, M. Lederer, Adsorption chromatography. IX. Chiral separation with aqueous solvents and liquid-liquid systems. *Journal of Chromatography A* **635** (1993) 346-348.
 178. T. Shibata, K. Mori, Y. Okamoto, Polysaccharide phases, in *Chiral separation by HPLC. Applications to pharmaceutical compounds*. (Ed. A.M. Krstulovic) *Ellis Horwood*, New York, 1989, p. 336.
 179. Y. Okamoto, R. Aburatani, K. Hatada, Chromatographic chiral resolution. XIV. Cellulose tribenzoate derivatives as chiral stationary phases for high performance liquid chromatography. *Journal of Chromatography A* **389** (1987) 95-102.
 180. E. Yashima, C. Yamamoto, Y. Okamoto. NMR studies of chiral discrimination relevant to liquid chromatographic enantioseparation by a cellulose phenyl carbamate derivative. *Journal of the American Chemical Society* **118** (1996) 4036-4048.

181. C.W. Amoss, N.M. Mayer, Separation of chiral compounds on Polysaccharide columns. In: Chiral separation methods for pharmaceutical and biotechnological products (Ed. S. Ahuja), *John Wiley & Sons, Inc.*, Hoboken, New Jersey, 2011, p. 57.
182. H. Ding, N. Grinberg, R. Thompson, D. Ellison. Enantiorecognition mechanism for derivatized cellulose under reversed phase conditions. *Journal of Liquid Chromatography & Related Technologies* **23** (2000) 2641-2651.
183. R. Cirilli, R. Costi, R. Di Santo, R. Ferretti, F. La Torre, L. Angiolella, M. Micocci. Analytical and semipreparative enantiomeric separation of azole antifungal agents by high-performance liquid chromatography on polysaccharide-based chiral stationary phases: Application to in vitro biological studies. *Journal of Chromatography A* **942** (2002) 107-114.
184. K.W. Phinney, L.C. Sander. Preliminary evaluation of a standard reference material for chiral stationary phases used in liquid and supercritical fluid chromatography. *Analytical and Bioanalytical Chemistry* **372** (2002) 101-108.
185. E. Lipka-Belloli, C. Len, G. Mackenzie, G. Ronco, J.P. Bonte, C. Vaccher. Diastereomeric resolution of nucleoside analogues, new potential antiviral agents, using high-performance liquid chromatography on polysaccharide-type chiral stationary phases. *Journal of Chromatography A* **943** (2002) 91-100.
186. D.A. Evans, J. Wu. Enantioselective Rare-Earth Catalyzed Quinone Diels-Alder Reactions. *Journal of the American Chemical Society* **125** (2003) 10162-10163.
187. N.S. Chowdari, D.B. Ramachary, C.F. Barbas. Organocatalytic Asymmetric Assembly Reactions: One-Pot Synthesis of Functionalized β -Amino Alcohols from Aldehydes, Ketones, and Azodicarboxylates. *Organic Letters* **5** (2003) 1685-1688.
188. S. Matsumura, Y. Maeda, T. Nishimura, S. Uemura. Palladium-Catalyzed Asymmetric Arylation, Vinylation, and Allenylation of *tert*-Cyclobutanols via Enantioselective C-C Bond Cleavage. *Journal of the American Chemical Society* **125** (2003) 8862-8869.
189. D.M. Hodgson, C.R. Maxwell, T.J. Miles, E. Paruch, I.R. Matthews, J. Witherington. Organolithium-induced enantioselective alkylative double ring-opening of epoxides: synthesis of enantioenriched unsaturated amino alcohols. *Tetrahedron* **60** (2004) 3611-3624.
190. E.N. Shapovalova, O.A. Shpigun, L.M. Nesterova, M.Y. Belov. Determination of the Optical Purity of Fungicides of the Triazole Series. *Journal of Analytical Chemistry* **59** (2004) 255-259.
191. B.H. Lipshutz, H. Shimizu. Copper(I)-Catalyzed Asymmetric Hydrosilylations of Imines at Ambient Temperatures. *Angewandte Chemie International Edition (English)* **43** (2004) 2228-2230.
192. Y. Yamashita, S. Kobayashi. Zirconium-Catalyzed Enantioselective [3+2] Cycloaddition of Hydrazones to Olefins Leading to Optically Active Pyrazolidine, Pyrazoline, and 1,3-Diamine Derivatives. *Journal of the American Chemical Society* **126** (2004) 11279-11282.
193. A. Hu, W. Lin. Ru-Catalyzed Asymmetric Hydrogenation of α -Phthalimide Ketones and 1,3-Diaryl Diketones Using 4,4'-Substituted BINAPs. *Organic Letters* **7** (2005) 455-458.
194. P.C. B. Page, B.R. Buckley, H. Heany, A.J. Blacker. Asymmetric Epoxidation of *cis*-Alkenes Mediated by Iminium Salts: Highly Enantioselective Synthesis of Levromakalim. *Organic Letters* **7** (2005) 375-377.

195. D.A. Evans, K.R. Fandrick, H.J. Song. Enantioselective Friedel–Crafts Alkylations of α,β -Unsaturated 2-Acyl Imidazoles Catalyzed by Bis(oxazoliny)pyridine–Scandium(III) Triflate Complexes. *Journal of the American Chemical Society* **127** (2005) 8942–8943.
196. N. Matthijs, M. Maftouh, Y. Vander Heyden. Screening approach for chiral separation of pharmaceuticals: IV. Polar organic solvent chromatography. *Journal of Chromatography A* **1111** (2006) 48–61.
197. I. Coldham, S. Dufour, T.F.N. Haxell, J.J. Patel, G. Sanchez-Jimenez. Dynamic Thermodynamic and Dynamic Kinetic Resolution of 2-Lithiopyrrolidones. *Journal of the American Chemical Society* **128** (2006) 10943–10951.
198. D. Wiktelius, E.K. Larsson, K. Luthman. Chemoenzymatic synthesis of enantiomerically enriched α -chiral 3-oxy-propionaldehydes by lipase-catalyzed kinetic resolution and desymmetrization. *Tetrahedron: Asymmetry* **17** (2006) 2088–2100.
199. G. Liu, X. Lu. Cationic Palladium Complex Catalyzed Highly Enantioselective Intramolecular Addition of Arylboronic Acids to Ketones. A Convenient Synthesis of Optically Active Cycloalkanols. *Journal of the American Chemical Society* **128** (2006) 16504–16505.
200. Y. Jin, X.Y. Liu, L.L. Jing, W. He, X.L. Sun, S.Y. Zhang. Investigation on 1,4-dichlorophthalazine-derivatized cinchona alkaloids-catalyzed asymmetric “interrupted” Feist–Bénary reaction. *Chirality* **19** (2007) 386–390.
201. H. Lebel, K. Huard. De Novo Synthesis of Troc-Protected Amines: Intermolecular Rhodium-Catalyzed C–H Amination with *N*-Tosyloxycarbamates. *Organic Letters* **9** (2007) 639–642.
202. S.Y. Wang, S.J. Ji, T.P. Loh. Cu(I) Tol-BINAP-Catalyzed Enantioselective Michael Reactions of Grignard Reagents and Unsaturated Esters. *Journal of the American Chemical Society* **129** (2007) 276–277.
203. A.V. Malkov, M. Figlus, S. Stoncius, P. Kocovsky. Organocatalysis with a Fluorous Tag: Asymmetric Reduction of Imines with Trichlorosilane Catalyzed by Amino Acid-Derived Formamides. *The Journal of Organic Chemistry* **72** (2007) 1315–1325.
204. J.-K. Jiang, K. Ghoreschi, F. Deflorian, Z. Chen, M. Perreira, M. Pesu, J. Smith, D.-T. Nguyen, E.H. Liu, W. Leister, S. Costanzi, J.J. O’Shea, C.J. Thomas. Examining the Chirality, Conformation and Selective Kinase Inhibition of 3-((3*R*,4*R*)-4-methyl-3-(methyl(7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl)amino)piperidin-1-yl)-3-oxopropanenitrile (CP-690,550). *Journal of Medicinal Chemistry* **51** (2008) 8012–8018.
205. M. Seto, J.L. Roizen, B.M. Stoltz. Catalytic Enantioselective Alkylation of Substituted Dioxanone Enol Ethers: Ready Access to C(α)-Tetrasubstituted Hydroxyketones, Acids, and Esters. *Angewandte Chemie International Edition (English)* **47** (2008) 6873–6876.
206. D. Minato, Y. Nagasue, Y. Demizu, O. Onomura. Efficient Kinetic Resolution of Racemic Amino Aldehydes by Oxidation with *N*-Iodosuccinimide. *Angewandte Chemie International Edition (English)* **47** (2008) 9458–9461.
207. X.-L. Huang, X.-Y. Chen, S. Ye. Enantioselective Synthesis of Aza- β -lactams via NHC-Catalyzed [2 + 2] Cycloaddition of Ketenes with Diazenedicarboxylates. *The Journal of Organic Chemistry* **74** (2009) 7585–7587.

208. M.K. Ghorai, D. Shukla, K. Das. Enantioselective Syntheses of Morpholines and Their Homologues via S_N2 -Type Ring Opening of Aziridines and Azetidines with Haloalcohols. *The Journal of Organic Chemistry* **74** (2009) 7013-7022.
209. X.-Y. Liu, C.-M. Che. Highly Enantioselective Synthesis of Chiral Secondary Amines by Gold(I)/Chiral Brønsted Acid Catalyzed Tandem Intermolecular Hydroamination and Transfer Hydrogenation Reactions. *Organic Letters* **11** (2009) 4204-4207.
210. S. Ma, S. Shen, H. Lee, M. Eriksson, X. Zeng, J. Xu, K. Fandrick, N. Yee, C. Senanayake, N. Grinberg. Mechanistic studies on the chiral recognition of polysaccharide-based chiral stationary phases using liquid chromatography and vibrational circular dichroism: Reversal of elution order of N-substituted alpha-methyl phenylalanine esters. *Journal of Chromatography A* **1216** (2009) 3784-3793.
211. D. Ding, C.-G. Zhao. Organocatalyzed synthesis of 2-amino-8-oxo-5,6,7,8-tetrahydro-4H-chromene-3-carbonitriles. *Tetrahedron Letters* **51** (2010) 1322-1325.
212. B. Yang, M.J. Miller. Iminonitroso ene reactions: experimental studies on reactivity, regioselectivity and enantioselectivity. *Tetrahedron Letters* **51** (2010) 328-331.
213. N. Kanbayashi, K. Onitsuka. Enantioselective Synthesis of Allylic Esters via Asymmetric Allylic Substitution with Metal Carboxylates Using Planar-Chiral Cyclopentadienyl Ruthenium Catalysts. *Journal of the American Chemical Society* **132** (2010) 1206-1207.
214. Y. Bereznitski, R. Lobrutto, N. Variankaval, R. Thompson, K. Thompmpson, P. Sajonz, L.S. Crocker, J. Kowal, D. Cai, M. Journet, T. Wang, J. Wyvratt, N. Grinberg. Mechanistic aspects of chiral discriminatino on an Amylose tris(3,5-dimethylphenyl)carbamate. *Enantiomer* **7** (2002) 305-315.
215. M. Matsushita, K. Yoshida, N. Yamamoto, P. Wirsching, R.A. Lerner, K.D. Janda. High-Throughput Screening by Using a Blue-Fluorescent Antibody Sensor. *Angewandte Chemie International Edition (English)* **42** (2003) 5984-5987.
216. H. Kanazawa, A. Okada, M. Higaki, H. Yokota, F. Mashige, K. Nakahara. Stereospecific analysis of omeprazole in human plasma as a probe for CYP2C19 phenotype. *Journal of Pharmaceutical and Biomedical Analysis* **30** (2003) 1817-1824.
217. S.P. Brown, M.P. Brochu, C.J. Sinz, D.W.C. MacMillan. The Direct and Enantioselective Organocatalytic α -Oxidation of Aldehydes. *Journal of the American Chemical Society* **125** (2003) 10808-10809.
218. T. Ooi, M. Kameda, M. Taniguchi, K. Maruoka. Development of Highly Diastereo- and Enantioselective Direct Asymmetric Aldol Reaction of a Glycinate Schiff Base with Aldehydes Catalyzed by Chiral Quaternary Ammonium Salts. *Journal of the American Chemical Society* **126** (2004) 9685-9694.
219. A. Cordova, H. Sunden, M. Engqvist, I. Ibrahem, J. Casas. The Direct Amino Acid-Catalyzed Asymmetric Incorporation of Molecular Oxygen to Organic Compounds. *Journal of the American Chemical Society* **126** (2004) 8914-8915.
220. A.S. Demir, P. Ayhan, A.A. Igdir, A.N. Duygu. Enzyme catalyzed hydroxymethylation of aromatic aldehydes with formaldehyde. Synthesis of hydroxyacetophenones and (S)-benzoins. *Tetrahedron* **60** (2004) 6509-6512.

221. Q. Fan, L. Lin, J. Liu, Y. Huang, X. Feng, G. Zhang. Highly Enantioselective Hetero-Diels–Alder Reaction of Brassard Diene with Aromatic Aldehydes. *Organic Letters* **6** (2004) 2185–2188.
222. T. Soeta, M. Kuriyama, K. Tomioka. Catalytic Asymmetric Conjugate Addition of Dialkylzinc Reagents to β -Aryl- α,β -unsaturated *N*-2,4,6-Triisopropylphenylsulfonylaldimines with Use of *N*-Boc-L-Val-Connected Amidophosphane-Copper(I) Catalyst. *The Journal of Organic Chemistry* **70** (2005) 297–300.
223. Y. Liu, K. Ding. Modular Monodentate Phosphoramidite Ligands for Rhodium-Catalyzed Enantioselective Hydrogenation. *Journal of the American Chemical Society* **127** (2005) 10488–10489.
224. S. Lou, P.N. Moquist, S.E. Schaus. Asymmetric Allylboration of Ketones Catalyzed by Chiral Diols. *Journal of the American Chemical Society* **128** (2006) 12660–12661.
225. X.M. Deng, P. Cai, S. Ye, X.L. Sun, W.W. Liao, K. Li, Y. Tang, Y.D. Wu, L.X. Dai. Enantioselective Synthesis of Vinylcyclopropanes and Vinylepoxides Mediated by Camphor-Derived Sulfur Ylides: Rationale of Enantioselectivity, Scope, and Limitation. *Journal of the American Chemical Society* **128** (2006) 9730–9740.
226. Y. Fukuta, T. Mita, N. Fukuda, M. Kanai, M. Shibasaki. De Novo Synthesis of Tamiflu via a Catalytic Asymmetric Ring-Opening of *meso*-Aziridines with TMSN_3 . *Journal of the American Chemical Society* **128** (2006) 6312–6313.
227. Y. Wu, Y. Zhang, M. Yu, G. Zhao, S. Wang. Highly Efficient and Reusable Dendritic Catalysts Derived from *N*-Prolylsulfonamide for the Asymmetric Direct Aldol Reaction in Water. *Organic Letters* **8** (2006) 4417–4420.
228. S.G. Kim, T.H. Park, B.J. Kim. Efficient total synthesis of (+)-*exo*-, (–)-*endo*-brevicomine and their derivatives via asymmetric organocatalysis and olefin cross-metathesis. *Tetrahedron Letters* **47** (2006) 6369–6372.
229. M. Lorin, R. Delepee, J.-C. Maurizot, J.P. Ribet, P. Morin. Sensitivity improvement of circular dichroism detection in HPLC by using a low-pass electronic noise filter: Application to the enantiomeric determination purity of a basic drug. *Chirality* **19** (2007) 106–113.
230. Y. Suto, M. Kanai, M. Shibasaki. Catalytic Enantioselective Mannich-type Reactions of Ketoimines. *Journal of the American Chemical Society* **129** (2007) 500–501.
231. C.A. Busacca, T. Bartholomeyzik, S. Cheekoori, N. Grinberg, H. Lee, S. Ma, A. Saha, S. Shen, C.H. Senanayake. On the Racemization of Chiral Imidazolines. *The Journal of Organic Chemistry* **73** (2008) 9756–9761.
232. R.R. Shaikh, A. Mazzanti, M. Petrini, G. Bartoli, P. Melchiorre. Proline-Catalyzed Asymmetric Formal α -Alkylation of Aldehydes via Vinylogous Iminium Ion Intermediates Generated from Arylsulfonyl Indoles. *Angewandte Chemie International Edition (English)* **47** (2008) 8707–8710.
233. S.S. Chimni, S. Singh, D. Mahajan. Protonated (*S*)-prolinamide derivatives—water compatible organocatalysts for direct asymmetric aldol reaction. *Tetrahedron: Asymmetry* **19** (2008) 2276–2284.
234. T. Tsubogo, S. Saito, K. Seki, Y. Yamashita, S. Kobayashi. Development of Catalytic Asymmetric 1,4-Addition and [3 + 2] Cycloaddition Reactions Using Chiral Calcium Complexes. *Journal of the American Chemical Society* **130** (2008) 13321–13332.

235. D. Almasi, D.A. Alonso, E. Gomez-Bengoa, C. Najera. Chiral 2-Aminobenzimidazoles as Recoverable Organocatalysts for the Addition of 1,3-Dicarbonyl Compounds to Nitroalkenes. *The Journal of Organic Chemistry* **74** (2009) 6163-6168.
236. M.R. Vishnumaya, V.K. Singh. Highly Efficient Small Organic Molecules for Enantioselective Direct Aldol Reaction in Organic and Aqueous Media. *The Journal of Organic Chemistry* **74** (2009) 4289-4297.
237. H. Jiang, M.W. Paixao, D. Monge, K.A. Jorgensen. Acyl Phosphonates: Good Hydrogen Bond Acceptors and Ester/Amide Equivalents in Asymmetric Organocatalysis. *Journal of the American Chemical Society* **132** (2010) 2775-2783.
238. H.X. Guo, S. Wu, K. Nadeau, G.A. Moniz, S. Caille. Effects of solvent on chiral and enantiomeric separation of Koga bases using derivatized amylose chiral stationary phase. *Chirality* **22** (2010) 50-55.
239. A. Ghanem. True and false reversal of the elution order of barbiturates on a bonded cellulose-based chiral stationary phase. *Journal of Chromatography A* **1132** (2006) 329-332.
240. S. Caccamese, G. Scivoli, S. Bianca, J.M. Lopez-Romero, F.J. Ortiz-Lopez. Chiral high-performance liquid chromatographic separation and circular dichroism spectra of the enantiomers of cytotoxic aristoclarine alkaloids. *Journal of Chromatography A* **1129** (2006) 140-144.
241. G. Nadalini, F. Dondi, A. Massi, A. Dondoni, T. Zhang, A. Cavazzini. High-performance liquid chromatographic separation of dihydropyrimidine racemates on polysaccharide-derived chiral stationary phases. *Journal of Chromatography A* **1126** (2006) 357-364.
242. J. Vachon, C. Lauper, K. Ditrich, J. Lacour. Enantioselective olefin epoxidation using novel biphenyl and binaphthyl azepines and azepinium salts. *Tetrahedron: Asymmetry* **17** (2006) 2334-2338.
243. A. Ghanem. Exploring solvent versatility in immobilized cellulose-based chiral stationary phase for the enantioselective liquid chromatographic resolution of racemates. *Journal of Separation Science*, **30** (2007) 1019-1028.
244. J.Y. Jin, W. Lee, J.H. Park, J.J. Ryoo. Liquid chromatographic enantiomer separation of N-phtaloyl protected α -amino acids on coated and immobilized chiral stationary phase derived from polysaccharide derivatives. *Journal of Liquid Chromatography & Related Technologies* **30** (2007) 1-9.
245. P. Grundmann, W.-D. Fessner. One-Pot, Regioselective Synthesis of Substituted Arylglycines for Kinetic Resolution by Penicillin G Acylase. *Advanced Synthesis and Catalysis* **350** (2008) 1729-1735.
246. T. Zhang, D. Nguyen, P. Franco. Enantiomer resolution screening strategy using multiple immobilised polysaccharide-based chiral stationary phases. *Journal of Chromatography A* **1191** (2008) 214-222.
247. J.Y. Jin, S.K. Bae, W. Lee. Comparative studies between covalently immobilized and coated chiral stationary phases based on polysaccharide derivatives for enantiomer separation of N-protected α -amino acids and their ester derivatives. *Chirality* **21** (2009) 871-877.
248. T. Saito, T. Kikuchi, H. Tanabe, J. Yahiro, T. Otani. Enantioselective synthesis of β -lactams via the IndaBox-Cu(II)-catalyzed Kinugasa reaction. *Tetrahedron Letters* **50** (2009) 4969-4972.

249. A.V. Malkov, K. Vrankova, S. Stoncius, P. Kocovsky. Asymmetric Reduction of Imines with Trichlorosilane, Catalyzed by Sigamide, an Amino Acid-Derived Formamide: Scope and Limitations. *The Journal of Organic Chemistry* **74** (2009) 5839-5849.
250. A. Ghanem. Direct enantioselective HPLC monitoring of lipase-catalyzed kinetic resolution of flurbiprofen. *Chirality* **22** (2010) 597-603.
251. B.M. Trost, V.S. Chan, D. Yamamoto. Enantioselective ProPhenol-Catalyzed Addition of 1,3-Diynes to Aldehydes to Generate Synthetically Versatile Building Blocks and Diyne Natural Products. *Journal of the American Chemical Society* **132** (2010) 5186-5192.
252. A. Ghanem, H.Y. Aboul-Enein. On the solvent versatility in immobilized amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase in high performance liquid chromatography: Application to the asymmetric cyclopropanation of olefins. *Analytica Chimica Acta* **548** (2005) 26-32.
253. A. Ghanem, H.Y. Aboul-Enein. Comparison, applications, advantages, and limitations of immobilized and coated amylase tris-(3,5-dimethylphenylcarbamate) chiral stationary phases in HPLC. *Journal of Liquid Chromatography & Related Technologies* **28** (2005) 2669-2680.
254. T. Zhang, C. Kientzy, P. Franco, A. Ohnishi, Y. Kagamihara, H. Kurosawa. Solvent versatility of immobilized 3,5-dimethylphenylcarbamate of amylose in enantiomeric separations by HPLC. *Journal of Chromatography A* **1075** (2005) 65-75.
255. T. Kawasaki, K. Suzuki, E. Licandro, A. Bossi, S. Maiorana, K. Soai. Enantioselective synthesis induced by tetrathia-[7]-helicenes in conjunction with asymmetric autocatalysis. *Tetrahedron: Asymmetry* **17** (2006) 2050-2053.
256. H. Suga, T. Suzuki, K. Inoue, A. Kakehi. Asymmetric cycloaddition reactions between 2-benzopyrylium-4-olates and 3-(2-alkenoyl)-2-oxazolidinones in the presence of 2,6-bis(oxazoliny)pyridine-lanthanoid complexes. *Tetrahedron* **62** (2006) 9218-9225.
257. P. Kasak, V.B. Arion, M. Widhalm. A chiral phosphepine-olefin rhodium complex as an efficient catalyst for the asymmetric conjugate addition. *Tetrahedron: Asymmetry* **17** (2006) 3084-3090.
258. A. Andersson, H. Nelander, K. Ohlen. Preparative chiral chromatography and chiroptical characterization of enantiomers of omeprazole and related benzimidazoles. *Chirality* **19** (2007) 706-715.
259. A. Ghanem, E. Al-Humaidi. Chiral recognition ability and solvent versatility of bonded amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase: Enantioselective liquid chromatographic resolution of racemic N-alkylated barbiturates and thalidomide analogs. *Chirality* **19** (2007) 477-484.
260. B. Saito, H. Egami, T. Katsuki. Synthesis of an Optically Active Al(salalen) Complex and Its Application to Catalytic Hydrophosphonylation of Aldehydes and Aldimines. *Journal of the American Chemical Society* **129** (2007) 1978-1986.
261. R. Cirilli, R. Ferretti, E. De Santis, B. Gallinella, L. Zanitti, F. La Torre. High-performance liquid chromatography separation of enantiomers of flavanone and 2'-hydroxychalcone under reversed-phase conditions. *Journal of Chromatography A* **1190** (2008) 95-101.

262. J. Shen, C.-H. Tan. Anthrone-derived NHPI analogues as catalysts in reactions using oxygen as an oxidant. *Organic and Biomolecular Chemistry* **6** (2008) 4096-4098.
263. C.M. Reisinger, X. Wang, B. List. Catalytic Asymmetric Hydroperoxidation of α,β -Unsaturated Ketones: An Approach to Enantiopure Peroxyhemiketals, Epoxides, and Aldols. *Angewandte Chemie International Edition (English)* **47** (2008) 8112-8115.
264. S. Shirakawa, T. Kimura, S.-I. Murata, S. Shimizu. Synthesis and Resolution of a Multifunctional Inherently Chiral Calix[4]arene with an ABCD Substitution Pattern at the Wide Rim: The Effect of a Multifunctional Structure in the Organocatalyst on Enantioselectivity in Asymmetric Reactions. *The Journal of Organic Chemistry* **74** (2009) 1288-1296.
265. S.L. Poe, A.R. Bogdan, B.P. Mason, J.L. Steinbacher, S.M. Opalka, D.T. McQuade. Use of Bifunctional Ureas to Increase the Rate of Proline-Catalyzed α -Aminoxylations. *The Journal of Organic Chemistry* **74** (2009) 1574-1580.
266. A. Ciogli, W. Bicker, W. Lindner. Determination of enantiomerization barriers of hypericin and pseudohypericin by dynamic high-performance liquid chromatography on immobilized polysaccharide-type chiral stationary phases and off-column racemization experiments. *Chirality* **22** (2010) 463-471.
267. T. Hashimoto, Y. Maeda, M. Omote, H. Nakatsu, K. Maruoka. Catalytic Enantioselective 1,3-Dipolar Cycloaddition of C,N-Cyclic Azomethine Imines with α,β -Unsaturated Aldehydes. *Journal of the American Chemical Society* **132** (2010) 4076-4077.
268. H.Y. Aboul-Enein, I. Ali, Chiral separation by liquid chromatography and related Technologies, *Marcel Dekker, Inc.* New York, 2003, p. 21.
269. G. Fogassy, A. Tungler, A. Lévai, G. Toth. Enantioselective hydrogenation of exocyclic α,β -unsaturated ketones: Part II. Hydrogenation in the presence of (S)-proline. *Journal of Molecular Catalysis A: Chemical* **179** (2002) 101-106.
270. M. Garzotti, M. Hamdan. Supercritical fluid chromatography coupled to electrospray mass spectrometry: a powerful tool for the analysis of chiral mixtures. *Journal of Chromatography B* **770** (2002) 53-61.
271. M.E. Andersson, D. Aslan, A. Clarke, J. Roeraade, G. Hagman. Evaluation of generic chiral liquid chromatography screens for pharmaceutical analysis. *Journal of Chromatography A* **1005** (2003) 83-101.
272. M.X. Wang, G.Q. Feng. Nitrile Biotransformation for Highly Enantioselective Synthesis of 3-Substituted 2,2-Dimethylcyclopropanecarboxylic Acids and Amides. *The Journal of Organic Chemistry* **68** (2003) 621-624.
273. Z. Tang, F. Jiang, X. Cui, L.-Z. Gong, A.-Q. Mi, Y.-Z. Jiang, Y.-D. Wu. *Asymmetric Catalysis Special Feature Part II: Enantioselective direct aldol reactions catalyzed by L-prolinamide derivatives*. Proceedings of the National Academy of Sciences of the USA **101** (2004) 5755-5760.
274. H. Guo, X. Wang, K. Ding. Assembled enantioselective catalysis for carbonyl-ene reactions. *Tetrahedron Letters* **45** (2004) 2009-2012.
275. N. Kato, S. Shimamura, S. Khan, F. Takeda, Y. Kikai, M. Hirama. Convergent approach to the maduropeptin chromophore: aryl ether formation of (R)-3-aryl-3-hydroxypropanamide and cyclization of macrolactam. *Tetrahedron* **60** (2004) 3161-3172.

276. Q. Fan, L. Lin, J. Liu, Y. Huang, X. Feng. A Mild and Efficient Asymmetric Hetero-Diels–Alder Reaction of the Brassard Diene with Aldehydes. *European Journal of Organic Chemistry* **2005** (2005) 3542-3552.
277. M. Nakajima, S. Kotani, T. Ishizuka, S. Hashimoto. Chiral phosphine oxide BINAPO as a catalyst for enantioselective allylation of aldehydes with allyltrichlorosilanes. *Tetrahedron Letters* **46** (2005) 157-159.
278. M. Marigo, T. Schulte, J. Franzen, K.A. Jorgensen. Asymmetric Multicomponent Domino Reactions and Highly Enantioselective Conjugated Addition of Thiols to α,β -Unsaturated Aldehydes. *Journal of the American Chemical Society* **127** (2005) 15710-15711.
279. Q. Yang, G. Shang, W. Gao, J. Deng, X. Zhang. A highly enantioselective, Pd-TangPhos-catalyzed hydrogenation of N-Tosylimines. *Angewandte Chemie International Edition (English)* **45** (2006) 3822-3835.
280. J. Xu, Y. Guan, S. Yang, Y. Ng, G. Peh, C.H. Tan. Asymmetric Baylis–Hillman Reactions Promoted by Chiral Imidazolines. *Chemistry - An Asian Journal* **1** (2006) 724-729.
281. C. Chen, S.F. Zhu, X.Y. Wu, Q.L. Zhou. Preparation and application of chiral spiro nitrogen-containing ligands for cobalt-catalyzed asymmetric Michael addition. *Tetrahedron: Asymmetry* **17** (2006) 2761-2767.
282. S. Kotani, S. Hashimoto, M. Nakajima. Chiral phosphine oxide BINAPO as a Lewis base catalyst for asymmetric allylation and aldol reaction of trichlorosilyl compounds. *Tetrahedron* **63** (2007) 3122-3132.
283. T.B. Poulsen, L. Bernardi, J. Aleman, J. Overgaard, K.A. Jorgensen. Organocatalytic Asymmetric Direct α -Alkynylation of Cyclic β -Ketoesters. *Journal of the American Chemical Society* **129** (2007) 441-449.
284. N. Byrne, E. Hayes-Larson, W.-W. Liao, C.M. Kraml. Analysis and purification of alcohol-sensitive chiral compounds using 2,2,2-trifluoroethanol as a modifier in supercritical fluid chromatography. *Journal of Chromatography B* **875** (2008) 237-242.
285. M. Tokizane, K. Sato, T. Ohta, Y. Ito. Asymmetric reduction of racemic 2-isoxazolines. *Tetrahedron: Asymmetry* **19** (2008) 2519-2528.
286. G.-S. Liu, Q.-L. Dong, Y.-S. Yao, Z.-J. Yao. Expedient total syntheses of camptothecin and 10-hydroxycamptothecin. *Organic Letters* **10** (2008) 5393-5396.
287. K. Murayama, T. Tanabe, Y. Ishikawa, K. Nakamura, S. Nishiyama. A synthetic study on gymnastatins F and Q: the tandem Michael and aldol reaction approach. *Tetrahedron Letters* **50** (2009) 3191-3194.
288. N. Tosa, A. Bende, R.A. Varga, A. Terec, I. Bratu, I. Grosu. H-Bond-Driven Supramolecular Architectures of the *Syn* and *Anti* Isomers of the Dioxime of Bicyclo[3.3.1]nonane-3,7-dione. *The Journal of Organic Chemistry* **74** (2009) 3944-3947.
289. P. Fang, X.-L. Hou. Asymmetric Copper-Catalyzed Propargylic Substitution Reaction of Propargylic Acetates with Enamines. *Organic Letters* **11** (2009) 4612-4615.
290. T. Kubota, N. Sawada, L. Zhou, C.J. Welch. Enantioseparation of benzazoles and benzanilides on polysaccharide-based chiral columns. *Chirality* **22** (2010) 382-388.
291. C. Wang, G. Yang, J. Zhuang, W. Zhang. From tropes to atropes: 5,5'-bridged 2,2'-bis(diphenylphosphino)biphenyls as chiral ligands for highly enantioselective

- palladium-catalyzed hydrogenation of α -phthalimide ketones. *Tetrahedron Letters* **51** (2010) 2044-2047.
292. Z. Weng, J. Li. Synthesis and antidepressant activity of optical isomers of 2-(4-benzylpiperazin-1-yl)-1-(5-chloro-6-methoxynaphthalen-2-yl) propan-1-ol (SIPI5056). *Bioorganic and Medicinal Chemistry Letters* **20** (2010) 1256-1259.
293. A. Natarajan, K. Wang, V. Ramamurthy, J.R. Scheffer, B. Patrick. Control of Enantioselectivity in the Photochemical Conversion of α -Oxoamides into β -Lactam Derivatives. *Organic Letters* **4** (2002) 1443-1446.
294. D.J. Spielvogel, S.L. Buchwald. Nickel-BINAP Catalyzed Enantioselective α -Arylation of α -Substituted γ -Butyrolactones. *Journal of the American Chemical Society* **124** (2002) 3500-3501.
295. C.Y. Chang, T.K. Yang. Asymmetric synthesis of ACE inhibitor Benazepril HCl via a bioreductive reaction. *Tetrahedron: Asymmetry* **14** (2003) 2239-2245.
296. K. Kuramochi, S. Nagata, H. Itaya, Y. Matsubarta, T. Sunoki, H. Uchiro, K.I. Takao, S. Kobayashi. A convergent total synthesis of epolactaene: an application of the bridgehead oxiranyl anion strategy. *Tetrahedron* **59** (2003) 9743-9758.
297. S. Ma, F. Yu, W. Gao. Studies on Pd(II)-Catalyzed Coupling-Cyclization of α - or β -Amino Allenes with Allylic Halides. *The Journal of Organic Chemistry* **68** (2003) 5943-5949.
298. Y. Yuasa, H. Tsuruta. Enantioselective synthesis of (*S*)-3-(4-thiazolyl)-2-*tert*-butoxycarbonylaminopropionic acid: A chiral building block for renin inhibitor. *Heterocycles* **63** (2004) 2385-2392.
299. Z. Zhang, G. Yang, G. Liang, H. Liu, Y. Chen. Chiral separation of Tamsulosin isomers by HPLC using cellulose Tris (3,5-dimethylphenylcarbamate) as a chiral stationary phase. *Journal of Pharmaceutical and Biomedical Analysis* **34** (2004) 689-693.
300. B.M. Trost, J. Xu. Palladium-Catalyzed Asymmetric Allylic α -Alkylation of Acyclic Ketones. *Journal of the American Chemical Society* **127** (2005) 17180-17181.
301. Y.K. Ye, R.W. Stringham. The effect of acidic and basic additives on the enantioseparation of basic drugs using polysaccharide-based chiral stationary phases. *Chirality* **18** (2006) 519-530.
302. Y.X. Chang, L.M. Yuan, F. Zhao. Effect of Chiral Additives in the Preparation of Cellulose-Based Chiral Stationary Phases in HPLC, and Effect on Enantiomer Resolution. *Chromatographia* **64** (2006) 313-316.
303. K. Kato, C. Matsuba, T. Kusakabe, H. Takayama, S. Yamamura, T. Mochida, H. Akita, T.A. Peganova, N.V. Vologdin, O.V. Gusev. 2,2'-Isopropylidenebis[(4*S*,5*R*)-4,5-di(2-naphthyl)-2-oxazoline] ligand for asymmetric cyclization-carbonylation of *meso*-2-alkyl-2-propargylcyclohexane-1,3-diols. *Tetrahedron* **62** (2006) 9988-9999.
304. C. Roussel, N. Vanthuyne, J.L. Jobert, A. Loas, A.E. Tanase, D. Gherase. HPLC on chiral support with polarimetric detection: Application to conglomerate discovery. *Chirality* **19** (2007) 497-502.
305. B.M. Trost, J. Xu, M. Reichle. Enantioselective Synthesis of α -Tertiary Hydroxyaldehydes by Palladium-Catalyzed Asymmetric Allylic Alkylation of Enolates. *Journal of the American Chemical Society* **129** (2007) 282-283.
306. B.M. Trost, J. Xu, T. Schmidt. Ligand Controlled Highly Regio- and Enantioselective Synthesis of α -Acetoxyketones by Palladium-Catalyzed Allylic Alkylation of 1,2-

- Enediol Carbonates. *Journal of the American Chemical Society* **130** (2008) 11852-11853.
307. R. Csuk, B. Woeste. A chemoenzymatic approach to (+)-pilocarpine. *Tetrahedron* **64** (2008) 9384-9387.
308. C. Roussel, N. Vanthuyne, M. Boucekara, A. Djafri, J. Elguero, I. Alkorta. Atropisomerism in the 2-Arylimino-*N*-(2-hydroxyphenyl)thiazoline Series: Influence of Hydrogen Bonding on the Racemization Process. *The Journal of Organic Chemistry* **73** (2008) 403-411.
309. J.-G. Kim, D.O. Jang. Trifluoroacetylation of amines with trifluoroacetic acid in the presence of trichloroacetonitrile and triphenylphosphine. *Tetrahedron Letters* **51** (2010) 683-685.
310. M.A. Andersson, R. Epple, V.V. Fokin, K.B. Sharpless. A New Approach to Osmium-Catalyzed Asymmetric Dihydroxylation and Aminohydroxylation of Olefins. *Angewandte Chemie International Edition (English)* **41** (2002) 472-475.
311. Y. Okumura, A. Ando, R.W. Stevens, M. Shimizu. Efficient and practical synthesis of both enantiomers of 3-phenylcyclopentanol derivatives. *Tetrahedron* **58** (2002) 8729-8736.
312. M. Ogasawara, K. Ueyama, T. Nagano, Y. Mizuhata, T. Hayashi. Palladium-Catalyzed Asymmetric Synthesis of Axially Chiral (Allenylmethyl)silanes and Chirality Transfer to Stereogenic Carbon Centers in S_E' Reactions. *Organic Letters* **5** (2003) 217-219.
313. F.J.M. de Santana, E.J. Cesarino, P.S. Bonato. New method for the chiral evaluation of mirtazapine in human plasma by liquid chromatography. *Journal of Chromatography B* **809** (2004) 351-356.
314. N. Aoyagi, S. Kawauchi, T. Izumi. Different recognitions of (*E*)- and (*Z*)-1,1'-binaphthyl ketoximes using lipase-catalyzed reactions. *Tetrahedron Letters* **45** (2004) 5189-5192.
315. B.H. Kim, S.U. Lee. Enantiomer Separation of a Novel Aminothiazolecarboxamide Fungicide Using Polysaccharide-Derived Chiral Stationary Phases. *Journal of Chromatography Science* **43** (2005) 501-504.
316. R. Shintani, W.-L. Duan, T. Nagano, A. Okada, T. Hayashi. Chiral Phosphine-Olefin Bidentate Ligands in Asymmetric Catalysis: Rhodium-Catalyzed Asymmetric 1,4-Addition of Aryl Boronic Acids to Maleimides. *Angewandte Chemie International Edition (English)* **44** (2005) 4611-4614.
317. N. Aoyagi, N. Ogawa, T. Izumi. Effects of reaction temperature and acyl group for lipase-catalyzed chiral binaphthol synthesis. *Tetrahedron Letters* **47** (2006) 4797-4801.
318. L. Cheng, L. Liu, D. Wang, Y.-J. Chen. Highly enantioselective and organocatalytic α -amination of 2-oxindoles. *Organic Letters* **11** (2009) 3874-3877.
319. T. Nishimura, J. Wang, M. Nagaosa, K. Okamoto, R. Shintani, F. Kwong, W. Yu, A.S.C. Chan, T. Hayashi. Rhodium-Catalyzed Asymmetric Addition of Arylboronic Acids to β -Phthaliminoacrylate Esters toward the Synthesis of β -Amino Acids. *Journal of the American Chemical Society* **132** (2010) 464-465.
320. H.Y. Aboul-Enein, I. Ali. Comparative study of the enantiomeric resolution of chiral antifungal drugs econazole, miconazole and sulconazole by HPLC on various

- cellulose chiral columns in normal phase mode. *Journal of Pharmaceutical and Biomedical Analysis* **27** (2002) 441-446.
321. J.H. Smitrovich, G.N. Boice, C. Qu, L. DiMichele, T.D. Nelson, M.A. Huffman, J. Murry, J. McNamara, P.J. Reider. Pseudoephedrine as a Chiral Auxiliary for Asymmetric Michael Reactions: Synthesis of 3-Aryl- δ -lactones. *Organic Letters* **4** (2002) 1963-1966.
322. R. Kuwano, K.I. Uchida, Y. Ito. Asymmetric Allylation of Unsymmetrical 1,3-Diketones Using a BINAP-Palladium Catalyst. *Organic Letters* **5** (2003) 2177-2179.
323. B. Zajc, R. Grahek, A. Kocijan, M.K. Lakshman, J. Kosmrlj, J. Lah. Evaluation of the Enantiomeric Resolution of 7,8-Dihydroxy-7,8-dihydrobenzo[a]-pyrene and Its 6-Fluoro and 6-Bromo Derivatives on Polysaccharide-Derived Stationary Phase. *The Journal of Organic Chemistry* **68** (2003) 3291-3294.
324. T.D. Nelson, C.J. Welch, J.D. Rosen, J.H. Smitrovich, M.A. Huffman, J.M. McNamara, D.J. Mathre. Effective use of preparative chiral HPLC in a preclinical drug synthesis. *Chirality* **16** (2004) 609-613.
325. M. Bandini, P.G. Cozzi, P. Melchiorre, A. Umani-Ronchi. Kinetic Resolution of Epoxides by a C-C Bond-Forming Reaction: Highly Enantioselective Addition of Indoles to *cis*, *trans*, and *meso* Aromatic Epoxides Catalyzed by [Cr(salen)] Complexes. *Angewandte Chemie International Edition (English)* **43** (2004) 84-87.
326. M. Bandini, A. Garelli, M. Rovinetti, S. Tommasi, A. Umani-Ronchi. Catalytic enantioselective addition of indoles to aryl nitroalkenes: An effective route to enantiomerically enriched tryptamine precursors. *Chirality* **17** (2005) 522-529.
327. K. Inoue, Y. Makino, N. Itoh. Production of (*R*)-chiral alcohols by a hydrogen-transfer bioreduction with NADH-dependent *Leifsonia* alcohol dehydrogenase (LSADH). *Tetrahedron: Asymmetry* **16** (2005) 2539-2549.
328. P.G. Cozzi, P. Kotrusz. Highly Enantioselective Addition of Me₂Zn to Aldehydes Catalyzed by ClCr(Salen). *Journal of the American Chemical Society* **128** (2006) 4940-4941.
329. L. Deng, H. Nakano, Y. Iwasaki. Direct separation of monoacylglycerol isomers by enantioselective high-performance liquid chromatography. *Journal of Chromatography A* **1198-1199** (2008) 67-72.
330. D. Leow, S. Lin, S.K. Chittimalla, X. Fu, C.-H. Tan. Enantioselective Protonation Catalyzed by a Chiral Bicyclic Guanidine Derivative. *Angewandte Chemie International Edition (English)* **47** (2008) 5641-5645.
331. V. Jurkauskas, S.L. Buchwald. Dynamic Kinetic Resolution via Asymmetric Conjugate Reduction: Enantio- and Diastereoselective Synthesis of 2,4-Dialkyl Cyclopentanones. *Journal of the American Chemical Society* **124** (2002) 2892-2893.
332. S. Ye, Z.Z. Huang, C.A. Xia, Y. Tang, L.X. Dai. A Novel Chiral Sulfonium Ylide: Highly Enantioselective Synthesis of Vinylcyclopropanes. *Journal of the American Chemical Society* **124** (2002) 2432-2433.
333. O. Azzolina, S. Collina, G. Brusotti, D. Rossi, A. Callegari, L. Linati, A. Barbieri, V. Ghislandi. Chemical and biological profile of racemic and optically active dialkylaminoalkyl naphthalenes with analgesic activity. *Tetrahedron: Asymmetry* **13** (2002) 1073-1081.
334. A. Kamal, M. Sandbhor, A.A. Shaik, V. Sravanthi. One-pot synthesis and resolution of chiral allylic alcohols. *Tetrahedron: Asymmetry* **14** (2003) 2839-2844.

