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

Klara Valko

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2.1. INTRODUCTION

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

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

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

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

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

2.2. THEORETICAL BACKGROUND OF HPLC

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

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

Figure 2.1. Schematic principles of chromatographic separation process

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

2.2.1. Direct measurement of lipophilicity by chromatographic methods

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

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

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

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

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

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

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

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

2.2.2. Measurements of lipophilicity using isocratic chromatographic retention

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

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

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

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

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

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

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

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

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

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

where d and e are regression coefficients.

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

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

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

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

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

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

2.2.3. Measurements of lipophilicity using gradient chromatographic retention

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

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

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

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

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

2.2.4. pH dependence of lipophilicity and reversed phase chromatographic retention

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

2.3. APPLICATION OF BIO-MIMETIC HPLC STATIONARY PHASES

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

2.3.1. Immobilised human serum albumin (HSA) stationary phase

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

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

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

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

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

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

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

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

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

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

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

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

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

2.3.3. Immobilised artificial membrane (IAM) stationary phase

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

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

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

2.4.1. Measurements of chromatographic hydrophobicity indices at three different pHs

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

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

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

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

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

2.4.2. Measurement of HSA binding by HPLC retention

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

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

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

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

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

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

*Plasma protein binding (PPB)

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

Figure 2.14. Typical calibration plot obtained on the HSA column

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

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

2.4.3. Measurement of AGP binding by HPLC retention

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

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

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

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

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

Figure 2.15. Typical calibration plot for AGP binding measurements

2.4.4. Measurement of IAM binding by HPLC retention

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

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

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

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

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

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

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

Figure 2.16. Typical calibration curve for the IAM measurements

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

2.5.1. Analysis of the effect of charge

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

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

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

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

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

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

2.5.2. Analysis by the solvation equation

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

2.6.1. Modelling steady state volume of distribution

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

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

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

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

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

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

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

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

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

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

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

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

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

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

2.6.2. Modelling unbound volume of distribution

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

2.6.4. General structure-property relationships for lead optimisation

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

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

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

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

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

2.7. ALTERNATIVE METHODS

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

2.7.1. Methods based on the principle of frontal chromatography

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

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

Figure 2.25. Typical chromatogram obtained by frontal chromatography

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

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

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

2.7.2. Methods based on solid particles

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

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

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

2.7.3. Methods based on equilibrium dialysis and ultra-filtration

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

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

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

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

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