*Journal of Chromatography A*, 656 (1993) 469–484
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 160

# Review

# Modeling oil–water partitioning and membrane permeation using reversed-phase chromatography

William J. Lambert

*Pfizer Central Research, Pharmaceutical Research and Development, Eastern Point Road, Groton, CT 06340 (USA)*

ABSTRACT

Reversed-phase chromatographic retention data are currently playing a significant role in the determination of one of the most important physical properties of compounds, the oil–water partition coefficient. The potential uses of partition coefficients cover many areas of chemistry and biology, and have been particularly important in the area of biomembrane transport. This review will address the theoretical foundation of the chromatographic method for the estimation of partition coefficients, with an emphasis on the origins of anomalous retention behavior. Deviations of chromatographically determined partition coefficients from true values may provide insight into reversed-phase retention mechanisms.

CONTENTS



0021-9673/93/$24.00 

## 1. INTRODUCTION

*Owing to the cardinal role of hydrophobic interactions in life processes, there is a need for a physical-chemical method to quantitatively measure the hydrophobicity of biologically active substances.*

In one sentence, the above quote captures the essence of the present review. Surprisingly, the quote comes not from a biology or pharmaceutical journal, but instead from this journal in 1976. Horváth *et al.* [1] were discussing their solvophobic theory of reversed-phase chromatography, and showed great insight into the potential application to biological systems. Apparently their thoughts were not lost. A recent search of the Pomona College partition coefficient database reveals that there are 116 references containing the determination of partition coefficients by high-performance liquid chromatography (HPLC) methods for over 1600 compounds. This figure represents almost 5% of the total entries for the database. While this database is certainly the most extensive of all partition coefficient databases, it is by no means all inclusive due to the fact that partition coefficients are often obtained for a particular purpose. Often the fact that partition coefficients have been determined as a part of the study may not be evident in the title, keywords, or abstract of the manuscript.

HPLC capacity factors are commonly used to estimate partition coefficients, and have therefore been the subject of many reviews [2–6]. The reason for the popularity of the HPLC method is that it is a very simple and efficient method relative to traditional "shake-flask" methods. The greatest shortcoming of the HPLC method is that outliers are often noted. These outliers may be due to the difference in the hydrogen bonding capability of the stationary phase relative to the bulk oil of interest, or they may be due to more specific interactions between the solute and the stationary phase (such as amines and free silanol groups on silica-based columns). Regardless of the cause, the existence of outliers reduces the predictability of the method, and casts doubt on the results. It is easy to understand then, how much of the literature on this subject has dealt with the alleviation or quantitation of anomalous retention behavior.

The present review will focus on the following questions: (1) How can partition coefficients be utilized by investigators, with particular attention to the pharmaceutical sciences? (2) What is the theoretical foundation of the chromatographic method? (3) What choices must be made and what pitfalls must be avoided by the investigator using this method? (4) Finally, in light of the theme for this issue, can information on reversed-phase retention mechanisms be gleaned from this approach?

## 2. SIGNIFICANCE OF OIL–WATER PARTITION COEFFICIENTS

Oil–water partition coefficients ($P$) are one of the most commonly reported physical properties of drugs, pesticides, and other chemicals. The literature base on partition coefficients crosses many disciplines ranging from synthetic to environmental chemistry. In the 1960s, Fujita *et al.* [7] introduced the $\pi$ substituent constant, which describes the difference in log $P$ between a substituted and an unsubstituted compound. While this approach could be applied to virtually any partitioning system, Hansch and co-workers concentrated on the now popular *n*-octanol–water system [8,9]. Other *a priori* methods of calculating partition coefficients have been developed based on either empirical fragment constants [10], or on molecular properties which can be calculated using quantum chemical calculations [11,12]. While these approaches have been quite useful, it is often difficult to account for various intramolecular interactions such as hydrophobic bonding which may alter the partition coefficient [13]. Thus, the experimental determination of log $P$ by either the traditional "shake-flask" method or by the HPLC method should be utilized on at least some representative members of a class of compounds.

Why are partition coefficients so important? The partition coefficient is defined as the ratio of the concentrations of a solute equilibrated be-

tween two immiscible phases, typically an oil and an aqueous phase. Thus, from a qualitative standpoint, the partition coefficient is a measure of the solutes preference for the oil phase over water (often referred to as the lipophilicity or hydrophobicity). Due to the ubiquitous nature of lipids and water in biological systems, it is not surprising that oil–water partition coefficients are of practical interest (see below). From a more fundamental standpoint, the partition coefficient may be described in terms of the cohesive and adhesive interactions of the solute and the respective solvents [14]. Thus, from a thermodynamic standpoint, partition coefficients provide information on the intermolecular forces of the solute in solution.