335. M. Benaglia, M. Cinquini, F. Cozzi, A. Puglisi, G. Celentano. Poly(ethylene-glycol)-supported proline: a recyclable aminocatalyst for the enantioselective synthesis of γ -nitroketones by conjugate addition. *Journal of Molecular Catalysis A: Chemical* (2003) 204-205.
336. C.A. Sandoval, T. Ohkuma, K. Muniz, R. Noyori. Mechanism of Asymmetric Hydrogenation of Ketones Catalyzed by BINAP/1,2-Diamine-Ruthenium(II) Complexes. *Journal of the American Chemical Society* **125** (2003) 13490-13503.
337. V. Pironti, S. Nicolis, E. Monzani, S. Colonna, L. Casella. Nitrite increases the enantioselectivity of sulfoxidation catalyzed by myoglobin derivatives in the presence of hydrogen peroxide. *Tetrahedron* **60** (2004) 8153-8160.
338. Y.G. Li, Q.S. Tian, J. Zhao, Y. Feng, M.J. Li, T.P. You. Asymmetric pinacol coupling of aromatic aldehydes catalyzed by a new titanium-Schiff base complex. *Tetrahedron: Asymmetry* **15** (2004) 1707-1710.
339. K. Micskei, C. Hajdu, L.A. Wessjohann, L. Mercs, A. Kiss-Szikszai, T. Patonay. Enantioselective reduction of prochiral ketones by chromium(II) amino acid complexes. *Tetrahedron: Asymmetry* **15** (2004) 1735-1744.
340. J.C. Kizirian, N. Cabello, L. Pinchard, J.C. Caille, A. Alexakis. Enantioselective addition of methylolithium to aromatic imines catalyzed by C_2 symmetric tertiary diamines. *Tetrahedron* **61** (2005) 8939-8946.
341. R.B. Bedford, M. Betham, M.E. Blake, A. Garces, S.L. Millar, S. Prashar. Asymmetric styrene dimerisation using mixed palladium-indium catalysts. *Tetrahedron* **61** (2005) 9799-9807.
342. H.F. Duan, J.H. Xie, W.J. Shi, Q. Zhang, Q.L. Zhou. Enantioselective Rhodium-Catalyzed Addition of Arylboronic Acids to Aldehydes Using Chiral Spiro Monophosphite Ligands. *Organic Letters* **8** (2006) 1479-1481.
343. J.V. Naubron, L. Giordano, F. Fotiadu, T. Burgi, N. Vanthuyne, C. Roussel, G. Buono. Chromatographic Resolution, Solution and Crystal Phase Conformations, and Absolute Configuration of *tert*-Butyl(dimethylamino)phenylphosphine-Borane Complex. *The Journal of Organic Chemistry* **71** (2006) 5586-5593.
344. S.S. Kim, S.H. Lee, J.M. Kwak. Enantioselective cyanosilylation of ketones catalyzed by Mn(salen)/Ph₃PO. *Tetrahedron: Asymmetry* **17** (2006) 1165-1169.
345. S. Liu, C. Wolf. Chiral Amplification Based on Enantioselective Dual-Phase Distribution of a Scalemic Bisoxazolidine Catalyst. *Organic Letters* **9** (2007) 2965-2968.
346. H.K. Luo, H.Y. Yang, T.X. Jie, O.S. Chiew, H. Schumann, L.B. Khim, C. Lim. Water-tolerant enantioselective carbonyl-ene reactions with palladium(II) and platinum(II) Lewis acid catalysts bearing BINAP. *Journal of Molecular Catalysis A: Chemical* **261** (2007) 112-119.
347. Y. Kobayashi, K. Soetrisno, K. Kodama, K. Saigo. Enantioselective inclusion of chiral alkyl aryl sulfoxides in a supramolecular helical channel consisting of an enantiopure 1,2-amino alcohol and an achiral carboxylic acid. *Tetrahedron: Asymmetry* **19** (2008) 295-301.
348. X. Liao, Z. Weng, J.F. Hartwig. Enantioselective α -Arylation of Ketones with Aryl Triflates Catalyzed by Difluorophos Complexes of Palladium and Nickel. *Journal of the American Chemical Society* **130** (2008) 195-200.

349. Y. Sohtome, N. Takemura, R. Takagi, Y. Hashimoto, K. Nagasawa. Thiourea-catalyzed Morita–Baylis–Hillman reaction. *Tetrahedron* **64** (2008) 9423-9429.
350. S. Facchetti, I. Cavallini, T. Funaioli, F. Marchetti, A. Iuliano. *Tropos* Ligands in Asymmetric Rhodium(I)-Catalyzed Addition of Arylboronic Acids to Enones: How a Tunable Coordination Gives Different Reaction Products. *Organometallics* **28** (2009) 4150-4158.
351. M. Szaleniec, A. Dudzik, M. Pawul, B. Kozik. Quantitative structure enantioselective retention relationship for high-performance liquid chromatography chiral separation of 1-phenylethanol derivatives. *Journal of Chromatography A* **1216** (2009) 6224-6235.
352. L.L. Machado, G. de Gonzalo, T.L.G. Lemos, M.C. de Mattos, M.C.F. de Oliveira, V. Gotor-Fernández, V. Gotor. Enantioselective acetylation of racemic alcohols by *Manihot esculenta* and *Passiflora edulis* preparations. *Journal of Molecular Catalysis B: Enzymatic* **60** (2009) 157-162.
353. Y.J. Zhang, J.H. Yang, S.H. Kim, M.J. Krische. *anti*-Diastereo- and Enantioselective Carbonyl (Hydroxymethyl)allylation from the Alcohol or Aldehyde Oxidation Level: Allyl Carbonates as Allylmetal Surrogates. *Journal of the American Chemical Society* **132** (2010) 4562-4563.
354. T. Seiser, N. Cramer. Rhodium-Catalyzed C–C Bond Cleavage: Construction of Acyclic Methyl Substituted Quaternary Stereogenic Centers. *Journal of the American Chemical Society* **132** (2010) 5340-5341.
355. F. Meyer, A. Laaziri, A.M. Papini, J. Uziel, S. Jugé. Efficient synthesis of β -halogeno protected L-alanines and their β -phosphonium derivatives. *Tetrahedron: Asymmetry* **14** (2003) 2229-2238.
356. S.I. Ueji, T. Taniguchi, T. Okamoto, K. Watanabe, Y. Ebara, H. Ohta. Flexibility of Lipase Brought About by Solvent Effects Controls Its Enantioselectivity in Organic Media. *Bulletin of the Chemical Society of Japan* **76** (2003) 399-403.
357. C. Bauduin, D. Moulin, E.B. Kaloun, C. Darcel, S. Juge. Highly Enantiomerically Enriched Chlorophosphine Boranes: Synthesis and Applications as P-Chirogenic Electrophilic Blocks. *The Journal of Organic Chemistry* **68** (2003) 4293-4301.
358. M.X. Wang, Y. Wu. Nitrile biotransformations for the synthesis of enantiomerically enriched Baylis–Hillman adducts. *Organic and Biomolecular Chemistry* **1** (2003) 535-540.
359. S. Mori, H. Yumoto, R. Matsumi, T. Nishigaki, Y. Ebara, S. Ueji. A method to greatly improve the enantioselectivity of lipase-catalyzed hydrolysis using sodium dodecyl sulfate (SDS) as an additive. *Tetrahedron: Asymmetry* **16** (2005) 3698-3702.
360. H.A. Wetli, E. Francotte. Automated screening platform with isochronal-parallel analysis and conditioning for rapid method development of chiral separation. *Journal of Separation Science* **30** (2007) 1255-1261.
361. Y. Hayashi, T. Itoh, S. Aratake, H. Ishikawa. A Diarylprolinol in an Asymmetric, Catalytic, and Direct Crossed-Aldol Reaction of Acetaldehyde. *Angewandte Chemie International Edition (English)* **47** (2008) 2082-2084.
362. D. Hazelard, H. Ishikawa, D. Hashizume, H. Koshino, Y. Hayashi. Proline-Mediated Enantioselective Construction of Tetrahydropyrans via a Domino Aldol/Acetalization Reaction. *Organic Letters* **10** (2008) 1445-1448.

363. H.A. Kennedy, S. Vietrich, H. Weinmann, D.E.A. Brittain. Synthesis of 7a-Substituted Hajos–Wiechert Ketone Analogues. *The Journal of Organic Chemistry* **73** (2008) 5151-5154.
364. S. Zhu, S. Yu, D. Ma. Highly Efficient Catalytic System for Enantioselective Michael Addition of Aldehydes to Nitroalkenes in Water. *Angewandte Chemie International Edition (English)* **47** (2008) 545-548.
365. R. Ferretti, B. Gallinella, F. La Torre, L. Zanitti, L. Turchetto, A. Mosca, R. Cirilli. Direct high-performance liquid chromatography enantioseparation of terazosin on an immobilised polysaccharide-based chiral stationary phase under polar organic and reversed-phase conditions. *Journal of Chromatography A* **1216** (2009) 5385-5390.
366. Y. Wang, G. Arsenault, N. Riddell, R. McCrindle, A. McAlees, J.W. Martin. Perfluorooctane Sulfonate (PFOS) Precursors Can Be Metabolized Enantioselectively: Principle for a New PFOS Source Tracking Tool. *Environmental Science and Technology* **43** (2009) 8283-8289.
367. J.S. Yadav, C.S. Reddy. Stereoselective synthesis of amphidinolide T1. *Organic Letters* **11** (2009) 1705-1708.
368. T. Zhang, D. Nguyen, P. Franco. Reversed-phase screening strategies for liquid chromatography on polysaccharide-derived chiral stationary phases. *Journal of Chromatography A* **1217** (2010) 1048-1055.
369. Z. Zheng, B.L. Perkins, B. Ni. Diarylprolinol Silyl Ether Salts as New, Efficient, Water-Soluble, and Recyclable Organocatalysts for the Asymmetric Michael Addition on Water. *Journal of the American Chemical Society* **132** (2010) 50-51.
370. B.-F. Shi, Y.-H. Zhang, J.K. Lam, D.-H. Wang, J.-Q. Yu. Pd(II)-Catalyzed Enantioselective C–H Olefination of Diphenylacetic Acids. *Journal of the American Chemical Society* **132** (2010) 460-461.
371. S. Ishikawa, F. Noguchi, H. Uno, A. Kamimura. Intramolecular Pauson-Khand reaction of optically active aza-Baylis-Hillman adducts. *Tetrahedron Letters* **51** (2010) 2329-2331.
372. A. Nakamura, S. Lectard, D. Hashizume, Y. Hamashima, M. Sodeoka. Diastereo- and Enantioselective Conjugate Addition of α -Ketoesters to Nitroalkenes Catalyzed by a Chiral Ni(OAc)₂ Complex under Mild Conditions. *Journal of the American Chemical Society* **132** (2010) 4036-4037.
373. D. Guieysse, C. Salagnad, P. Monsan, M. Remaud-Simeon. Lipase-catalysed enantioselective transesterification toward esters of 2-bromo-tolylacetic acids. *Tetrahedron: Asymmetry* **14** (2003) 317-323.
374. H. Jin, Z.Y. Li, X.W. Dong. Enantioselective hydrolysis of various substituted styrene oxides with *Aspergillus Niger* CGMCC 0496. *Organic and Biomolecular Chemistry* **2** (2004) 408-414.
375. G. Zhong, Y. Yu. Enantioselective Synthesis of Allylic Alcohols by the Sequential Aminooxylation–Olefination Reactions of Aldehydes under Ambient Conditions. *Organic Letters* **6** (2004) 1637-1639.
376. R. Kino, K. Daikai, T. Kawanami, H. Furuno, J. Inanaga. Remarkable effect of tris(4-fluorophenyl)phosphine oxide on the stabilization of chiral lanthanum complex catalysts. A new and practical protocol for the highly enantioselective epoxidation of conjugated enones. *Organic and Biomolecular Chemistry* **2** (2004) 1822-1824.

377. K. Matsui, S. Takizawa, H. Sasai. Enantioselective Morita–Baylis–Hillman (MBH) reaction promoted by a heterobimetallic complex with a Lewis base. *Tetrahedron Letters* **46** (2005) 1943–1946.
378. F. Foubelo, B. Moreno, T. Soler, M. Yus. Reductive ring opening of dihydrodibenzothiepine and dihydrodinaphtho-oxepine and -thiepine. *Tetrahedron* **61** (2005) 9082–9096.
379. M. Shi, J.W. Shi. Chiral thiophosphoramidate and selenophosphoramidate ligands in the Cu(I)-promoted catalytic enantioselective 1,3-dipolar cycloaddition reactions of azomethine ylides and pyrrole-2,5-dione derivatives. *Tetrahedron: Asymmetry* **18** (2007) 645–650.
380. C. Wang, X. Wu, L. Zhou, J. Sun. A Highly Enantioselective Organocatalytic Method for Reduction of Aromatic *N*-Alkyl Ketimines. *Chemistry - A European Journal* **14** (2008) 8789–8792.
381. E. Lipka, C. Vaccher, J.P. Bonte. Enantioseparation of benzoxazolinone aminoalcohols and their aminoketone precursors, potential adrenergic ligands, by analytical and preparative liquid chromatography on amylose chiral stationary phases and characterization of the enantiomers. *Chirality* **21** (2009) 769–776.
382. P. Krumlinde, K. Bogar, J.-E. Bäckvall. Synthesis of a Neonicotinoide Pesticide Derivative via Chemoenzymatic Dynamic Kinetic Resolution. *The Journal of Organic Chemistry* **74** (2009) 7407–7410.
383. X. Jiang, Y. Zhang, X. Liu, G. Zhang, L. Lai, L. Wu, J. Zhang, R. Wang. Enantio- and Diastereoselective Asymmetric Addition of 1,3-Dicarbonyl Compounds to Nitroalkenes in a Doubly Stereocontrolled Manner Catalyzed by Bifunctional Rosin-Derived Amine Thiourea Catalysts. *The Journal of Organic Chemistry* **74** (2009) 5562–5567.
384. F. Dong, X. Liu, Y. Zheng, Q. Cao, C. Li. Stereoselective degradation of fungicide triadimenol in cucumber plants. *Chirality* **22** (2010) 292–298.
385. R. Yazaki, N. Kumagai, M. Shibasaki. Direct Catalytic Asymmetric Addition of Allyl Cyanide to Ketones via Soft Lewis Acid/Hard Brønsted Base/Hard Lewis Base Catalysis. *Journal of the American Chemical Society* **132** (2010) 5522–5531.
386. D.L. Boger. Vancomycin, Teicoplanin and Ramoplanin Synthetic and mechanistic studies. *Medicinal Research Reviews* **21** (2001) 356–381.
387. K.H. Ekborg-Ott, L. Youbang, D.W. Armstrong. High enantioselective HPLC separations using the covalent bonded macrocyclic antibiotic, Ristocetin A, Chiral stationary phase. *Chirality* **10** (1998) 434–483.
388. P.J. Loll, P.H. Axelsen. The structural biology of molecular recognition by vancomycin. *Annual Review of Biophysics and Biomolecular Structure* **29** (2000) 265–289.
389. Y. Liu, A.W. Lantz, D.W. Armstrong. High efficiency liquid and super-/subcritical fluid-based enantiomeric separation: an overview. *Journal of Liquid Chromatography & Related Technologies* **27** (2005) 1121–1178.
390. G. Boatto, M. Nieddu, M.V. Faedda, P. De Caprariis. Enantiomeric separation by HPLC of 1,4-dihydropyridines with vancomycin as chiral selector. *Chirality* **15** (2003) 494–497.
391. K. Pihlainen, R. Kostianen. Effect of the eluent on enantiomer separation of controlled drugs by liquid chromatography–ultraviolet absorbance detection–

- electrospray ionisation tandem mass spectrometry using vancomycin and native β -cyclodextrin chiral stationary phases. *Journal of Chromatography A* **1033** (2004) 91-99.
392. R. Patel, L. Chu, V. Nanduri, J. Li, A. Kotnis, W. Parker, M. Liu, R. Mueller. Enantioselective microbial reduction of 6-oxo-8-[4-[4-(2-pyrimidinyl)-1-piperazinyl]butyl]-8-azaspiro[4.5]decane-7,9-dione. *Tetrahedron: Asymmetry* **16** (2005) 2778-2783.
 393. R. Berkecz, R. Torok, I. Ilisz, E. Forro, F. Fulop, D.W. Armstrong, A. Peter. LC Enantioseparation of β -Lactam and β -Amino Acid Stereoisomers and a Comparison of Macrocyclic Glycopeptide- and β -Cyclodextrin-Based Columns. *Chromatographia* **63** (2006) S37-S43.
 394. Y. Zhang, C. Holliman, D. Tang, D. Fast, S.B. Michael. Development and validation of a direct enantiomeric separation of pregabalin to support isolated perfused rat kidney studies. *Journal of Chromatography B* **875** (2008) 148-153.
 395. C.G. Evans, J.E. Gestwicki. Enantioselective Organocatalytic Hantzsch Synthesis of Polyhydroquinolines. *Organic Letters* **11** (2009) 2957-2959.
 396. Y. Liu, R.V. Rozhkov, R.C. Larock, T.L. Xiao, D.W. Armstrong. Fast Super/Subcritical Fluid Chromatography Enantiomeric Separations of Dihydrofurocoumarin Derivatives with Macrocyclic Glycopeptide Stationary Phases. *Chromatographia* **58** (2003) 775-779.
 397. P. Busca, F. Paradisi, E. Moynihan, A.R. Maguire, P.C. Engel. Enantioselective synthesis of non-natural amino acids using phenylalanine dehydrogenases modified by site-directed mutagenesis. *Organic and Biomolecular Chemistry* **2** (2004) 2684-2691.
 398. C.G. Avila-Ortiz, G. Reyes-Rangel, E. Juaristi. Preparation of both enantiomers of β^2 -(3,4-dihydroxybenzyl)- β -alanine, higher homologues of Dopa. *Tetrahedron* **61** (2005) 8372-8381.
 399. K. Vedha-Peters, M. Gunawardana, J.D. Rozzell, S.J. Novick. Creation of a Broad-Range and Highly Stereoselective D-Amino Acid Dehydrogenase for the One-Step Synthesis of D-Amino Acids. *Journal of the American Chemical Society* **128** (2006) 10923-10929.
 400. I. Ilisz, S. Ballet, K. Van Rompaey, R. De Wachter, D. Tourwé, D.W. Armstrong, A. Péter. High-performance liquid chromatographic separation of stereoisomers of *N*-phthaloyl-protected amino acids and dipeptidomimetics. *Journal of Separation Science* **30** (2007) 1881-1887.
 401. Z. Pataj, I. Ilisz, R. Berkecz, A. Misicka, D. Tymecka, F. Fulop, D.W. Armstrong, A. Peter. Comparison of performance of Chirobiotic T, T2 and TAG columns in the separation of β^2 - and β^3 -homoamino acids. *Journal of Separation Science* **31** (2008) 3688-3697.
 402. Z. Pataj, R. Berkecz, I. Ilisz, A. Misicka, D. Tymecka, F. Fülöp, D.W. Armstrong, A. Peter. High-performance liquid chromatographic chiral separation of β^2 -homoamino acids. *Chirality* **21** (2009) 787-798.
 403. X. Zhang, Y. Bao, K. Huang, K.L. Barnett-Rundlett, D.W. Armstrong. Evaluation of dalbavancin as chiral selector for HPLC and comparison with teicoplanin-based chiral stationary phases. *Chirality* **22** (2010) 495-513.
 404. A. Peter, E. Vekes, D.W. Armstrong, D. Tourwe. Enantioseparation by HPLC of imino acids on macrocyclic glycopeptide stationary phases and as their (*S*)-*N*-(4-

- Nitrophenoxycarbonyl)phenylalanine methoxyethyl ester derivatives. *Chromatographia* **56** (2002) S41-S47.
405. A. Peter, E. Vekes, D.W. Armstrong. Effects of temperature on retention of chiral compounds on a ristocetin A chiral stationary phase. *Journal of Chromatography A* **958** (2002) 89-107.
 406. S.M. Staroverov, M.A. Kuznetsov, P.N. Nesterenko, G.G. Vasiarov, G.S. Katrukha, G.B. Fedorova. New chiral stationary phase with macrocyclic glycopeptide antibiotic eremomycin chemically bonded to silica. *Journal of Chromatography A* **1108** (2006) 263-267.
 407. R. Berkecz, I. Ilisz, G. Benedek, F. Fulop, D.W. Armstrong, A. Peter. High-performance liquid chromatographic enantioseparation of 2-aminomono- and dihydroxycyclopentanecarboxylic and 2-aminodihydroxycyclohexanecarboxylic acids on macrocyclic glycopeptide-based phases. *Journal of Chromatography A* **1216** (2009) 927-932.
 408. H.J. Bohm, G. Klebe. What can we learn from molecular recognition in protein-ligand complexes for the design of new drugs? *Angewandte Chemie International Edition (English)* **35** (1996) 2588-2614.
 409. A.W. Adamson, A.P. Gast. Physical chemistry of surfaces. *John Wiley & Sons, Inc.*, New York, 1997.
 410. V. Sundaresan, R. Abrol. Towards a general model for protein-substrate stereoselectivity. *Protein Science* **11** (2002) 1330-1339.
 411. R. Thompson, V. Prasad, N. Grinberg, D. Ellison, J. Wyvratt. Mechanistic aspects of the stereospecific interactions of immobilized α 1-acid glycoprotein. *Journal of Liquid Chromatography & Related Technologies* **24** (2001) 813-825.
 412. R. Ledger. Nonaromatic hydroxylation of bupivacaine during continuous epidural infusion in man. *Journal of Biochemical and Biophysical Methods* **57** (2003) 105-114.
 413. M.E. Rodriguez Rosas, S. Patel, I.W. Wainer. Determination of the enantiomers of ketamine and norketamine in human plasma by enantioselective liquid chromatography-mass spectrometry. *Journal of Chromatography B* **794** (2003) 99-108.
 414. D. Whittington, P. Sheffels, E.D. Kharasch. Brainstem carbachol injections in the urethane anesthetized rat produce hippocampal theta rhythm and cortical desynchronization: a comparison of pedunclopontine tegmental versus nucleus pontis oralis injections. *Journal of Chromatography B* **809** (2004) 313-321.
 415. J. Prabhakaran, V.J. Majo, J.J. Mann, J.S.D. Kumar. Chiral synthesis of (2*S*,3*S*)-2-(2-morpholin-2-yl-2-phenylmethoxy)phenol. *Chirality* **16** (2004) 168-173.
 416. M.E. Rodriguez-Rosas, J.G. Medrano, D.H. Epstein, E.T. Moolch, K.L. Preston, I.W. Wainer. Determination of total and free concentrations of the enantiomers of methadone and its metabolite (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine) in human plasma by enantioselective liquid chromatography with mass spectrometric detection. *Journal of Chromatography A* **1073** (2005) 237-248.
 417. A. Garcia-Arieta, F. Abad-Santos, M.A. Rodriguez-Martinez, Y. Varas-Polo, J. Novalbos, N. Laparidis, S. Gallego-Sandin, K. Orfanidis, J. Torrado. An eutomer/distomer ratio near unity does not justify non-enantiospecific assay methods in bioequivalence studies. *Chirality* **17** (2005) 470-475.

418. T. Benincori, G. Celentano, T. Pilati, A. Ponti, S. Rizzo, F. Sannicolo. Configurationally Stable Molecular Propellers: First Resolution of Residual Enantiomers. *Angewandte Chemie International Edition (English)* **45** (2006) 6193-6196.
419. Y. Zhang, K. Hidajat, A.J. Ray. Enantio-separation of racemic pindolol on α_1 -acid glycoprotein chiral stationary phase by SMB and Varicol. *Chemical Engineering Science* **62** (2007) 1364-1375.
420. O. Illa, M. Arshad, A. Ros, E.M. McGarrigle, V.K. Aggarwal. Practical and Highly Selective Sulfur Ylide Mediated Asymmetric Epoxidations and Aziridinations Using an Inexpensive, Readily Available Chiral Sulfide. Applications to the Synthesis of Quinine and Quinidine. *Journal of the American Chemical Society* **132** (2010) 1828-1830.
421. M.X. Wang, G. Lu, G.J. Ji, Z.T. Huang, O. Meth-Cohn, J. Colby. Enantioselective biotransformations of racemic α -substituted phenylacetone nitriles and phenylacetamides using *Rhodococcus* sp. AJ270. *Tetrahedron: Asymmetry* **11** (2000) 1123-1135.
422. K. Harada, Q. Yuan, M. Nakayama, A. Sugii. Effects of organic modifiers on the chiral recognition by different types of silica-immobilized bovine serum albumin. *Journal of Chromatography A* **740** (1996) 207-213.
423. W.H. Lee, K.J. Kim, M.G. Kim, S.B. Lee. Enzymatic resolution of racemic ibuprofen esters: Effects of organic cosolvents and temperature. *Journal of Fermentation and Bioengineering* **80** (1995) 613-615.
424. E. Brandsteterova, K. Marcincinova, J. Lehotay, A. Zbojova, J. Halko. HPLC analysis of optical isomers of leucovorin and methotrexate using achiral-chiral system. *Neoplasma* **40** (1993) 241-245.
425. R.J. Mullin, D.S. Duch. Preparation of a chiral matrix Resolution of (6R,\$)-N5-formyltetrahydrofolate. *Journal of Chromatography A* **555** (1991) 254-259.
426. J. Haginaka, H. Takehira. Separation of enantiomers on a chiral stationary phase based on ovoglycoprotein: II. Comparison of chiral recognition properties with crude ovomucoid. *Journal of Chromatography A* **777** (1997) 241-247.
427. M. Suzuki, Y. Yanagawa, H. Iwasaki, H. Kanda, K. Yanagihara, H. Matsumoto, Y. Ohara, Y. Yazaki, R. Sakoda. First systematic chiral syntheses of two pairs of enantiomers with 3,5-dihydroxyheptenoic acid chain, associated with a potent synthetic statin NK-104. *Bioorganic and Medicinal Chemistry Letters* **9** (1999) 2977-2982.
428. J.S. Albert, D. Aharony, D. Andisik, H. Barthlow, P.R. Bernstein, R.A. Bialecki, R. Dedinas, B.T. Dembofsky, D. Hill, K. Kirkland, G.M. Koether, B.J. Kosmider, C. Ohnmacht, W. Palmer, W. Potts, W. Rumsey, L. Shen, A. Shenvi, S. Sherwood, P.J. Warwick, K. Russell. Design, synthesis and SAR of Tachykinin antagonists: modulation of balance in NK₁/NK₂ receptor antagonist activity. *Journal of Medicinal Chemistry* **45** (2002) 3972-3983.
429. D. Ohman, B. Norlander, C. Peterson, F. Bengtsson. Simultaneous determination of reboxetine and *O*-desethylreboxetine enantiomers using enantioselective reversed-phase high-performance liquid chromatography. *Journal of Chromatography A* **947** (2002) 247-254.
430. F. Pellati, S. Benvenuti, M. Melegari. Enantioselective LC analysis of synephrine in natural products on a protein-based chiral stationary phase. *Journal of Pharmaceutical and Biomedical Analysis* **37** (2005) 839-849.

THE IMPORTANCE OF CHOOSING THE OPTIMAL SOLID FORM OF A DRUG

Rolf Hilfiker

Contents

7.1. INTRODUCTION.....	352
7.2. THERMODYNAMICS	355
7.2.1. Polymorphs.....	355
7.2.2. Hydrates.....	360
7.2.2.1. Example for the Selection of a Hydrate or Anhydrate.....	363
7.3. FORM SCREENING.....	366
Screening for Salts/Co-Crystals.....	366
7.3.1.1 Example of a Co-Crystal Screen.....	367
7.3.2. Screening for Polymorphs	369
7.3.3. Types of polymorph screens.....	372
7.4. CHARACTERISATION.....	374
7.4.1. X-ray diffraction (XRD).....	374
7.4.2. Raman spectroscopy	376

7.5. CONCLUSION	378
ABBREVIATIONS.....	381
REFERENCES	382

7.1. INTRODUCTION

The majority of drugs are administered as oral dosage forms, and by far the most common oral dosage forms are tablets and capsules. This means that in the formulated product (drug product, DP), the active pharmaceutical ingredient (API) or drug substance (DS) will be in most cases in the solid state. This fact presents at the same time big challenges and big opportunities to drug developers, since a particular active molecule can in most cases exist in many different solid forms with very different physical properties.

First of all, the active molecule itself can often be crystallised as different *polymorphs*. According to McCrone's definition [1], "The polymorphism of any element or compound is its ability to crystallise as more than one distinct crystal species," we will call different crystal arrangements of the same chemical composition polymorphs. Due to the different spatial arrangement of the molecules in the crystal lattice, different inter- and intra-molecular interactions such as van der Waals interactions and hydrogen bonds will be encountered in different crystal structures. Therefore, different polymorphs will have different free energies and consequently different physical properties such as solubility, chemical stability, melting point, density, etc. It is very important to note that while this definition is used most commonly, some authors, in particular the International Conference on Harmonisation (ICH), use the term "polymorph" more broadly, including both the amorphous state and solvates [2].

If long-range order is absent in the solid phase, the substance is called *amorphous*. According to the third law of thermodynamics, the stable form at 0 K should always be a perfectly ordered one. Therefore, amorphous forms are metastable, i.e. have a higher free energy and a higher solubility than the stable crystalline phase. Generally, they are also chemically much less stable than a crystalline form. Due to their higher solubility, they may be an attractive choice to solve solubility issues of poorly soluble drugs. But, the challenge is then to find ways to ensure that the amorphous form is sufficiently kinetically stable, so that crystallisation does not occur during storage.

Many compounds can also form *solvates*, where solvent molecules are incorporated in the crystal lattice. Different types of solvates exist and they can be stoichiometric or non-stoichiometric. In stoichiometric solvates, the solvent molecule is usually an integral part of the crystal structure and is essential for the maintenance of the molecular network (see Figure 7.1). Non-stoichiometric

solvates can, for example, be channel solvates, where the solvent molecules reside in channels in the crystal structure. Of particular practical importance are hydrates, where the incorporated solvent is water. Solvates, with the exception of hydrates, are normally not a preferred form for the API. The first reason is that unnecessary addition of solvents to the drug product is undesirable; the second, and probably more important one, is that solvates are never thermodynamically stable at ambient conditions, since the vapour pressure of any solvent in the atmosphere is zero, which means that under equilibrium conditions, the solvate will desolvate, i.e. the solvent molecules will leave the crystal structure.

Figure 7.1. Schematic depiction of various types of solid forms

This situation is different for *co-crystals*. In co-crystals, the additional molecule which is incorporated in the crystal lattice is a solid at room temperature. Therefore, its equilibrium vapour pressure is negligible for practical purposes, and a co-crystal can be thermodynamically stable at ambient conditions, which can make co-crystals very viable candidates as drug substances.

When a compound is acidic or basic, it is generally possible to create a *salt* with a suitable base or acid, and such a salt can in turn often be crystallised. Since salts usually have higher water solubility and bioavailability than the corresponding uncharged molecule, they are popular choices for drug substances. About half of all active molecules are marketed as salts [3,4].

Obviously, salts may also crystallise as solvates or co-crystals; solvates, co-crystals and salts, together with their solvates and co-crystals, may all be able to exist as different polymorphs. This means that the potential number of solid forms of an API is indeed very large, which implies on one hand that significant effort is needed to produce and characterise all relevant forms, but on the other hand, it also means that there is a huge potential to optimise the properties of the solid according to the specific requirement of the drug. Of fundamental importan-

ce to the decision as to which form to develop is the understanding of the thermodynamic relationship between the forms.

Therefore, the following steps should typically be followed in terms of solid-state investigations during the development of a new drug:

- screening for salts and/or co-crystals if the properties of the parent molecule are not optimal,
- screening for polymorphs, hydrates and solvates of the parent molecule, salt or co-crystal,
- characterisation of the various forms and elucidating their thermodynamic and kinetic relationships,
- selection of the optimal form,
- formulation development,
- development of analytical methods to assess form purity,
- development of a stable and efficient crystallisation process to produce the desired form.

Polymorphism is a very common phenomenon in connection with drug substances where the API is a small organic molecule with a molecular weight below 600 g/mol [3,5]. Literature values for the prevalence of polymorphs, solvates and hydrates vary. Figure 7.2a is the result of 180 focused polymorph screens carried out at a contract research organisation [6] and Figure 7.2b shows the statistical evaluation of entries in the European Pharmacopoeia with regard to solid-state information [7]. It is not surprising that the prevalence of different solid forms found in the European Pharmacopoeia is lower than for the recently carried out polymorph screens, since some of the API in the Pharmacopoeia were discovered a long time ago, when polymorph screens were not always carried out thoroughly. The data show that the prevalence of substances which can exist in various solid forms is high and that if considerable effort is put into polymorph screening, several solid forms will be discovered for the vast majority (>85%) of substances.

