PHYSICOCHEMICAL PROFILING IN DRUG RESEARCH AND DEVELOPMENT

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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)
—NH ₂	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* (*S*₀) 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, $K_{\text{sp}} = [A^-] [Y^+]$ for an acid and $K_{\text{sp}} = [BH^+] [X^-]$ 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.

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

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

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

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

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} .

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

For monoprotic acid and base:

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

For diprotic ampholyte:

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

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

¹ min/comp.

† Spectral Gradient Analysis

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 Profiler^{PLUS} (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_0) value was measured at pH 6.0 using the SSF method [81].

First the S_0 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: $S_0 = 556 \pm 13.2$ 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_0 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 $\log S_{\text{pH}}/\text{pH}$ profil 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 precisely 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 ($\text{pK}_a = 9.6$), 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 $37 \pm 0.1^\circ\text{C}$ 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: $\log P_{\text{TLC}} = 4.02 \pm 0.05$ ($n = 3$).

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.

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