### 2.1. Solubility estimation

Data on the interactive forces of various solutes have a number of potential applications, and most notably, the estimation of aqueous solubility [15–19]. In fact, a number of authors have utilized HPLC capacity factors to estimate aqueous solubility directly [20–22]. However, there are three caveats for these approaches. First, the activity coefficient for the solute in the oil phase is often set to unity by assuming that the solute behaves much like the oil. This certainly limits the range of solutes which may be tested by a particular approach. For example, octanol is often used for pharmaceuticals since most drugs have a similar solubility parameter to octanol [16]. Secondly, the approach is only applicable to non-electrolytes. The ionization of weak electrolytes must be accounted for separately. Finally, it should be noted that crystalline solids have an additional solute–solute interaction which is not applicable to liquids. A number of approximations may be applied in order to estimate the aqueous solubility of crystalline compounds [16,23,24]. Nonetheless, some knowledge of the crystal lattice energy is needed, and this can not possibly be accomplished by a solution state property such as partitioning. Thus, the crystal lattice energy must be independently measured or estimated if the solubility of a crystalline solid is to be correlated with the partition coefficient.

### 2.2. Pharmacological activity

The use of quantitative structure–activity relationships (QSARs) are now well ingrained in the pharmaceutical sciences. The demonstration of the importance of partition coefficients in QSARs has been particularly significant, and has been exhaustively reviewed [25–28]. The concept of utilizing chromatographic studies to correlate directly with bioactivity (sometimes referred to as quantitative retention–activity relationships) has been promoted by several authors [29–33].

Much of the partition coefficient effect in bioactivity studies is due to transport through biomembranes to the active site, which will be discussed further below. However, $\pi$ values have been utilized directly in assessing bioactivity using linear free energy relationships describing the hydrophobic interaction of drug substituents with active sites and enzymes [25,34], in analogy to Hammett $\sigma$ and Taft $E_s$ constants describing electronic and steric effects. Thus, the bioactivity ($B$) of a substituted compound may be described as

$$\log B = k\pi + k'\sigma + k''E_s + k''' \quad (1)$$

The active site may be an enzyme, in which case the above equation could be applied to enzyme inhibition or even the metabolism of the drug [35,36].

In addition to intrinsic bioactivity, several other pharmaceutical uses of partition coefficients have been made. The central nervous system toxicity of more lipophilic drugs has been explained based on the ability of these compounds to more readily cross the blood–brain barrier [37], which is made up of relatively pore-free capillaries. These authors suggest that drugs should be designed so as to minimize their lipophilicity, and thus, minimize central nervous system side effects. The hydrolysis of red blood cells represents a toleration problem for some injectable drugs, and has been shown to be predicted based on oil–water partition coefficients [38]. The biodistribution, protein binding,

and metabolism of drugs may also be altered by their lipophilicity [18,39].

### 2.3. Biomembrane permeability

Biological membranes are thin layers of lipid and protein that compartmentalize the living organism and permit selective passage of metabolites and nutrients [40]. For the present discussion, the term membrane will be used loosely. The permeation of solutes will be considered not only for individual cells, but also for barriers such as the nasal mucosa, the cornea of the eye, the vaginal mucosa, the rectal mucosa, the blood–brain barrier, the stratum corneum (the outer layer of the skin), and enterocytes of the intestine. These complex and heterogeneous barriers are nonetheless often modelled using partition coefficients, including those obtained chromatographically [41–56].

Most biomembranes consist of phospholipids arranged in bilayers [57]. The permeation of solutes in membranes has been considered at length by Diamond and Katz [58], who stress the anisotropic nature of the bilayer. They suggest that the head-group region of bilayers may present the most resistance to the permeation of lipophilic solutes, while the alkane-like interior of the bilayer may limit the permeation of polar solutes (see Fig. 1). Most compounds of pharmaceutical interest contain at least one polar functionality. This has led some investigators to suggest that alkanes may be more relevant to biomembrane transport than octanol since most drugs contain multiple polar functionalities [59,60]. An alternate approach would be to avoid the use of bulk solvents altogether, and instead use structured lipids such as liposomes. Partitioning into liposomes may correlate better with biological activity and membrane transport since surface group interactions and bilayer organization effects are not present in bulk solutions [61,62]. One could even suggest that reversed-phase HPLC capacity factor may be preferred over bulk oil–water partition coefficients, since the alkyl chains of the column are structured and the insertion of solutes into these chains would include an entropic cost not associated with transfer into a bulk oil [63]. However,