Figure 7.2. Frequency of occurrence of solid forms of organic molecules, (a) result of 180 focused polymorph screens; (b) from data in the European Pharmacopoeia

The importance, implications and investigation of polymorphism is described in-depth in several recent monographs and edited books [8-12]. An excellent book about salts in the pharmaceutical industry is the one by Stahl & Wermuth [4].

Even if the envisaged market form of the drug is not a solid but a solution, information about the solid-state properties of the drug substance may still be necessary [13]. If different forms have significantly different solubilities, it may be possible to unintentionally create a supersaturated solution with respect to the least soluble form by preparing a concentrated solution of a metastable form. Furthermore, the drug substance will in most cases be handled as a solid in some stages of the manufacturing process, and its handling and stability properties may depend critically on the solid form.

In addition to the crystalline, amorphous and liquid states, condensed matter can exist in various mesophases. These mesophases are characterised by exhibiting partial order between that of the crystalline and amorphous states [14,15]. Several drug substances are known to form liquid crystalline phases, which can be either thermotropic, where liquid crystal formation is induced by temperature, or lyotropic, where the transition is solvent-induced [16-18]. Such mesophases as well as the amorphous form will not be further discussed in this chapter.

7.2. THERMODYNAMICS

Thermodynamics describes which form is stable under which condition as a function of external conditions (temperature, pressure, humidity, *etc.*). It is clearly essential to know which form is stable under given conditions, since any other form may convert to this stable form during storage. Such a transition will change the properties of the drug and may therefore affect its efficacy or safety. In the absence of humidity, there will be exactly one non-solvated polymorph of a particular compound (active molecule, salt or co-crystal) which is thermodynamically stable at a certain temperature and pressure (except at the transition temperature of an enantiotropic system; see below). Under ambient conditions, *i.e.* at a certain temperature and humidity, a hydrate may be the thermodynamically stable form. This is the reason why, in most cases, either a hydrate or the thermodynamically stable non-solvated form is developed. It ensures that no conversion takes place during storage. The disadvantage of the stable form is that it will have the lowest solubility and therefore also the lowest bioavailability if it is a BCS (Biopharmaceutical Classification System) class II or IV drug [19].

7.2.1. Polymorphs

At constant pressure, the stability of a compound or a mixture of compounds is determined by its Gibbs free energy (G). The temperature dependence of the basic thermodynamic state functions enthalpy (H), entropy (S) and Gibbs free energy is given by Equations 7.1 to 7.3, where $c_{p,m}$ is the molar heat capacity at

constant pressure, T is the absolute temperature, H^0 is the enthalpy at 0 K and S^0 is the entropy at 0 K [20]. According to the third law of thermodynamics, S^0 is zero for a perfect crystal (see Introduction).

$$H = H^0 + \int_0^T c_{p,m} dT \quad (7.1)$$

$$S = S^0 + \int_0^T \frac{c_{p,m}}{T} dT \quad (7.2)$$

$$G = H - TS \quad (7.3)$$

The temperature dependence of H , S , TS and G of any solid is depicted schematically in Figure 7.3.

Figure 7.3. Temperature dependence of enthalpy (H), free energy (G), entropy (S) and TS

Using these equations, we can now calculate the stability of polymorphs and the amorphous form (or melt) as a function of temperature. These could be either polymorphs of a single molecule, a particular solvate, salt or co-crystal. In Figure 7.4, the enthalpy and free energy of two polymorphs and the amorphous form/melt are plotted as a function of temperature. This is called an **energy-temperature diagram** or ET diagram. The phase with the lowest free energy at any temperature is the thermodynamically stable one at that particular temperature. Thus, the melting point (T_m) of a polymorph is defined as the temperature where the free energy of that polymorph is equal to the free energy of the melt. If two different polymorphs (α and β) exist, two different situations are now possible:

- (i) the free energy functions of polymorph α and polymorph β do not intersect at a temperature below the melting point of polymorph β (Figure 7.4a) and
- (ii) the free energy functions of polymorph α and polymorph β do intersect at a temperature which is lower than the melting point of polymorph β (Figure 7.4b).

If (i) is the case, the system of polymorph α and β is called a **monotropic** system, which means that polymorph α is more stable than polymorph β at all temperatures below the melting point of the higher melting form. In situation (ii), the system of polymorph α and β is called an **enantiotropic** system. In such a situation, polymorph β is the thermodynamically stable form from 0 K to the transition temperature (T_t) and polymorph α is the stable form from the transition temperature to its melting point ($T_{\text{melt},\alpha}$). If more than two polymorphs exist, the above arguments can be extended in an analogous way.

Figure 7.4. Qualitative energy-temperature (ET) diagram for (a) a monotropic and (b) an enantiotropic system. The temperature regions of the stable forms are marked

It is very important to know if a system is monotropic or enantiotropic and what the transition temperature is in an enantiotropic system. If, for example, the transition temperature is 40°C and the low temperature form is chosen for development, it has to be ascertained that form conversion to the high temperature form will not occur during the processing steps or storage where higher temperatures may occur. It will also impact the design of the crystallisation process. If crystallisation starts at a temperature above the transition temperature, it must be ensured that the metastable form nucleates first, if the stable form at room temperature is the target and if a phase transformation during the crystallisation process is to be avoided, which is generally the case.

Constructing quantitative energy-temperature diagrams of the form in Figure 7.4 is experimentally difficult, since it requires knowledge of the enthalpies and heat capacities starting from 0 K, a range which is very difficult to access. If, however,

the enthalpy and free energy difference between the polymorph and amorphous form/melt is plotted as a function of temperature, quantitative energy-temperature diagrams are readily accessible. Taking into account that the free energy difference between the melt and the polymorph at the melting point of the polymorph is zero, Equations 7.4 and 7.5 are obtained, which allow accurate calculation of enthalpy and free energy differences in the temperature range where the heat capacity can be determined. Naturally, the range close to 0 K will still be difficult to access, but it is normally of no practical interest anyway. From such a quantitative diagram, the transition temperature, the heat of transition between polymorphs (see Chapter 8) and the free energy difference and hence solubility ratio of polymorphs are obtained. $\Delta H_{m,\alpha} = H_{liq} - H_{\alpha}$ at $T_{m,\alpha}$ is the melting enthalpy of polymorph α .

$$H_{\alpha} - H_{liq} = -\Delta H_{m,\alpha} + \int_{T_m}^T (c_{P,m,\alpha} - c_{P,m,liq}) dT \quad (7.4)$$

$$G_{\alpha} - G_{liq} = -\Delta H_{m,\alpha} + \int_{T_m}^T (c_{P,m,\alpha} - c_{P,m,liq}) dT - T \left(\frac{-\Delta H_{m,\alpha}}{T_{m,\alpha}} + \int_{T_m}^T \frac{c_{P,m,\alpha} - c_{P,m,liq}}{T} dT \right) \quad (7.5)$$

If we make the further simplifying assumption that the heat capacity differences between solid and liquid are zero in the temperature range of interest, Equation 7.6 follows.

$$G_{\alpha} - G_{liq} \approx -(\Delta H_{m,\alpha} / T_{m,\alpha}) (T_{m,\alpha} - T) \quad (7.6)$$

Therefore, using experimentally determined values of T_m and ΔH_m (see Chapter 8), quantitative energy-temperature diagrams can be conveniently constructed with the simplifying assumption stated above (see Figure 7.5). The slopes of the lines are equal to the ratio of melting enthalpy and melting temperature of the corresponding polymorph.

From Equation 7.6, the transition temperature of enantiotropic systems can then also be easily calculated (Equation 7.7); see also Chapter 8.

$$T_t = \frac{T_{m,\alpha} T_{m,\beta} (\Delta H_{m,\beta} - \Delta H_{m,\alpha})}{T_{m,\alpha} \Delta H_{m,\beta} - T_{m,\beta} \Delta H_{m,\alpha}} \quad (7.7)$$

Figure 7.5. Quantitative ET diagram (difference of enthalpy/energy between a polymorph and melt) for (a) a monotropic and (b) an enantiotropic system (heat capacity differences neglected)

It is important, however, to realise that Equation 7.7 neglects the heat capacity difference between the solid and the melt. This can lead to errors in the calculated transition temperature, especially if the melting temperatures of the two polymorphs are far apart, and if the difference between the heat capacities of the melt and polymorph are not negligible, which is often the case. In such a situation, Equation 7.5 should be applied. The effect of heat capacity differences on calculated transition temperatures is demonstrated in Figures 7.6a and 7.6b. For that particular substance, the melting points were 28 K apart and the heat capacity difference between the melt and the solid was about $0.4 \text{ J K}^{-1} \text{ g}^{-1}$. Without taking the heat capacity difference between melt and solid into account, a

transition temperature of 295 K was calculated (Figure 7.6a). Considering the measured heat capacities led to a shift of the calculated transition temperature to 340 K (Figure 7.6b). This value was later confirmed by slurry experiments of the two polymorphs at various temperatures. Performing such slurry experiments is highly recommended to confirm the calculated values of transition temperatures.

Figure 7.6. Quantitative ET diagram. Example for a certain API: $T_{\text{melt,I}}=413\text{K}$, $\Delta H_{\text{melt,I}} = 97 \text{ J g}^{-1}$; $T_{\text{melt,II}}=441 \text{ K}$, $\Delta H_{\text{melt,I}} = 84 \text{ J g}^{-1}$. (a) heat capacity difference between the melt and solid neglected, (b) measured heat capacity difference between the melt and solid ($c_{p,\text{I}} - c_{p,\text{melt}} \approx c_{p,\text{I}} - c_{p,\text{melt}} \approx -0.4 \text{ J K}^{-1} \text{ g}^{-1}$) taken into account. Note that units are not in mole units but in mass units. This has no effect on Equations 7.4 to 7.7 as long as the same set of units is used throughout

7.2.2. Hydrates

As mentioned in Section 7.2, the form chosen for the final drug product is in most cases either the non-solvated polymorph, which is thermodynamically stable at room temperature, or a hydrate. Therefore, hydrates deserve special attention and both their thermodynamic and kinetic properties have to be elucidated.

Both stoichiometric and non-stoichiometric hydrates exist. In stoichiometric hydrates, the ratio between the number of water molecules and the number of compound molecules in the crystal is well-defined and constant over a certain range of water activities. The water molecules are then normally located at specific positions in the crystal lattice as schematically depicted in Figure 7.1. Non-stoichiometric hydrates, however, contain an amount of water which can continuously vary within a certain range when the water activity of the environment is varied. In such cases, the water molecules often reside within channels, larger voids or layers of the crystal lattice [21]. Compounds may also form multiple stoichiometric hydrates. An example is shown in Figure 7.7. This particular substance can exist as a mono-, sesqui-, di-, hemipenta- and trihydrate.

Figure 7.7. Dynamic vapour sorption (DVS) of a substance which can form an anhydrate, as well as a mono-, sesqui-, di-, hemipenta- and trihydrate

While the thermodynamic properties of non-solvated forms depend only on temperature and pressure, the free energy of hydrates (and solvates) is influenced by the water (or solvent) activity of the environment. The formation of a stoichiometric hydrate with n molecules of water per compound molecule can be described by Equation 7.8.

The equilibrium constant K_h for hydrate formation can be expressed by Equation 7.9, where $a_{H_2O,eq}$ is the water activity at equilibrium. The activities of solids are defined as unity and therefore do not appear in Equation 7.9.

$$K_h = a_{H_2O,eq}^{-n} \quad (7.9)$$

Equation 7.9 shows that there is one very well-defined water activity, where the hydrate and anhydrate are in equilibrium. If the water activity (or relative humidity) is slightly below this critical water activity, only the anhydrate is stable, and if it is slightly above, only the hydrate is stable, *i.e.* the typical situation of a step-wise phase transition is observed. Knowledge of this critical water activity is of great importance in order to be able to decide which form is the preferred one for development.

The standard free energy of hydrate formation can then be calculated using Equation 7.10.

$$\Delta G_h^0 = -RT \ln(K_h) \quad (7.10)$$

Knowing ΔG_h^0 , which can be calculated from the experimentally determined $a_{H_2O,eq}$, the free energy difference between hydrate and anhydrate (ΔG_h) can be calculated as a function of water activity using Equation 7.11.

$$\Delta G_h = \Delta G_h^0 + RT \ln(a_{H_2O}^{-n}) \quad (7.11)$$

From the free energy difference of the hydrate and anhydrate, important parameters like the ratio of solubilities (L) and intrinsic dissolution rates (J) between the hydrate and anhydrate can be calculated (Equation 7.12). For the derivation of Equation 7.12, the assumption is made that the activity coefficient is unity. This is generally a very good approximation as long as the solubility is not very high.

$$\Delta G_h = \Delta G_h^0 + RT \ln(a_{H_2O}^{-n}) = RT \ln \frac{L_{hydrate}}{L_{anhydrate}} = RT \ln \frac{J_{hydrate}}{J_{anhydrate}} \quad (7.12)$$

In water, the water activity is 1 and Equation 7.12 can be simplified obtaining Equation 7.13.

$$\Delta G_h^0 = RT \ln \frac{L_{hydrate}}{L_{anhydrate}} = RT \ln \frac{J_{hydrate}}{J_{anhydrate}} \quad (7.13)$$

Combining Equation 7.12 with Equations 7.9 and 7.10 yields Equation 7.14, which describes the relationship between the critical water activity for hydrate formation, solubility and the dissolution rate in water.

$$\frac{L_{hydrate}}{L_{anhydrate}} = \frac{J_{hydrate}}{J_{anhydrate}} = a_{H_2O,eq}^n \quad (7.14)$$

It can be seen that the solubility and dissolution rate of the hydrate in water are smaller compared to the anhydrate if the critical water activity is smaller than 1. The reduction of solubility and dissolution rate is higher if the critical water activity is lower and if the stoichiometry of the hydrate is higher.

7.2.2.1. Example for the Selection of a Hydrate or Anhydrate

Carbamazepine (CBZ) can be used as an example how the characterisation and selection process should be carried out. Many solid forms of CBZ are known. In addition to many solvates and co-crystals, four non-solvated forms and a dihydrate are described in the literature. A brief description of the latter is given in Table 1 [22,23]. Different authors use different nomenclature for the forms of CBZ. It is therefore recommended that the crystal system is listed at the same time, since only that is unambiguous.

Table 1. Properties of various forms of carbamazepine (CBZ).

	Form I	Form IIa (IV)	Form IIb	Form III	Dihydrate
Crystal System	triclinic	C-monoclinic	trigonal	P-monoclinic	orthorhombic
Melting behaviour	~ 194°C	~ 188°C	135 – 170°C → Form I	162 – 175°C → Form I	n.a.
Properties	thermodynamically stable above ~ 73°C			thermodynamically stable below ~ 73°C	

According to what was mentioned above, the two forms which should be considered for development are the thermodynamically stable form at room temperature (Form III) and the dihydrate. In order to be able to make this choice, both the thermodynamics and kinetics of hydrate formation and dehydration have to be elucidated. A dynamic vapour sorption (DVS) experiment on Form III (Figure 7.8) showed no indication of hydrate formation within the timeframe of the experiment.

Figure 7.8. DVS of CBZ Form III. The thick black line represents the humidity and the thin black line the mass of the sample. It can be seen that Form III does not convert to the dihydrate, even though it is exposed to humidities higher than $a_{\text{H}_2\text{O,eq}}$ for one day

Figure 7.9. DVS of CBZ dihydrate. Water loss is observed at about 5 % relative humidity, which is well below $a_{\text{H}_2\text{O},\text{eq}}$

Figure 7.9 is the corresponding DVS of the dihydrate. It shows that dehydration occurs at about 5% relative humidity. These two experiments reveal that the critical water activity for the formation of the dihydrate is above 0.05, but they fail to provide a reasonable estimate for the actual value. This is generally the case for DVS experiments, since hydration and dehydration can be very slow, such that kinetic effects dominate.

An appropriate method to determine the critical water activity is to perform slurry experiments on mixtures of the hydrate and anhydrate in water/solvent mixtures of known water activity. Solvents which do not form solvates with the particular substance should be chosen, so that solvate formation will not compete with the formation of hydrates and non-solvated forms. If the water activity of this solvent/water mixture is now below the critical water activity of hydrate formation, the hydrate will dehydrate and, at the end, a pure anhydrate will be obtained and *vice versa*. A correction may have to be applied for the water removed from the solvent/water mixture due to hydrate formation or the water released to the solvent/water mixture due to dehydration of the hydrate.

In Figure 7.10, the resulting Raman spectra of another substance which can form an anhydrate and a hemihydrate in slurries of various water activities are shown. It can clearly be seen that the characteristic peak of the hemihydrate is observed at water activities of 0.8 and higher, *i.e.* the critical water activity for hemihydrate formation is about 0.75 in that particular case.

Figure 7.11 shows the result of such slurry experiments of CBZ Form III and the dihydrate in a variety of water/solvent mixtures. It shows that the critical water activity for dihydrate formation is about 0.65 at 23°C. Within experimental error,

it is independent of the solvent, as it should be. Using this value, the standard free energy difference between the dihydrate and Form III can be calculated as -2.4 kJ/mol (Equations 7.9 & 7.10). From this, it follows that Form III is about 2.6 times more soluble in water than the dihydrate.

Figure 7.10. Raman spectra of the anhydrate (bottom) and hemihydrate (top) of an API. The spectra in between were recorded after mixtures of the anhydrate and hemihydrate were slurried for two days in water/solvent mixtures with water activities of 0.6, 0.7, 0.8 and 0.9. It can be seen that for $a_w=0.8$ and 0.9, complete conversion of the anhydrate to the hemihydrate occurred, while the opposite was the case for $a_w=0.7$ and 0.6

Figure 7.11. Results of slurry experiments of mixtures of CBZ Form III and the dihydrate in various water/solvent mixtures with water activities of 0.1, 0.2, 0.3, etc. ($T=23^{\circ}\text{C}$). Grey denotes that the resulting form was Form III, black denotes that the resulting form was the dihydrate. (MeOH – methanol, EtOH – ethanol, 2PrOH – 2-propanol, THF – tetrahydrofuran)

It has to be considered that the critical water activity for hydrate formation is also temperature dependent and increases with temperature in the vast majority of cases. At 37 °C, the corresponding values for CBZ are: $a_{\text{H}_2\text{O}, \text{eq}} \approx 0.8$, $\Delta G_{\text{h}}^0 \approx -1.5$ kJ/mol, solubility ratio in water ≈ 1.8 . Based on these data gathered for the two forms of CBZ, Form III would be favoured, since

- it has an approximately two-fold higher water solubility and CBZ is BCS Class II, i.e. solubility is a bottleneck,
- it is thermodynamically stable in dry conditions and up to fairly humid conditions (65 % r.h.),
- if it is exposed to high humidities for many hours, it will still be *kinetically* stable.

Of course, other aspects which might affect the ease of manufacturing, processing and formulation have to be taken into account as well.

Knowledge of the critical water activity as a function of temperature is also very helpful for the design of the crystallisation process of Form III, since a water activity of the crystallisation medium exceeding this critical water activity should always be avoided.

7.3. FORM SCREENING

Clearly, before being able to select the optimal form, the relevant forms have to be identified and prepared, *i.e.* form screening has to be carried out. Most substances will be able to form salts, co-crystals or both, and therefore salt or co-crystal screening is often the first step in form selection.

7.3.1. Screening for Salts/Co-Crystals

In general, salt formation will be possible if the molecule contains acidic or basic groups, which is the case for a large fraction of active molecules. Since making a salt will normally involve an additional step in the synthesis and since the molecular weight of a salt will always be higher than that of the neutral molecule, salts will only be chosen if they promise to have clear advantages compared to the free acid/base. As a rule, a salt is chosen if the free acid/base has at least one of the following undesirable properties:

- very low solubility in water,
- low melting point,
- high hygroscopicity,
- low chemical stability,
- problematic solid forms, such as channel hydrates or enantiotropic polymorphs with a transition temperature close to room temperature,
- other undesirable properties such as unfavourable habit,
- intellectual property (IP) issues.

The potential acids or bases which might be able to form a salt with the API under investigation are chosen based on pK_a differences, counterion toxicity (preferably Generally Recognized As Safe (GRAS)) status, see also the classification scheme in [4]), *etc.* As a rule of thumb, a pK_a difference of 2-3 is sufficient for salt formation. It is advisable to perform salt screening and salt selection in stages, starting on the microscopic scale (e.g. a 96-well plate format) where a large number of salts is produced and characterised using a limited number of methods (e.g. birefringence, Raman spectroscopy, X-ray powder diffraction (XRPD)) in order to identify a few promising candidates. Suitable methods for producing salts in such a high-throughput format include very slow evaporation of stoichiometric quantities of the acid and base in a variety of solvents where the solubilities of the acid and the base are sufficiently high, and slurring of the acid and base in solvents where the solubility of the salt is not very high. In the second stage, the successful candidates of the high-throughput screening are then produced on the scale of a few hundred milligrams and characterised in more detail in order to select the best ones. Desirable properties of the salts include crystallinity, high water solubility, low hygroscopicity, good chemical stability and a high melting point. The relative importance of these properties may vary from project to project.

Co-crystals can offer valuable alternatives, especially for very weak bases or acids. Reasons for attempting to make co-crystals are the same as for salts.

7.3.1.1 Example of a Co-Crystal Screen

A certain API had a needle-like morphology with an extremely large aspect ratio (Figure 7.12). Due the corresponding low bulk density, this posed considerable problems during formulation and processing, since the API required a high dosage strength. Attempts to change the morphology by changing crystallisation conditions and the solvents for crystallisation failed, and salts were not an option, because the API was neither acidic nor basic. Therefore, co-crystal formation was attempted.

Figure 7.12. Microscopic image of an API with an unfavourable needle-like morphology.

The API had an amide group which can form very strong interactions with carboxylic acids by forming eight-membered rings with two hydrogen bonds (see Figure 7.13), making carboxylic acids promising candidates for co-crystal formation.

Figure 7.13. Possible interaction motif between an amide and a carboxylic acid.

The following 24 compounds were selected as potential co-crystal formers based on their potential interaction with the API and their toxicity:

- acetylsalicylic acid
- *L*-aspartic acid
- benzenesulphonic acid
- benzoic acid
- citric acid
- ethanedisulphonic acid
- fumaric acid
- gentisic acid
- *L*-lactic acid
- maleic acid
- *L*-malic acid
- malonic acid
- *L*-mandelic acid
- methanesulphonic acid
- oxalic acid
- pamoic acid
- saccharin
- succinic acid
- *L*-tartaric acid
- ethyl maltol
- methyl 4-hydroxybenzoate
- urea
- *L*-proline
- *L*-tryptophan

In order to optimise the chances of success of a co-crystal screen, an understanding of phase diagrams of the co-crystal former, API and solvent is very beneficial [24,25]. Depending on the respective behaviour, different strategies are optimal.

In this particular case, equimolar amounts of the API and co-crystal former were mixed in four different solvents where the API and co-crystal former had similar solubilities. These 96 (4x24) solutions were then evaporated very slowly under a controlled nitrogen stream in a 96-well plate. The resulting solids were analysed by Raman spectroscopy and XRPD. In a second set of experiments, equimolar amounts of the API and co-crystal former were mixed in four different solvents which did not dissolve the API and co-crystal former. These slurries were temperature-cycled for 4 days, then the solvent was evaporated and the solids were analysed in the same way. The XRPD and Raman spectra of the 192 samples showed that, in many cases, a crystalline API and/or crystalline co-crystal former was obtained in the end. For benzoic, ethanedisulphonic, maleic, *L*-malic, *L*-tartaric acid and ethyl maltol, Raman and XRPD spectra were recorded which differed from the components, *i.e.* they were promising candidates for co-crystal formation (see also Section 7.4.2). These were then scaled-up to about 100 mg and first characterised by Raman spectroscopy and XRPD in order to verify that the same form was obtained in the scale-up experiment as in the microscopic high-throughput experiment. They were further characterised by NMR, elemental analysis, differential scanning calorimetry (DSC), thermogravimetry-Fourier transform IR (TG-FTIR), DVS, aqueous solubility and light microscopy in order to judge if their physical properties were favourable. It turned out that co-crystals with benzoic acid and maleic acid had good physical properties (high melting point, low hygroscopicity and sufficient aqueous solubility) and fulfilled the target of the study, *i.e.* they had a much more desirable morphology.

7.3.2. Screening for Polymorphs

Screening for polymorphs and other relevant solid forms is not an easy task and requires a lot of experience, since it has to be ensured that the largest possible variety in crystallisation conditions is used, both in terms of the type of crystallisation methods and solvent choice. The identification of solvates is also important, not because they might be used in the dosage form, but because it is advisable not to use solvents which can form solvates with a particular compound in a crystallisation process to produce that compound.

The fact that different polymorphic forms can be obtained when the crystallisation conditions are changed can be explained by Ostwald's rule of stages which states that, "When leaving a given state and in transforming to another state, the state which is sought out is not the thermodynamically stable one, but the state nearest in stability to the original state." [26]. The rule implies that during a crystallisation process, several forms may crystallise in sequence starting from the least stable form and ending up in the stable form. Or, in other words, if a very fast crystallisation method, such as a rapid precipitation, is applied, one is likely to obtain a metastable form, while a very slow

crystallisation process, such as a slow cooling or slurry ripening, would yield the stable form. But, true to its name, Ostwald's rule of stages is a rule and not a law, and many violations of this rule have been reported. Nevertheless, it is a useful concept for the design of polymorphism screens.

A more mechanistic view of the formation of different polymorphs takes into account cluster formation, nucleation and crystal growth [27,28]. This model also readily explains the influence of the solvent (if crystallisation is performed from a solution) and additives in addition to the crystallisation speed. Clusters and nuclei are small, and therefore the surface free energy significantly influences the value of the total free energy of the cluster or nucleus. This is depicted schematically in Figure 7.14. The free energy of individual molecules, *i.e.* very small clusters, is equal to the free energy in solution (G_{solution}). Due to the surface energy, the free energy then increases with increasing cluster radius up to a critical cluster radius (r^*) and the corresponding free energy (G_{nucleus}). Further growth of the nucleus leads to a decrease in free energy and is therefore spontaneous. When the cluster is large enough such that the surface energy becomes negligible, the free energy of the crystal (G_{α} or G_{β}) is adopted, which is smaller than G_{solution} if the solution is super-saturated. Solvent and/or additive molecules can preferentially adsorb on the surface of the nuclei and can therefore alter the heights of the activation barriers $\Delta G_{\alpha}^* = G_{\text{nucleus},\alpha} - G_{\text{solution}}$ and $\Delta G_{\beta}^* = G_{\text{nucleus},\beta} - G_{\text{solution}}$ for polymorph α and β , respectively. Since the surfaces of different polymorphs may be different, this preferential adsorption may also depend on the polymorph. Therefore, a situation may be encountered where ΔG_{α}^* is larger than ΔG_{β}^* in a certain solvent A, while in another solvent B, ΔG_{β}^* is larger than ΔG_{α}^* , such that polymorph β is kinetically favoured in solvent A while polymorph α preferentially forms in solvent B. A type of additive that can have a particularly large effect is impurities [29-31] with a similar structure to the main component, since they may adsorb very strongly on the surface of certain nuclei due to their chemical similarity. Such impurities are often formed during the final steps of the synthetic process. It is therefore crucial to perform crystallisations with a variety of solvents in order to increase the chance of successful competition of solvent molecules with impurity molecules for preferential adsorption.

The relative thermodynamic stability of polymorphs of non-solvated forms, on the other hand, is independent of the solvent it is formed in, since the solvent can only contribute to the surface free energy of the crystal. While the surface free energy is an important contribution to the total free energy of nuclei which are less than a few nanometres in diameter, the surface free energy is negligible compared to the bulk free energy if the crystal size is larger than about 100 nm. The formation and stability of solvates, however, is of course influenced by the choice of solvent. This is yet another reason why various solvents should be used in a polymorph screen.

Therefore, in order to find all possible polymorphs and solvates, a large number of crystallisation methods in a large number of diverse environments have to be carried out.

Figure 7.14. Energy barriers for polymorph crystallisation

Classical crystallisation methods have been reviewed by Guillory [32] and are the following:

- crystallisation by cooling a solution,
- crystallisation by evaporation,
- crystallisation by precipitation,
- crystallisation by vapour diffusion,
- suspension equilibration (often also called “slurry ripening”),
- crystallisation from the melt,
- heat induced transformations,
- crystallisation by sublimation,
- desolvation of solvates,
- crystallisation by salting out,
- crystallisation by pH change,
- lyophilisation.

Within these methods, further diversity can be introduced by changing solvents, temperatures, pressure, rates of cooling, evaporation, *etc.* Several types of crystallisation experiments are also influenced by the initial solid form that is used (*i.e.* polymorph, solvate, hydrate or the amorphous form) as this can affect the solubility and hence the degree of supersaturation. As explained above, the choice of solvent is of particular importance. First, it has to be chosen such that the solubility is in a suitable range for the selected type of crystallisation

experiment (reasonably high solubility for cooling experiments, very low solubility in solvents used for precipitation, *etc.*). Second, it is important to use solvents with diverse physical properties in order to explore the entire parameter space of possible environments. There are several approaches in order to classify solvents in terms of dissimilarity. A very useful one is the method applied by Gu et al. [33]. They examined 96 solvents in terms of eight relevant solvent properties: hydrogen bond acceptor propensity, hydrogen bond donor propensity, polarity/dipolarity, dipole moment, dielectric constant, viscosity, surface tension and cohesive energy density and sorted the 96 solvents into 15 groups according to their statistical similarity using principal component analysis. As expected, solvent molecules with the same functional groups were often found within the same group. In a thorough polymorphism screen, where the highest diversity is desired, crystallisations using at least one solvent of each group should therefore be carried out, so that the whole spectrum of solvent properties is used in the crystallisation experiments.

Of course, one can never be sure that one has found all relevant polymorphs unless one carries out an infinite number of experiments. This essentially paraphrases McCrone's famous statement, "It is at least this author's opinion that every compound has different polymorphic forms and that, in general, the number of forms known for a given compound is proportional to the time and money spent in research on that compound" [1].

It may also be difficult to reproduce the crystallisation of a metastable form and there are accounts of so-called "disappearing polymorphs", *i.e.* forms which could be obtained at some stage but not crystallised at a later date. As nucleation is a stochastic process, it is possible that two crystallisation experiments which are performed in exactly the same way may lead to a different outcome. If conditions are chosen properly, however, and any potential seeds of other forms are carefully removed, it should always be possible to reproduce any form if a large enough number of experiments is performed.

7.3.3. Types of polymorph screens

Given the fact that it is likely that the more solid forms are found, the more effort is invested in the search and that one can never be sure that all relevant forms are experimentally discovered, two questions arise immediately, *i.e.* how extensive a search should be carried out and when in the development process a polymorph screen should be carried out. The answers to these questions must take both economic and safety criteria into account.

Regulatory authorities have set some criteria in order to ensure the safety and efficacy of drug products. Therefore, ICH guidelines [2] demand that a polymorph screen has to be carried out in order to obtain regulatory approval for pharmaceutical products. There is, however, no guideline given on how such a screen should be performed.

Economic criteria would suggest that a polymorph screen is conducted late in the development process of a drug, since the attrition rate of drugs during clinical development is large. Only on the order of 10% of the drugs entering Clinical Phase I will gain final approval as drug products [34]. On the other hand, it would be an unacceptable risk to develop a dosage form without knowing which form is thermodynamically stable under certain conditions of temperature and humidity. Therefore, a good strategy which balances economic considerations and risk is to carry out several polymorph screens during the development process which are appropriate for the corresponding phase.

Often, the formulation used in Clinical Phase II is close to the final formulation of the drug. Therefore, by that time, the optimal solid form should have been identified with a reasonably high probability. A limited polymorph screen in the preclinical phase or Clinical Phase I will therefore generally have the aim of identifying the thermodynamically stable polymorph and hydrates with a confidence level of 70 – 90 %. If the drug is successful in Clinical Phase II, this confidence level should be increased and the search should be extended to metastable forms, since they may be both important for the development of a crystallisation process of the desired stable form and they may also improve the patent protection of the drug. The relevance of metastable forms for the development of a crystallisation process stems from the fact that metastable forms might be produced inadvertently if their existence and their properties are not known and no appropriate measures are taken so that they are not formed.

A polymorph screen should proceed in a well-structured and standardised way, while recognising that the procedure has to be tailored with respect to the substance under investigation. An approach that is not adapted to the substance is prone to inefficiency and overlooking relevant forms. The procedure used should also be tunable to permit various degrees of thoroughness as stated above.

In essence, a polymorph screen should at least contain the following elements:

- Characterisation of the starting material by methods such as powder or single-crystal XRD, DSC, TG-FTIR or thermogravimetry-mass spectrometry (TG-MS), DVS, Raman or IR, magic-angle spinning (MAS) NMR, solubility measurements, microscopy and HPLC (purity).
- If the substance does not degrade at the melting point, hot-stage microscopy or hot-stage Raman microscopy can be a very efficient way for creating other polymorphs [35].
- Crystallisation experiments from solution, using several techniques with a variety of solvents and solvent mixtures. Due to the practical relevance of hydrates, water and water/solvent mixtures should always be included. Suspension equilibration and slow cooling experiments have particular importance in finding the thermodynamically stable form, which is often the most important aim of a polymorph screen.

- All new forms have to be characterised by the methods used to characterise the starting material and their thermodynamic relationships have to be elucidated.
- Other techniques such as desolvation of solvates, mechanical stress (pressure, grinding) are necessary to obtain the information needed for the processing and manufacturing steps.

Very useful tools for polymorphism (and salt and co-crystal) screens are high-throughput screening systems, where crystallisation experiments are carried out in an array format, using *e.g.* 96-well plates. Such systems have been developed by research foundations [36], big pharmaceutical companies [37-39] and companies specialising in solid-state research and development [40-44]. They commonly allow crystallisation by cooling, evaporation, precipitation and slurry conversion. Primary characterisation of the solid is generally performed by Raman microscopy and/or XRPD.

High-throughput screens should always be complemented by larger scale experiments, however, where more of the corresponding form is produced in order to allow a full physicochemical characterisation. It may also be necessary to subject the substance to conditions which are not accessible in a high-throughput screening system.

7.4. CHARACTERISATION

Many physicochemical and physical methods are suitable to characterise solids. They include thermal methods like DSC, TG, thermo-mechanical analysis (TMA), thermally stimulated current (TSC), microcalorimetry and thermomicroscopy; spectroscopic methods like IR, Raman, near IR (NIR), NMR and Terahertz spectroscopy; coupled methods like TG-FTIR, TG-MS and hot-stage Raman microscopy; scattering methods like XRD; particle sizing methods like Fraunhofer diffraction, sieving, etc.; and mechanical tests to assess hardness, flow properties, etc.

Other chapters describe many of these important methods in detail (thermal methods which are extremely useful for understanding the thermodynamic properties of solids in Chapter 8, NIR spectroscopic method in Chapter 9). Therefore, we will focus here on only two very important methods for the characterisation of solid forms. Both X-ray diffraction and Raman spectroscopy are very sensitive and specific for the identification of different solid forms.

7.4.1. X-ray diffraction (XRD)

The physical basis of both single crystal X-ray diffraction and XRPD is Bragg's law (Equation 7.15), which describes the relationship between the angle for constructive interference (Θ), layer spacing (d), wavelength (λ) and order of the interference (n).

$$n\lambda = 2d \sin(\Theta) \quad (7.15)$$

Figure 7.15. Principle of Bragg's law: constructive interference when the path difference is λ or multiples of λ

In single crystal XRD, the crystal is placed in an X-ray beam and the spatial intensity of the diffracted X-ray light is recorded while the angular position of the single crystal is varied. This allows the determination of the crystal structure, *i.e.* assignment of the space group and location of all atoms in the unit cell.

In XRPD, the sample exposed to the X-ray beam is a powder, and if the particles are randomly oriented, all crystal planes in the crystal lattice will be suitably oriented with respect to the X-ray beam (see Figure 7.15) and give rise to constructive interference at the respective angle. Therefore, a pattern in the form depicted in Figure 7.16 will be obtained. Different polymorphs will have different crystal plane spacings and will therefore lead to a different XRPD patterns (see Figure 7.16). Rare exceptions exist where the differences are so small that they may not be discernible using standard instrumentation.

Figure 7.16. XRPD patterns of paracetamol Form 1 (top, offset) and Form 2 (bottom)

An experimental problem with XRPD is so-called preferential orientation. If the particles are non-spherical (e.g. plates or needles), they may not be randomly oriented. In that case, some lines in the XRPD may become greatly attenuated or even disappear. When comparing the XRPD patterns of various samples, good sample preparation is therefore essential. Orientation effects can, for example, be reduced by gentle grinding of the substance. Investigating the sample by optical microscopy may also be very helpful to see if the particles are preferentially oriented. When a sample is ground, care must be taken that the solid form is not changed due to the grinding. The absence of such changes can be confirmed if the positions of the lines in the XRPD diagram are not shifted by grinding when the intensity of the grinding is varied.

Due to the physical principle of XRD, layer spacings in the crystal are measured and the signal will be the stronger when more layers with a constant spacing are present in the solid, *i.e.* the farther the spatial order extends. XRPD is therefore sensitive to **long range order**.

7.4.2. Raman spectroscopy

Like IR and NIR, Raman is a vibrational spectroscopy technique. Vibrational spectra are mainly governed by vibrations within the molecule, but since these vibrations are influenced by the environment of the molecule and hence the crystal packing, they will also differ for different polymorphs of the same molecule. In contrast to X-ray diffraction, these techniques primarily probe **short range order**.

Raman and IR are complementary methods. They differ in their selection rules (change of dipole moment or polarisability during vibration) and in practical aspects. The main advantages of Raman compared to IR in connection with solid-state characterisation are:

- no sample preparation is necessary (IR often uses KBr pellets; modern attenuated total reflectance IR (ATR-IR) also eliminates sample preparation),
- in general, narrower bands are found,
- Raman microscopy requires less sample preparation and has better spatial resolution than IR-microscopy,
- Raman spectroscopy can easily be applied for online monitoring (see Chapter 9),
- low frequencies are more readily attainable (this is the range of lattice vibrations, where the difference between polymorphs is often very pronounced),
- measurements can be performed through glass/quartz containers,
- little interference from water (weak Raman scatterer).

The advantages of IR are lower instrument cost, wider availability and no problems with samples that exhibit fluorescence.

Given the advantages of Raman spectroscopy, the focus of this section is placed on that technique. Compared to XRPD, differences in the Raman spectra of two polymorphs will in general not be as obvious, since the spectra are basically determined by the structure of the corresponding molecule. Nevertheless, the specificity of spectroscopic and diffraction methods with respect to the differentiation of polymorphs is similar if a high quality Raman spectrometer with very good wavenumber resolution is used.

In Figure 7.17a, the Raman spectra of three forms of CBZ are depicted (see Table 1). While the spectra look essentially similar, clear line shifts can, for example, be observed between 1550 and 1650 cm^{-1} (Figure 7.17b).

Figure 7.17. Raman spectra of CBZ Form I (top), Form III (middle) and Form IV (bottom); (a) in the whole wavenumber range and (b) wavenumber range from 1650 to 1550 cm^{-1} . Raman intensities scaled and shifted

In some cases, such as isomorphous solvates (solvates which are identical in structure, except that different solvents occupy a certain lattice space), the specificity of IR and Raman is often superior to XRPD.

The usefulness of Raman spectroscopy as a characterisation method for high-throughput screening (HTS) experiments is demonstrated in Figure 7.18. A co-crystal screen was performed in a 96-well plate quartz plate and the resulting solids were characterised by Raman microscopy directly in the wells. Raman microscopy allows spatial resolution in the order of 10 microns, such that spectra of individual crystals can easily be recorded. The second spectrum from the bottom is the Raman spectrum of a crystal in a particular well. It can be seen that both characteristic lines of the co-crystal former (spectrum at the bottom) and API (spectrum at the top) can be discerned in the solid which formed, indicating the presence of both molecules in the solid. On the other hand, it can be seen that some lines (highlighted by the ovals) are shifted with respect to the components (API and co-crystal former), indicating a different solid structure and thus potential co-crystal formation. This solid was therefore classified as a “co-crystal lead” and the co-crystal was reproduced on a larger scale (third spectrum from the bottom). It had the same Raman spectrum as the small crystal in the well, indicating that it was the same form, and further characterisation of this larger sample then proved that it was in fact a co-crystal.

Figure 7.18. Raman spectra of the co-crystal former (L-tartaric acid), the co-crystal in a 96-well plate (recorded with a Raman microscope), the scaled-up co-crystal and the API (piracetam).

7.5. CONCLUSION

If an active ingredient is formulated as a solid dosage form, it is possible to choose the form of the API in the drug product from a very large number of

possible forms. Which one of these forms is chosen may have a tremendous impact on the quality of the drug product. Bioavailability may be affected, which will have an impact on both drug efficacy and safety. Varying physical and chemical stability as well as hygroscopicity will affect the required storage conditions and maximal storage life. Non-stoichiometric hydrates may lead to problems in terms of API content, since the drug substance may contain various amounts of water, *i.e.* API content may not be proportional to API mass. Parameters such as bulk density, flowability, compressibility, the influence of mechanical stress on the solid form and melting point will affect the formulation process. The taste of the drug product may even be influenced (*e.g.* salt forms). Finally, the cost of the drug product may be influenced, since the efficiency of the crystallisation process (yield, purity, speed and removal of residual solvents) may be different for different forms.

In terms of polymorph selection, stability is generally the dominant property for the pharmaceutical industry, such that in most cases the stable polymorph will be chosen for development. Having the lowest free energy, the stable form also has the generally undesired property of being the least soluble one, but that is often considered a smaller problem than the potential conversion of a metastable form to the stable form during storage. If hydrates exist, the decision becomes more difficult, as outlined in Section 7.2.2.1.

Therefore, in order to be able to select the optimal form, a thorough characterisation is necessary, including the determination of solubility as a function of pH and temperature, stability, morphology, hygroscopicity, chemical purity, particle size, melting point, *etc.* Which of these parameters are most important will depend on the particular drug and the intended application of the drug. It makes sense to list the relevant properties for a particular drug in descending order of importance and then to compile the corresponding values of the available polymorphs, salts and co-crystals. The respective property of each form could then be graded with colours or shades of grey as “good”, “intermediate” or “bad”, so that a look at the corresponding matrix (Figure 7.19) would allow for choosing the optimal form.

	free drug, polym. α	free drug, polym. β	Salt A, polym. I	Salt B, polym. I	Co-cr X, Polym. A	Co-cr Y, Polym. A	
Solubility	0.01	0.04	1	0.08	0.5	0.7	
Thermodyn. stability	yes	no	yes	yes	yes	yes	
Hygroscopicity	+0.1% @ 70%r.h.	+0.1% @ 70%r.h.	+0.1% @ 70%r.h.	+2.0% @ 70%r.h.	channel hydrate	+1.5% @ 70%r.h.	
Melting Point	80°C	70°C	150°C	170°C	150°C	60°C	
.....							

Figure 7.19. Scheme for selecting the optimal form. White indicates good, light grey intermediate and dark grey bad properties. Therefore, “Salt A, Polymorph I” would be the optimal choice here

Naturally, in order to be able to select the optimal form, it is necessary to first produce the various forms and then to understand their properties in detail. Therefore, thorough form screening and characterisation is essential. This requires experimental effort, experience, theoretical insight and understanding. But, we certainly think that the effort invested in form screening and understanding more than pays off, since it will ensure that a better product in terms of efficacy, safety, ease of use, cost and patent protection will result in the end.

ABBREVIATIONS

API	active pharmaceutical ingredient
ATR-IR	attenuated total reflectance infrared
BCS	Biopharmaceutics Classification System
CBZ	carbamazepine
DP	drug product
DS	drug substance
DSC	differential scanning calorimetry
DVS	dynamic vapour sorption
ET-diagram	energy-temperature diagram
GRAS	generally recognised as safe
HPLC	high performance liquid chromatography
ICH	International Conference on Harmonisation
IP	intellectual property
IR	infrared
NIR	near infrared
NMR	nuclear magnetic resonance
r.h.	relative humidity
TG	thermogravimetry
TG-FTIR	thermogravimetry coupled to Fourier transform IR
TG-MS	thermogravimetry coupled to mass spectroscopy
TMA	thermomechanical analysis
TSC	thermally stimulated current
XRD	X-ray diffraction
XRPD	X-ray powder diffraction

REFERENCES

1. W.C. McCrone. Polymorphism. In: *Physics and Chemistry of the Organic Solid State*. (Eds. D. Fox, M.M. Labes and A. Weissberger) *Interscience Publishers*, London (1965) vol. 2, pp. 725-767.
2. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonised Tripartite Guideline. Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Product.
3. Ulrich J. Griesser, J.G. Stowell. Solid-state analysis of polymorphism. In *Pharmaceutical Analysis*. (Eds: D.C. Lee, M. L.Webb) *Blackwell Publishing Ltd.*, Oxford (2003) p. 240-294.
4. P. H. Stahl, C.G. Wermuth. Handbook of Pharmaceutical Salts: Properties, Selection, and Use. *Wiley-VCH*, Weinheim (2002).
5. C.A. Lipinski, F. Lombarda, B.W. Dominy and P.J. Feeney. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* **46**(1) (2001) 3-26.
6. G.P. Stahly. Presentation at 22nd *SCI Process Development Symposium*, Cambridge, December 2004.
7. U. J. Griesser. The importance of solvates. In "Polymorphism in the Pharmaceutical Industry" (Ed. R. Hilfiker) *Wiley-VCH*, Weinheim (2006) p 221-234.
8. H.G. Brittain. Polymorphism in Pharmaceutical Solids. *Marcel Dekker, Inc.*, New York (1999).
9. S.R. Byrn, R.R Pfeiffer, J.G. Stowell. Solid State Chemistry of Drugs. 2nd Ed., *SSCI Inc.*, West Lafayette (1999).
10. J. Bernstein. Polymorphism in Molecular Crystals. *Oxford Science Publications*, Oxford (2002).
11. R. Hilfiker. Polymorphism in the Pharmaceutical Industry. *Wiley-VCH*, Weinheim (2006).
12. H.G. Brittain. Polymorphism in Pharmaceutical Solids. 2nd edition, *Informa Healthcare*, New York (2009).
13. W.H. DeCamp. The impact of polymorphism on drug development: A regulator's viewpoint. *American Pharmaceutical Review* **4** (2001) 70-77.
14. B. Wunderlich and J. Grebowicz. Thermotropic mesophases and mesophase transitions of linear, flexible macromolecules. *Advances in Polymer Science* **60/61** (1984) 1-59.
15. B. Wunderlich. A classification of molecules, phases, and transitions as recognized by thermal analysis. *Thermochimica Acta* **340** (1999) 37-52.
16. E.B. Vadas, P. Toma and G. Zografi. Solid-state transitions initiated by water vapor sorption of crystalline L-660,711, a leukotriene D₄ receptor antagonist. *Pharmaceutical Research* **8**(2) (1991) 148-155.
17. K.R. Morris, A.W. Newman, D.E. Bugay, S.A. Ranadive, A.K. Singh, M. Szyper, S.A., Varia, H.G. Brittain and A.T.M. Serajuddin. Characterization of humidity-dependent changes in crystal properties of a new HMG-CoA reductase inhibitor in support of

- its dosage form development. *International Journal of Pharmaceutics* **108**(3) (1994) 195-206.
18. C.L. Stevenson, D.B. Bennett and D. Lechuga-Ballesteros. Pharmaceutical liquid crystals: The relevance of partially ordered systems. *Journal of Pharmaceutical Sciences* **94**(9) (2005) 1861-1880.
19. G.L. Amidon, H. Lennernas, V.P. Shah and J.R. Crison. A theoretical basis for a biopharmaceutic drug classification: The correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharmaceutical Research* **12**(3) (1995) 413-420.
20. S. Lohani, D.J.W. Grant. Thermodynamics of polymorphs. In *Polymorphism in the Pharmaceutical Industry* (Ed. R. Hilfiker), Wiley-VCH, Weinheim (2006) p21-42.
21. K.R. Morris. Structural aspects of hydrates and solvates. In *Polymorphism in Pharmaceutical Solids*. (Ed. H.G. Brittain), Marcel Dekker, Inc., New York, (1999) p 125-181.
22. A.L. Grzesiak, M. Lang, K. Kim and A.J. Matzger. Comparison of the four anhydrous polymorphs of carbamazepine and the crystal structure of form I. *Journal of Pharmaceutical Sciences* **92**(11) (2003) 2260-2271.
23. R.J. Behme and D. Brooke. Heat of fusion measurement of a low melting polymorph of carbamazepine that undergoes multiple phase changes during DSC. *Journal of Pharmaceutical Sciences* **80**(10) (1991) 986-990.
24. T. Rager and R. Hilfiker. Stability domains of multi-component crystals in ternary phase diagrams. *Zeitschrift für Physikalische Chemie* **223** (2009) 793-813.
25. T. Rager and R. Hilfiker. Co-crystal formation from solvent mixtures. *Crystal Growth and Design* **10**(7) (2010) 3237-3241.
26. W. Ostwald. Studien über die Bildung und Umwandlung fester Körper. *Zeitschrift für Physikalische Chemie* **22** (1897) 289-330.
27. A. Myerson. Handbook of Industrial Crystallization. 2nd edition. Butterworth-Heinemann, Boston (2002).
28. R.J. Davey, J. Garside. From Molecules to Crystallizers. Oxford University Press, Oxford (2000).
29. N. Blagden and R. Davey. Polymorphs take shape. *Chemistry in Britain* **35**(3) (1999) 44-47.
30. W. Beckmann, W. Otto and U. Budde. Crystallization of the stable polymorph of hydroxytriendione: Seeding process and effects of purity. *Organic Process Research and Development* **5**(4) (2001) 387-392.
31. T. Mukuta, A.Y. Lee, T. Kawakami and A.S. Myerson. Influence of impurities on the solution-mediated phase transformation of an active pharmaceutical ingredient. *Crystal Growth & Design* **5**(4) (2005) 1429-1436.
32. J.K. Guillory. Generation of polymorphs, hydrates, solvates and amorphous solids. In *Polymorphism in Pharmaceutical Solids*. (Ed. H.G. Brittain) Marcel Dekker, New York (1999) 183-226.
33. C.-H. Gu, H. Li, R.B. Gandhi and K. Raghavan. Grouping solvents by statistical analysis of solvent property parameters: implication to polymorph screening. *International Journal of Pharmaceutics* **283**(1) (2004) 117-125.
34. R.J. Harman. The drug development process. 1. Introduction and overview. *Pharmaceutical Journal* **262**(7035) (1999) 334-337.

35. M. Szelagiewicz, C. Marcolli, S. Cianferani, A.P. Hard, A. Vit, A., Burkhard, M.von Raumer, U.C. Hofmeier, A. Zilian, E. Francotte and R. Schenker. In situ characterization of polymorphic forms. *Journal of Thermal Analysis and Calorimetry* **57**(107) (1999) 23-43.
36. W.-F. Maier, J. Klein, C. Lehmann, H.W. Schmidt. Offenlegungsschrift DE 198 22 077 A1, "Kombinatorisches Verfahren zur Herstellung und Charakterisierung von kristallinen und amorphen Materialbibliotheken im Mikrogramm-Massstab" (1999).
37. S. Balbach and C. Korn. Pharmaceutical evaluation of early development candidates "the 100 mg-approach". *International Journal of Pharmaceutics* **275**(1) (2004) 1-12.
38. R. Storey, R. Docherty, P. Higginson, C. Dallman, C. Gilmore, G. Barr and W. Dong. Automation of Solid Form Screening Procedures in the Pharmaceutical Industry - How to Avoid the Bottlenecks. *Crystallography Reviews* **10** (2004) 45-56.
39. D.L. Carlton, O.P. Dhingra, P.W. Waters. "High Throughput Crystal Form Screening Workstation and Method of Use", US patent 7,008,599 (2006).
40. S.L. Morissette, Ö. Almarsson, M.L. Peterson, J.F. Remenar, M.J. Read, A.V. Lemmo, S. Ellis, M.J. Cima and C.R. Gardner. High-throughput crystallization: polymorphs, salts, co-crystals and solvates of pharmaceutical solids. *Advanced Drug Delivery Reviews* **56**(3) (2004) 275-300.
41. C.R. Gardner, C.T. Walsh and Ö. Almarsson. Drugs as materials: Valuing physical form in drug discovery *Nature Reviews Drug Discovery* **3**(11) (2004) 926-934.
42. A. Van Langevelde and E. Blomsma. High-throughput screening in solid form selection. *Acta Crystallographica Section A: Foundations* **58** (2002) C9 (Supplement).
43. P. Desrosiers, E. Carlson, W. Chandler, H. Chau, P. Cong, R. Doolen, C. Freitag, S. Lin, C. Masui, E. Wu, T. Crevier, D. Mullins, L. Song, R. Lou, J. Zhan, A. Tangkilisan, Q. Ung and K. Phan. High throughput technologies for preformulation: Salt selection and polymorph studies. *Acta Crystallographica Section A: Foundations* **58** (2002) C9 (Supplement).
44. R. Hilfiker, J. Berghausen, F. Blatter, A. Burkhard, S.M. De Paul, B. Freiermuth, A. Geoffroy, U. Hofmeier, C. Marcolli, B. Siebenhaar, M. Szelagiewicz, A. Vit and M. von Raumer. Polymorphism – Integrated approach from high-throughput screening to crystallization optimization. *Journal of Thermal Analysis and Calorimetry* **73**(2) (2003) 429-440.

**THERMAL ANALYSIS AND CALORIMETRIC
METHODS APPLIED TO PHARMACEUTICAL
SOLID FORMS**

Danièle Giron

Contents

8.1. INTRODUCTION.....	388
8.2. BASIC PRINCIPLES OF THERMAL METHODS AND THEIR IMPORTANCE IN PHARMACEUTICAL ANALYSIS	389
8.2.1. Thermal analysis methods.....	389
8.2.2. Microcalorimetry	389
8.2.3. Overview of applications	389
8.3. FACTORS TO BE CONSIDERED IN THE INSTRUMENTATION	392
8.3.1. Heat flux differential scanning calorimetry (DSC) and power compensation DSC.....	392
8.3.2. Thermogravimetry	398
8.4. WHAT ARE THE AREAS OF APPLICATION FOR THE STUDY OF SOLID FORMS?	401
8.4.1. Thermodynamic data	401
	386

8.4.1.1. Two polymorphic solid phases	401
8.4.1.2. The phase diagrams of salts, solvates and hydrates.....	405
8.4.1.3. Amorphous state	408
8.4.2. Purity determination	409
8.4.3. Kinetic aspects	411
8.4.4. Sorption isotherms and hydrates.....	414
8.4.5. Combined techniques	417
8.4.5.1. Combination of DSC and XRD	417
8.4.5.2. Thermogravimetry-mass spectrometry (TG-MS) and thermogravimetry-infrared spectroscopy (TG-IR).....	417
8.4.6. Isothermal microcalorimetry and solution calorimetry	422
8.4.6.1. Microcalorimetry.....	423
8.4.6.2. Solution calorimetry	425
8.5. SOME EXAMPLES OF APPLICATIONS	425
8.5.1. The selection of the right form	425
8.5.2. Sensitivity of DSC.....	426
8.5.3. Disappearing solvates	427
8.5.4. Selection of the best form considering the pharmaceutical environment.....	427
8.6. CONCLUSION	429
REFERENCES	429

8.1. INTRODUCTION

Thermal analysis techniques, in which a physical property is monitored as function of temperature or time while the analyte is heated or cooled under controlled conditions, are fundamental techniques for the characterisation of drug substances and products. They are fundamental as processing or aging conditions may be simulated, and because the methods give access to thermodynamic data. Due to the different information conveyed, thermal analysis methods are complementary to other analytical techniques, such as spectroscopy, chromatography, melting point determination, loss on drying, assays for identification, purity and quantitation. Thermal analysis techniques are basic methods in the field of solid state analysis, in physical and chemical characterisation of pure substances, as well as for mixtures. They find the best application in pre-formulation, processing and control of the drug product. The introduction of sophisticated, automated, robust and sensitive instruments considerably increased the advantages of these methods. New horizons have been opened with the availability of combined techniques and microcalorimetry, an emerging technique which is now used routinely.

Since changes in temperature and moisture might induce the changes in the solid state, processing and storage may have a considerable effect on activity, toxicity and stability of compounds. The pharmaceutical industry is faced with the new challenges of quicker development and higher performance, in terms of technology, reliability and up-scale in the international current Good Manufacturing Practise (cGMP) environment. Current requirements set by the International Conference of Harmonisation (ICH) for the characterisation and the quantitation of polymorphism in new entities [1], re-enforce the position of thermal analysis and microcalorimetric techniques, which can deliver the correct information concerning the thermodynamic relationships between phases, for the proper selection of salt and crystal forms. The amorphous state is better understood and determinable. Differential scanning calorimetry (DSC) purity analysis is a fast, absolute, orthogonal purity technique for organic compounds. Microcalorimetry, DSC and thermogravimetry (TG) are advantageous in the process of design of drug products.

8.2. BASIC PRINCIPLES OF THERMAL METHODS AND THEIR IMPORTANCE IN PHARMACEUTICAL ANALYSIS

8.2.1. Thermal analysis methods

When a material is heated or cooled, there is a change in its structure or composition. These transformations are connected with a heat exchange. The first application of thermal analysis was presented by Le Chatelier in 1887 [2]. In differential thermal analysis (DTA), the temperature induced in the sample is measured. DSC, which determines the heat flow into and out of the sample, as well as the temperature of the thermal phenomenon during a controlled change of temperature, is the basic thermal technique used for solid forms. Thermal analysis techniques also cover all other techniques in which a physical property is monitored as a function of temperature or time, while the sample is heated or cooled under controlled conditions. For the solid state, the phase diagram rules for single compounds, as well as for mixtures of several components, have to be considered.

The most common methods used in solid state characterisation are: DSC and TG, and they are often combined with mass spectrometry, X-ray diffraction (XRD), IR, Raman spectroscopy and microscopy. Books and reviews dealing with the principles, instrumentation and applications are given in references [3-10]. Specific applications of thermal analysis and calorimetric methods for pharmaceutical polymorphism are given in references [11-19].

8.2.2. Microcalorimetry

A calorimeter measures the heat flow into or out of a sample, whereas a differential calorimeter measures the heat of a sample relative to a reference. A differential scanning calorimeter combines these two methods, and additionally, heats the sample with a linear temperature ramp.

Microcalorimetry in isothermal mode is a growing technique, complementary to DSC for the characterisation of pharmaceuticals [20]. Larger sample volume and higher sensitivity mean that phenomena of very low energy, immeasurable by DSC, may be studied. The output of the instrument is measured by the rate of heat change (dq/dt), as a function of time, with a sensitivity better than 0.1 μ W. Microcalorimetry can be applied to isolated systems in specific atmospheres, or for batch mode, where reactants are mixed in the calorimeter. The most useful application currently, thanks to high throughput microcalorimeters [21], is the routine quantitation of undesirable amorphous content down to 0.1 - 0.3 % [22-25].

Solution calorimetry can be used in adiabatic or isoperibol modes in microcalorimeters at constant temperature. This method is used for polymorphic interpretation and for quantitation [12].

8.2.3. Overview of applications

The processing of the drug substances and drug products involves solvent(s), temperature and pressure changes, as well as mechanical stress and, as a result,

different solid phases may coexist in the drug product. Organic substances show supersaturation behaviour and unstable solid phases which should not exist at a defined temperature, pressure and humidity, but may behave like stable forms. These solid metastable phases, obtained outside of their domains of stability, will convert to their thermodynamically stable forms at given temperatures, pressures and relative humidities, in response to changes in environmental conditions, processing, or over time. These conversions, driven by thermodynamics, are governed by kinetics and are influenced by impurities, particle size, crystal defects and presence of seeds.

The market withdrawal of Ritonavir in 1998, one year after its launch, was caused by it having a rate of dissolution that was far too slow, due to an insoluble stable polymorph not found during development [26]. The current focus of research in the solid-state area is to understand the origin of polymorphism at the molecular level, and to predict and prepare the most stable form at the start of development. The selection of a metastable form should result from targeted choice, rather than from chance. The advantage of thermal and microcalorimetric techniques is that their sensitivity allows a sample size of considerably less than 1 mg. Table 8.1 summaries the analysis methods detailed, including coupled and combined techniques.

Table 8.1. Main thermal analysis techniques and calorimetry

Technique	Measurements	Thermal events
Differential Scanning Calorimetry (DSC) Derivative DSC (DDSC) Micro-DSC Hyper-DSC	Heat flow into and out of the sample as a function of temperature/time	Every thermal event associated with energy change during heating or cooling melting, boiling, sublimation and crystallisation glass transition polymorphic phase transition desolvation, dehydration and isomerisation complex phase transition thermal degradation mixtures; phase diagrams purity determination oxidation, catalysis chemical reaction kinetic analysis coupling with TG, microscopy or X-ray
Modulated DSC (MDSC)	Total heat flow, irreversible and reversible heat flow	every transition distinction between overlapping thermal events study of relaxation of glass transition
Thermogravimetry (TG) Derivative thermogravimetry (DTG)	Change in sample mass as a function of temperature and/or time	Every change of mass desolvation, dehydration sorption-desorption isotherms degradation compositional analysis chemical reaction kinetic analysis drying optimisation coupling with DSC, GC, MS, IR, Raman

Technique	Measurements	Thermal events
Thermomechanical Analysis (TMA), Thermodilatometry	Dimensional changes during compression, penetration, tension, flexure or torsion versus temperature	Mechanical properties, dimensions expansion, dilatation glass transition gelification, swelling
Dynamic mechanical analysis (DMA)	Dimensions versus frequency and temperature	Young modulus polymer analysis
Thermally Stimulated Current (TSC) Dielectric Thermal Analysis (DETA)	Depolarisation current curve versus temperature Dielectric constant	relaxation alpha and beta of glasses quantification of amorphous
Thermal Conductivity (TC)	Thermal conductivity	thermal conductivity, thermal diffusivity on line analysis of processes
Thermomicroscopy	Microscopical observation under different modes of light illumination during heating or cooling	melting, crystallisation, evaporation.. eutectic formation solubility studies crystallinity using polarised light morphological studies
TG-MS, TG-Raman, TG-IR	Coupled spectroscopic analysis with TG	analysis of volatiles investigation of drying processes investigation of solvates, hydrates interpretation of thermal events
Evolved gas analysis (EGA)	TG coupled with GC and possibly MS, IR	Identification and quantification of volatile components
Combined spectroscopic methods	Programmed heating cell with spectroscopic analysis (IR, Raman, NMR, Terahertz))	spectroscopic data of phases obtained in situ during combined with Thermomicroscopy interpretation of thermal events
Temperature resolved XPRD or coupled DSC/XRD	X-ray powder diffraction versus temperature	interpretation of thermal events identification of phases in situ XPRD of unstable forms combined with modelling
Microcalorimetry	Heat flux out of the sample in Isothermal mode	heat of solution heat of transformation, heat of reaction quantification of amorphous stability prediction kinetic
Nano-DSC	Heat flux out of the sample	Specially designed for life science, folding of proteins, siRNA used for quality control in biological science
Calorimetry Accelerated calorimetry Reaction calorimetry	Heat measurement	safety simulation of processes simulation reactors
Titration calorimetry	Heat measurement during addition of reactant	binding constant study of molecular binding of proteins, native cells drug-cells interactions
Micro-thermal analysis	Heat flux and atomic force microscopy	Topography, with conductivity and thermal diffusivity interfaces studies

8.3. FACTORS TO BE CONSIDERED IN THE INSTRUMENTATION

Modern instruments used for thermal analysis usually consist of four parts:

- the sample and sample holder,
- sensors to detect and measure a property of the sample, as well as the temperature,
- a system within the instrument that allows the experimental parameters to be controlled,
- a computer to control data collection and processing.

8.3.1. Heat flux differential scanning calorimetry (DSC) and power compensation DSC

DSC measures the temperature and the heat flow associated with transitions in materials, as a function of time and temperature, in a controlled atmosphere and relative to a reference. These measurements provide quantitative and qualitative information about physical and chemical changes that involve endothermic or exothermic processes, or changes in heat capacity.

The principle of DSC is as follows: two ovens are heated in a linear manner. One oven contains the sample in a pan; the other contains an empty pan as a balance, termed a reference pan. If nothing occurs to the sample then the sample pan and reference pan are at the same temperature during heating. If a change, such as melting occurs in the sample, then energy is used by the sample and the temperature remains constant in the sample pan during melting. Therefore, a difference of temperature occurs between the sample pan and reference pan.

Two methods of measurement are used by manufacturers. The first method is termed "heat flux DSC", in which the instrument measures the temperature difference (DTA). Through calibration, this temperature difference is transformed into heat flow (dq/dt). The instrument contains a furnace, which is a block for both sample and reference cells. The sample holder contains the sample pan, and the reference pan is connected by a low resistance heat flow path; thermocouples are used for determining the temperature difference between sample and reference.

In the second method, which is termed "power compensation DSC", two individual heaters are used in order to monitor the individual heating rates of the two individual ovens. A system controls the temperature difference between sample and reference. If any difference is detected, the specific heater will be corrected so that the temperature is kept the same in both pans. That is, when an endothermic or exothermic process occurs, the instrument delivers the compensation energy which must be given in order to maintain equal temperatures in both pans.

In the first method (heat flux DSC), temperature is primarily measured, in the second case (power compensation DSC), energy is primarily measured. Despite their different modes of operation, both instruments deliver the same information: heat flow as a function of temperature (or time).

For first order transitions, such as melting, crystallisation, sublimation, boiling *etc.*, integration of the curve gives the energy involved in the transition. Melting, boiling, sublimation and desolvation are endothermic, which means they need energy. Crystallisation is exothermic, which means that it supplies energy. Solid-solid phase transition and decomposition may be endothermic or exothermic. For second order transitions, the signal gives the change in the specific heat; for example, in the glass transitions of amorphous materials. Manufacturers represent the heat flow differently: endotherms have the positive values in power compensation DSC, and negative values for heat flux DSC. Figure 8.1 shows typical transitions for a single compound: with a power compensation DSC with an endotherm rising, or for heat flux DSC with an endotherm falling. Both representations are accepted if the signs for endotherm or exotherm are given on the curves. Generally the temperature is plotted on the x abscissa (usual given in $^{\circ}\text{C}$) and the heat flow (dQ/dt , dq/dt or dH/dt) is plotted on the y ordinate in J s^{-1} . Most recent curves are given in mWatts (mW), which is equivalent, and in this chapter both expressions are used. Melting, crystallisation and phase transitions are first order transitions. The intersection between the baseline and the slope of the peak is the extrapolated temperature onset of the transition (T_e). The corresponding energy is the area under the peak. The peak temperature (T_m) is very dependent on instrument and measurement parameters. The glass point, which is a second order transition, is determined as an inflexion point.

Figure 8.1. Examples of a power compensation and heat flux DSC curves for a single compound

Modern instruments permit the heating, cooling and isotherm curves between sub-ambient temperatures, with use of a cooling device, and higher temperatures in the range of 1200 - 1500 °C. Curves made using different instruments cannot be compared to one another; corrections must be calculated according to each instrument. Great care must be given to the temperature, with the temperature plotted on the abscissa being the programmed temperature, not the real temperature of the sample. The actual temperature depends on the thermal resistance and heating rate of the instrument, which is called the "thermal lag" of the system. The correlation between apparent melting point and heating rate depends on the manufacturer. Generally, pure indium (> 99.9999 %) is used with each series of measurements. Modern instruments, with data handling, enable the true temperature of each point of the DSC curve to be calculated.

Figure 8.2 exemplifies the effect of the heating rate on the DSC curves. An example of the DSC curves of indium using different heating rates on a DSC-2 Perkin-Elmer instrument is given in Figure 8.2a. Since energy is the integration of the signal as a function of the time, the DSC curves are not identical at every heating rate. The "thermal lag" is instrument specific. Since the melting temperature of indium is 156.6 °C, the extrapolated onset has to be corrected for each heating rate. Figure 8.2b shows the plot of the extrapolated onset of the curves for indium versus heating rate at 1.25, 2.0, 5.0, 10.0 and 20 °C min⁻¹. This effect has to be taken into consideration when comparing curves obtained using different heating rates, and especially if measured with different instruments.

Figure 8.2. Effect of heating rate on the melting point of indium.

(a) DSC curves at 1.25, 2.0, 5.0, 10.0 and 20.0 °C min⁻¹;

(b) corresponding linear relationship between melting point and heating rate

Figure 8.3 shows the influence of heating rate on the resolution of polymorphs of temazepam. The best resolution is obtained with a low heating rate. Figure 8.4 exemplifies the influence of the sample mass on the resolution for the same compound. The heating rate (sensitivity of the instrument), and the sample mass have to be chosen carefully to detect thermal effects which are very close to one another.

Figure 8.3. DSC curves showing the effect of heating rate on the resolution of polymorphs of temazepam

Figure 8.4. DSC curves showing the effect of sample mass on the resolution of polymorphs of temazepam. Heating rate $1.25\text{ }^{\circ}\text{C min}^{-1}$

Figure 8.5 deals with the measurement of the glass transition with and without thermal effect. The thermal effect appearing as a maximum on DSC curve depends on the thermal history of the sample, and is commonly used in the study of amorphous materials and polymers. At high heating rates the signal is higher because, for the glass transition the change in c_p is dq/dt or dH/dt , and is easier to measure.

Figure 8.5. DSC curves for the glass transition (a) without thermal effect and (b) with thermal effect

Great efforts have been made in recent years to validate different instruments, not only in comparing principles and results, but also in determining heating and cooling rates, particle size, weight, resolution, atmosphere and type of pans. The type and the size of pans is of great importance for comparing results; types of pan may include open pan, crimped pan, sealed pan or pin hole. The same type of pans must be used for both sample and reference. If pans are hermetically closed then the solvent cannot escape, and the curves are not the same as for pans with a pin hole. Figure 8.6 exemplifies the problem for a monohydrate. In a sealed pan, the DSC melting peak of the monohydrate is observed. Whereas, if a pin hole is made in the cover of the pan, water can escape and the transition into the anhydrous form is observed as endothermic/exothermic phenomenon before the melting of the anhydrous form occurs. Unlike the curves for monohydrate, the anhydrous form is not affected by the type of the pan.

The use of flowing purge gas (nitrogen or argon) is required as the waste products from sublimation or decomposition have to be removed. Due to the lubricant used in the manufacture of aluminium pans, these pans must be preheated in order to avoid artefacts. The homogeneity of the sample, the sample preparation, any contaminations, the time between the manufacture of the sample and its measurement, the sample mass and thermal lag are the main source of errors. In pharmaceutical development, instruments must be calibrated and routinely checked. It should be emphasised that melting points of polymorphs may differ by less than 1 °C, and that differences in melting energy are often less than 5 %. Therefore, all variables of an automated device have to be controlled.

Table 8.2 deals with examples of standards, including IUPAC and WHO standards, proposed for temperature calibration. The standards must be of certified purity. For heat calibration, the melting energies of certified substances with melting points between 60 °C and 230 °C are proposed in Table 8.2.

Figure 8.6. Effect of the type of pan on DSC curves for a monohydrate (—) and for an anhydrous form (---). With the sealed pan, one may measure the melting of the hydrate. With pin hole, water escapes and a recrystallisation into the anhydrous form is observed

Table 8.2. Certified substances for the calibration of DSC instruments

Certified substance	Melting temperature, °C	Standard substance	Melting energy, J g ⁻¹
Iodobenzene	-31.3	Naphthalene	148.6
H ₂ O	0.0	Benzil	112.0
4-nitrotoluene	51.5	Benzoic acid	147.2
Biphenyl	69.3	Biphenyl	120.4
Naphthalene	80.2	Diphenylacetic acid	146.9
Benzil	94.7	Indium	28.7
Acetanilide	114.0	Tin	60.2
Benzoic acid	122.1		
Diphenylacetic acid	146.5		
Indium	156.6		
Anisic acid	183.1		
2-chloro-anthraquinone	210.0		
Tin	231.9		
Antraquinone	284.5		
Lead	327.5		
Zinc	418.9		

In modulated DSC (MDSC), the normally linear heating ramp is overlaid with a sinusoidal function, or an oscillating function defined by a frequency and an amplitude, to produce a sine wave shaped temperature versus time function. Using Fourier mathematics, the DSC signal is then split into two components: one reflecting non-reversible events (kinetic), and the other reversible events. This method is generally not applied in the characterisation of solid pharmaceutical forms. For details on this application, see [3-8].

8.3.2. Thermogravimetry

In thermogravimetry (TG), the change in sample mass is determined as a function of temperature and/or time. The instrument used is a thermobalance that allows the continuous weighing of a sample as a function of time. The sample holder and a reference holder are bonded to each side of a microbalance, in a vertical or horizontal design. The sample holder is situated in a furnace, without direct contact with the sample, the temperature of which is controlled by a temperature programmer. The balance portion is maintained at a constant temperature and the instrument is able to record the loss or gain in mass of the sample as a function of temperature and time [$m = f(T)$]. Frequently, the mass change is given as percentage of the sample. Most instruments also record the derivative of the mass change [$dm/dt = f(T)$].

The derivative TG (DTG) allows a better distinction of overlapping steps, and the determination of the temperature at which the rate of mass change is maximal or minimal. The area under the DTG curve is proportional to this mass change, and the height of the DTG peak at any temperature gives the rate of mass change. DSC and DTG performed on hydrates show the same outcome.

Balance is adjusted with certified mass at ambient temperature and it is recommended to check the mass at temperatures of use. Therefore, the author proposes three standards: calcium oxalate dihydrate (water content 12.3 %) (used for TG in European Pharmacopoeia), disodium tartrate dihydrate (water content 15.7 %) (used as standard for Karl Fischer analysis) and copper sulphate pentahydrate (water content 36.1 %). Figure 8.7a shows such calibration curves at 20 °C min⁻¹ heating rate. The dehydrations cover a temperature range of 50 to 270 °C. In this figure we plot for comparison the common excipient lactose monohydrate.

For temperature calibration, the standards recommended by ICTA are ferromagnetic standards exhibiting loss of ferromagnetism at their Curie point temperature within a magnetic field: Nickel (354 °C), Permanorm 3 (266 °C), Numetal (386 °C), Permanorm 5 (459 °C) and Trafoperm (754 °C). A thermobalance with horizontal plates is proposed by manufacturers as the melting of standards is easier for the user.

Tables 8.3a and 8.3b show calibrations of a thermobalance at different heating rates. In order to check the stability of the system, a baseline at the highest sensitivity has to be performed for all heating rates in the temperature range of analysis; the highest deviation will be observed at the highest heating rate.

Figure 8.7. Thermogravimetry of standard organic substances at 20 °C min⁻¹.
(a) TG curves; (b) DTG compared to TG of copper(II) sulphate pentahydrate

Figure 8.7b shows the use of DTG for copper sulphate pentahydrate, at the same heating rate as given in Figure 8.7a. The DTG determines precisely the end of each step at rapid heating rates, which is an advantage when desolvation and decomposition overlap. Figure 8.8 exemplifies the effect of heating rate on TG

curves of disodium maleate dihydrate. The results obtained at the $5\text{ }^{\circ}\text{C min}^{-1}$ heating rate comply with the theoretical values of water content of the dihydrate (18.45 %).

Table 8.3a. Examples of the mass calibration of a thermobalance at different heating rates

Substance	Theoretical amount of water, %	Amount of water found, %		
		$5\text{ }^{\circ}\text{C min}^{-1}$	$10\text{ }^{\circ}\text{C min}^{-1}$	$20\text{ }^{\circ}\text{C min}^{-1}$
Disodium tartrate dihydrate	15.65	15.73	15.60	15.73
Calcium oxalate monohydrate	12.32	12.55	12.51	12.48
Copper sulphate pentahydrate	36.07	36.08	36.03	36.04

Table 8.3b. Examples of the temperature calibration of a thermobalance at different heating rates

Substance	Theoretical melting point, $^{\circ}\text{C}$	Determined melting point, $^{\circ}\text{C}$		
		$5\text{ }^{\circ}\text{C min}^{-1}$	$10\text{ }^{\circ}\text{C min}^{-1}$	$20\text{ }^{\circ}\text{C min}^{-1}$
4-nitrotoluene	51.50	51.49	51.64	53.78
Indium	156.60	157.62	157.38	157.74
Tin	231.93	233.44	233.42	233.68

Figure 8.8. TG curves of disodiummaleate dihydrate at two different heating rates

8.4. WHAT ARE THE AREAS OF APPLICATION FOR THE STUDY OF SOLID FORMS?

8.4.1. Thermodynamic data

Once solid form is obtained, several questions arise:

- Is the form a mixture of polymorphs?
- What is the chemical purity?
- What is the degree of crystallinity?
- Is the salt form pure?
- What are the thermodynamic windows:
 - for true polymorphs?
 - for solvated forms?
 - for different hydrated forms?
 - for the amorphous form?