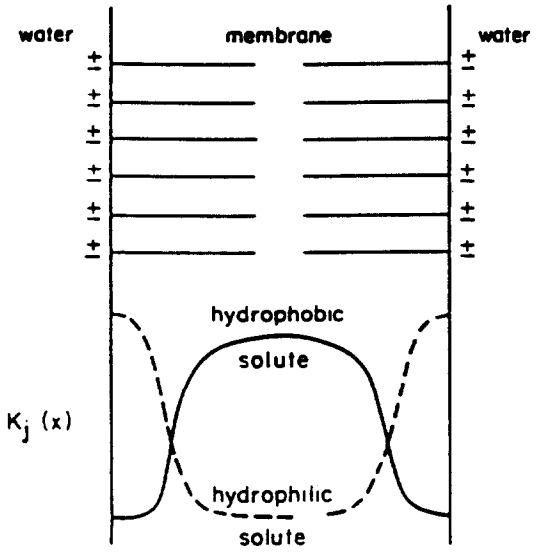


Fig. 1. Dependence of solute partition coefficient ($K_j$) on position within a lipid bilayer. From Diamond and Katz [58].

the density of alkyl chains in most common monomeric RP-HPLC columns generally do not approach that of phospholipid bilayers, which typically have densities of 5 $\mu$mol/m$^2$ [63,64].

To simplify the discussion of diffusion of solutes through membranes, physical chemists often treat the membrane of interest as a simple homogeneous membrane (see Fig. 2). The flux ($J$) of material through the membrane will be driven by the concentration gradient ($-dC/dx$) according to Fick's law

$$J = -D\left(\frac{dC}{dx}\right) \quad (2)$$

where $D$ is the diffusion coefficient. At steady

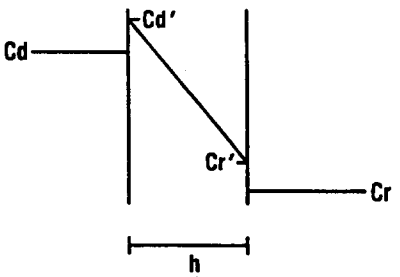


Fig. 2. Schematic of solute concentration ($C$) in a homogeneous membrane of thickness $h$ at steady state. The subscripts d and r refer to the donor and receiver sides of the membrane, respectively.

state, the flux through a membrane of thickness $h$ can be described by

$$J = \frac{P_{mw} D (C_d - C_r)}{h} \quad (3)$$

where $P_{mw}$ is the membrane–water partition coefficient and $C_d$ and $C_r$ represent the permeant concentrations on the donor and receiver sides, respectively. The quantity $P_{mw}D/h$ is often referred to as $k_P$, the permeability coefficient. It is clear from the above equation that partitioning is essential to membrane permeation. Hansch and Fujita [65] have explained the general applicability of relating log $P$ to biological activity in terms of the necessity of a compound to travel from some extracellular phase to some site of action in the cellular phase. They realized that the nature of most membranes are "more or less organic phases", and that the key process is partitioning.

The importance of partitioning is probably best demonstrated by homologous series of compounds with varying alkyl chain length ($n$). The increase in log $k_P$ due to the methylene group, $\pi_{CH_2}$, assuming no change in $D$ through the series is

$$k_{P,n} = k_{P,0}\, 10^{\pi n} \quad (4)$$

where $k_{P,0}$ is the permeability coefficient of the hypothetical reference congener [66]. The methylene group contribution to HPLC retention ($\tau_{CH_2}$) is discussed in detail elsewhere in this volume [67]. $\tau$ and $\pi$, like $k'$ and $P$, are directly related to each other. For mouse stratum corneum, values of 0.263 and 0.220 have been reported for $\pi_{CH_2}$ for alkanol and hydrocortisone-21-ester permeabilities, respectively [68,69]. This good agreement between two dramatically different series of compounds supports the concept that both are permeating through a lipophilic barrier. Under the same conditions, however, 5′-esters of the nucleotide antagonist, vidarabine, have a $\pi_{CH_2}$ value of 0.0893 [70]. This suggests that vidarabine is permeating through a non-lipophilic pathway, which would be consistent with temperature effects on permeability for these classes of compounds [71]. It is not clear if the pathway taken by vidarabine is various appendages in the skin (*e.g.*, sweat glands, hair follicles) or molecular level channels in the stratum corneum lipid barrier. However, this example demonstrates the challenge of treating a complex membrane as a homogeneous lipid.

### 2.4. Other uses

Partition coefficients have been used with much success in environmental chemistry. Bioconcentration potential, the ability of a chemical to accumulate in an animal (particularly fish) from its environment, is critical not only for the animal, but also other members of the food chain. The bioconcentration of compounds from water to fish has been shown to correlate with octanol–water partition coefficients [72,73]. The adsorption of chemicals from ground water to soil has also been correlated with partition coefficients [74]. Typically the adsorption coefficient is corrected for the percent of organic matter before the correlation is attempted [75]. While this reduces the variation observed between different type of soils, the method still has a fair degree of variability.

The sorption of solutes to various polymers has been modeled using partition coefficients. Jenke [76] observed an excellent correlation between octanol–water partition coefficients and the adsorption of various solutes to polyolefin intravenous administration bags from aqueous solutions. RP-HPLC capacity factors have also been used directly for this purpose [77].

### 2.5. Choice of the reference solvent

The above examples demonstrate that partition coefficients have played an important role in many areas of chemistry and biology for many decades. Octanol has by far become the most commonly used oil for oil–water partition coefficient determinations, in large part due to the extensive literature base developed by Hansch and his co-workers. While octanol–water partition coefficients have been shown to correlate with many properties, the choice of octanol is in some respects unfortunate. The mutual solubilities of octanol and water are high relative to

alkanes, and the hydrogen-bonding capability of octanol causes deviation when comparing linear free energy relationships between different solvent systems [9]. It is also not clear that octanol has the appropriate characteristics to correlate with all membranes and physicochemical properties. It is likely that these arguments will persist, leaving the investigator to make the appropriate choice of the reference solvent for his or her application.

## 3. THE RP-HPLC METHOD

### 3.1. Basis of the method

If one assumes that retention in RP-HPLC is due to partitioning between the mobile and stationary phases, it should be possible to find a correlation between bulk-phase partitioning and HPLC capacity factor ($k'$). The capacity factor is related to the stationary phase–mobile phase partition coefficient ($P_{SM}$) through the volumes ($V$) of the respective phases [78]

$$k' = P_{SM} \frac{V_S}{V_M} \quad (5)$$

The correlation of partition coefficients between various partitioning systems has long been suggested by Collander [79], who showed that $P$ for one solvent system could be related to another $P$ as

$$\log P_1 = a \log P_2 + b \quad (6)$$

where $a$ and $b$ are constants. While the above equation has been applied extensively over the last forty years, the premise is too simplistic. The solvents included in Collander's study were all alcohols. In attempting to extend the relationship to more non-polar systems such as alkanes, it is necessary to account for compounds with hydrogen bonding capability [34]. By assigning a substituent constant ($I_H$) to account for the effect of hydrogen bonding, Seiler [80] found that Collander's relationship between alkane and octanol partition coefficients was improved when the sum of all $I_H$ were added. The challenge to investigators attempting to correlate HPLC capacity factor with a specific partition coefficient is to determine if the Collander equation holds, and if not, can a correction factor such as Seiler's be applied. Much of the literature on this subject has dealt with the alleviation or quantitation of anomalous retention behavior.

### 3.2. Advantages over the traditional "shake-flask" method

Since anomalous retention behavior has the possibility of leading to erroneous determinations of partition coefficients, why not simply use the traditional "shake-flask" method to determine the partition coefficient? The major advantage to the HPLC method is speed. It is possible to test a whole series of compounds overnight with very little preparation time. On the other hand, the "shake-flask" method can be fairly tedious and time consuming. Prior to the start of the experiment, the two phases must be mutually saturated, which can take several days to accomplish [81]. The concentration of solute should be measured after equilibrium is achieved (preferably in both phases). This requires the existence of an analytical method, which if not available, must be developed, and possibly some trial and error in order to determine the optimal phase volume ratio. It is thus clear to see why the HPLC method has gained such popularity.

There are a number of other advantages of the HPLC method. First, only a small amount of compound is needed for the measurement, typically on the order of 1 mg. Second, the method is generally insensitive to impurities or degradation products which might either affect bulk partitioning or analysis. However, a word of caution is appropriate. If an impurity with a high molar absorptivity is present, its peak may be mistaken for the true peak. This situation would argue for the use of a more specific means of detection (mass spectrometry or photodiode array). Third, it is reasonable to utilize the HPLC method over a broader range of lipophilicities than is practically possible with the shake-flask method. Finally, solutes which are surface active often form emulsions on shaking, necessitating the use of centrifugation, which is avoided by the HPLC method. The major disadvantage to the HPLC method is that some sort

of calibration with compounds of known partition coefficient is necessary. If these standards behave differently on the column from the solute of interest, then an erroneous prediction of the partition coefficient is expected.

### 3.3. Choice of the mobile phase

One of the most difficult decisions that the investigator must make when beginning to utilize HPLC for the determination of partition coefficients is which mobile phase is appropriate. These choices include (1) which organic modifier to use, (2) the level or range of modifier to use, (3) whether to extrapolate capacity factor to 0% organic (often referred to as $k'_0$ or $k'_w$) or to choose a particular percent organic, (4) should a pH buffer or buffers be employed and (5) should stationary phase modifiers be included. Many of these choices will depend on the particular situation and intended use of the partition coefficient. This review will attempt to glean some insight on these decisions from the literature.