These questions need to be answered quickly in order to measure correctly the physicochemical parameters of the isolated form.

Polymorphism is the ability of a substance to crystallise into different crystalline phases that have different arrangements, and/or conformations of the molecules, in the crystal lattice. These crystalline forms are called polymorphs, or crystalline modifications. Polymorphs have the same liquid or gaseous state, but they behave differently in the solid state. The amorphous state is characterised by solidification in a disordered or random manner, structurally similar to the liquid state. The expression pseudo-polymorphism applies to hydrates and solvates. Hydrates or solvates do not have the same chemical structure as the anhydrous forms, though polymorphism between hydrates or solvates does occur.

The relationships between different phases are governed by the Gibbs phase rule:

$$V = C + 2 - \Psi \quad (8.1)$$

where V = variance, C = number of components, Ψ = number of phases.

8.4.1.1. Two polymorphic solid phases

In the case of two polymorphic solid phases, C is equal to one. If two solid phases are present, and if both pressure and temperature vary, the variance is unity. If pressure is fixed, then variance is zero. Phase diagrams of pressure versus temperature illustrate the different equilibrium curves for polymorphism (Figure 8.9).

For each solid form, there is a solid-liquid equilibrium curve and a solid-vapour equilibrium curve. In the case of enantiotropy, there is an equilibrium curve (BF in Figure 8.9a), where both polymorphs are in equilibrium and undergo reversible transition at a specific temperature and at a fixed pressure. In the case of monotropy (Figure 8.9b), there is no thermodynamic transition between two phases, since only one solid form is thermodynamically stable. The dashed lines correspond to the metastable equilibrium curves which have to be taken into consideration.

Figure 8.9. Phase diagrams P, T for a single compound showing (a) enantiotropic and (b) monotropic polymorphism

The ability of a system to perform work, and to undergo a spontaneous change at constant pressure is measured by the Gibbs energy, ΔG . ΔG has been defined in terms of enthalpy and entropy changes, ΔH and ΔS , at temperature T as:

$$\Delta G = \Delta H - T\Delta S \quad (8.2)$$

Gibbs energy (G), enthalpy (H) and entropy (S) are state functions and the term Δ can be omitted.

$$G = H - TS \quad (8.3)$$

At 0 Kelvin, $G = H$. Since the entropy S is always positive, G decreases with increasing temperature. The energy diagram, G versus temperature at a given pressure, reflects the transition observed between both solid phases, and between solid and liquid phases. If a transition between phases occurs then at this temperature both phases have the same Gibbs energy, G .

In general, the thermodynamic relationship between two polymorphic phases is represented by plotting Gibbs energy as a function of temperature for each form (Figure 8.10). If the two curves intersect below the melting point of each polymorph, a reversible transition occurs at the temperature T_t of the intersection. At temperatures below T_t , polymorph A has the lower Gibbs energy and is therefore the thermodynamically stable form, while at temperatures above T_t , polymorph B is stable. The transition point can be low, even below ambient temperature, or in other cases, very high (above 200 °C). In the case of monotropy, there is no intersection of the Gibbs energy of both forms, and the higher melting form is always the thermodynamically stable form.

Figure 8.10. Energy diagrams depicting dependences of H and G vs. temperature for (a) enantiotropic and (b) monotropic compound

The relative position of the G -isobars ($\Delta G_{A \rightarrow B}$) of different modifications can be determined by solubility experiments in a given solvent:

$$\Delta G_{A \rightarrow B} = RT \ln(S_A/S_B) \quad (8.4)$$

where S_A is the saturation solubility of the modification A and S_B is the saturation solubility of the modification B.

Each crystal form has its own heat capacity, which is a function of the enthalpy H and the temperature. The heat capacity of solids at constant volume and constant pressure are about the same. The H isobars of the two modifications are parallel and their distance is the transition enthalpy ΔH_t .

Burger [27-28], proposed to plot energy/temperature diagrams of the Gibbs energy (G) and the enthalpy (H), as functions of temperature. This proposal is the fundamental tool for the solution of complex polymorphic systems. As shown in Figures 8.10a and 8.10b, a notable difference between enantiotropy and monotropy is the melting enthalpy of the higher melting form. In the case of enantiotropy, the higher melting form has the lower melting enthalpy. In the case of monotropy, the higher melting form has the higher melting enthalpy.

DSC, which measures every heat flow change upon heating or cooling, is the most appropriate technique, since the method allows us to determine the melting points and the melting enthalpies, as well as the transition points and the corresponding energy. The influence of kinetics can be followed by using different heating rates and tempering *in situ* [12].

The relationship between melting enthalpies of two solid phases A and B, and the heat of transition is approximately:

$$\Delta H_t = \Delta H_{fA} - \Delta H_{fB} \quad (8.5)$$

The temperature of transition between two enantiotrops can be calculated according to Equation 8.6 by neglecting the differences in specific heat.

$$T_t = T_A T_B (\Delta H_{fA} \Delta H_{fB}) / (\Delta H_{fA} T_B - \Delta H_{fB} T_A) \quad (8.6)$$

In the case of enantiotropy, the low melting form is the thermodynamic stable form below the transition point, and above this point the high melting form is the thermodynamic stable form. The transition point can be low; close to 40 °C in the case of tolbutamide, close to 100 °C in the case of propyphenazone or even higher than 200 °C. Figure 8.11 illustrates the case of the reversible transition between polymorphs of tolbutamide, with reversible DSC heating and cooling curves.

Figure 8.11. DSC curve for the reversible enantiotropic transition; Inset: heating and cooling curves in the temperature range of the transition

In the case of monotropy, there is only the high melting form present, which is the thermodynamic stable form within the entire range of temperatures. Sometimes a metastable form can behave as a stable form, and the DSC curve shows an exothermic transition before the melting of the stable form, helping to define the thermodynamically stable anhydrous form (see Figure 8.12).

Due to kinetics, metastable forms can appear and because different cases have to be taken into account, it is mandatory to use different heating rates for DSC experiments (see Section 8.4.3). For solid form selection it is absolutely necessary to know the temperature of the transition of enantiotropic systems, since it is a thermodynamic value. Does the transition take place at 20 °C, 30 °C, 70 °C or at 120 °C? What are the consequences for drug development? The temperature of the process, the temperature of storage and transport are important factors in production.

Figure 8.12. DSC curve of a metastable form which transforms in the stable form with exothermic transition (monotropy)

Due to kinetic behaviour, the temperature of transition observed in DSC is higher than the temperature observed by solubility measurements or slurry experiments in the presence of solvents. Table 8.4 shows some data obtained by calculation, according to Equation 8.6, compared to experimental data.

Table 8.4. Determination of enantiotropic transition temperatures for example substances by calculation and experiment

Substance	Transition temperature, °C	Method
Carbamazepine	71	Calculation
	73	Solubility
Nimodipine	82	Calculation
	88	Slurry experiment
Substance in development	39	Calculation
	25	Slurry experiment

8.4.1.2. The phase diagrams of salts, solvates and hydrates

The phase diagrams of salts, solvates and hydrates are more complex because binary mixtures are often of different compositions. The new compound may undergo congruent melting (Figure 8.13a), or non-congruent melting (Figure 8.13b). Through heating, the melting of the solvate may be observed, followed by transformation to an anhydrous form, or the solvent may be involved in an endothermic transition into the anhydrous form. A series of such binary phase diagrams has to be considered if several compounds are formed. These diagrams are fundamental to the understanding of crystallisation and drying steps. Analytical investigations of solvates are not possible without thermogravimetry. Examples of DSC and TG curves corresponding at these behaviours are given in Figures 8.14 and 8.15.

Figure 8.13. Binary phase diagrams (solvates, salts); (a) stable compound C and 2 eutectics; (b) non-congruent melting, peritectic behaviour, the compound dissociates

Figure 8.14. DSC and TG curves for congruent melting of the hydrate followed by loss of water and crystallisation into the anhydrous form

The effect of pressure on dehydration steps is exemplified by Figure 8.16 for copper sulphate pentahydrate. Depending on the pressure, dehydration can give the anhydrous form, the monohydrate or the trihydrate. This explains the coexistence of different steps of hydration during drying, milling or tableting.

Figure 8.15. DSC and TG curves for a peritectic behaviour of a solvate: endothermic transition between the ethanol solvate and the anhydrous form

Figure 8.16. Effect of the pressure on the dehydration steps of copper sulphate pentahydrate: into trihydrate, monohydrate and anhydrous. Adapted from reference [29]

8.4.1.3. Amorphous state

If a physical property of a crystalline substance is plotted against temperature, a sharp discontinuity occurs at the melting point. For amorphous substances there is no melting point, and a change of the baseline of the DSC curve occurs at the so-called glass transition temperature T_g . Below this temperature, the amorphous phase has certain properties of a crystalline solid (*e.g.* plastic deformation) and is termed “glassy”. At temperatures above T_g , the substance retains some of the properties of a liquid, *e.g.* molecular mobility, and is termed “rubbery”. Above T_g , the increase in molecular mobility facilitates spontaneous crystallisation, with an exothermic enthalpy change after the glass transition (see Figure 8.17). The glass transition temperature is decreased by water, or other solvents and additives, facilitating crystallisation. The amorphous state is unstable, and the study of the glass transition with excipients under humidity, is a part of the pre-formulation process [30]. Amorphous solid phases are obtained either by melt quenching, if no degradation occurs during the melting, or by lyophilisation. They are easily detected by DSC, since the glass transition can be followed by the exothermic crystallisation into the crystalline state, or can be detected by XRD. Figure 8.18 shows a case where a very small amount of amorphous form was detected by DSC alone.

Figure 8.17. DSC curves of a crystalline and an amorphous hydrochloride salt of a substance. Inset: temperature range of the glass transition of the amorphous compound

Figure 8.18. Detection of an amorphous part down to 1% in a drug substance with polymorphic behaviour.

8.4.2. Purity determination

As already described, the method for purity determination previously proposed [31] is particularly attractive as it does not require a reference standard. This method delivers absolute purity or assay value and results are obtained in less than 30 minutes. The determination of purity by means of DSC is based on the assumption that impurities depress the melting point of a pure material according to eutectic phase diagram behaviour. Figure 8.19a shows the phase diagram for the two component mixture with a eutectic point. At the eutectic point, for example 40 % A, 60 % B, the crystals A and B melt together at the temperature T_E (Figure 8.19b), below the melting temperature of the pure compounds. If a mixture of A and B (containing e.g. 90 % A) is heated, the melting of the eutectic mixture (which is 40 % A) is observed initially, until all of B is melted. During the melting of the eutectic mixture (40 % A, 60 % B) a part of A is melted with B, with the corresponding amount of A ($\frac{2}{3} \times 10\%$, *i.e.* 6.66 % of A). As the temperature increases, pure A melts between T_E and T_m (T_m being the end of the melting). For the corresponding DSC curve, if an endothermic heat flow at the eutectic temperature is observed, then the melting of crystals of A occurs.

Figure 8.19. Purity determination by DSC

- (a) phase diagram of the mixture of two compounds forming eutectic mixture,
 (b) DSC curve of the eutectic mixture,
 (c) broadening of DSC curve during the melting of a substance after eutectic point,
 (d) a plot of observed analyte temperature vs. reciprocal value of the fraction melted.

The effect of impurity on the DSC curve is a melting depression and a broadening of the melting curve. The amount of impurities is calculated from the melting point depression $\Delta T = T_0 - T_m$. The van't Hoff's law for diluted solutions is:

$$x = \frac{-\Delta T \Delta H_f}{RT_0^2} \quad (8.7)$$

where x is the mole fraction of impurities, ΔT the melting point depression, ΔH_f the melting point of pure material, T_m the melting of the analyte, T_o the melting point of the pure compound and R the gas constant.

The DSC procedure does not directly measure ΔT , but can be used to calculate it from the melting curve. At the eutectic point all of B is in the liquid phase. During the melting of A, after the eutectic point, the concentration of B varies in the liquid phase. This causes the broadening of the DSC curve (Figure 8.19c). With no solid solution formation, the concentration of impurity in the liquid phase at any temperature during the melting is inversely proportional to the fraction melted at that temperature, and the melting-point depression is directly proportional to the mole fraction of impurity. A plot of the observed analyte temperature, T_i , versus the reciprocal of the fraction melted $1/F_i$ at temperature T_i , should yield a straight line with the slope equal to the melting-point depression ($T_o - T_m$) (Figure 8.19d). The theoretical melting point of the pure compound is obtained by extrapolation to $1/F_i = 0$:

$$T_i = T_o - \frac{RT_o^2 \left(\frac{1}{F_i} \right)}{\Delta H_f} x \quad (8.8)$$

The correction, K , has to be made corresponding to the part already melted in the eutectic, and the detected beginning of the melting curve. Figure 8.19d depicts such a determination. Software from manufacturers mostly uses iterative linearisation which gives the best value of K .

Characteristics of the purity determination are as follows:

- impurities which have eutectic behaviour are measured (i.e. soluble in the liquid phase and insoluble in the solid phase),
- the sum of impurities should be $< 2\%$,
- the result is expressed in mole % without knowledge of impurities,
- pure material is not needed,
- small amounts (0.1 - 2 mg) of material are used,
- if decomposition occurs during melting it can give erroneous results.

The calculation of purity allows the interpretation of the complex curves given by endotherms preceding the melting. It has been used successfully in stability studies at the beginning of development. An example is given in Section 8.4.4, Table 8.5.

8.4.3. Kinetic aspects

Every DSC study should include scans at different heating rates due to DSC being a dynamic method, and solid-state transformations, while being thermodynamically driven, are kinetically controlled. The DSC scans will differ if the sample under study is stable or metastable at ambient temperature. A typical set of DSC scans illustrates the study of the polymorphic relationship of two forms A and B.

In the case of enantiotropy (Figure 8.20a):

- Scan 1. The sample studied is the stable form A, which gives the endothermic solid phase transition $A \rightarrow B$ followed by the melting endotherm of form B.
- Scan 2. The sample studied is the stable form A, but for kinetic reasons the solid transformation $A \rightarrow B$ does not occur. Instead form A melts.
- Scan 3. The sample studied is the stable form A which melts. Form B crystallises from the melt with an exothermic peak and form B melts at a higher temperature.
- Scan 4. The sample studied is the metastable form B, which becomes stable at a temperature above the transition temperature. An exothermic peak corresponds to the solid transformation $B \rightarrow A$, followed by successive transformation $A \rightarrow B$, and melting of B.
- Scan 5. The sample studied is the metastable form B. The DSC scan shows its melting endotherm.

Figure 8.20. Possible DSC scans for 2 polymorphs. (a) enantiotropy; (b) monotropy

In the case of monotropy (Figure 8.20b):

- Scan 1. The sample studied is the stable form A, and its melting endotherm is observed.
- Scan 2. The sample studied is the metastable form B, which transforms exothermically in the solid state into the stable form A. Form A melts at a higher temperature.
- Scan 3. The sample studied is the metastable form B, which does not transform into A, but melts endothermically. From the melt the stable crystalline form A appears, with an exothermic peak, then A melts at a higher temperature.

Similar interpretations apply to all methods that involve heating (*e.g.* hot stage optical microscopy, hot stage infrared or Raman microscopy, temperature resolved or variable temperature XRD).

It is difficult to distinguish between enantiotropy and monotropy in the cases of Figure 8.20a, Scan 5 and Figure 8.20b, Scan 1, as well as in Figure 8.20a, Scan 3 and Figure 8.20b, Scan 3. The interpretation of the DSC curves is facilitated by Burger's enthalpy of fusion rule [27-28]: if the higher melting form has the lower melting enthalpy, then both forms are related enantiotropically. As demonstrated in Figure 8.21, for a benzoquinoline hydrochloride [12], the melting enthalpy of the higher melting form B is lower than the melting enthalpy of A. Therefore, the two forms are enantiotropically related, with form A being stable below the transition point. Modification B is hygroscopic and undergoes a solvent-mediated transition to form A in alcohols at ambient temperature.

Figure 8.21. Polymorphic behaviour of a benzoquinoline hydrochloride. Each form has its melting curve. Enantiotropy demonstrated by the Burgers' rule. Form A on the left side has a melting point of 304 °C and a melting energy of 50 kJ mol⁻¹. Form B has a melting point of 311 °C and a melting energy of 46 kJ mol⁻¹ [14]

Figure 8.22 shows DSC curves of propyphenazone. Samples from two manufacturers were investigated in our laboratory since the second sample did not comply with melting requirements. An unexpectedly high melting point was not due to higher purity, but to the enantiotropic behaviour. The second sample contained traces of a high melting form. A slower heating rate showed for both samples two endotherms. DSC experiments allowed the manufacture *in situ* of the

high melting form, and to characterise it completely including solubility and dissolution rate [12,13]. For examples of kinetic effects due to polymorphism and salts, see references [32,33]. Kinetics also governs the steps of hydration and dehydration in the solid state, giving rise to complex conversion relationships [34-36], (see example in Figure 8.25).

Figure 8.22. Effect of heating rate on the DSC of propyphenazone enantiotropic system. Batch 1 and 2 of two manufacturers have different melting points. At high heating rates (20, 10, 5, or 2.5 °C min⁻¹) batch 1 shows only one peak. Batch 1 is a pure form, batch 2 contains very small amounts of high melting form which acts as seed. With slow heating rates transformation to the high melting form occurs for both batches [12]

8.4.4. Sorption isotherms and hydrates

Most drug substances crystallise in the form of solvates or hydrates. The DSC/TG curves allow us to follow the desolvation process. It is possible to measure water sorption isotherms by TG in controlled atmospheres. Often the instrument used is an automatic multi-vapour gravimetric sorption analyser, a so-called “dynamic vapour system” (DVS), *e.g.* those made by Surface Measurement Systems Ltd. Figure 8.23 shows such curves for an enantiotropic drug substance which absorbs water to form a trihydrate. The desorption occurs with hysteresis, allowing us to define the relative humidity range for each form. According to the curves, the trihydrate form would be possible to develop. However, it was not possible to manufacture it as a pure form in the process. The enantiotropic form, which was stable at room temperature, could be manufactured and stored in tight containers [14].

Figure 8.23. Example of a water sorption-desorption isotherm for two enantiotrops (A and B) and a trihydrate of the investigated compound

Figure 8.24 shows the use of sorption isotherm measurement for a salt selection. The hydrochloride first selected was too hygroscopic for easy development, and therefore new salts were studied. The DSC purity was determined for stability comparison (Table 8.5). The hydrogen fumarate and hydrogen tartrate were attractive due to their good hygroscopic behaviour, however the stability results clearly showed that hydrogen tartrate was chemically stable. This example demonstrates the advantageous use of thermal techniques for quick decisions in early development.

Table 8.5. DSC purity results for the stability comparison of salts given in Figure 8.24.

DSC time	DSC purity, %		
	Hydrogen fumarate	Hydrogen maleate	Hydrogen tartrate
Initial	99.8	99.8	99.9
1 week	99.4	no peak	99.9
Luminous exposure: 1200 klux h	99.1	97.6	99.5

Figure 8.24. Salt selection. The hygroscopic hydrochloride is replaced by the hydrogen tartrate [18]. (a) Dynamic vapour system (DVS) curves of hydrochloride, (b) DVS curves for hydrogenmaleate, hydrogenfumarate and hydrogentartrate salts

It is recommended that several cycles of sorption and desorption are performed when a hydrate is produced. Figure 8.25 shows the kinetic effect of seeds for a drug substance. The anhydrous form is converted to the monohydrate at a relative humidity of $RH > 90\%$. After the desorption, traces of monohydrate are present and the substance absorbs water very fast. The behaviour was also found in stability studies of batches of the drug [32].

Recently introduced automatic systems bring a faster understanding of complex hydration and dehydration behaviour, as well as their kinetics with monitored relative humidity (RH %). Instruments for research have $0.1\ \mu\text{g}$ sensitivity, and less than $10\ \text{mg}$ of sample can be used. By using solvent vapours, solvated forms can be manufactured and studied. These studies are a prerequisite for the microcalorimetric analysis of amorphous content.

Figure 8.25. Water sorption-desorption isotherms and kinetics. In the first cycle the substance takes up water only at RH > 90%. After the desorption cycle the third absorption cycle shows the transformation into the monohydrate already at 30 % RH

8.4.5. Combined techniques

8.4.5.1. Combination of DSC and XRD

XRD instruments are equipped with heating cells and are routinely used in most research laboratories. X-ray patterns of pure forms obtained *in situ* can be used for modelling, and in favourable cases, for crystal structure determination. Figure 8.26 exemplifies the combination of DSC/XRD for an enantiotropic system with heating and cooling curves. The advantage of coupled instruments developed in a research laboratory has been demonstrated [37]. The trehalose monohydrate dehydration and hydration were studied in a humid atmosphere as an application of a new commercial instrumentation [38].

8.4.5.2. Thermogravimetry-mass spectrometry (TG-MS) and thermogravimetry-infrared spectroscopy (TG-IR)

An example of TG-MS is given for calcium oxalate in Figure 8.27, where water, CO and CO₂ are positively identified. Figure 8.28 is an example of the successful interpretation of a crystallisation into solvates followed by desolvation to a metastable form. The DSC and TG-MS in this figure correspond to the methanol solvate. Similar pictures are obtained with other solvates *e.g.* acetone and ethanol. The DSC/TG/TG-MS and temperature resolved XRD experiments, as well as slurry experiments, were necessary to understand the crystallisation of this drug substance and to change the solvent of crystallisation [32].

Figure 8.29a shows the DSC/TG for aspartame hemihydrate and corresponding IR spectra (Figure 8.29b). The first endotherm at around 130 °C is the loss of

Figure 8.26. Use of combined DSC-XRD for the detection of a reversible enantiotropic transition of a purine derivative; (a) successive DSC heating up to 120 $^{\circ}\text{C}$ (1) cooling (2) and heating up to 140 $^{\circ}\text{C}$ showing the reversible phase transition and the melting (3); (b) reversible transition between 50 and 125 $^{\circ}\text{C}$ followed by XRD

water, and the sharp endotherm is due to decomposition and formation of methanol [14]. Solvates can be successfully characterised [39], and quantitative information of the spectroscopic signal is possible [40].

An example is given in Figure 8.30 of the interpretation of the behaviour of a malonate using several combined technologies: DSC, TG, TG-MS, temperature resolved XRD and IR. Through drying, the malonic acid can sublime and the

Figure 8.27. (a) TG curve of calcium oxalate and (b) MS signals of H_2O , CO and CO_2

base formed [14]. Another example, given in Figure 8.31, deals with the loss of water from a drug substance during drying. The decomposition product formed was at first believed to be a new form. DSC/TG/XRD and IR were then successfully used for the identification of the substance with two enantiotrops [15]. The DSC of the stable form shows an enantiotropic transition. After the melting of the second form, a degradation into the lactam with the loss of a molar quantity of water was detected by TG-MS and temperature resolved XRD. The IR-heating cell allowed the structure of the lactam to be confirmed.

Figure 8.28. Identification of a methanol solvate in a complex crystallisation behaviour by TG-MS; (a) DSC curve and (b) TG curve and corresponding MS signal [14]

Figure 8.29. Use of TG-IR for the detection of methanol in the degradation of aspartame; (a) TG and DSC curves, (b) corresponding IR spectra

Figure 8.30. Use of combined techniques for the interpretation of thermal events of a malonate. (a) DSC and TG curves. Three different DSC peaks are interpreted as melting (1) and decomposition (2) of the malonate followed by melting of the base (3); (b) temperature resolved XRD shows the irreversible transformation into the base according to (c); (c) XRD patterns of the free base and malonate; (d) TG-MS experiment indicates the evolution of water and CO₂ during heating of malonic acid; (e) IR spectra taken at different temperatures confirm the presence of CO₂ and the chemical structure of the base [14]

Figure 8.31. Combined techniques elucidating the decomposition of an enantiotropic drug into a lactam [15]. (a) DSC curve; (b) TG-MS confirms the degradation with loss of water; (c) the temperature resolved XRD shows the 3 steps: form A, form B and lactam; (d) IR spectra in heating cell confirm the enantiotropic transition and show the chemical transformation.

8.4.6. Isothermal microcalorimetry and solution calorimetry

8.4.6.1. Microcalorimetry

The decrease in the glass transition temperature, T_g , by water and solvents, is the principle behind the microcalorimetric method of amorphous content determination. The substance is subjected to vapours of water, or organic solvent, in an isothermal microcalorimeter (Figure 8.32). The heat flow of crystallisation which is proportional to the amorphous content is measured. The sorption-desorption isotherm presented in Figure 8.33 exemplifies the process. The un-milled sample is not hygroscopic but after micronisation, the powder absorbs water (cycle 2), and an increase in mass is observed. An abrupt decrease is observed in cycle 3 as the drug crystallises and loses water. The corresponding exothermic energy of crystallisation can then be measured.

Isothermal Microcalorimetry

Type:	Thermal Activity Monitor, Thermometrics
Sample volume:	3 ml, glass ampoule
Temperature:	20 – 80 °C
Precision:	0.1 % (at 300 μ W)
Baseline stability:	$\pm 0.2 \mu$ W (8 h)
Humidity control:	e.g. 57 % RH (sat. NaCl-solution)

Figure 8.32. Setting of isothermal calorimeter for quantitation of amorphous content [17, 21]

Figure 8.33. Sorption-desorption isotherms (a) before and (b) after micronisation. Crystallisation of the amorphous part in humid atmosphere

Figure 8.34 shows an example of the determination of amorphous content in a batch of micronised substance for inhalation, by using two different solvent vapours, and the methods were validated. The same result is obtained for this

batch: 12.9 % compared to 12.7 % amorphous content. However, as demonstrated in Figure 8.34, the time of crystallisation is quite different. With vapours from a mixture of ethanol and water, the crystallisation is rapid, but the start of crystallisation cannot be measured for low amounts of amorphous form. This method can be used in the range 7 - 15 % of amorphous content. In order to analyse lower contents, dimethylformamide (DMF) used as vapour, can act slowly, and the method has been validated in the range 2 - 30 % amorphous content. An automatic instrument with 48 channels (TA instruments), allows us to perform routine analysis with a limit of detection at the 1 % level.

Figure 8.34. Example of quantitative validated methods for the determination of amorphous content by isothermal microcalorimetry; (a) and (b) microcalorimetric curves for a batch with two different methods, (c) automatic isothermal microcalorimetric instrument (TA analysis), (d) comparison of curves with very low amorphous form content

8.4.6.2. Solution calorimetry

Solution calorimetry has been described in United States Pharmacopeia (USP) for the determination of the amorphous form, but has also been used for quantitation of polymorphs [14]. This method supposes that no other crystalline form may interfere. In the case of enantiotropy:

$$\Delta H_t = \Delta H_{SA} - \Delta H_{SB} \quad (8.9)$$

where ΔH_{SA} is the heat of solution of form A, and ΔH_{SB} the heat of solution of form B.

This method can be used for polymorphs, as demonstrated in Figure 8.35 for an enantiotropic drug. The same results for the heat of transition are found by DSC and solution calorimetry [12,16,18]. Solution calorimetry is very helpful when the substance decomposes on melting. This approach allows us to choose between forms and select the most stable [14,18].

Figure 8.35. Solution calorimetry. Determination of the heat of transition between 2 enantiomers [16.] Before and after the break of the ampoule containing the substance in water the instrument is calibrated by Joule effect. Heat of transition found by DSC: 10.4 kJ mol⁻¹; Heat of transition found by solution microcalorimetry: 9.7 kJ mol⁻¹

8.5. SOME EXAMPLES OF APPLICATIONS

8.5.1. The selection of the right form

The following example, given in Figure 8.36, is a situation often found in early development. We received a sample, obtained by precipitation, for which the DSC scan exhibits dual melting. By using different heating rates it was possible to

calculate the melting enthalpies and it was concluded that the sample is an enantiotropic system. The polymorphism study of slurries in different solvents of a purer sample gave a third form. According to Burger's rule, and the equilibration study, C is the stable form, A and B are enantiotrops, and both are monotrops to C. Consequently, form C was selected for further study [32].

Figure 8.36. Case of a drug candidate with two enantiotropic metastable forms obtained in research laboratory. A third form was thermodynamically stable [32].
Summary of their relationships.

8.5.2. Sensitivity of DSC

Figure 8.37 deals with a drug substance for which traces of a stable form (form B) acted as a catalyst for quick transformation if the samples were not kept at very low temperatures. DSC proved more sensitive and allowed monitoring [15,18].

Figure 8.37. Example of quantitative determination of undesirable form by the melting DSC curve. Sensitivity of DSC: traces of a stable form acting as seeds by aging are determinable [15,17]

8.5.3. Disappearing solvates

Solvates are very often formed in the presence of volatile solvents. By isolation in the air they easily transform into anhydrous forms, stable or unstable, in amorphous powder, or in hydrated forms. The best way to detect them would be direct measurement in the crystallisation vessel. Figure 8.38 shows the results of a large number of experiments to understand the complex situation found with a drug substance.

- Crystallisation in ethanol <40 °C form A, >50 °C form B
Which form to be selected?
- ➡ Burger's rule? A: one DSC peak B: two DSC peaks with decomposition
 - ➡ X-ray heating cell: B $\Rightarrow$ A before melting and decomposition

Figure 8.38. Disappearing solvates. Example of experiments conducted to select the right form [18]

8.5.4. Selection of the best form considering the pharmaceutical environment

Water is present in the air and in excipients, and is often used in mixtures for crystallisation purposes. The TG curves of hydrates can be considered fingerprints. Figure 8.39 is an example of the coexistence of monohydrate and trihydrate in the solvent mixture of a process. The TG curve permitted the evaluation of the presence of the trihydrate, since trihydrate and monohydrate had separated steps for the loss of water [16,18]. Figure 8.40 is an example of the relationships needed to select thermodynamic windows for hydrates [12].

Figure 8.39. Detection of impure hydrates by TG.

- (a) TG curves of the monohydrate, the trihydrate and of an impure sample containing both;
 (b) solubility behaviour of the monohydrate and of the trihydrate [18]

Figure 8.40. Example of relationships in a complex problem of hydrates and solvates.

- (a) As demonstrated on the DSC curves the dehydration of two polymorphs of the hydrate occur differently.
 A: DSC of anhydrous form I with transformation into anhydrous III.
 B: polymorphic hydrate A loses water and gives the anhydrous form I.
 C: polymorphic hydrate B loses water into the anhydrous form II;
 (b) A scheme of the relationships between solvates, hydrates and anhydrous form

8.6. CONCLUSION

In conclusion, thermal analysis and calorimetric techniques are necessary for the selection and the characterisation of solid forms. They are versatile and offer quick results. As exemplified in Figure 8.41, characterisation of the relationship between all forms is the objective of development. For this challenging purpose a range of methods is necessary, and when this is achieved, process analytical technology will be the future for upscale and manufacture.

Figure 8.41. Relationships between the solid phases of tetracaine hydrochloride according to [41]. For the study, DSC, TG and temperature resolved XRD as well as solubility and slurries experiments were performed. 6 crystalline anhydrous forms were identified, an amorphous form, a tetrahydrate, a monohydrate and a hemi-hydrate. At room temperature only anhydrous forms 1 and 5 could be obtained for a long period of time. Form 1 is the stable form and corresponds to the commercial quality

REFERENCES

1. Guideline Specification Q6A. Investigating the need to set acceptance criteria for polymorphism in drug substances and drug products. *International Conference on Harmonization (ICH)* (1999).
2. L. Le Chatelier. *Compte Rendu*. **104** (1886) 1243.
3. G. Höhne, W. Hemminger, H.J. Flammersheim. *Handbook of Thermal Analysis and Calorimetry*. Springer, 2003.
4. P.J. Haines. *Principles of Thermal Analysis and Calorimetry*. RCS Paperback, 2002.
5. P. Gabbott. *Principles and Applications of Thermal Analysis*. Wiley-Blackwell Publ. (2007).

6. D.Q.M. Craig, M. Reading. Thermal Analysis of Pharmaceuticals. *CRC Press, UK* 2006.
7. W-P. Pan, L. Judovits. Techniques in thermal Analysis, hyphenated techniques, Thermal Analysis of surface, fast rate analysis. *ASTM*, 2007.
8. D. Giron, Thermal Analysis of Drugs and Drug Products. In: *Encyclopedia of Pharmaceutical Technology*. (Eds. J. Swarbrick, J.C. Boylan) *Marcel Dekker*, 2006.
9. D. Giron. Thermal Analysis and microcalorimetric methods in the industry: essential techniques for proper development of pharmaceuticals. *European Pharmaceutical Review* 2006 (5) 84-90.
10. D.Giron. Latest Development in the area of thermal analysis and calorimetry that are impacting on pharmaceutical manufacturing. *European Pharmaceutical Review* 2008 (2) 71-76.
11. B. Rodriguez-Spong, C.P. Price, A. Jayasankar, A.J. Matzger, N. Rodriguez-Hornedo. General principles of pharmaceutical solid polymorphism: A supramolecular perspective. *Advanced Drug Delivery Reviews* **56**(3) (2004) 241-274.
12. D. Giron. Thermal analysis and calorimetric methods in the characterization of polymorphs and solvates. *Thermochimica Acta* **248** (1995) 1-59.
13. D. Giron. Le polymorphisme. *Labo-Pharma Problèmes et techniques*. **307** (1981) 151-160.
14. D. Giron. Investigations of polymorphism and pseudo-polymorphism in pharmaceuticals by combined thermoanalytical techniques. *Journal of Thermal Analysis and Calorimetry* **64**(1) (2001) 37-60.
15. D. Giron. Monitoring of polymorphism from detection to quantification. *Engineering in Life Sciences* **3**(3) (2003) 103-112.
16. D. Giron, M. Mutz, S. Garnier. Solid-state of pharmaceutical compounds: Impact of the ICH Q6 guideline on industrial development. *Journal of Thermal Analysis and Calorimetry* **77**(2) (2004) 709-747.
17. D. Giron, S. Monnier, M. Mutz, T. Buser, F. Stowasser, K. Schulze, M. Bellus. Comparison of quantitative methods for analysis of polyphasic pharmaceuticals. *Journal of Thermal Analysis and Calorimetry* **89**(3) (2007) 729-743.
18. D. Giron, S. Monnier, M. Mutz. Challenging characterization and monitoring of polymorphs. Latest developments. *Lecture presented at AFCAT-GEFTA-STK meeting, Mulhouse*, 2008.
19. I.M. Vitez. Utilization of DSC for pharmaceutical crystal form quantitation. *Journal of Thermal Analysis and Calorimetry* **78**(1) (2004) 33-45.
20. S. Gaisford, M. A.A. O'Neil, Pharmaceutical Isothermal Calorimetry, *Informa Healthcare*, 2007.
21. M. Mutz, A. Motreff, M. Monnier, T. Buser, P. Schwab, D. Giron. Use of high throughput microcalorimeter for faster determination of amorphous content. *STK annual meeting*, June 2006, Fribourg, Switzerland.
22. G. Buckton, P.Darcy, A.J. Mackellar. The use of isothermal microcalorimetry in the study of small degrees of amorphous content of powders. *International Journal of Pharmaceutics* **117**(2) (1995) 253-256.
23. D. Giron, P. Remy, S.Thomas, E. Vilette. Quantitation of amorphicity by microcalorimetry. *Journal of Thermal Analysis and Calorimetry* **48**(3) (1997) 465-470.
24. B.Shah, V.K. Kakumanu, A.K. Bansal. Analytical techniques for quantification of amorphous/crystalline phases in pharmaceutical solids. *Journal of Pharmaceutical Science* **95**(8) (2006) 1641-1665.

25. M. Savolainen, K. Jouppila, O. Pajamo, L. Christiansen, C. Strachan, M. Karjalainen, J. Rantanen. Determination of amorphous content in the pharmaceutical process environment. *Journal of Pharmacy and Pharmacology* **59**(2) (2007) 161-170.
26. S.R. Chemburkar, J.B.K. Deming, H. Spiwek, K. Patel, J. Morris, R. Henry, S. Spanton, W. Dziki, W. Porter, J. Quick, P. Bauer, J. Donaubauer, B.A. Narayanan, M. Soldani, D. Riley, K. McFarland. Dealing with the impact of ritonavir polymorphs on the late stages of bulk drug process development. *Organic Process Research and Development* **4**(5) (2000) 413-417.
27. A.Burger, R. Ramberger. On the polymorphism of pharmaceuticals and other molecular crystals. Theory of thermodynamic rules. *Mikrochimica Acta [Wien]* **11** **72**(3) (1979) 259-271.
28. Burger, U.J. Griesser. *Scientia Pharmaceutica*. **58** (1990) 423-430.
29. M. Soustelle. In: Handbook of powder technology, Vol.9. *Powder Technology and Pharmaceutical Processes* (Eds. D. Chulia, M. Deleuil, Y. Pourcelot) Elsevier 1994.
30. B.C. Hancock, G. Zografi. Characteristics and significance of the amorphous state in pharmaceutical systems. *Journal of Pharmaceutical Science* **86**(1) (1997) 1-12.
31. D. Giron, C. Goldbronn. Place of DSC purity analysis in pharmaceutical development. *Journal of Thermal Analysis and Calorimetry* **44**(1) (1995) 217-251.
32. D. Giron. Polymorphism: Thermodynamic and kinetic factors to be considered in chemical development. *American Pharmaceutical Review* **8** (2005) 32-37 and 72-79.
33. D. Giron. Characterisation of salts of drug substances. *Journal of Thermal Analysis and Calorimetry* **73**(2) (2003) 441-450.
34. D. Giron, C. Golbronn, M. Mutz, S. Pfeffer, P. Piechon, P. Schwab. Solid state characterization of pharmaceutical hydrates. *Journal of Thermal Analysis and Calorimetry* **68**(2) (2002) 453-465.
35. W.Chongcharoen, S.R. Byrn, N. Sutanthavinu. Solid state interconversion of norfloxacin hydrates. *Journal of Pharmaceutical Science* **97**(1) (2008) 473-487.
36. A.K. Salameh, L.S. Taylor. Physical stability of crystal hydrates and their anhydrides in the presence of excipients. *Journal of Pharmaceutical Science* **95**(2) (2006) 446-461.
37. M. Ollivon, G. Keller, C. Bourgaux, D. Kalnin, P. Villeneuve, P. Lesieur. *Journal of Thermal Analysis and Calorimetry* **85**(1) (2006) 219-224.
38. Kichi, H. Toraya. Simultaneous measurements of X-ray diffraction (XRD) and differential scanning calorimetry data under controlled humidity conditions: instrumentation, and application to studies on hydration, dehydration and re-hydration process of pharmaceutical compounds. *Advances in X-ray Analysis* **47** 240-248.
39. Rodriguez, D.E. Bugay. Characterization of pharmaceutical solvates by combined gravimetric and infrared analysis. *Journal of Pharmaceutical Science* **86**(2) (1997) 263-266.
40. F. Eigenmann, M. Maciejewski, A. Baiker. Quantitative calibration of spectroscopic signals in combined TG-FT-IR systems. *Thermochimica Acta* **440**(1) (2006) 81-92.
41. D. Giron, M. Draghi, C. Goldbronn, S. Pfeffer, P. Piechon. Study of polymorphism of tetracaine hydrochloride. *Journal of Thermal Analysis and Calorimetry* **49**(2) (1997) 913-927.

**NEAR INFRARED SPECTROSCOPY IN DRUG
DISCOVERY AND DEVELOPMENT PROCESSES**

Yves Roggo, Klara Dégardin and Michel Ulmschneider

Contents

9.1. INTRODUCTION.....	434
9.2. NIRS AND IMAGING: THEORY AND INSTRUMENTATION	435
9.2.1. General introduction	435
9.2.2. Characteristics of NIRS.....	436
9.2.3. NIR instrumentation.....	437
9.3. CHEMOMETRICS AND VALIDATION OF NIR METHODS.....	438
9.3.1. Development and validation of NIR methods	438
9.3.2. Mathematical preprocessing of spectroscopic data	442
9.3.3. Principal component analysis (PCA).....	443
9.3.4. Pattern recognition	444
9.3.5. Regression methods	446

9.4. QUALITATIVE ANALYSES BY NIRS	447
9.4.1. Identification and qualification	447
9.4.2. Polymorphism.....	449
9.4.3. Other applications.....	449
9.5. QUANTITATIVE ANALYSES BY NIRS.....	450
9.5.1. Physical parameters.....	450
9.5.2. Polymorphs determination.....	451
9.5.3. Moisture determination	451
9.5.4. Content determination.....	452
9.6. ON-LINE CONTROL BY MEANS OF NIRS.....	455
9.6.1. Powder Blending	455
9.6.2. Granulation	457
9.6.3. Drying	458
9.6.4. Crystallinity and polymorphism	458
9.6.5. Coating	459
9.6.6. Biotechnology	459
9.7. APPLICATIONS OF NIR SPECTRAL IMAGING	461
9.7.1. General use of NIR chemical imaging for pharmaceutical applications.....	461
9.7.2. Tablet composition analysis with NIR imaging	461
9.7.3. Application of NIR imaging to process optimization.....	462
9.8. CONCLUSION	464
REFERENCES	464

9.1. INTRODUCTION

Process analytical technology (PAT) has existed for many years in various industries. It was one of the objectives contained in the Pharmaceutical cGMPs for the 21st Century initiative launched in 2002 by the Food and Drug Administration (FDA). The FDA guideline defined PAT as a system for designing, analyzing and controlling pharmaceutical manufacturing through the measurement of critical quality and performance parameters. The measurements performed on raw and in-process materials or process parameters were intended to enhance final product quality. Both the FDA and the industry anticipated benefits over conventional manufacturing practices: higher final product quality, increased production efficiency, decreased operating costs, better process capacity, and fewer rejects [1].

Building quality into a pharmaceutical product has to be considered from the moment of a product's conception. PAT provides a motivating framework. If product quality requirements are understood and implemented from the outset, process failure after scale-up to commercial manufacturing will be much less likely. PAT greatly enhances process understanding. It continuously improves product quality, extends the acquired knowledge base for new projects, and shortens time to market. Trends in PAT were reviewed in 2011 [2], while Herdling and Lochmann have described and discussed PAT implementation in solid dosage form production [3]. PAT draws on the relevant basic sciences and a complex multiplicity of engineering and control technologies.

Near infrared spectroscopy (NIRS) is an important tool for PAT implementation [4], as it is increasingly used in pharmaceutical research and development.

In 1800, William Herschel discovered radiation beyond visible red light. However, it was many years before the NIR region was used for spectroscopy [5]. A wide overlap was observed in its bands, making them difficult to interpret. Karl Norris, an engineer at the U.S. Department of Agriculture, demonstrated the potential value of the NIR region for quantitative work by making measurements of agricultural products in the 1960s. The basic idea was to provide various research and production facilities with online NIR measurements of agricultural products, which was the field of interest at the time [5,6]. NIRS and chemometrics then spread to other domains, such as food [7], and the chemical [8] and oil industries [9], proving effective for both qualitative and quantitative analyses.

NIRS is widely used in the pharmaceutical industry, with multiple practical applications and a massively increased presence in specialized journals.

NIRS is generally chosen for its speed, low cost, and non-destructiveness. It is a selective technique that is sensitive to the physical chemistry of the samples it analyzes. These can be measured intact or through packaging such as glass or plastic bags. NIR imaging is derived from NIRS and involves the acquisition of spatially located spectra that display compound distribution in the sample for analysis along with features such as moisture or particle size. Several reviews of NIRS are now available [10-13], together with its application to the development of solid dosage forms [14]. Interest in NIR has increased because of improved instrumentation and the development of fibre optics allowing non-contact measurement; it has also been boosted by the progress in computer science and data processing that has facilitated interpretation of NIR spectra, notably involving chemometrics, the body of mathematical and statistical techniques developed for the processing of chemical analytic data. Mid-IR (MIR) spectra, on the other hand, especially the absorbance bands, are directly readable thanks to chemical peak specificity.

The aim of this chapter is to present the theory and instrumentation of NIRS and NIR imaging, briefly describe some of the chemometric tools used in calibrating and validating NIR methods, and finally to focus on some NIR applications in the development process and drug discovery.

9.2. NIRS AND IMAGING: THEORY AND INSTRUMENTATION

9.2.1. General introduction

Infrared (IR) spectroscopy is a versatile tool applied to the qualitative and quantitative determination of molecular compounds of all types. The IR domain is subdivided into NIR, MIR, and far IR (FIR) with the following range limits (Figure 9.1):

Near IR: 780 nm to 2 500 μm (12 800 to 4 000 cm^{-1})

Mid IR: 2 500 μm to 50 μm (4 000 to 200 cm^{-1})

Far IR: 50 μm to 500 μm (200 to 20 cm^{-1})

100	400	800	2 500	50 000	5·10 ⁵	3·10 ⁶	nm
	Ultraviolet	Visible	Infrared			Microwave	
			near	middle	far		
10 ⁵	25 000	12 500	4 000	200	20	3.3	cm ⁻¹

Figure 9.1: Electromagnetic spectrum – the infrared range

MIR spectroscopy is by far the most widely used, with absorption, reflection, and emission spectra employed for both qualitative and quantitative analyses. FIR is primarily used for absorption measurements of inorganic and metal-organic samples.

The NIR region is particularly used for routine quantitative determination in complex samples containing functional groups of hydrogen bonded to carbon, nitrogen, or oxygen. This is of interest in agriculture, feed, food, and, more recently, pharmaceutical industries. NIRS was first used for the rapid screening of feed and food products for protein, moisture, starch, oil, lipid, and cellulose content.

9.2.2. Characteristics of NIRS

The main vibrations observed in the NIR region are those of -CH, -OH, -SH, and -NH bonds. All the absorption bands result from overtones or combinations of the fundamental MIR bands [15,16]. The overtone and combination molecular absorptions found within the NIR region are much less intense than the fundamental IR absorptions.

Once the appropriate chemometric tools were developed, the NIR region turned out to be of great interest for industrial applications. NIRS is fast: once a method has been developed and validated, measurement only takes seconds. Samples require no preparation and can be measured as such, intact. They are available for further analysis since NIRS is non-destructive. Measurements can be performed on- and at-line. NIR spectrometers tend to be very robust. Glass fibre optics can be used to perform remote analysis by bringing radiation directly to the sample. The fibre optic probe can be in contact with the sample or immersed in it. As NIR measurements can be done through glass, this material can be used for windows, lenses, and any other optical components, which simplifies sampling.

Table 9.1 compares some pros and cons of NIR, IR and Raman spectroscopy.

Table 9.1. Comparison of near infrared (NIR), infrared (IR) and Raman spectroscopy

Spectroscopy	NR	IR	Raman
Signal range, cm ⁻¹	12 000 – 4 000	4 000 – 400	4 000 - 50
Signal intensity	++	+++	+
Microscopic analysis	No	Yes	Yes
Fiber optic interfacing	Yes	Yes (limit length)	Yes
Sampling through glass	Yes	0	Yes
Instrument robustness	+++	+	++
Chemical interpretation	With chemometric tools	Direct	Direct

NIRS has many more practical advantages in the pharmaceutical process thanks to numerous applications enabled by developments in instrumentation and sampling.

9.2.3. NIR instrumentation

The instrument design first depends on how measurements are performed. Diffuse reflectance and transmittance measurements are both used, although diffuse reflectance much more widely because of its ease of use. In the diffuse reflectance mode, radiation penetrates the particle surface layer, excites the vibrational modes of the analyte molecule, and is then scattered in all directions. Reflectance measurements penetrate only 1 mm to 4 mm into the solid sample surface. In this case the ordinate is the logarithm of the reciprocal of reflectance R , $\log (1/R)$, where R is the ratio of the intensity of radiation reflected from the sample to reflectance from a standard reflector. In transmittance mode, the entire path through the sample is integrated into the spectral measurement, thereby reducing error due to sample non-homogeneity. Transmittance is suitable for measuring through compact samples, like intact tablets, but surface scattering induces a loss of transmitted energy with the net effect being a decrease in the signal-to-noise ratio.

Many spectrometers have been specifically designed for the NIR range. The ideal instrument has both transmittance and reflectance capabilities. However, the choice is wide, especially when compared with MIR spectrometers. Grating, diode array, and Fourier transform (FT) instruments are the most sophisticated. FT spectrometers are mostly based on the Michelson interferometer, although other types of optical systems are also encountered. Tungsten-halogen lamps with quartz windows are used as sources while detectors are usually made of lead sulfide (PbS) or arsine-gallium (AsGa). Handheld NIR spectrometers have also been developed that allow measurement in the field rather than in the lab [17-19]. FT spectrophotometers are preferred for many applications because of their speed, reliability, and convenience. They appear to have better signal-to-noise ratios and a much larger energy throughput than dispersive instruments. Interferometric instruments also feature high resolutions, high accuracy, and reproducible frequency determination. Other designs include diode array detectors and NIR emitting diode sources. Acousto-optic tunable filters (AOTF) capable of microsecond scanning speeds are devices based on diffraction. Other techniques are available, such as ultrafast-spinning interference filter wheels, interferometers with no moving parts, and tunable laser sources.

Specific instruments are used for chemical imaging. A complete spectrum is acquired for each pixel, meaning that a hyperspectral image is in fact a data cube, *i.e.* a 3D matrix (Figure 9.2). Chemical imaging experiments yield an $X \times Y \times \lambda$ matrix or data cube, where X and Y are the spatial dimensions and λ the spectral dimension [20]. In principle, it is possible to collect hyperspectral images with single-point detectors, *i.e.* classical mapping with microscopes. However, array

detectors measure all pixels simultaneously, reduce recording time, provide uniform background, and improve the signal to noise ratio [21]. Figure 9.3 presents an example of a NIR image analyzer.

Figure 9.2. Near infrared (NIR) imaging concept [1]

Figure 9.3. Near infrared (NIR) imaging [1]

9.3. CHEMOMETRICS AND VALIDATION OF NIR METHODS

Chemometrics [22-24] is used to select the optimal experimental procedure and processing technique for chemical analytic data. It draws on a variety of specialties, including experimental design, data extraction (modeling, regression, classification, hypothesis testing), and techniques for understanding chemical mechanisms (see review by Lavine [25] and many textbooks [26-28]).

9.3.1. Development and validation of NIR methods

Sample selection

A NIR model is built and validated with several sample sets, beginning with the calibration set used to compute the model. The second validation set is used to evaluate the model's ability to predict unknown samples. Both sets must be independent, *i.e.* they must consist of samples from different batches. Set selection and preparation are critical issues. For instance, for quantitative methods the analyst must collect or prepare samples which span the complete range of constituent concentrations spread out as evenly as possible. Calibration sets must comprise a correctly distributed number of sample types. Spectro-

scopic measurements with calibration samples should be performed under conditions approximating to these for routine samples.

Table 9.2 offers an example of a quantitative NIR validation plan. Qualitative methods require testing only for specificity and robustness.

Table 9.2. Tests for quantitative near infrared (NIR) validation parameters

Parameters for validation	Tests
Specificity	Predict all samples of the external validation set using the calibrated model
	Predict other samples (challenge samples)
Linearity	Plot and determine slope and intercept of NIR predicted values (y) versus reference values (x) of calibration and validation sets
	Determine the standard error of prediction (SEP) with validation set Compare SEP and accuracy of the reference method.
Accuracy	Compare reference and calibration set NIR results using paired sample t-tests to test for significant differences
	Recovery experiments
Precision	Repeatability: e.g. 10 times on the same day
	Intermediate precision: 3 different analysts assaying the same production sample on 3 separate days
Robustness	Test errors associated with sampling speed, temperature, sample position, fibre optic parameters, etc.

Once the model has been constructed and validated it can be routinely used. It can be run on the device used during development or on another device; in which case transferability must be ensured (some adjustment is usually necessary).

Use of regression for NIR quantitative methods

Before a quantitative model is computed, the purpose of the calibration and its minimal accuracy and limits of validity need to be established. A robust model presupposes the design of a range of samples comprising adequate lateral variation [29-31].

Calibration is the fitting stage: the main data set, containing only the calibration samples, is used to compute model parameters such as principal components (PCs) and regression coefficients. The models must be validated to get an idea of how well a regression model performs if used to predict new, unknown samples. A validation set consisting of samples with known variable values is used. The most common distribution allocates two-thirds of the samples to the calibration set and one third to the internal validation set. Sample selection studies have compared Kennard-Stone and successive projections algorithms, random sampling, and full cross-validation for use with multiple linear regression (MLR) and partial least squares (PLS) models [32,33].

During the validation step, only the spectral information is introduced into the model, from which response values are predicted and compared to the known

values of the reference method. If the uncertainty of prediction is reasonably low, the model can be considered usable. Independent test-set validation and cross-validation are the most current methods of estimating prediction error. With cross-validation, the same samples are used for both model estimation and testing. This represents an alternative form of validation if sample numbers are small. The method consists in leaving out a few samples from the calibration set and calibrating the model on the remaining data. The left-out sample values are then predicted and the corresponding prediction residuals computed. The process is repeated with another calibration subset, and so on until every object has been left out. Figure 9.4 illustrates the steps required for complete modeling.

Figure 9.4. Principle of multivariate calibration (NIR - Near infrared)

The predicted Y values are then compared with the observed Y values (Figure 9.5). This generates a prediction residual that can be used to compute a validation residual variance, or a measure of the uncertainty of future predictions.

Goodness of fit of a prediction model can be evaluated by further criteria such as the low standard error of calibration (SEC), low SEP, high correlation coefficient (R^2), and low bias. SEC, standard error of cross-validation (SECV), SEP, bias, slope, and SEP with bias correction (SEP(C)) are the criteria of model accuracy (formu-

las in Figure 9.6). Naes and co-workers have described the statistical strategies available [34] and there are other examples of NIR validation methods [35].

Figure 9.5. Example of a regression performed on near infrared (NIR) spectra and reference results (KF - Karl Fischer titration)

$$s_y = \sqrt{\frac{\sum_{j=1}^m (y_j - \bar{y})^2}{m-1}} \quad \text{cov}(y, y') = \sqrt{\frac{\sum_{j=1}^m (y_j - \bar{y})(y'_j - \bar{y}')}{m-1}} \quad R = \frac{\text{cov}(y', y)}{\sigma_{y'} \cdot \sigma_y}$$

$$\text{SEC} = \sqrt{\frac{\sum_{j=1}^m (y_j - y'_j)^2}{m-1-q}} \quad \text{SEP} = \sqrt{\frac{\sum_{j=1}^n (y_j - y'_j)^2}{n}} \quad \text{Bias} = \frac{\sum_{j=1}^n (y_j - y'_j)}{n}$$

where

- y_j = reference value for the sample #j
- y'_j = predicted value for the sample #j
- m = number of samples in calibration
- n = number of samples in validation
- q = PC number
- σ_y = standard deviation

Figure 9.6. Formulas for goodness of fit criteria (SEC - standard error of calibration; SEP - standard error of prediction)

9.3.2. Mathematical preprocessing of spectroscopic data

Spectral raw data may have a distribution or shape that is not optimal for analysis. Background effects, baseline shifts, or measurements under different conditions can complicate the extraction of information. It is thus important to minimize the noise introduced by such effects with preprocessing operations. These include centering, weighting, and numerous mathematical transformations.

Mean-centering consists of subtracting average spectra from each spectrum to ensure that all results will be interpretable in terms of variation around the mean. Weights based on the standard deviation (SDev, square root of the variance) may also be used for scaling. A possible weighting option is the $1/\text{SDev}$ standardization, which gives all variables the same variance so that they have the same chance to influence estimation of the components. This is advisable if the variables are measured with different units, have different ranges, or are of different types. It is also possible to fix a constant weight for each variable manually. This involves stretching and shrinking by measuring a position relative to the extremes. However, this emphasizes the relative influence of unreliable or noisy attributes. Smoothing is relevant for variables which themselves are a function of some underlying variable, for instance time. It is also one of the first operations performed on recorded NIR spectra. It removes as much noise as possible without degrading important information content. Polynomial smoothing, also called Savitzky-Golay smoothing, involves least square fitting of a polynomial equation to a window of n sequentially selected spectral data points. Normalization is a family of transformations which are computed sample-wise, in this case to improve specific properties. Mean normalization is the classic algorithm. It consists in dividing each row of a data matrix by its average, thus neutralizing the influence of possible hidden factors. Maximum normalization is an alternative procedure which divides each row by its maximum absolute value instead of the average. Multiplicative scatter correction (MSC) is a transformation method used to compensate for additive and/or multiplicative effects in spectral data. It successfully treats multiplicative scattering and similar effects such as path length problems, offset shifts, and interference. Derivation is typically relevant for spectral data that are a function of some underlying variable influencing absorbance at various wavelengths. It is also a simple but powerful technique for magnifying fine structure in raw spectra lacking structure, which is common in NIRS. By increasing the order of derivation, band structure resolution is increased. The Savitzky-Golay algorithm permits computation to higher order derivatives, including a smoothing factor which determines how many adjacent variables will be used to estimate the polynomial approximation for derivation. Norris derivation is an alternative algorithm for computing first derivatives. A baseline correction algorithm is the standard normal variate (SNV) method which does not affect overall spectra layout.

9.3.3. Principal component analysis (PCA)

Large tables contain information partly hidden by data complexity; a feature typical of NIR spectra collection. PCA is a projection method that visualizes all the information contained in a data table. It can be used to show in what respect one sample differs from another, which variables contribute the most to that difference, and whether these variables contribute in the same way, and are correlated or independent from each other. It also reveals sample patterns and outliers.

PCA modeling forms the basis for several classification and regression methods. The underlying idea is to replace a complex data set by a simpler version having fewer dimensions, but still fitting the original data closely enough to be considered a good approximation. Two samples can be described as similar if they have close values for most variables. From a geometric perspective, in the case of close coordinates in the multidimensional space of variables, the two points are located in the same area (Figure 9.7).

Figure 9.7. The geometric concept of principal component analysis (PCA)
A, B, C - three samples; λ - wavelength; PC - principal component

PCA consists in finding the directions in space – known as principal components (PCs) – along which the data points are furthest apart. It requires a linear combination of the initial variables that contribute the most to making the samples different from each other. PCs are computed iteratively, with the first PC carrying the most information, *i.e.* the most explained variance, and the second PC carrying most of the residual information not taken into account by the previous PC, and so on. This process can go on until as many PCs have been computed as there are potential variables in the data table. At that point, all between-sample variation has been accounted for, and the PCs form a new set of axes. Usually, only the first PCs contain pertinent information, with the last PCs being more likely to describe noise. Deciding on the number of components to retain in a PCA model involves a compromise between simplicity, completeness, and effectiveness.

Each PCA component is characterized by attributes, *e.g.* variances, loadings, and scores. Variances are data scattering measure showing how much information

the PCs take into account. Residual variance designates the variation in the data that remains to be explained once the current PC has been taken into account, while explained variance measures the variation in the data accounted for by the current PC. Loadings describe the data structure in terms of variable correlations. Variables with high loadings for a given PC contribute greatly to the meaning of that particular PC. Scores describe the data structure in terms of sample patterns and emphasize differences or similarities. Each sample has a score on each PC which is the coordinate of the sample on the PC. It describes the major features of the sample, relative to the variables with high loadings on the same PC. Samples with close scores along the same PC are considered as similar because they have close values for the corresponding variables.

Once NIR spectra have been measured, building and using a PCA model involves three steps: selecting the appropriate preprocessings, running the PCA algorithm and diagnosing the model, and interpreting the loading and score plots (Figure 9.8) [36].

Figure 9.8. Principal component analysis (PCA) score plot identifying seven clusters with the first three principal components (PCs) [36]

9.3.4. Pattern recognition

Pattern recognition methods are often applied in chemistry [37], biology [38], and food science [39]. The main goal is to assign new samples reliably to preexisting classes. Classification results can also be used as a diagnostic tool to identify the most important variables or find outliers. Applications include predicting whether a pharmaceutical product meets specified quality

requirements or more generally confirming substance identity. PCA and discriminant analysis are techniques that have found extensive use in NIR analysis for this purpose.

The classification techniques can be divided into two categories: unsupervised and supervised. In unsupervised classification, samples are classified without prior knowledge, except the spectra. The spectroscopist's job is to explain the clusters obtained. Many clustering algorithms can be used, such as the hierarchical method (Figure 9.9) [36].

Figure 9.9. Hierarchical cluster analysis (HCA) dendrogram [36]

Supervised methods are those requiring prior knowledge, *i.e.* the category membership of samples. Thus, the classification model is developed on a training set of samples with known categories [28]. Then the model performance is evaluated by comparing the classification predictions to the true categories of the validation samples. Therefore, mathematical models are computed in a first step with a calibration set containing spectra and class information. Feature extraction methods such as PCA are often applied before cluster analysis.

Current methods for supervised pattern recognition are numerous. Typical linear methods are linear discriminant analysis (LDA) based on distance calculation, soft independent modeling of class analogy (SIMCA), which emphasizes similarities within a class, and PLS discriminant analysis (PLS-DA), which performs regression between spectra and class memberships. More advanced methods are based on non-linear techniques, such as neural networks or support vector machines (SVM).

9.3.5. Regression methods

From univariate to multivariate regression

Regression concerns all methods attempting to fit a model to the observed data. In spectroscopy the simplest method of quantitative calibration is based on a single independent variable, *e.g.* wavelength, since a sample attribute such as analyte concentration is a linear function of absorbance at a given wavelength. This is called univariate regression. In this approach, a wavelength is selected when it shows a high degree of correlation between concentration and absorbance. Correlation is an indicator of how well the calibration describes the data set. The linear relationship permits direct and visual estimation of goodness of fit, thus enhancing the analyst's trust in their data collection. Where pharmaceutical samples are concerned, the linear approach rapidly reaches its limits and another approach, multivariate regression, is required.

Multivariate regression takes several predictive variables simultaneously into account for greater accuracy. The fitted model may thus be used to describe the relationship between two groups of variables, or to predict the values of unknown samples.

Multiple linear regression (MLR)

MLR extends linear regression to one wavelength by least squares, with more than one wavelength selected to perform a calibration. The method requires independent variables in order to explain the data set. More samples than predictors are necessary and no missing values must be present in the data table. If it complies with these conditions, MLR will approximate the response values by a linear combination of predictor values, yielding regression coefficients. It is worth mentioning that MLR is the only multivariate method for which formal statistical tests of significance for regression coefficients are available. Y variances provide the relevant measure of MLR model performance, showing how much variation remains in the observed response after the modeled part is removed, and acting as an overall measure of misfit.

Principal component regression (PCR) and PLS regression

With PLS multivariate regression, spectral and constituent data are modeled simultaneously according to an iterative algorithm. PCR and PLS are both projection methods, like PCA. PLS1 deals with only one response variable at a time (like MLR and PCR). PLS2 handles several responses simultaneously. The unexplained part of the data set is made up of residuals. The original data are combined in factors or PCs. A critical step in PLS modeling is the selection of the number of factors. Selecting too few factors will provide an inadequate explanation of variability, while too many factors will cause overfitting and instability in the resulting calibration. Coefficients, loadings and scores are calculated to indicate the extent of original data involvement in the computation of each factor. In a final step, the amount of variance modeled is maximized for

each factor and the residuals are minimized. Either an additional and independent data set is used or the training data set is split into subsets for continuous internal validation at each iterative step (cross-validation). In addition, the resulting PLS calibration must prove able to predict unknown samples

9.4. QUALITATIVE ANALYSES BY NIRS

Qualitative analyses mean classifications of samples based on pattern recognition methods and their NIR spectra. Classification is simply a matter of finding out whether new samples are similar to classes of samples that have been used to make the model. If a new sample fits a particular model well, it is said to be a member of that class. Many analytical tasks fall into this category. The aim of this part is to present an overview of pharmaceutical applications for qualitative analyses, especially the identification and qualification of raw and final material.

9.4.1. Identification and qualification

Analysis of raw materials and pharmaceutical intermediates

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines describe the importance of identity tests [40-43]. Pharmacopoeias [44] have selected analytical methods like HPLC, optical rotation, and colorimetry to identify raw materials. The NIR application for qualitative analysis is, however, now also described by the European Pharmacopoeia in chapter 2.2.40. The qualification of a sample will determine whether it is within the normal variability range or subject to overlimit deviations. Key quality parameters can be evaluated for this purpose [45]. Distance based methods are often applied for the qualification of products. If the sample belongs to the same population as the reference product, then there is a probability of 99.7 % that the distance will be less than three times the standard deviation. If the maximum distance is higher than that value, then the sample is from a different population.

The identification of incoming raw materials is now a common NIRS application [46-48] thanks to the limited sample preparation it requires (Figure 9.10). A lot of publications describe the application of NIRS for the control of excipients, active pharmaceutical ingredients (APIs) and final products. Ulmschneider and co-workers applied NIRS to identify different types of starch, sugar, cellulose, intermediates and APIs with PCA and cluster calibration [49-52]. NIRS was used by Ebube and co-workers to differentiate between Avicel products [53]. The discrimination of cellulose [54] is indeed statistically significant. Cellulose ethers were identified by NIRS, but the separation of methylcellulose and cellulose ethers with methyl or hydroxyalkyl groups was not possible. Several types of poly(vinylpyrrolidone) (povidones) are characterized by their viscosity measured in water. Kreft and co-workers [55] have developed a SIMCA method for their differentiation by NIRS. The identification of raw materials can be

performed directly at-line in the dispensing or at the reception in the warehouse. NIRS is now used in the manufacture of solids and also in biotechnolgy production for identification of cell culture medium [56]. NIRS identification is used in manufacturing plants and during process development.

Figure 9.10. Raw material identification through plastic bags with a fibre optic probe in a warehouse

Final product: identification and counterfeit detection

Discriminating substances in tablet matrixes is possible and was studied by Chong and co-workers [57]. NIR can be and is commonly used for identity tests in quality control departments [58]. NIR transmission spectroscopy combined with chemometric methods is moreover applicable to confirm the identity of clinical trial tablets [59].

NIRS is besides a suitable method for the fast detection of counterfeit medicines [60-66]. In Figure 9.11 a discriminant analysis model is presented that was computed on NIR spectra of authentic and suspect capsules. For example, genuine capsules labelled as types 1 and 2 could be separated after pretreatment. Counterfeits of both types of capsules were successfully separated from the genuine capsules as a result of Mahalanobis distance. The same procedure can be applied to detect placebos in clinical studies.

Figure 9.11. Discriminant Analysis performed on near infrared (NIR) spectra of genuine (reference) and counterfeit capsules

9.4.2. Polymorphism

The ability of a substance to be present in different crystalline forms is called polymorphism. The solid state properties have an influence on the stability and dissolution properties of the pharmaceutical product. This needs to be controlled by analytical methods, which could also help in galenical production and development. Raman spectroscopy has been successfully applied to characterize API polymorphic forms, but this pharmaceutical issue can be solved by NIRS as well. Information concerning the crystalline form of miokamycin could, *e.g.* be determined by NIRS [67]. This tool could moreover improve the understanding of physical forms of theophylline [68] and polymorphic transformation of pazopanib hydrochloride [69]. In addition, the characterization and analysis of azithromycin, an antibiotic derived from erythromycin A, was studied by Blanco and co-workers [70]. The suitability of NIRS to follow changes in both the amorphous and crystalline forms of lactose at room temperature was also investigated [71]. Also, their differentiation was possible by studying NIR frequencies of water peaks. A comprehensive review dealing with polymorph analysis was recently published [72].

9.4.3. Other applications

After the presentation of the main qualitative applications of NIRS, other studies can be mentioned as well, such as the discrimination of production sites of tablets. This is valuable to manufacturers, customers and industry regulations. Yoon and co-workers [73] used PCA for this purpose, which score plots showed that spectra of tablets originating from different manufacturing sites are statistically different.

NIRS and chemometrics are besides combined to understand process (Figure 9.12) and dissolution issues [74]. This shows how NIRS can be useful for understanding batch differences due to variations in process conditions. Beginning with a qualitative analysis of the potential application of NIRS and IR imaging for solids analysis, the ability of NIRS to detect the effects of melt granulation time-temperature gradient, compaction force, coating formulation and coating time was tested on pilot production samples.

As a new field of application, NIR qualitative analyses of biotechnology products are also increasingly performed such as identification of bacteria strains [75,76].

Figure 9.12. Comparison of production sites before and after process harmonization

9.5. QUANTITATIVE ANALYSES BY NIRS

Once the classification of samples has been achieved it can be useful to know more precisely to what extent they differ. Therefore, the development of a quantitative model appears useful. Historically, the first models were computed for the determination of sample moisture, according to two strong water bands absorbing at 1450 and 1940 nm [16].

9.5.1. Physical parameters

NIR spectra contain information about the chemical and physical properties of the analyzed samples. NIRS is nowadays used to determine a large panel of physical parameters on powders and tablets. Various biopharmaceutical parameters can be quantitatively analyzed by NIRS, such as hardness (for

instance, tablet hardness [77]), particle size [78-80], compaction force, flow properties [81] and dissolution rate [82,83].

Tablet hardness is determined in different studies with PLS and MLR methods. Morisseau and co-workers [84] used these well established regression methods for this purpose and concluded that the accuracy of the results are highly dependent on the products and their formulation. In a study on drug content and tablet hardness by Chen and co-workers [85], good results were achieved on two different models computed by an artificial neural network (ANN). The correlation between compression force and NIR spectra at a specific wavelength is demonstrated by Guo [86]. Blanco and co-workers [87] have more recently shown the possibility of predicting the pressure of compaction on a lab sample by using a PLS model.

The use of different regression methods enable chemists to follow the percentage of the drug released in the medium by a tablet. The dissolution profile was determined with NIRS by Donoso and Ghaly [88].

Berntsson published results on the determination of particle size when measuring powder blends with NIRS in reflectance [89,90]. In 2004, Otsuka analyzed the scattering effect due to particle size that was measured with a PCR model [91].

9.5.2. Polymorphs determination

The polymorphic form of a product is a key parameter as it influences its dissolution properties. The determination of the ratio between amorphous and crystalline forms of products is usually performed by X-Ray diffraction. Several studies showed that this analysis can also be done by NIRS [92-94]. Bai [95] observed great agreement between NIRS and X-Ray in the analysis of glycine crystallinity. The SEP obtained by NIRS was 3.2 % and this tool was proven to detect crystallized glycine at a lower content than X-Ray diffraction. NIRS combined with regression methods has already been used for several polymorphism or crystallization applications [67,70,96-99].

9.5.3. Moisture determination

The quantitative analysis of moisture is one of the very first applications of NIRS in the pharmaceutical field. The existence of water in medicines is critical as it ensures stability. Water content is indicated by the presence of two important water bands at 1450 nm and especially around 1940 nm (Figure 9.13).

NIR spectroscopy is now used to determine the water content in powders or granules [100-102], tablets or capsules [103-105], as well as in lyophilised vials or solutions [106]. Use of NIRS in moisture determination is long established, therefore most of the relevant applications for this purpose are now implemented on-line (Figure 9.14).

Figure 9.13. Water content evolution in lyophilised vials (peak at 1940 nm)

Figure 9.14. Near infrared (NIR) inspection machine for 100 % on-line water content determination of lyophilised vials

9.5.4. Content determination

Many studies have been published during the last few years concerning the determination of the chemical content of compounds such as API, excipients or moisture in medicines. Potential samples for analysis can be of various forms like powders, granulates, tablets, liquids, gels, films or lyophilised vials [107-113].

A study was published comparing NIR spectrometers, such as FT-NIR, FTIR-PAS (Photoacoustic Spectroscopy), FTIR-ATR (Attenuated Total Reflectance), DRIFTS (Diffuse Reflectance Infrared Fourier Transform Spectroscopy) and FT-Raman for the determination of vitamin C in powders and solutions [114].

Chalus and co-workers presented different data pretreatments and regression methods for prediction models of API in low-dosage tablets [115]. An example of the quantitative analysis of API is presented in Figure 9.15 where the results of the PLS regression can be observed. Figure 9.15a presents the pretreated NIR spectra, while the selection of 13 factors from the cross validation is shown in Figure 9.15b. The results of the validation with reference and NIR predicted data are displayed in Figure 9.15c, and the statistics of the method in Figure 9.15d [116].

Figure 9.15. Determination of API content by NIRS (range: 1 - 8 mg API/tablet)[116].

PC - principal component; SEC - standard error of calibration; SEP(C) - SEP with bias correction; API - active pharmaceutical ingredient; NIRS - near infrared spectroscopy

An increasing number of papers are published about the use of NIRS for the follow-up of the tablet production process, from the raw materials to the final product, with tablets being coated or not or packaged or not [74,106,117,118]. Successful implementation during early stage formulation development was also presented by Li [119]. NIRS spectroscopy allows a 100 % packaging check of tablets with a built-in PCA model to sort up to 12 000 tablets per minute [120]. Many other quantitative applications are summarised in Table 9.3.

Table 9.3. Examples of quantitative applications of NIRS.

Sample form	Analyte	Regression Method*	Remarks†	Ref.
Tablets	Coating thickness	PLS	Measurements are made directly in a fluidised bed and the coating thickness is followed.	[121]
	Coating thickness	PLS	Tablets are composed of two different chemical compositions.	[122]
	Ibuprofen 800 mg	PLS	Tablets of 7.6 mm thickness have to be reduced to 3.6 mm. The transmittance measurement is thus usable on a dedicated device.	[123]
	Metformin	PLS Linear regression	PLS appeared to be more accurate than single wavelength regression.	[124]
	Caffeine	PLS	The range of caffeine content is 0-100 % m/m.	[125]
	Steroid	PLS	Transmittance of the tablets is measured. The SEP allowed the use of NIRS for the assay of tablets for batch release.	[126]
	Gemfibrozil	PLS	Tablets of two related preparations are identified by a classification model. Their content is predicted for 751 mg/g and 810 mg/g formulation.	[127]
	Paracetamol	MLR, PLS	Two wavelength selection modes were tried for the MLR.	[128]
	Paracetamol	MLR	The MLR model is computed on two wavelengths.	[129]
	Acetylsalicylic acid	PLS	This study assays acetylsalicylic acid in three different formulations: only API, API combined with vitamin C, or with vitamin C and paracetamol. Measurements are performed in reflectance and transmittance on intact tablets and reflectance on milled tablets.	[130]
Tablets Powders	Paracetamol Amantadine Hydrochloride	ANN	Assays of paracetamol and amantadine hydroxide are determined by an ANN model. Models are based on tablets and powders.	[131]
	Diphenhydramine	PLS	Tablets of diphenhydramine are measured in reflectance and transmittance. Their milled form is measured in reflectance. Results are comparable for the three kinds of measurements.	[132]
	Mirtazapine	PLS	The PLS model for determination of content is based on lab powder samples and production tablets. The first factors of the model had to be excluded and the final selected model used 4 factors.	[87]
Powder	Paracetamol Diphenhydramine Hydrochloride Caffeine	ANN, PLS	The study compares different pretreatments and PLS to ANN. ANN improves the results compared to classical PLS.	[133]
	Amylose	Peak ratio	The computed model presents a RMSEP of 1.2 %.	[134]
Powder Granulate	Ferrous lactate Dihydrate	PLS	The concentration range was 650-850 mg/g. Identification is first performed on the samples. The lab samples are powders while production ones are granulates and both are included in the model.	[135]

Sample form	Analyte	Regression Method*	Remarks†	Ref.
Lyophilised samples	Water	PLS	Two PLS models are built, the first one for water content of 1 – 40 % w/w and the second one for content between 1 and 10 % w/w.	[136]
Extruded film	Clotrimazole	PLS	The drug content range was 0-20 % in a hot melt extruded film of polyethylene oxide.	[137]
Translucent gel	Ketoprofen	MLR, PLS	MLR is preferred to PLS because it gives a good idea of the ability of NIR to predict content.	[138]

* Partial least squares (PLS); multiple linear regression (MLR); artificial neural network (ANN)

† Near infrared spectroscopy (NIRS); active pharmaceutical ingredient (API); root mean square error of prediction (RMSEP)

9.6. ON-LINE CONTROL BY MEANS OF NIRS

9.6.1. Powder Blending

The blending of API with excipients is a critical step in the manufacturing of pharmaceutical solids. Without a homogenous blend it is impossible to get uniform doses with the right content of API in the final product. However, the determination of homogeneity is problematic. Currently, samples are mostly removed from the blender and analyzed by conventional methods like HPLC or UV/VIS-spectroscopy. The API distribution is thus determined and the homogenous distribution of the excipients assumed if the API is homogeneously distributed. Moreover, the sampling often changes the powder distribution and is consequently the source of significant sampling errors. Classical methods are besides destructive, time and cost consuming, labor intensive, require solvents and are responsible for long cycle times as they are performed off-line. Therefore, the use of a fast, non-destructive method is advisable. NIRS offers these advantages and enables the analysis of all the compounds of a powder mixture. On-line or in-line application is possible for homogeneity (Figure 9.16) and end point determination.