#### 3.3.1. Organic modifier

Methanol is by far the most commonly used organic modifier in the determination of partition coefficients [3,4,6]. There are two likely reasons for this. First, methanol is the most water-like of all commonly used RP-HPLC solvents. It is capable of hydrogen bond acceptance and donation. Methanol has a solubility parameter of 14.5 $(cal/cm^3)^{1/2}$, while water has a value of 23.4 $(cal/cm^3)^{1/2}$ [82] (1 cal = 4.14 J). In comparison, acetonitrile and tetrahydrofuran have solubility parameters of 11.9 and 9.1 $(cal/cm^3)^{1/2}$, respectively. Not only the mobile phase, but also the stationary phase, will be affected less with methanol. McCormick and Karger [83] have shown that the adsorption of acetonitrile and tetrahydrofuran to alkyl-bonded silica occurs to a greater degree than with methanol. An interesting comparison of acetonitrile and methanol was made by Sanchez-Moyano *et al.* [54] who compared $k'_0$ using a $C_{18}$ column for both solvents to heptane–water partition coefficients for a homologous series of solutes with varying chain length. A plot of log $P$ (or log $k'_0$) *versus* $n$ demonstrates that the heptane partition coefficient has very similar slope to $k'_0$ for methanol, but not for acetonitrile. In analogy to eqn. 4, the slopes of these plots are equal to $\pi_{CH_2}$. Thus, the sensitivity of the partitioning to the methylene group is similar for bulk heptane–water partitioning when methanol is used as the modifier, but not when acetonitrile is used. Other investigators have also found a superior octanol–water log $P$ correlation using methanol rather than acetonitrile or tetrahydrofuran as the modifier [84,85].

The second reason for the use of methanol relates to the quadratic nature of the relationship between capacity factor and the volume ratio ($\varphi$) of mobile phase (see below). Schoenmakers *et al.* [86] have shown that for plots of log $k'$ *versus* $\varphi$, methanol generally has significantly less curvature than either tetrahydrofuran or acetonitrile, which is consistent with the solubility parameters for these solvents. Thus, the use of methanol minimizes errors in extrapolation to 0% organic.

The use of any organic solvent at high concentrations in the mobile phase may raise questions of whether the mobile phase resembles pure water at all. Leo [87] suggests that at high methanol concentrations (>50%), the HPLC method is fairly insensitive to hydrophobicity. As an example, Leo cites the work of Spencer *et al.* [88] who found a poor correlation between partition coefficients (determined by HPLC using 70 or 90% methanol) and elastase inhibition by 4-hydroxy-2-pyrones. By reanalyzing the data using calculated partition coefficients, a positive correlation was found. Thus, investigators using the HPLC method may wish to minimize the percentage of organic solvent, possibly by adjusting column length [3,6,89].

#### 3.3.2. To extrapolate or not to extrapolate

Plots of log $k'$ *versus* the percent of organic are generally linear over a fairly broad range, particularly for methanol [90]. This linearity is often used to justify the practice of extrapolating to 0% organic when correlating $k'$ to partition coefficients. Conceptually, this approach would minimize the effect of the solvent on the nature of the aqueous phase. However, the relationship

between capacity factor and the volume ratio ($\varphi$) of mobile phase is quadratic in nature, and may be expressed as

$$\log k' = A\varphi^2 + B\varphi + C \quad (7)$$

This relationship, which is observed experimentally, has been predicted theoretically based on statistical mechanical [64] or thermodynamic arguments [91,92]. The quadratic relationship between log $k'$ *versus* $\varphi$ causes severe problems for the extrapolation method. First, as noted above, organic modifiers such as acetonitrile have significant curvature even at moderate percentages [86]. Second, for methanol, curvature is typically observed at volume fractions below 30% and above 70% [90]. Thus, extrapolated $k'$ values determined at moderate volume fractions would deviate from the true $k'_0$ value. This would also be true for investigators using the same range of organic solvent with different solvents [64,86,92], which is intuitively troubling. Third, deviations of classes of compounds are observed even when extrapolation is performed by one set of investigators. This is exemplified by the work of Hammers *et al.* [93] who compared log $P$ estimates using extrapolated log $k_0$ values *versus* log $k'$ values at 50% methanol. While the extrapolated values yielded a slightly improved correlation for many compound classes, certain classes (particularly polar compounds) appeared to have anomalous behavior. Finally, extrapolation requires significantly more experimental study and data analysis. This additional amount of work begins to reduce one of the main advantages of the HPLC method over the "shake-flask" method; namely, time. Thus, many investigators utilize the $k'$ value at only one mobile phase condition [2,3,6,94,95].

Some authors have criticized the use of a single mobile phase condition, suggesting that a different rank ordering of log $P$ values may be obtained as a function of the percent organic [4,96]. Stated in another way, plots of log $k'$ *versus* volume fraction of the organic modifier for two solutes would have an intersection somewhere between 0 and 100% organic. The paper by Geng *et al.* [90] in this volume provides significant insight into this matter. Plots of log $k'$ *versus* volume fraction typically converge near a volume fraction of 1. If convergence generally occurs only at very high percentages of methanol, a change in rank order would not be anticipated. The convergence can be quantitated by noting that the slope ($-S$) of such plots for related series of compounds can be related to $k_w$ by the relationship

$$S = p + q \log k_w \quad (8)$$

where $p$ and $q$ are constants for a given set of conditions [90]. Assuming that these plots are linear over the entire range of volume of fraction, the lines for the various compounds will intersect at the point where log $k'$ equals $-p/q$. The values for $p$ and $q$ for a wide variety of compounds with methanol as the organic modifier are provided by Geng *et al.* Analysis of this data suggests that the intersection occurs at 130% methanol in 10 of the 14 data sets (with a 95% confidence interval for the mean of 7%). The remaining 4 sets of data had values of 67% or greater. These data would appear to suggest that in general, one would not expect a change in the rank ordering of partition coefficients based on capacity factors determined at a specific percent organic. (This statement assumes that the alteration of the percentage of organic is not affecting secondary equilibria on the column.) Geng *et al.* also argue that since to a first approximation log $k'$ varies linearly with percent organic, the choice of percent organic to utilize in partition coefficient determinations can be somewhat arbitrary. An attempt should be made to utilize as low a methanol concentrations as is reasonable in order to maximize selectivity (the log $k'$ values converge at a high percentage of organic). However, the methanol concentration used should be above 25–30% so as to remain in the linear portion of log $k'$ *versus* volume fraction plots. The experimental observation that the dependence of the calculated log $P$ does not appear to vary dramatically with the percent organic for most substances, particularly if a mobile phase of at least 25% water is utilized [3,97], would appear to support the above conclusions.

An interesting insight into extrapolation can

be gleaned from the approach taken by Yamagami and Takao [95], who investigated actual log $k'_0$ values (no extrapolation). They found an extremely poor correlation with log $P$, whereas a much better correlation was found using log $k'_{50}$ with methanol (Fig. 3). This would suggest that silica-based reversed-phase columns predict octanol–water partition coefficients better under conditions where adsorbed methanol modifies the stationary phase. Thus, even though a quadratic extrapolation (see eqn. 7) may yield an improved estimate of log $k_0$, such an extrapolation should not be used for estimating log $P$. Furthermore, Geng *et al.* [90] suggest that while interpolation using eqn. 7 is more accurate than linear interpolation, quadratic extrapolation often leads to significant errors.

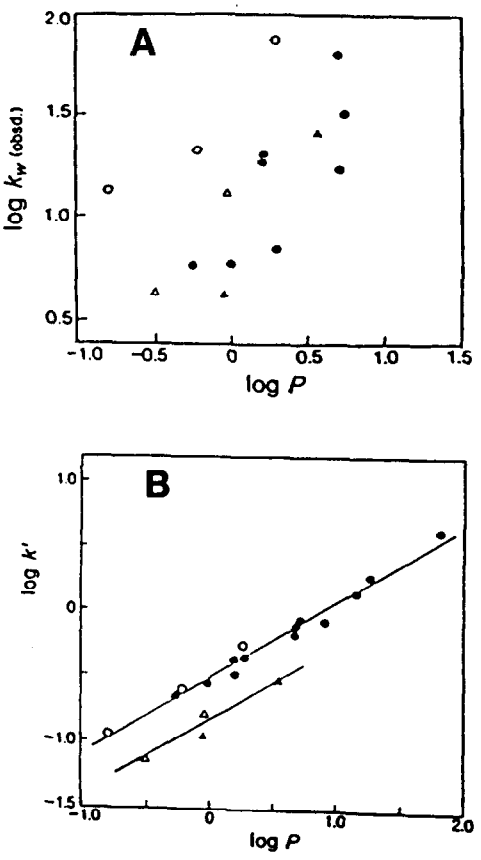


Fig. 3. (A) Plot of actual log $k_w$ *versus* log $P$ showing the poor correlation in the absence of methanol. (B) Plot of the same relationship as in A, except that the capacity factor was determined at 50% methanol. ▲ = amphiprotics; ● = non-hydrogen bonders and H-acceptors; the open symbols represent amides and esters. From Yamagami and Takao [95].

### 3.3.3. *Buffers and other additives*

For weak electrolytes, a decision must be made with respect to the pH of the mobile phase. It is often tempting to buffer the mobile phase at the pH of interest. For example, pH 7.4 is often used for pharmaceutical applications since this is the physiologic pH [98]. There are a number of problems with this approach. First, the pH is only an apparent pH since the mobile phase is not entirely made of water. Secondly, the altered dielectric constant of the mobile phase will affect the acid dissociation constants ($K_a$) of the solutes of interest. The $K_a$ values of various functionalities may be affected to varying degrees, and possibly opposite directions of change. Thus, it may be more sound to buffer the mobile phase so as to ensure that the compound is in its neutral state (if possible), yielding the intrinsic partition coefficient [3,6]. The apparent partition coefficient can then be calculated at the appropriate pH based on knowledge of the acid dissociation constants, which can be determined experimentally or estimated based on substituent constants [99].