Figure 9.16. Implementation of an on-line near infrared (NIR) spectrometer in solid development for blending monitoring

Numerous studies have been carried out to explore the use of NIRS for powder blending control. The fact that NIRS has great potential in this application has been shown by Wargo and Drennen [139-142]. Cho and co-workers dealt with the effective mass that is sampled by NIR fibre-optic reflectance probes in blending processes and demonstrated that the sampled mass met FDA requirements [143]. Hailey and co-workers showed that by using a fibre-optic reflectance probe it is possible, either in a y-cone or blender, to use NIRS for in-line blend analysis [144]. Sekulić and co-workers also evaluated NIRS for on-line monitoring of powder blending processes by using a fibre-optic reflectance probe and showed its feasibility with a model-free approach [145]. NIR imaging was used by El-Hagrasy and co-workers who demonstrated the possibility of using it for on-line blending control. However, they pointed out the fact that multiple sampling points are necessary for correct process control [21]. Sekulić and co-workers focused on qualitative approaches of blend evaluation in a study using a small blender and a reflectance fibre optic probe. Different blends were produced, monitored via NIRS and different mathematical pre-processing performed on the resulting data [146]. Skibsted and co-workers presented a qualitative and quantitative method and control charts, with which they were able to monitor the homogeneity of a blend [147]. The use of NIRS for the quantification of the drug content has also been used by Popo and co-workers. However, they measured samples obtained by stream-sampling instead of taking spectra directly in the blender [148]. Berntsson and co-workers described the quantitative in-line monitoring in a mixer, both in the lab and at the production scale. With high speed sampling, average content and distribution of the mixture content were assessed [149].

Figure 9.17 presents an example of a blend process monitoring of API using the moving block method. In Figure 9.18, a quantitative determination of API is performed during the blend process. Since 2007, a large number of publications have been written about NIRS and the blend process [150-160]. The use of this spectroscopy for blend monitoring has therefore been fully demonstrated.

Figure 9.17. Moving block standard deviation at a specific wavelength of active pharmaceutical ingredient (API) during the blend process

Figure 9.18. On-line quantitative determination of the active pharmaceutical ingredient (API) content during the blend process.

9.6.2. Granulation

In many cases, powder blending is followed by a granulation step, which is often necessary for tablet compression or capsule filling. These are produced either by dry granulation, like roller compaction, wet granulation, such as fluid bed spray, or high shear mixer granulation. The moisture content of granulates is important for the rest of the process as it influences their properties and, for example, the hardening of tablets during the storage. Classical measurement methods such as infrared dryers for moisture content determination require time and consequently slow down the manufacturing on the development process. As NIRS can be performed in real time, the process might be monitored more efficiently, resulting in greater process reliability and optimized product characteristics. NIR can be used during the process optimization step. Rantanen and co-workers used NIR-reflectance spectroscopy for in-line moisture content determination in fluidised bed granulation. They followed spraying and drying phases by NIRS and were able to determine drying end points [102,161] and the effects of binder and particle size on moisture determination [162]. A non-linear calibration model was developed with a combination of NIRS and other process measurements [163]. Frake and co-workers applied in-line NIRS to a fluidised bed granulation to control the granule moisture content and changes in particle size [164]. Findlay and co-workers showed that this spectroscopy enables the control of a fluidised bed granulation. They determined the drying end and time points when binder addition should be stopped [100]. Gupta and co-workers determined content uniformity, moisture content and strength of compacts [165]. Ultimately, NIRS is a useful tool for the monitoring of the different phases of the granulation process and determination of particule size and API content [166]. The granulation rate can be estimated by NIRS as well [167].

9.6.3. Drying

Drying is most of the time another critical step in the production of medicines. It is used at different stages of the process, such as granulation or lyophilisation. As the O-H bands are characteristic by NIRS, the first on-line applications of this spectroscopy were mainly for the monitoring of drying processes. In Figure 9.19, the evolution of NIR spectra during a drying process can be observed. Indeed at the position of the water bands, especially at 1950 nm, the analyzed spectra present a variation of the intensity, which can be directly correlated to the moisture of the samples.

Figure 9.19. On-line water content determination during the active pharmaceutical ingredient (API) drying step. In Figure 9.19A, the analyzed spectra present a variation of the intensity of the water peak at 1950 nm. The differences between the spectra are highlighted in Figure 9.19B

Brülls and co-workers presented the possibility of following the transition in the cake during a freeze drying step. The measurement, performed by introducing a fibre optic probe in a vial, showed a good correlation with the classical method [168]. Sukowski and Ulmschneider studied the analysis of 100 % production vials directly in-line [169]. The use of NIR for the drying process was validated and transferred by Peinado and co-workers [170].

9.6.4. Crystallinity and polymorphism

During the drying phase of wet granulation, polymorphic changes can occur in an API or in some of the excipients. The polymorphic changes of glycine involve important modifications in the hydrogen bonding of crystals. They were quantified by Davis and co-workers with NIRS during wet granulation [171].

Crystallisation may also be followed in an earlier step, *i.e.* the production of an API. Févotte and co-workers used a fibre optic probe to monitor crystallisation by NIRS. This method showed the possibility of using this type of spectroscopy to follow the API crystallisation on-line [98].

9.6.5. Coating

One of the last steps in the preparation of a drug may involve the coating of some tablets or granulates. It is important to ensure the integrity and good quality of the drug because coating may influence the release of the drug or assure its stability. NIR diffuse reflectance spectroscopy was used with a fibre optic probe to determine the film coating thickness of pellets by means of a PLS model. The probe was inserted on a side port of the fluidized bed reactor, and located vertically to the pellet bed [121]. In the case of Pérez-Ramos and co-workers who dealt with tablets, the probe was placed directly in the coating pan for diffuse reflectance measurement. A univariate model was used, which followed the decrease and increase of specific bands of a core compound and the coating, respectively [172]. NIR and Raman spectroscopy were used simultaneously during the fluid bed pellet coating process by Dogomolov and co-workers [173]. The coating thickness of pellets [174,175] or tablets [176] was proven to be measurable by NIRS.

9.6.6. Biotechnology

A new field of application for NIRS, following trends in biotechnological manufacturing processes, has lately emerged in the pharmaceutical field. This tool, combined with chemometrics, enables cell culture monitoring, for instance, as in the system presented in Figure 9.20.

Figure 9.20. Near infrared (NIR) and cell culture monitoring
Probe and instrumentation

In 2002 and 2003, Arnold and co-workers presented the acquisition, calibration, validation and implementation of the fermentation process including controlling and monitoring. In these studies, the authors managed to collect NIR spectra in transmittance and reflectance modes. For the direct on-line or in-line implementation, development was advised because the transfer from at-line to in-line analyses would be a challenge [177,178]. Cimander and Mandenius in 2002 applied the methodology on the fermentation of *Escherichia coli* to produce antibiotics. Spectra were acquired with an immersion probe and PCA and PLS were used as chemometric tools. Models to track the amounts of biomass, tryptophan, phosphate, glucose and acetate were developed and validated [179]. *Vibrio cholerae* fermentations used to produce toxins or plasmids were measured by Navratil and co-workers in 2004. NIR spectra were acquired with a fibre optic probe and PLS computed to develop calibrations of biomass, glucose and acetate. Interference problems that occurred when applying the chosen models in the bioreactor were discussed and corrected. Finally, the authors applied NIR prediction models in the production [180]. In 2003, Tamburini and co-workers tried to monitor the fermentation of *Staphylococcus* and *Lactobacillus*. Spectra were also acquired with a fibre optic probe and PLS regression was applied to develop models for glucose, lactic acid, acetic acid and biomass. These models were then used for automatic control [181]. The determination of biomass, glucose, lactic acid and acetic acid during fermentations of *Staphylococcus xylosus* was performed by Tosi and co-workers. Models were developed by PLS for glucose, biomass, lactic acid and acetic acid. The SEC and SEP were satisfactory and the models were then applied to other microorganisms in the same medium [182]. The study of Yeung and co-workers [183] compared two strategies for the preparation of calibration samples of a *Saccharomyces cerevisiae* bioprocess. PLS was applied to the NIR spectra to observe cell debris, protein and RNA. The calibration models selected according to the SEP values were finally validated.

Traditionally, many fermentation products come from microbial bioprocesses. However, lately, mammalian and insect cell cultivations were also exploited for the high-cost products they can be engineered to produce. Arnold and co-workers [184] developed a method to monitor mammalian cell cultivation. NIR spectra were acquired with an immersion probe and models were constructed for glucose, lactate, glutamine and ammonia. External and internal validation was performed. The monitoring of insect cell culture was already developed by Riley and co-workers in 1996 [185]. Calibration models were established for glutamine and glucose with PLS and the models could be used for high concentrations. Studies about cell culture media were made Lewis and co-workers in 2000 [186] and Jung and co-workers in 2002 [187]. In the first study, the authors developed models to predict glucose production using PLS. The results were compared according to the SEC, SEP and mean percent error (MPE) and the best model for the control of culture was retained. This demonstrated the ability of NIRS to monitor on-line fermentations and cell cultures. In the second study, the system was coupled with a lab-system to provide a real-time spectral background

reference. Smoothing and PLS were applied to develop calibration models for glucose and lactate.

During the last 3 years, the number of publications dealing with fermentation or cell culture monitoring has significantly increased [188-193], some of them being of very good quality like the report by Henriques and Buziol [194]. NIR can therefore be used for the development of bioprocess.

9.7. APPLICATIONS OF NIR SPECTRAL IMAGING

9.7.1. General use of NIR chemical imaging for pharmaceutical applications

The chemical compound homogeneity is an important issue for the development of pharmaceutical solids. A classical NIR spectrometer integrates spatial information [130,195,196]. However, the use of a mean spectrum on the surface can be a drawback in solid form analysis. On the contrary, hyperspectral imaging provides information that is spatial and spectral, and both qualitative and quantitative. It can map chemical compound distribution and determine particle size. In the pharmaceutical industry, it is for instance important to map the distribution of APIs and excipients in a tablet as this reveals physical interaction between components and helps solve homogeneity issues. This explains the increasing number of spectroscopic imaging studies on the visualization of chemical component homogeneity [21,197-201]. The method was applied to quality control and to process problems affecting pharmaceutical tablets: dissolution, polymorph distribution, moisture content determination, API localization and characterization, counterfeits' detection, blending, and granulation.

Spectral imaging is a complex and multidisciplinary field. The introduction of new detectors is making its use increasingly powerful and attractive. It has proven its potential for qualitative pharmaceutical analyses and can be used when spatial information becomes relevant for an analytical application. Even if online applications and regulatory method validation require further study, the potential contribution of imaging to quality control and PAT needs no further demonstration. A detailed overview of the pharmaceutical applications of NIR imaging and chemometric tools for image analysis is available in several reviews [202,203]. Only two examples will be discussed here.

9.7.2. Tablet composition analysis with NIR imaging

An example of the reconstruction of a tablet by NIR spectral imaging is presented below. A tablet was cut lengthwise with a trimmer to get a plane surface and the coating was then removed. The sample and references were analyzed using a chemical imaging NIR spectrometer (Sapphire™, Malvern) with the following acquisition parameters: detector size of 320 × 256 array, spectral range of 1 100 – 2 450 nm and a spatial resolution of 40 µm/pixel. The acquisition lasted about 5 minutes.

After the pretreatment of the spectra with a second derivative, wavelengths were selected to give contrast images and display the localization of mannitol, API and crospovidone with NIR images at specific wavelengths (Figure 9.21).

After the aforementioned steps were performed, tablet reconstruction by NIR imaging was then possible. This highlights the main advantage of imaging: the large area of analysis. The images are indeed more representative of the sample than a mean spectrum of an entire tablet.

Figure 9.21. Use of multivariate curve resolution–alternating least squares (MCR-ALS) in order to obtain distribution maps – near infrared (NIR) imaging [1]
API – active pharmaceutical ingredient

9.7.3. Application of NIR imaging to process optimization

The aim of this study was to use NIR imaging to solve granulation issues in a new formulation development. Undesired powder agglomerations were indeed developed during the granulation step and imaging was applied to characterize the structure.

The measured sample contained API, starch, Avicel®, crospovidone, and sodium lauryl sulfate. The sample and references were analyzed by NIR imaging (Malvern system) with 20 co-adds on a spectral range of 1 100 – 2 450 nm. The full image size was 320 × 256 pixels (or 4.1 × 3.3 mm). The images were interpreted and sample raw materials mapped using PLS classification with five loadings. This was based on the reference spectra of starch, API, Avicel®, crospovidone, and sodium lauryl sulfate.

The PLS model allowed the identification all five chemical compounds. The PLS multivariate analysis showed that the core contained Avicel® and API and starch and crospovidone in the periphery (Figure 9.22). Thanks to this information, a solution to the granulation issue could be found consisting of the addition of a premixing step to avoid agglomeration. NIR imaging proved to be useful in the improvement of process understanding. In our case, the powder agglomeration was heterogeneous and the layers had high excipient content, making it possible to apply supervised classification.

Figure 9.22. Images obtained by classification methods – near infrared (NIR) imaging [1]. API - active pharmaceutical ingredient

9.8. CONCLUSION

The potential of NIRS for process development needs no further demonstration, as NIRS is a powerful way to discriminate pharmaceutical compounds. This method can be used qualitatively to detect, identify and qualify as well as control raw materials and final products. Additionally, it is a suitable tool for the classification and quantification of pharmaceutical samples. NIRS is moreover a potentially precious diagnostic technique in process trouble-shooting and can provide spectral profiles of pharmaceutical products. Besides, it can support the development of new processes and drug discovery. NIRS can be applied across the pharmaceutical production process in chemistry, biotechnology and galenic fields [204,205]. The success of this analytical technique relies on key advantages [46]. As previously observed, NIRS is influenced by the chemical and the physical properties of the samples. This spectroscopy requires limited or no sample preparation and is non-destructive. Moreover, the measurement is fast with performance in less than a second for on-line applications and NIR frequencies are transmitted through glass. Other vibrational techniques like Raman spectroscopy [206] and mid-IR should be mentioned. These techniques can also be applied successfully to solve pharmaceutical issues in order to support the development of new drugs and new processes. Finally, NIR imaging systems were developed in recent years. Namely, a hyper-spectral imaging spectrometer records simultaneously spectra and spatial information of samples. NIR imaging [207] completes NIR spectroscopy and is used when spatial distribution is an important issue of the analysis.

REFERENCES

1. Y. Roggo, M. Ulmschneider. Chemical Imaging and Chemometrics: Useful Tools for Process Analytical Technology. *Wiley*, 2009, Chapter 4.3.
2. W. Chew, P. Sharratt. Trends in process analytical technology. *Analytical Methods* **2**(10) (2010) 1412-1438.
3. T. Herdling, D. Lochmann. Implementation of Process Analytical Technology (PAT) in the production of solid dosage. Implementierung von Process Analytical Technology (PAT) in der solida-produktion. *Pharmazeutische Industrie* **72**(3) (2010) 402-408.
4. S.H. Tabasi, R. Fahmy, D. Bensley, C. O'Brien, S.W. Hoag. Quality by design, part II: Application of NIR spectroscopy to monitor the coating process for a pharmaceutical sustained release product. *Journal of Pharmaceutical Sciences* **97**(9) (2008) 4052-4066.
5. K.H. Norris. History of NIR. *Journal of Near Infrared Spectroscopy* **4**(1-4) (1996) 31-37.
6. Y. Roggo, L. Duponchel, J.-P. Huvenne. Quality evaluation of sugar beet (*beta vulgaris*) by near-infrared spectroscopy. *Journal of Agricultural and Food Chemistry* **52**(5) (2004) 1055-1061.

7. P. Williams, K. Norris, Editors, Near-Infrared Technology in the Agricultural and Food Industries. American Association of Cereal Chemists, St. Paul, MN (1987) 143-167.
8. M.S. Larrechi, M.P. Callao. Strategy for introducing NIR spectroscopy and multivariate calibration techniques in industry. *TrAC Trends in Analytical Chemistry* **22**(9) (2003) 634-640.
9. M. Blanco, S. MasPOCH, I. Villarroya, X. Peralta, J.M. González, J. Torres. Geographical origin classification of petroleum crudes from near-infrared spectra of bitumens. *Applied Spectroscopy* **55**(7) (2001) 834-839.
10. H.P.R. Aenugu, D.S. Kumar, S.N. Parthiban, S.S. Ghosh, D. Banji. Near infra red spectroscopy- An overview. *International Journal of ChemTech Research* **3**(2) (2011) 825-836.
11. Y. Roggo, P. Chaluz, L. Maurer, C. Lema-Martinez, A. Edmond, N. Jent. A review of near infrared spectroscopy and chemometrics in pharmaceutical technologies. *Journal of Pharmaceutical and Biomedical Analysis* **44**(3) (2007) 683-700.
12. J. Luybaert, D.L. Massart, Y. Vander Heyden. Near-infrared spectroscopy applications in pharmaceutical analysis. *Talanta* **72**(3) (2007) 865-883.
13. D. Cozzolino. A review on the use of near infrared spectroscopy for plant analysis. *Medicinal Plants - International Journal of Phytomedicines and Related Industries* **2**(1) (2010) 13-20.
14. E. Räsänen, N. Sandler. Near infrared spectroscopy in the development of solid dosage forms. *Journal of Pharmacy and Pharmacology* **59**(2) (2007) 147-159.
15. L. Bokobza. Near infrared spectroscopy. *Journal of Near Infrared Spectroscopy* **6**(1-4) (1998) 3-17.
16. D.A. Burns, E.W. Ciurczak. Handbook of Near-Infrared Analysis. 2nd Edition, *Marcel Dekker*, 2001, p. 814.
17. S. Morimoto, W.F. McClure, D.L. Stanfield. Hand-Held NIR Spectrometry: Part 1: An Instrument Based upon Gap-Second Derivative Theory. *Applied Spectroscopy* **55**(2) (2001) 182-189.
18. L.F. Capitán-Vallvey, A.J. Palma. Recent developments in handheld and portable optosensing-A review. *Analytica Chimica Acta* **696**(1-2) (2011) 27-46.
19. K. Dégardin, Y. Roggo, P. Margot. Evaluation de spectromètres portables raman, infrarouge et proche infrarouge pour la détection de contrefaçons de médicaments. *Spectra Analyse* **276** (2010) 46-52.
20. E.N. Lewis, J.W. Schoppelrei, E. Lee, L.H. Kidder. Near-Infrared Chemical Imaging as a Process Analytical Tool. In: Process Analytical Technology, (Editor: K.A. Bakeev), *Blackwell publishing Ltd.*, 2005, p. 187-225.
21. A.S. El-Hagrasy, H.R. Morris, F. D'Amino, R.A. Lodder, J.K. Drennen III. Near-infrared spectroscopy and imaging for the monitoring of powder blend homogeneity. *Journal of Pharmaceutical Sciences* **90**(9) (2001) 1298-1307.
22. L. Breiman, J.H. Friedman, R.A. Olsen, C.J. Stone. Classification and Regression Trees 1984. *Wadsworth*, 1983.
23. D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers-Verbeke. Handbook of Chemometrics and Qualimetrics: Part A. Data Handling in Science and Technology, Vol. 20A. *Elsevier*, 1997.

24. B.G.M. Vandeginste, D.L. Massart, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers-Verbeke. Handbook of Chemometrics and Qualimetrics: Part B. Data Handling in Science and Technology. *Elsevier*, 1998.
25. B.K. Lavine. Chemometrics. *Analytical Chemistry* **72**(12) (2000) 91-98.
26. M. Otto. Chemometrics Statistics and Computer Application in Analytical Chemistry. *Wiley-VCH*, 1999.
27. R.G. Brereton. Chemometrics Data Analysis for the Laboratory and Chemical Plant. *John Wiley & Sons*, 2003.
28. D.L. Massart, B.G.M. Vandeginste, S.N. Deming, Y. Michotte, L. Kaufman. Chemometrics: a textbook. *Journal of Chemometrics* **2**(4) (1988) 298-299.
29. M. Blanco, M.A. Romero, M. Alcalá. Strategies for constructing the calibration set for a near infrared spectroscopic quantitation method. *Talanta* **64**(3) (2004) 597-602.
30. M. Blanco, J. Coello, H. Iturriaga, S. MasPOCH, C. de la Pezuela. Strategies for constructing the calibration set in the determination of active principles in pharmaceuticals by near infrared diffuse reflectance spectrometry. *Analyst* **122**(8) (1997) 761-765.
31. J.H. Kalivas, Basis sets for multivariate regression. *Analytica Chimica Acta* **428**(1) (2001) 31-40.
32. H.A.D. Filho, R.K.H. Galvão, M.C.U. Araújo, E.C. da Silva, T.C.B. Saldanha, G.E. José, C. Pasquini, I.M. Raimundo Jr., J.J.R. Rohwedder. A strategy for selecting calibration samples for multivariate modelling. *Chemometrics and Intelligent Laboratory Systems* **72**(1) (2004) 83-91.
33. R.K.H. Galvão, M.C.U. Araujo, G.E. José, M.J.C. Pontes, E.C. Silva, T.C.B. Saldanha. A method for calibration and validation subset partitioning. *Talanta* **67**(4) (2005) 736-740.
34. T. Naes, T. Isaksson, T. Fearn, T. Davies. Multivariate Calibration and Classification. *NIR Publications*, 2002.
35. M.C. Sarraguça, J.A. Lopes. The use of net analyte signal (NAS) in near infrared spectroscopy pharmaceutical applications: Interpretability and figures of merit. *Analytica Chimica Acta* **642**(1-2) (2009) 179-185.
36. F. Been, Y. Roggo, K. Dégardin, P. Esseiva, P. Margot. Profiling of Counterfeit Medicines by Vibrational Spectroscopy. *Forensic Science International* **211**(2011) 83-100.
37. D. González-Arjona, V. González-Gallero, F. Pablos, A.G. González. Authentication and differentiation of irish whiskeys by higher-alcohol congener analysis. *Analytica Chimica Acta* **381**(2-3) (1999) 257-264.
38. L. Simon, M. Nazmul Karim. Probabilistic neural networks using Bayesian decision strategies and a modified gompertz model for growth phase classification in the batch culture of bacillus subtilis. *Biochemical Engineering Journal* **7**(1) (2001) 41-48.
39. F. Marini, F. Balestrieri, R. Bucci, A.D. Magri, A.L. Magri, D. Marini. Supervised pattern recognition to authenticate Italian extra virgin olive oil varieties. *Chemometrics and Intelligent Laboratory Systems* **73**(1) (2004) 85-93.
40. International Conference on Harmonisation Q2A, ICH Q2A: Text on Validation of Analytical Procedures. *FDA Federal Register* 60(96) (1995) 11260.
41. International Conference on Harmonisation Q2B, ICH Q2B: Validation of Analytical Procedures: Methodology. *FDA Federal Register* 62(96) (1997) 27463-27467.

42. International Conference on Harmonisation Q6A, ICH Q6A, Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances. *FDA Federal Register* 65(251) (2000) 83041-83063.
43. International Conference on Harmonisation Q7A, ICH Q7A: Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients. 66(186) (2001) 49028-49029.
44. EMEA, European Pharmacopeia (2011).
45. J. Märk, M. Andre, M. Karner, C.W. Huck. Prospects for multivariate classification of a pharmaceutical intermediate with near-infrared spectroscopy as a process analytical technology (PAT) production control supplement. *European Journal of Pharmaceutics and Biopharmaceutics* 76(2) (2010) 320-327.
46. C. van der Vlies. The use of NIR spectrometry in the pharmaceutical QC laboratory. *European Journal of Pharmaceutical Sciences* 2(1-2) (1994) 79-81.
47. H.R.H. Ali, H.G.M. Edwards, I.J. Scowen. Noninvasive in situ identification and band assignments of some pharmaceutical excipients inside USP vials with FT-near-infrared spectroscopy. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy* 72(4) (2009) 890-896.
48. G. Chen, F. Kamal, J. Phillips. A comprehensive strategy to develop and implement a multi-site NIR method for identification of raw materials. *American Pharmaceutical Review* 12(6) (2009) 54-61.
49. M. Ulmschneider, G. Barth, E. Trenka. Building transferable cluster calibrations for the identification of different solid excipients with near-infrared spectroscopy. *Pharmazeutische Industrie* 62(5) (2000) 374-376.
50. M. Ulmschneider, E. Penigault. Assessing the transfer of quantitative NIR calibrations from a spectrometer to another one. *Analisis* 28(1) (2000) 83-87.
51. M. Ulmschneider, E. Penigault, Non-invasive confirmation of the identity of tablets by near-infrared spectroscopy. *Analisis* 28(4) (2000) 336-346.
52. M. Ulmschneider, A. Wunenburger, E. Penigault, Using near-infrared spectroscopy for the noninvasive identification of five pharmaceutical active substances in sealed vials. *Analisis* 27(10) (1999) 854-856.
53. N.K. Ebube, N.K. Ebube, S.S. Thosar, R.A. Roberts, M.S. Kemper, R. Rubinovitz, D.L. Martin, G.E. Reier, T.A. Wheatley, A.J. Shukla. Application of near-infrared spectroscopy for nondestructive analysis of Avicel powders and tablets. *Pharmaceutical Development and Technology* 4(1) (1999) 19-26.
54. K. Kramer, S. Ebel. Application of NIR reflectance spectroscopy for the identification of pharmaceutical excipients. *Analytica Chimica Acta* 420(2) (2000) 155-161.
55. K. Kreft, B. Kozamernik, U. Urleb. Qualitative determination of polyvinylpyrrolidone type by near-infrared spectrometry. *International Journal of Pharmaceutics* 177(1) (1999) 1-6.
56. A.O. Kirdar, G. Chen, J. Weidner, A.S. Rathore. Application of near-infrared (NIR) spectroscopy for screening of raw materials used in the cell culture medium for the production of a recombinant therapeutic protein. *Biotechnology Progress* 26(2) (2010) 527-531.
57. X.-M. Chong, C.-Q. Hu, Y.-C. Feng, H.-H. Pang. Construction of a universal model for non-invasive identification of cephalosporins for injection using near-infrared diffuse reflectance spectroscopy. *Vibrational Spectroscopy* 49(2) (2009) 196-203.

58. L. Alvarenga, D. Ferreira, D. Altekruze, J.C. Menezes, D. Lochmann. Tablet identification using near-infrared spectroscopy (NIRS) for pharmaceutical quality control. *Journal of Pharmaceutical and Biomedical Analysis* **48**(1) (2008) 62-69.
59. R. De Maesschalck, T. Van den Kerkhof. Implementation of a simple semi-quantitative near-infrared method for the classification of clinical trial tablets. *Journal of Pharmaceutical and Biomedical Analysis* **37**(1) (2005) 109-114.
60. P. de Peinder, M.J. Vreedenbregt, T. Visser, D. de Kaste. Detection of Lipitor® counterfeits: A comparison of NIR and Raman spectroscopy in combination with chemometrics. *Journal of Pharmaceutical and Biomedical Analysis* **47**(4-5) (2008) 688-694.
61. Y.C. Feng, X.B. Zhang, C.Q. Hu. Construction of identification system for non-invasive analysis of macrolides tablets using near infrared diffuse reflectance spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis* **51**(1) (2010) 12-17.
62. R. Kalyanaraman, G. Dobler, R. Rubick, Portable spectrometers for pharmaceutical counterfeit detection. *American Pharmaceutical Review* **13**(3) (2010) 38-45.
63. A.C. Moffat, S. Assi, R.A. Watt. Identifying counterfeit medicines using near infrared spectroscopy. *Journal of Near Infrared Spectroscopy* **18**(1) (2010) 1-15.
64. A.J.O. Neil, R.D. Jee, G. Lee, A. Charvill, A.C. Moffat. Use of a portable near infrared spectrometer for the authentication of tablets and the detection of counterfeit versions. *Journal of Near Infrared Spectroscopy* **16**(3) (2008) 327-333.
65. O.Y. Rodionova, A.L. Pomerantsev. NIR-based approach to counterfeit-drug detection. *TrAC Trends in Analytical Chemistry* **29**(8) (2010) 795-803.
66. I. Storme-Paris, H. Rebiere, M. Matoga, C. Civade, P.-A. Bonnet, M.H. Tissier, P. Chaminade. Challenging near infrared spectroscopy discriminating ability for counterfeit pharmaceuticals detection. *Analytica Chimica Acta* **658**(2) (2010) 163-174.
67. M. Blanco, J.Coello, H. Iturriaga, S. Maspocho, C. Pérez-Maseda. Determination of polymorphic purity by near infrared spectrometry. *Analytica Chimica Acta* **407**(1-2) (2000) 247-254.
68. K.L. Vora, G. Buckton, D. Clapham. The use of dynamic vapour sorption and near infra-red spectroscopy (DVS-NIR) to study the crystal transitions of theophylline and the report of a new solid-state transition. *European Journal of Pharmaceutical Sciences* **22**(2-3) (2004) 97-105.
69. S.E. Barnes, T. Thurston, J.A. Coleman, A. Diederich, D. Ertl, J. Rydzak, P. Ng, K. Bakeev, D. Bhanushali. NIR diffuse reflectance for on-scale monitoring of the polymorphic form transformation of pazopanib hydrochloride (GW786034); Model development and method transfer. *Analytical Methods* **2**(12) (2010) 1890-1899.
70. M. Blanco, D. Valdés, M.S. Bayod, F.Fernández-Marí, J.Llorente. Characterization and analysis of polymorphs by near-infrared spectrometry. *Analytica Chimica Acta* **502**(2) (2004) 221-227.
71. G. Buckton, E. Yonemochi, J. Hammond, A. Moffat. The use of near infra-red spectroscopy to detect changes in the form of amorphous and crystalline lactose. *International Journal of Pharmaceutics* **168**(2) (1998) 231-241.
72. N. Chieng, T. Rades, J. Aaltonen. An overview of recent studies on the analysis of pharmaceutical polymorphs. *Journal of Pharmaceutical and Biomedical Analysis* **55**(4) (2011) 618-644.

73. W.L. Yoon, R.D. Jee, A. Charvill, G. Lee, A.C. Moffat. Application of near-infrared spectroscopy to the determination of the sites of manufacture of proprietary products. *Journal of Pharmaceutical and Biomedical Analysis* **34**(5) (2004) 933-944.
74. Y. Roggo, N. Jent, A. Edmond, P. Chalus, M. Ulmschneider. Characterizing process effects on pharmaceutical solid forms using near-infrared spectroscopy and infrared imaging. *European Journal of Pharmaceutics and Biopharmaceutics* **61**(1-2) (2005) 100-110.
75. L.E. Rodriguez-Saona, F.M. Khambaty, F.S. Fry, E.M. Calvey. Rapid detection and identification of bacterial strains by fourier transform near-infrared spectroscopy. *Journal of Agricultural and Food Chemistry* **49**(2) (2001) 574-579.
76. L.E. Rodriguez-Saona, F.M. Khambaty, F.S. Fry, J. Dubois, E.M. Calvey. Detection and identification of bacteria in a juice matrix with Fourier transform-near infrared spectroscopy and multivariate analysis. *Journal of Food Protection* **67**(11) (2004) 2555-2559.
77. M. Otsuka, I. Yamane. Prediction of tablet properties based on near infrared spectra of raw mixed powders by chemometrics: Scale-up factor of blending and tableting processes. *Journal of Pharmaceutical Sciences* **98**(11) (2009) 4296-4305.
78. J. Muselík, K. Krejčová, M. Rabišková, A. Bartošíková, M. Dračková, L. Vorlová. Pellet coating thickness determination by near-infrared reflectance spectroscopy: Comparison of two reference methods. *Current Pharmaceutical Analysis* **6**(4) (2010) 225-233.
79. J. Muselík, K. Krejčová, M. Rabišková, A. Bartošíková, M. Dračková, L. Vorlová. Determination of the thickness of tablet coating by near-infrared spectroscopy. Stanovení tloušťky obalu tablet blízkou infračervenou spektroskopií. *Chemické listy* **104**(1) (2010) 41-45.
80. M.C. Sarraguça, A.V. Cruz, H.R. Amaral, P.C. Costa, J.A. Lopes. Comparison of different chemometric and analytical methods for the prediction of particle size distribution in pharmaceutical powders. *Analytical and Bioanalytical Chemistry* **399**(6) (2011) 2137-2147.
81. M.C. Sarraguça, A.V. Cruz, S.O. Soares, H.R. Amaral, P.C. Costa, J.A. Lopes. Determination of flow properties of pharmaceutical powders by near infrared spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis* **52**(4) (2010) 484-492.
82. M.P. Freitas, A. Sabadin, L.M. Silva, F.M. Giannotti, D.A. do Couto, E. Tonhi, R.S. Medeiros, G.L. Coco, V.F.T. Russo, J.A. Martins. Prediction of drug dissolution profiles from tablets using NIR diffuse reflectance spectroscopy: a rapid and nondestructive method. *Journal of Pharmaceutical and Biomedical Analysis* **39**(1-2) (2005) 17-21.
83. H. Mou, X. Wang, T. Lv, L. Xie, H. Xie. On-line dissolution determination of Baicalin in solid dispersion based on near infrared spectroscopy and circulation dissolution system. *Chemometrics and Intelligent Laboratory Systems* **105**(1) (2011) 38-42.
84. K.M. Morisseau, C.T. Rhodes. Near-infrared spectroscopy as a nondestructive alternative to conventional tablet hardness testing. *Pharmaceutical Research* **14**(1) (1997) 108-111.
85. Y. Chen, S.S. Thosar, R.A. Forbess, M.S. Kemper, R.L. Rubinovitz, A.J. Shukla. Prediction of drug content and hardness of intact tablets using artificial neural network and near-infrared spectroscopy. *Drug Development and Industrial Pharmacy* **27**(7) (2001) 623-631.

86. J.-H. Guo, G.W. Skinner, W.W. Harcum, J.P. Malone, L.G. Weyer. Application of near-infrared spectroscopy in the pharmaceutical solid dosage form. *Drug Development and Industrial Pharmacy* **25**(12) (1999) 1267-1270.
87. M. Blanco, M. Alcala. Content uniformity and tablet hardness testing of intact pharmaceutical tablets by near infrared spectroscopy: A contribution to process analytical technologies. *Analytica Chimica Acta* **557**(1-2) (2006) 353-359.
88. M. Donoso, E.S. Ghaly. Prediction of drug dissolution from tablets using near-infrared diffuse reflectance spectroscopy as a nondestructive method. *Pharmaceutical Development and Technology* **9**(3) (2004) 247-265.
89. O. Berntsson, L.G. Danielsson, S. Folestad. Estimation of effective sample size when analyzing powders with diffuse reflectance near-infrared spectrometry. *Analytica Chimica Acta* **364**(1-3) (1998) 243-251.
90. O. Berntsson, T. Burger, S. Folestad, L.-G. Danielsson, J. Kuhn, J. Fricke. Effective sample size in diffuse reflectance near-IR spectrometry. *Analytical Chemistry* **71**(3) (1999) 617-623.
91. M. Otsuka. Comparative particle size determination of phenacetin bulk powder by using Kubelka-Munk theory and principal component regression analysis based on near-infrared spectroscopy. *Powder Technology* **141**(3) (2004) 244-250.
92. I. Fix, K.-J. Steffens. Quantifying low amorphous or crystalline amounts of alpha-lactose-monohydrate using X-ray powder diffraction, near-infrared spectroscopy and differential scanning calorimetry. *Drug Development and Industrial Pharmacy* **30**(5) (2004) 513-523.
93. A. Gombas, I. Antal, P. Szabó-Révész, S. Marton, I. Erős. Quantitative determination of crystallinity of alpha-lactose monohydrate by near infrared spectroscopy (NIRS). *International Journal of Pharmaceutics* **256**(1-2) (2003) 25-32.
94. W. Li, G.D. Worosila, W. Wang, T. Mascaro. Determination of polymorph conversion of an active pharmaceutical ingredient in wet granulation using NIR calibration models generated from the premix blends. *Journal of Pharmaceutical Sciences* **94**(12) (2005) 2800-2806.
95. S.J. Bai, M. Rani, R. Suryanarayanan, J.F. Carpenter, R. Nayar, M.C. Manning. Quantification of glycine crystallinity by near-infrared (NIR) spectroscopy. *Journal of Pharmaceutical Sciences* **93**(10) (2004) 2439-2447.
96. J.J. Seyer, P.E. Luner. Determination of indomethacin crystallinity in the presence of excipients using diffuse reflectance near-infrared spectroscopy. *Pharmaceutical Development and Technology* **6**(4) (2001) 573-582.
97. O. Berntsson, L.-G. Danielsson, M.O. Johnansson and S. Folestad. Quantitative determination of content in binary powder mixtures using diffuse reflectance near infrared spectrometry and multivariate analysis. *Analytica Chimica Acta* **419**(1) (2000) 45-54.
98. G. Fevotte, J. Calas, F. Puel, C. Hoff. Applications of NIR spectroscopy to monitoring and analyzing the solid state during industrial crystallization processes. *International Journal of Pharmaceutics* **273**(1-2) (2004) 159-169.
99. A.D. Patel, P.E. Luner, M.S. Kemper. Quantitative analysis of polymorphs in binary and multi-component powder mixtures by near-infrared reflectance spectroscopy. *International Journal of Pharmaceutics* **206**(1-2) (2000) 63-74.