Other mobile phase additives have also been proposed, such as lipids, ion-pairing agents [4,100], and silanol masking agents [2,96]. The first topic is discussed below under the section on stationary phases. Retention mechanisms in ion-pairing chromatography are quite complex since they involve numerous factors such as ionic and hydrophobic interactions between the solutes and the ion-pairing agent [101]. A good correlation between log $P$ and log $k'$ has been observed for a series of amines using dodecyl-sulphate as an ion-pairing agent on a phenylsilica column [100]. However, it should be noted that the amines were all structurally related, and had nearly identical p$K_a$ values and steric factors. Since a single ion-pairing agent will interact to varying degrees with structurally unrelated amines due to steric and other factors, it is difficult to imagine that these complex interactions can model partition coefficients for a wide variety solutes. Furthermore, these interactions

will vary dramatically as organic modifier percentage is varied. Thus, caution must be used in applying ion-pairing agents for partition coefficient determination.

Since free silanols in silica-based reversed-phase columns are able to specifically interact with various functionalities, it seems obvious that any method which could mask their effect would be advantageous. Many early papers on partition coefficient correlations concentrated on methods of covalently blocking these groups (*e.g.*, silylation with hexamethyldisilazane or trimethylsilyl chloride [33]). As alkyl binding chemistry improved, such efforts were no longer productive. However, unreacted silanol groups are still present in modern day columns, as evidenced by anomalous peak broadening of amine containing compounds. While the addition of agents such as triethylamine and *n*-decylamine can lessen peak broadening for analytical HPLC procedures, they should be used with caution for partition coefficient correlations since they may act as ion-pairing agents with acidic solutes. Unfortunately, compounds of interest often contain various acidic and basic functionalities. In such cases, silanol masking agents should not be used.

### 3.4. Solute concentration and association

The amount of compound injected on the column should be considered from several points of view. Obviously, the concentration should be adequate to allow quantitation without overloading the column. The European Chemical Industry Ecology and Technology Centre [6] has recommended the use of 1 mg/ml solutions, which should be sufficient to allow detection of most solutes. Unfortunately this paper does not recommend an injection volume, and column overload is typically caused by the mass of solute injected. An injection volume of approximately 20 $\mu$l is generally acceptable at this concentration. One should also consider the possibility of solute association, which may vary as a function of solute concentration. For example, the dimerization of carboxylic acids in organic phases is well known, necessitating the study of partitioning as a function of solute concentration [102].

### 3.5. Void marker

Some thought must be given to the choice of the void marker so that the capacity factor may be calculated. This is particularly important if some solutes of interest have retention volumes approaching that of the void volume. A number of solutes have been utilized including thiourea, formamide, sodium nitrate, sodium iodide, and methanol [3]. It is also possible to utilize a homologous series in order to determine the void retention time [3,103].

## 4. STATIONARY PHASES USED IN PARTITION COEFFICIENT CORRELATIONS

### 4.1. Silica-based columns

Octadecyl silica (ODS) columns are used in the vast majority of log $P$ correlations [2–4,6], although a similar if not better correlation has been observed with $C_8$ columns [96]. When used with methanol–water mobile phases, capacity factors on these columns generally correlate well with literature octanol–water partition coefficients. A poor correlation is typically found for alkane–water partition coefficients [104], unless substituents are limited to non-polar functionalities [93].

Despite attempts to decrease the effects of free silanol groups through chemical modification [33], the addition of a lipophilic amine to the mobile phase [96,104], or saturation of the stationary phase with octanol (see below), outliers due to specific interactions between the free silanol groups and various functionalities are commonly observed [3,6,31,104–106]. Amines which are not sterically hindered are particularly problematic as outliers [98,107].

Hydrogen bond donors, acceptors, and non-hydrogen bonders have been differentiated into classes on ODS systems with methanol as the organic modifier [2,97,106], however, this is not always the case [3]. An example of differentiation by solute class is shown in Fig. 4. If the series of compounds being investigated contains

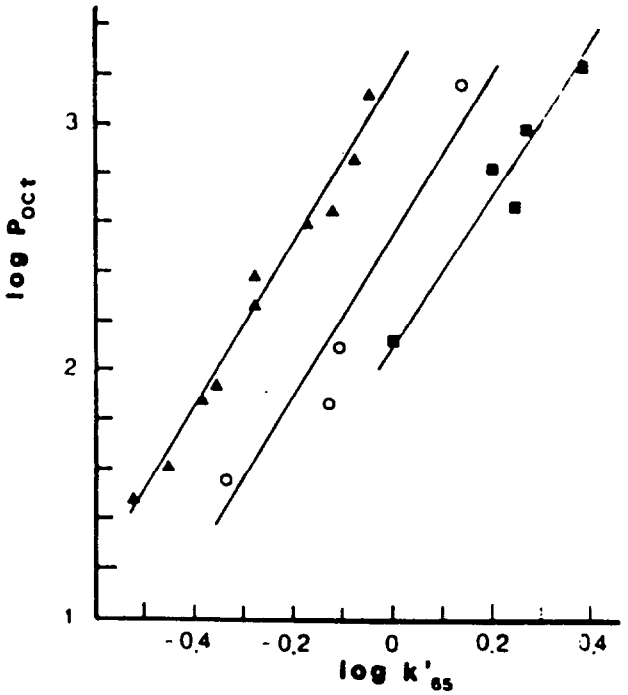


Fig. 4. Example of differentiation of solute class using a silica-based reversed-phase column. ○ = Hydrogen bonders; ■ = non-hydrogen bonders; ▲ = amphiprotics. From Miyake *et al.* [106].