100. W.P. Findlay, G.R. Peck, K.R. Morris. Determination of fluidized bed granulation end point using near-infrared spectroscopy and phenomenological analysis. *Journal of Pharmaceutical Sciences* **94**(3) (2005) 604-612.
101. X.P. Zhou, P. Hines, M.W. Borer. Moisture determination in hygroscopic drug substances by near infrared spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis* **17**(2) (1998) 219-225.
102. J. Rantanen, O. Antikainen, J.-P. Mannermaa, J. Yliruusi. Use of the near-infrared reflectance method for measurement of moisture content during granulation. *Pharmaceutical Development and Technology* **5**(2) (2000) 209-217.
103. R.G.Jr. Buice, T.B. Gold, R. A. Lodder, G. A. Digenis. Determination of moisture in intact gelatin capsules by near-infrared spectrophotometry. *Pharmaceutical Research* **12**(1) (1995) 161-163.
104. O. Berntsson, G. Zackrisson, G. Ostling. Determination of moisture in hard gelatin capsules using near-infrared spectroscopy: applications to at-line process control of pharmaceuticals. *Journal of Pharmaceutical and Biomedical Analysis* **15**(7) (1997) 895-900.
105. C.C. Corredor, D. Bu, D. Both. Comparison of near infrared and microwave resonance sensors for at-line moisture determination in powders and tablets. *Analytica Chimica Acta* **696**(1-2) (2011) 84-93.
106. N.W. Broad, R.D. Jee, A.C. Moffat, M.J. Eaves, W.C. Mann, W. Dziki. Non-invasive determination of ethanol, propylene glycol and water in a multi-component pharmaceutical oral liquid by direct measurement through amber plastic bottles using Fourier transform near-infrared spectroscopy. *Analyst* **125**(11) (2000) 2054-2058.
107. M. Alcalà, J. Leon, J. Roperó, M. Blanco, R.J. Románach. Analysis of low content drug tablets by transmission near infrared spectroscopy: Selection of calibration ranges according to multivariate detection and quantitation limits of PLS models. *Journal of Pharmaceutical Sciences* **97**(12) (2008) 5318-5327.
108. J. Ali, K. Pramod, S.H. Ansari. Near-infrared spectroscopy for nondestructive evaluation of tablets. *Systematic Reviews in Pharmacy* **1**(1) (2010) 17-23.
109. R. López-Arellano, E.A. Santander-García, J.M. Andrade-Garda, G. Alvarez-Avila, J.A. Garduño-Rosas, E.A. Morales-Hipólito. Quantification of lysine clonixinate in intravenous injections by NIR spectroscopy. *Vibrational Spectroscopy* **51**(2) (2009) 255-262.
110. P. Singh, D.K. Jangir, R. Mehrotra, A.K. Bakhshi. Development and validation of an infrared spectroscopy-based method for the analysis of moisture content in 5-fluorouracil. *Drug Testing and Analysis* **1**(6) (2009) 275-278.
111. M.C. Sarraguça, J.A. Lopes. Quality control of pharmaceuticals with NIR: From lab to process line. *Vibrational Spectroscopy* **49**(2) (2009) 204-210.
112. A.W. Sobanska. Rapid determination of nifuroxazide in tablets by near-infrared spectroscopy. *Chemia Analityczna* **54**(5) (2009) 1021-1033.
113. X. Wang, Q. Fu, J. Sheng, X. Yang, J. Jia, W. Du. Construction of a universal quantitative model for ibuprofen sustained-release capsules from different manufacturers using near-infrared diffuse reflection spectroscopy. *Vibrational Spectroscopy* **53**(2) (2010) 214-217.
114. H. Yang, J. Irudayaraj. Rapid determination of vitamin C by NIR, MIR and FT-Raman techniques. *The Journal of Pharmacy and Pharmacology* **54**(9) (2002) 1247-1255.

115. P. Chalus, Y. Roggo, S. Freitag, M. Ulmschneider. Comparaison de spectromètres proche infrarouge pour la détermination de principe actif dans des comprimés à faible dosage. *Spectra Analyse* **247** (2005) 44-49.
116. P. Chalus, Y. Roggo, S. Walter, M. Ulmschneider. Near-infrared determination of active substance content in intact low-dosage tablets. *Talanta* **66**(5) (2005) 1294-1302.
117. M. Blanco, J. Coello, H. Iturriaga, S. Maspocho, D. Serrano. Near-infrared analytical control of pharmaceuticals. A single calibration model from mixed phase to coated tablets. *Analyst* **123**(11) (1998) 2307-2312.
118. S.M. Han, P.G. Faulkner. Determination of SB 216469-S during tablet production using near-infrared reflectance spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis* **14**(12) (1996) 1681-1689.
119. W. Li, L. Bagnol, M. Berman, R.A. Chiarella, M. Gerber. Applications of NIR in early stage formulation development. Part II. Content uniformity evaluation of low dose tablets by principal component analysis. *International Journal of Pharmaceutics* **380**(1-2) (2009) 49-54.
120. T. Herkert, H. Prinz, K.-A. Kovar. One hundred percent online identity check of pharmaceutical products by near-infrared spectroscopy on the packaging line. *European Journal of Pharmaceutics and Biopharmaceutics* **51**(1) (2001) 9-16.
121. M. Andersson, S. Folestad, J. Gottfries, M.O. Johansson, M. Josefson, K.G. Wahlund. Quantitative analysis of film coating in a fluidized bed process by in-line NIR spectrometry and multivariate batch calibration. *Analytical Chemistry* **72**(9) (2000) 2099-2108.
122. M. Andersson, M. Josefson, F.W. Langkilde, K.-G. Wahlund. Monitoring of a film coating process for tablets using near infrared reflectance spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* **20**(1-2) (1999) 27-37.
123. J.L. Ramirez, M.K. Bellamy, R.J. Romanach. A novel method for analyzing thick tablets by near infrared spectroscopy. *AAPS PharmSciTech* (2001) 2:E11.
124. I.H.I. Habib, M.S. Kamel. Near infra-red reflectance spectroscopic determination of metformin in tablets. *Talanta* **60**(1) (2003) 185-190.
125. M. Laasonen, T. Harmia-Pulkkinen, C. Simard, M. Rasanen, H. Vuorela. Development and validation of a near-infrared method for the quantitation of caffeine in intact single tablets. *Analytical Chemistry* **75**(4) (2003) 754-760.
126. N.W. Broad, R.D. Jee, A.C. Moffat, M.R. Smith. Application of transmission near-infrared spectroscopy to uniformity of content testing of intact steroid tablets. *Analyst* **126**(12) (2001) 2207-2211.
127. M Blanco, J Coello, H Iturriaga, S Maspocho, N Pou. Development and validation of a near infrared method for the analytical control of a pharmaceutical preparation in three steps of the manufacturing process. *Fresenius' Journal of Analytical Chemistry* **368**(5) (2000) 534-539.
128. A. Eustaquio, M. Blanco, R.D. Jee, and A.C. Moffat. Determination of paracetamol in intact tablets by use of near infrared transmittance spectroscopy. *Analytica Chimica Acta* **383**(3) (1999) 283-290.
129. A.D. Trafford, R.D. Jee, A.C. Moffat, P. Graham. A rapid quantitative assay of intact paracetamol tablets by reflectance near-infrared spectroscopy. *Analyst* **124** (5) (1999) 163-167.

130. P. Merckle, K.A. Kovar. Assay of effervescent tablets by near-infrared spectroscopy in transmittance and reflectance mode: acetylsalicylic acid in mono and combination formulations. *Journal of Pharmaceutical and Biomedical Analysis* **17**(3) (1998) 365-374.
131. Y. Dou, Y. Sun, Y. Ren, P. Ju, Y. Ren. Simultaneous non-destructive determination of two components of combined paracetamol and amantadine hydrochloride in tablets and powder by NIR spectroscopy and artificial neural networks. *Journal of Pharmaceutical and Biomedical Analysis* **37**(3) (2005) 543-549.
132. P. Corti, E. Dreassi, G. Ceramelli, S. Mattii. Near infrared transmittance analysis for the assay of solid pharmaceutical dosage forms. *Analyst* **124**(5) (1999) 755-758.
133. Y. Dou, Y. Sun, Y. Ren, Y. Ren. Artificial neural network for simultaneous determination of two components of compound paracetamol and diphenhydramine hydrochloride powder on NIR spectroscopy. *Analytica Chimica Acta* **528**(1) (2005) 55-61.
134. C.C. Fertig, F. Podczek, R.D. Jee, M.R. Smith. Feasibility study for the rapid determination of the amylose content in starch by near-infrared spectroscopy. *European Journal of Pharmaceutical Sciences* **21**(2-3) (2004) 155-159.
135. M. Blanco, J. Coello, A. Eustanquio, H. Iturriaga, S. Maspoek. Development and validation of a method for the analysis of a pharmaceutical preparation by near-infrared diffuse reflectance spectroscopy. *Journal of Pharmaceutical Sciences* **88**(5) (1999) 551-556.
136. G.X. Zhou, Z. Ge, J. Dorwart, B. Izzo, J. Kukura, G. Bickel, J. Wyvratt. Determination and differentiation of surface and bound water in drug substances by near infrared spectroscopy. *Journal of Pharmaceutical Sciences* **92**(5) (2003) 1058-1065.
137. S.V.S. Tumuluri, S. Prodduturi, M.M. Crowley, S.P. Stodghill, J.W. McGinity, M.A. Repka, B.A. Avery. The use of near-infrared spectroscopy for the quantitation of a drug in hot-melt extruded films. *Drug Development and Industrial Pharmacy* **30**(5) (2004) 505-511.
138. M.S. Kemper, E.J. Magnuson, S.R. Lowry, W.J. McCarthy, N. Aksornkoae, D.C. Watts, J.R. Johnson, A.J. Shukla. Use of FT-NIR transmission spectroscopy for the quantitative analysis of an active ingredient in a translucent pharmaceutical topical gel formulation. *AAPS PharmSci.* **3**(3) (2001) E23.
139. D.J. Wargo, J.K. Drennen. Near-infrared spectroscopic characterization of pharmaceutical powder blends. *Journal of Pharmaceutical and Biomedical Analysis* **14**(11) (1996) 1415-1423.
140. R.P. Cogdill, C.A. Anderson, M. Delgado-Lopez, D. Molseed, R. Chisholm, R. Bolton, T. Herkert, A.M. Afán, J.K.3rd Drennen. Process analytical technology case study part I: feasibility studies for quantitative near-infrared method development. *AAPS PharmSciTech* **6**(2) (2005) E262-E272.
141. R.P. Cogdill, C.A. Anderson, M. Delgado-Lopez, D. Molseed, R. Chisholm, R. Bolton, T. Herkert, A.M. Afán, J.K.3rd Drennen. Process analytical technology case study: part II. Development and validation of quantitative near-infrared calibrations in support of a process analytical technology application for real-time release. *AAPS PharmSciTech* **6**(2) (2005) E273-E283.
142. R.P. Cogdill, C.A. Anderson, J.K. Drennen. Process Analytical Technology Case Study, Part III: Calibration Monitoring and Transfer. *AAPS PharmSciTech.* **6**(2) (2005) E284-E297.

143. J. Cho, P.J. Gemperline, P.K. Aldridge, S.S. Sekulic. Effective mass sampled by NIR fiber-optic reflectance probes in blending processes. *Analytica Chimica Acta* **348**(1-3) (1997) 303-310.
144. P.A. Hailey, P. Doherty, P. Tapsell, T. Oliver, P.K. Aldridge. Automated system for the on-line monitoring of powder blending processes using near-infrared spectroscopy part I. System development and control. *Journal of Pharmaceutical and Biomedical Analysis* **14**(5) (1996) 551-559.
145. S.S. Sekulic, H.W. Ward, D.R. Brannegan, E.D. Stanley, C.L. Evans, S.T. Sciavolino, P.A. Hailey, P.K. Aldridge. On-line monitoring of powder blend homogeneity by near-infrared spectroscopy. *Analytical Chemistry* **68**(3) (1996) 509-513.
146. S.S. Sekulic, J. Wakenman, P. Doherty, P.A. Hailey. Automated system for the on-line monitoring of powder blending processes using near-infrared spectroscopy. Part II. Qualitative approaches to blend evaluation. *Journal of Pharmaceutical and Biomedical Analysis* **17**(8) (1998) 1285-1309.
147. E.T.S. Skibsted, H.F.M. Boelens, J.A. Westerhuis, D.T. Witte, A.K. Smilde. Simple assessment of homogeneity in pharmaceutical mixing processes using a near-infrared reflectance probe and control charts. *Journal of Pharmaceutical and Biomedical Analysis* **41**(1) (2006) 26-35.
148. M. Popo, S. Romero-Torres, C. Conde, R.J. Romañach. Blend uniformity analysis using stream sampling and near infrared spectroscopy. *AAPS PharmSciTech* **3**(3) (2002) E24.
149. O. Berntsson, L.-G. Danielsson, B. Lagerholm, S. Folestad. Quantitative in-line monitoring of powder blending by near infrared reflection spectroscopy. *Powder Technology* **123**(2-3) (2002) 185-193.
150. Y. Ghebremeskel, R.A. Weiss. Near infrared analysis of blends of sulfonated polystyrene ionomers and poly(ϵ -caprolactam). *Journal of Polymer Science, Part B: Polymer Physics* **46**(15) (2008) 1602-1610.
151. H. Ma, C.A. Anderson. Characterization of pharmaceutical powder blends by NIR chemical imaging. *Journal of Pharmaceutical Sciences* **97**(8) (2008) 3305-3320.
152. Z. Shi, R.P. Cogdill, S.M. Short, C.A. Anderson. Process characterization of powder blending by near-infrared spectroscopy: Blend end-points and beyond. *Journal of Pharmaceutical and Biomedical Analysis* **47**(4-5) (2008) 738-745.
153. I. Storme-Paris, J. Clarot, S. Esposito, J.C. Chaumeil, A. Nicolas, F. Brion, A. Rientord, P. Chaminade. Near infrared Spectroscopy homogeneity evaluation of complex powder blends in a small-scale pharmaceutical preformulation process, a real-life application. *European Journal of Pharmaceutics and Biopharmaceutics* **72**(1) (2001) 189-198.
154. C.C. Corredor, D. Jayawickrama, G. McGeorge, D.B.B.M. Squibb, Monitoring of blending uniformity: Form conversion and fluid bed drying by near infrared and Raman spectroscopy. *American Pharmaceutical Review* **13**(1) (2010) 66-72 January/February 2010.
155. S.Kim, H. Nakagawa, M. Kano, S. Hasebe. Estimation of active pharmaceutical ingredients content in blending process for drug products manufacturing. AIChE Annual Meeting, paper 445d, Salt Lake City, USA, Nov. 7-12 (2010).
156. C.V. Liew, A.D. Karande, P.W.S. Heng. In-line quantification of drug and excipients in cohesive powder blends by near infrared spectroscopy. *International Journal of Pharmaceutics* **386**(1-2) (2010) 138-148.

157. T. Puchert, C.-V. Holzhauser, J.C. Menezes, D. Lochmann, G. Reich. A new PAT/QbD approach for the determination of blend homogeneity: Combination of on-line NIRS analysis with PC Scores Distance Analysis (PC-SDA). *European Journal of Pharmaceutics and Biopharmaceutics* **78**(1) (2011) 173-182.
158. P. Doležel, J. Muselík, K. Dvořáčková, K. Šustová. A study of the process of homogenization of powder mixtures using NIR spectroscopy. Studium procesu homogenizace práškových směsí s využitím NIR spektroskopie. *Česká a slovenská farmacie* **59**(6) (2010) 263-271.
159. D.M. Koller, A. Posch, G. Hörl, C. Voura, S. Radl, N. Urbanetz, S.D. Fraser, W. Tritthart, F. Reiter, M. Schlingmann, J.G. Khinast. Continuous quantitative monitoring of powder mixing dynamics by near-infrared spectroscopy. *Powder Technology* **205**(1-3) (2011) 87-96.
160. A.U. Vanarase, M. Alcalà, J.I. Jerez Rozo, F.J. Muzzio, R.J. Romañach. Real-time monitoring of drug concentration in a continuous powder mixing process using NIR spectroscopy. *Chemical Engineering Science* **65**(21) (2010) 5728-5733.
161. J. Rantanen, S. Lehtola, P. Rämetsä, J.-P. Mannermaa, J. Yliruusi. On-line monitoring of moisture content in an instrumented fluidized bed granulator with a multi-channel NIR moisture sensor. *Powder Technology* **99**(2) (1998) 163-170.
162. J. Rantanen, E. Räsänen, J. Tenhunen, M. Käsäkoski, J.-P. Mannermaa, J. Yliruusi. In-line moisture measurement during granulation with a four-wavelength near infrared sensor: an evaluation of particle size and binder effects. *European Journal of Pharmaceutics and Biopharmaceutics* **50**(2) (2000) 271-276.
163. J. Rantanen, E. Rasanen, O. Antikainen, J.-P. Mannermaa, J. Yliruusi. In-line moisture measurement during granulation with a four-wavelength near-infrared sensor: an evaluation of process-related variables and a development of non-linear calibration model. *Chemometrics and Intelligent Laboratory Systems* **56**(1) (2001) 51-58.
164. P. Frake, D. Greenhalgh, S.M. Grierson, J.M. Hempenstall, D.R. Rudd. Process control and end-point determination of a fluid bed granulation by application of near infrared spectroscopy. *International Journal of Pharmaceutics* **151**(1) (1997) 75-80.
165. A. Gupta, G.E. Peck, R.W. Miller, K.R. Morris. Real-time near-infrared monitoring of content uniformity, moisture content, compact density, tensile strength, and young's modulus of roller compacted powder blends. *Journal of Pharmaceutical Sciences* **94**(7) (2005) 1589-1597.
166. M. Alcalà, M. Blanco, M. Bautista, J.M. González. On-line monitoring of a granulation process by NIR spectroscopy. *Journal of Pharmaceutical Sciences* **99**(1) (2010) 336-345.
167. A.T. Tok, X. Goh, W.T. Ng, R.B. Tan. Monitoring granulation rate processes using three PAT tools in a pilot-scale fluidized bed. *AAPS PharmSciTech* **9**(4) (2008) 1083-1091.
168. M. Brülls, S. Folestad, A. Sparén, A. Rasmuson. In-Situ Near-Infrared Spectroscopy Monitoring of the Lyophilization Process. *Pharmaceutical research* **20**(3) (2003) 494-499.
169. L. Sukowski, M. Ulmschneider. In-line process analytical technology based on qualitative near-infrared spectroscopy modeling. *Pharmazeutische Industrie* **67**(7) (2005) 830-835.

170. A. Peinado, J. Hammond, A. Scott. Development, validation and transfer of a near infrared method to determine in-line the end point of a fluidised drying process for commercial production batches of an approved oral solid dose pharmaceutical product. *Journal of Pharmaceutical and Biomedical Analysis* **54**(1) (2011) 13-20.
171. T.D. Davis, G.E. Peck, J.G. Stowell, K.R. Morris, S.R. Byrn. Modeling and Monitoring of Polymorphic Transformations During the Drying Phase of Wet Granulation. *Pharmaceutical Research* **21**(5) (2004) 860-866.
172. J.D. Pérez-Ramos, W. P. Findlay, G. Peck, K.R. Morris. Quantitative analysis of film coating in a pan coater based on in-line sensor measurements. *AAPS PharmSciTech*, **6**(1) (2005) E127-E136.
173. A. Bogomolov, M. Engler, M. Melichar, A. Wigmore. In-line analysis of a fluid bed pellet coating process using a combination of near infrared and Raman spectroscopy. *Journal of Chemometrics* **24**(7-8) (2010) 544-557.
174. M. Bürmen, M. Možina, F. Pernuš, B. Likar. Coating film thickness of pharmaceutical pellets measured by near infrared spectroscopy. *Industrial Technology (ICIT), 2010 IEEE International Conference* (2010) 199-204.
175. M.-J. Lee, D.-Y. Seo, H.-E. Lee, I.-C. Wang, W.-S. Kim, M.-Y. Jeong, G.J. Choi. In line NIR quantification of film thickness on pharmaceutical pellets during a fluid bed coating process. *International Journal of Pharmaceutics* **403**(1-2) (2011) 66-72.
176. M.J. Lee, C.R. Park, A.Y. Kim, B.S. Kwon, K.H. Bang, Y.S. Cho, M.Y. Jeong, G.J. Choi. Dynamic calibration for the in-line NIR monitoring of film thickness of pharmaceutical tablets processed in a fluid-bed coater. *Journal of Pharmaceutical Sciences* **99**(1) (2010) 325-335.
177. Arnold, et al., Employing Near-Infrared Spectroscopic Methods of Analysis for Fermentation Monitoring and Control Part 1, Method Development. BioPharm International, 2002. November 2002. 26-34.
178. Arnold, et al., Employing Near-Infrared Spectroscopic Methods of Analysis for Fermentation Monitoring and Control Part 2, Implementation Strategies. BioPharm International, 2003. January 2003. 47-70.
179. C. Cimander, C.-F. Mandenius. Online monitoring of a bioprocess based on a multi-analyser system and multivariate statistical process modelling. *Journal of Chemical Technology & Biotechnology* **77**(10) (2002) 1157-1168.
180. M. Navrátil, A. Norberg, L. Lembrén, C.-F. Mandenius. On-line multi-analyzer monitoring of biomass, glucose and acetate for growth rate control of a vibrio cholerae fed-batch cultivation. *Journal of Biotechnology* **115**(1) (2005) 67-79.
181. E. Tamburini, G. Vaccari, S. Tosi, A. Trilli. Near-Infrared Spectroscopy: A Tool for Monitoring Submerged Fermentation Processes Using an Immersion Optical-Fiber Probe. *Applied Spectroscopy* **57**(2) (2003) 132-138.
182. S. Tosi, M. Rossi, E. Tamburini, G. Vaccari, A. Amaretti, D. Matteuzzi. Assessment of in-line near-infrared spectroscopy for continuous monitoring of fermentation processes. *Biotechnology Progress* **19**(6) (2003) 1816-1821.
183. K.S.Y. Yeung, M. Hoare, N. F. Thornhill, T. Williams, J.D. Vaghjiani. Near-Infrared Spectroscopy for Bioprocess Monitoring and Control. *Biotechnology and Bioengineering* **63**(6) (1998) 684-693.
184. S. Alison Arnold, J. Crowley, N. Woods, L.M. Harvey, B. McNeil. In-situ near infrared spectroscopy to monitor key analytes in mammalian cell cultivation. *Biotechnology and Bioengineering* **84**(1) (2003) 13-19.

185. M.R. Riley, M. Rhiel, X. Zhou, M.A. Arnold, D.W. Murhammer. Simultaneous measurement of glucose and glutamine in insect cell culture media by near infrared spectroscopy. *Biotechnology and Bioengineering* **55**(1) (1996) 11-15.
186. C.B. Lewis, R.J. McNichols, A. Gowda, G.L. Coté. Investigation of Near-Infrared Spectroscopy for Periodic Determination of Glucose in Cell Culture Media in Situ. *Applied Spectroscopy* **54**(10) (2000) 1453-1457.
187. B. Jung, S.Lee, J.H. Yang, T. Good, G.L. Coté. Automated On-Line Noninvasive Optical Glucose Monitoring in a Cell Culture System. *Applied Spectroscopy* **56**(1) (2002) 51-57.
188. D. Landgrebe, C. Haake, T. Höpfner, S. Beutel, B. Hitzmann, T. Scheper, M. Rhiel, K. Reardon. On-line infrared spectroscopy for bioprocess monitoring. *Applied Microbiology and Biotechnology* **88**(1) (2010) 11-22.
189. A.S. Rathore, Bhambure, V. Ghare. Process analytical technology (PAT) for biopharmaceutical products. *Analytical and Bioanalytical Chemistry* **398**(1) (2010) 137-154.
190. E.K. Read, R.B. Shah, B.S. Riley, J.T. Park, K.A. Brorson, A.S. Rathore. Process analytical technology (PAT) for biopharmaceutical products: Part II. Concepts and applications. *Biotechnology and Bioengineering* **105**(2) (2010) 285-295.
191. P. Roychoudhury, R.O'Kennedy, B. McNeil, L.H. Harvey. Multiplexing fibre optic near infrared (NIR) spectroscopy as an emerging technology to monitor industrial bioprocesses. *Analytica Chimica Acta* **590**(1) (2007) 110-117.
192. Z.I.T.A. Soons, M. Streefland, G. van Straten, A.J.B. van Boxtel. Assessment of near infrared and "software sensor" for biomass monitoring and control. *Chemometrics and Intelligent Laboratory Systems* **94**(2) (2008) 166-174.
193. A.P. Teixeira, R. Oliveira, P.M. Alves, M.J. T. Carrondo. Advances in on-line monitoring and control of mammalian cell cultures: Supporting the PAT initiative. *Biotechnology Advances* **27**(6) (2009) 726-732.
194. J.G. Henriques, S. Buziol, E. Stocker, A. Voodg, J.C. Menezes. Monitoring mammalian cell cultivations for monoclonal antibody production using near-infrared spectroscopy. *Advances in Biochemical Engineering / Biotechnology* **116** (2010) 73-97.
195. Y. Roggo, C. Roeseler, M. Ulmschneider. Near infrared spectroscopy for qualitative comparison of pharmaceutical batches. *Journal of Pharmaceutical and Biomedical Analysis* **36**(4) (2004) 777-786.
196. Y. Roggo, L. Duponchel, J.-P. Huvenne. Comparison of supervised pattern recognition methods with McNemar's statistical test: Application to qualitative analysis of sugar beet by near-infrared spectroscopy. *Analytica Chimica Acta* **477**(2) (2003) 187-200.
197. C. Beleites, G. Steiner, M.G. Sowa, R. Baumgartner, S. Sobottka, G. Schackert, R. Salzer. Classification of human gliomas by infrared imaging spectroscopy and chemometric image processing. *Vibrational Spectroscopy* **38**(1-2) (2005) 143-149.
198. J.M. Chalmers, N.J. Everall, M.D. Schaeberle, I.W. Levin, E.Neil Lewis, L.H. Kidder, J. Wilson, R. Crocombe. FT-IR imaging of polymers: an industrial appraisal. *Vibrational Spectroscopy* **30**(1) (2002) 43-52.
199. A.d. Juan, R. Tauler, R. Dyson, C. Marcolli, M. Rault, M. Maeder. Spectroscopic imaging and chemometrics: a powerful combination for global and local sample analysis. *TrAC Trends in Analytical Chemistry* **23**(1) (2004) 70-79.
200. E.N. Lewis, J. Schoppelrei, E. Lee. Near-infrared chemical Imaging and the PAT initiative. *Spectroscopy* **19**(4) (2004) 26-36.

201. E.N. Lewis, E. Lee, L.H. Kidder. Combining Imaging and Spectroscopy: Solving Problems with Near Infrared Chemical Imaging. *Microscopy Today* **12**(6) (2004) 8-12.
202. C. Gendrin, Y. Roggo, C. Collet. Pharmaceutical applications of vibrational chemical imaging and chemometrics: A review. *Journal of Pharmaceutical and Biomedical Analysis* **48**(3) (2008) 533-553.
203. R.A. Lodder. NIR spectrometric imaging. *European Journal of Pharmaceutical Sciences* **2**(1-2) (1994) 84.
204. M. Blanco, R. Cueva-Mestanza, A. Peguero. Controlling individual steps in the production process of paracetamol tablets by use of NIR spectroscopy. *Journal of Pharmaceutical and Biomedical Analysis* **51**(4) (2010) 797-804.
205. M. Blanco, M. Bautista, M. Alcalà. API determination by NIR spectroscopy across pharmaceutical production process. *AAPS PharmSciTech* **9**(4) (2008) 1130-1135
206. Y. Roggo, K. Dégardin, P. Margot. Identification of pharmaceutical tablets by Raman spectroscopy and chemometrics. *Talanta* **81**(3) (2010) 988-995.
207. Y. Roggo, A. Edmond, P. Chalut, M. Ulmschneider. Infrared hyperspectral imaging for qualitative analysis of pharmaceutical solid forms. *Analytica Chimica Acta* **535**(1-2) (2005) 79-87.

INDEX

1

1D NMR..... 179

2

2D NMR..... 179

A

α -1-acid-glycoprotein 81
Acetaminophen 10, 98, 101, 150, 155
Acetanilide..... 89
Acetophenone 85, 89
Acetylsalicylic acid 7, 453
Acyclovir 8, 98, 101, 156
Alpha-1-acid-glycoprotein 108
Amantadine..... 453
Amdinocillin 156
Amorphous..... 351
Amoxicillin 14, 98, 101
Ampicillin..... 8, 98, 101
Amrinone 98, 101
Anhydrate..... 361, 362, 363
Antiarrhythmic drugs 16
Antibiotic Phases 308
Antipyrine..... 150, 155
APP cleaving enzyme 183
Aspirin..... 98, 101
Atenolol..... 150, 155
Azithromycin..... 197, 217

B

Barbital 10
Benzapril..... 156, 158
Benzimidazole..... 85
Benzisoquinoline 412
Benzocaine 11

Benzoic acid 10
Betamethasone 98, 101, 150, 155
Bioavailability..... 123
Biological membranes..... 6
Biopharmaceutical classification system..... 123
Blood/brain distribution 108
Bromazepam..... 86, 88, 98, 101
Budesonide..... 86
Bumetanide 98, 101
Bupivacaine 98
Bupivacaine HCl..... 101
Butyrophenone 85, 89

C

Caco-2 cells..... 126
CADD 3
Caffeine 453
Capillary Electrophoresis..... 29
Captopril..... 8, 10
Carbamazepine 86, 362
Cefixime 150, 155
Ceftazidime..... 98, 101, 156
Cellulose 273
Cephalexin 98, 101
Cephalothin 150, 155
Cerivastatin 156
Cetirizine 150, 156
Charge transfer complexes..... 259
Chemometrics 437
Chiral separation..... 237
Chirality 236
Chloropyramine..... 11
Chlorpheniramine..... 98, 101, 150, 155
Chlorpromazine..... 7, 49, 88
Chlortetracycline..... 156
Chlorthalidone 155
Chromatographic hydrophobicity index..... 72, 83
Cimetidine..... 98, 101
Ciprofloxacin..... 98, 101, 156
Clonazepam 98, 101

Clotrimazole	454
Co-Crystal Screen	366
Colchicine	85, 150, 155
Congruent melting	404
Co-solvent method	30
Counterfeit detection	447
Crospovidone	462
Crown ethers	245
Cyclodextrins	243
Cyclosporins	168
Cytarabine	98, 101

D

Daunorubicin	156
Debrisoquine	11
Demeclocycline	156
Deramciclane	50
Diastereomers	236
Diazepam	99, 101
Diclofenac	86, 150, 155
Differential scanning calorimetry	387
Digitoxin	99, 101
Diltiazem	99, 102
Dioleoylphosphatidylcholine	136, 137
Diphenhydramine	99, 102
Dissolution template titration	34
Distribution coefficient	21
DNA gyrase	181
DOSY	215
Double-sink method	136, 137
Doxepin	99, 102
Doxycycline	156
DVS	See Dynamic vapour sorption
Dynamic vapour sorption	360

E

Effective permeability	130, 149, 154
Effective solubility	17
Egg lecithin	136
Electrostatic interactions	173, 260
Enalapril	8
Enantiomers	236
Enantiotropic system	356
Enantiotropy	411
Endotherm	392
Energy-temperature diagram	355
Enoxacin	156
Ephedrine	11
Eprosartan	150, 155
Equilibrium dialysis	112
Erythromycin	195, 217
Ethosuximide	99, 102
Eutectic point	408
Exotherm	392

F

Famotidin	8
Famotidine	156
FaSSIF	20, 37
Felbamate	99, 102
FeSSIF	20, 37
Fexofenadine	8, 150, 156
Finasteride	99, 102
Flumazenil	99, 102
Flurbiprofen	86
Form screening	365
Fragment-based drug design	213
Frontal chromatography	108

G

Ganciclovir	99, 102, 156
Gatifloxacin	150, 156
Gemfibrozil	99, 102, 453
Glipizide	99, 102
Gradient chromatographic retention	73

H

Haloperidol	99, 102
Henderson-Hasselbalch equation	18, 74
Heptanophenone	89
Hexanophenone	89
HPLC	35, 37, 39, 65, 69, 70
HSQC	194, 203, 217
HTS	3
Human serum albumin	79
Hydrates	359, 413
Hydrochlorothiazide	44, 99, 102
Hydrogen bonding	173, 237, 260
Hydrophobic interactions	173

I

Ibuprofen	7, 453
Imidocarbamates	214
Imipramine	88, 99, 102
Immobilised artificial membrane	83
Immobilised artificial membrane affinity	107
Inclusion compounds	243
Indole	85
Indomethacin	86, 99, 102
Intestinal absorption	108
Intrinsic solubility	17
Ionic strength	16
Ionization	8, 16
Ionization macroconstants	13
Ionization microconstants	13
Isocratic chromatographic retention	70
Isocytosines	183
Isothermal microcalorimetry	422
Isothermal titration calorimetry	174
Isradipine	99, 102

K

Ketoprofen..... 99, 102, 454
Kinetic solubility17, 18, 32

L

LADME6
Lag-time analysis..... 144
Levocarnitine 156
Lidocaine..... 11, 16
Ligand exchange chromatography..... 270
Lipophilicity 16, 20, 68, 70, 73, 74
Lisinopril..... 43, 156
log P.....5
log S.....5
Lomefloxacin 156
Lorazepam..... 99, 102
Lyophilized solubility assay 36
Lyotropic..... 354

M

Macrolide-ribosome interactions..... 217
Maprotiline..... 47
Mass Spectrometry 180
MDCK cells..... 126
MEK protein kinase..... 184
Meloxicam 156
Melfhalan 156
Mesalamine 156
Mesophases..... 354
Metamizole..... 10
Metastable form 354, 356
Metformin..... 8, 453
Methylprednisolone 99, 102
Metoclopramide 99, 102
Metolazone..... 150, 155
Metronidazole 99, 102
Mexiletine..... 16
Microcalorimetry..... 387, 388
Midazolam 144, 145, 155
Minocycline..... 156
Mirtazapine..... 453
Monotropic system 356
Monotropy..... 403, 411
Moxifloxacin..... 28
Multiple linear regression..... 445
Multivariate regression..... 445

N

Nabumetone 99, 102
Nafillin..... 150, 155
Naproxen..... 99, 102, 150, 155
Near infrared spectroscopy..... 433
Nephelometric 32
Nicardipine..... 86, 88, 99, 102
Nifedipine 99, 102

Nitrofurantoin 42
Nizatidine..... 86, 88, 99, 102
NMR/pH titration..... 28
NOE..... 193, 195
NOESY..... 195, 205, 215
Noradrenaline 11
Norfloxacin..... 14, 22, 150, 156
Nuclear Magnetic Resonance 178

O

Octanol/water partition coefficient..... 68, 69, 70
Octanophenone..... 89
Ofloxacin..... 156
Olbutamide..... 10
Ondansetron 99, 102
Optical waveguide grating..... 178
Oxybutynin..... 150, 155

P

Paclitaxel 168
PAMPA..... 126, 127, 129, 130, 131
Papaverine..... 7
Paracetamol 89, 453
Partition coefficient..... 20
Pefloxacin..... 156
Permeability..... 16, 123, 130, 148
Permeability coefficient..... 134, 148, 149
P-glycoprotein..... 8, 124
P-gp efflux..... 8
pH partition hypothesis..... 128
Pharmaceutical profiling..... 5
Phase chromatographic retention..... 74
Phenobarbital 99, 102
Phenyltetrazole..... 85
Phenyltheophylline 85
Phenytoin 99, 102
PHOGSY..... 199
pH-partition hypothesis 7
Physicochemical profiling..... 5
Pindolol..... 150, 155
Piperacillin..... 99, 102
Piroxicam 10, 86, 99, 102, 156
pK_a..... 5
Polymorph screen..... 371, 372
Polymorphism..... 353, 400, 448, 457
Polymorphs 351, 354
Polysaccharide phases 273
Potentiometric method..... 26, 34, 38
Potentiometry 28
Prazosin 99, 102
Prednisolone..... 99, 102
Prednisone..... 99, 102
Prilocaine 210
Principal component 445
Principal component analysis 442
Procainamide..... 16, 99, 102
Process analytical technology 433
Propafenone..... 16

Propiophenone.....85, 89
 Propranolol..... 88, 99, 102, 144, 145, 146, 147, 150, 155
 Propyphenazone.....403, 412
 Prostaglandin E₁-ethylester 51
 Protein Phases.....312
 Protein–ligand binding.....169, 172
 Pyridoxine150

Q

QSAR.....3
 Quinidine16, 99, 102
 Quinine100, 102, 144, 145, 150, 155

R

Racemates236
 Raman spectroscopy375
 Retention factor66, 70
 Reversed-phase HPLC retention107
 Rifampin.....100, 102
 Risperidone.....150, 155
 Ristocetin.....311
 Ritonavir389
 ROESY200

S

Salicylic acid150, 155
 Saquinavir100, 102
 Saturation shake-flask method33
 Savitzky-Golay smoothing.....441
 Serum albumin binding.....108
 Shake-flask method35, 37
 Solubility.....16, 17, 18
 Solution calorimetry.....422
 Solvates351, 352
 Sorption isotherms413
 Sparfloxacin156
 Spectral Gradient Analysis.....29
 Starch273
 Statins168
 STD.....199, 203, 204, 205, 219
 Steroid.....453
 Sulfadiazine.....156
 Sulfadimidine10
 Sulfamethoxazole100, 103
 Surface Plasmon Resonance.....110, 175

T

Teicoplanin310
 Telithromycin217
 Telmisartan.....47
 Temazepam393
 Temocaprilat.....8
 Terbutaline100, 103, 156

Tetracaine.....11
 Tetracycline.....156
 Theophylline.....85, 150, 155
 Thermal analysis.....387, 388
 Thermogravimetry.....387, 397
 Thermogravimetry-infrared spectroscopy.....416
 Thermogravimetry-mass spectrometry416
 Thermotropic354
 Thioflavones215
 Tolbutamide.....100, 103, 150, 155, 403
 Torsemide.....156
 Trazodone.....100, 103
 Trimethoprim.....100, 103
 trNOESY.....219
 TROSY.....192, 207
 Trovafloxacin.....156

U

Ultra-filtration.....112
 Ultrafiltration-LC/MS.....32
 Unbound volume of distribution103, 104, 108
 Unstirred water layer.....See UWL
 UV/pH titration.....27
 UWL.....132, 134, 135

V

Valerophenone.....85, 89
 Valsartan10
 Van der Waals interactions.....173
 van't Hoff's law.....409
 Varfarin149
 Venlafaxine.....48
 Verapamil...100, 103, 143, 144, 145, 148, 149, 150, 155
 Volume of distribution.....98, 108

W

Warfarin88, 100, 103, 143, 148, 150, 155
 Water/skin partition108
 WaterLOGSY.....199, 203

X

X-ray diffraction.....373

Z

Zidovudine.....100, 103
 Zolmitriptan.....100, 103
 Zolpidem100, 103
 Zopiclone.....150, 155