similar functionalities, this effect may not be of concern as long as the standards utilized are similar in nature. However, if the series contains multiple classes of compounds (*e.g.*, bases, hydrogen bonders, non-hydrogen bonders), one should carefully select the column and conditions. Columns with fewer free silanols are known to produce plots of log $k'$ *versus* volume fraction that are less curved [90]. It is also reasonable to expect improved log $P$ correlation with less differentiation by solute class with better columns.

Attempts have been made to account for hydrogen bonding differences between compounds, making the technique more applicable to broad classes of compounds. Miyake *et al.* [106] found that for ODS columns with methanol–water mobile phases, hydrogen bonding differences between octanol–water partition coefficients and retention could be accounted for by the following equation

$$\log P_{oct} = a + b \log k' + cA + dD \quad (9)$$

where $a$, $b$, $c$ and $d$ are constants, $A$ is an indicator variable which equals 1 for compounds which are hydrogen bonding acceptors (0 if not), and $D$ is an indicator variable for hydrogen bonding donors. This approach led to a greatly improved correlation, particularly at moderate percentages of methanol (50–70%). However, when used with $k'_w$, this approach only caused a slight improvement, suggesting that hydrogen bonding differences between the stationary phase and octanol are minimal at low methanol percentages.

### 4.2. Polymeric columns

A number of authors have proposed utilizing polymeric reversed-phase columns in place of ODS columns [94,107–109]. The use of polymeric columns offers several potential advantages. First, these columns avoid silanol group interactions with solutes, probably the most common cause of outliers. In addition, they do not contain the trace metals which are associated with silica and are known to cause anomalous retention behavior [110]. Finally, these columns are typically stable over a wide range of pH. Thus, they can be used to measure the intrinsic partition coefficients of acids and bases at extreme pHs which might not be possible with a less stable bonding chemistry.

Polystyrene–divinylbenzene (PS–DVB)-based columns have been studied extensively. While an improvement in octanol–water partition coefficient prediction has been reported with PS–DVB columns over ODS columns [107], other investigators have reported significant deviations of classes of compounds (*e.g.*, acids from bases) [108,109]. It is likely that the specific interactions between solutes and the PS–DVB stationary phase are due to the electron-rich $\pi$-orbitals present in the column [111–114].

Benson and Woo [111] have suggested that $C_{18}$ derivatization of the PS–DVB columns eliminate $\pi$-orbital interactions of the polymer with solutes through steric hindrance. If these $\pi$-orbital interactions are significant, dipole (solute)–dipole (column), dipole (column)–induced dipole (solute), and hydrogen bonding retention interactions would be occurring which would not occur

in bulk alkane. To test this hypothesis, Lambert *et al.* [89] analyzed the deviations of HPLC partition coefficient values using the Act-I column from literature alkane–water partition coefficient values as a function of the compound's (or substituent's) molar refractivity (related to polarizability), dipole moment, the Hammett substituent constant ($\sigma_p$), or the hydrogen bonding acceptance ($pK_{HB}$). A significant correlation of the deviations was not observed for any parameter, suggesting that no specific interactions were occurring with the stationary phase. Thus, it appears that the $C_{18}$-derivatized PS–DVB column could be used for alkane–water partition coefficient determinations for compounds containing dramatically varying functionalities.

Octadecylpolyvinyl (ODPV) columns provide an alternative method to avoiding $\pi$-orbital interactions associated with PS–DVB columns. Capacity factors using an ODPV column have been shown to have a good correlation with octanol–water partition coefficients for a broad range of compounds [109]. This column was found to provide a correlation which is superior to PS–DVB columns, and similar to that provided by ODS columns.

### 4.3. Oil-loaded phases

An interesting approach to predict oil–water partition coefficients by HPLC is to load the stationary phase with the oil of interest, and determine the capacity factor with an oil-saturated mobile phase [2,98,105,115]. This approach generally yields a value for a in eqn. 6 of approximately 1, suggesting that the stationary phase is indeed much like the oil [2]. Two disadvantages of this approach are that retention times become prohibitively long for very lipophilic compounds [2] and that it is often difficult to maintain the stationary phase precisely at the saturation level [98].

Terada and co-workers [106,116] have proposed using glycerol-coated controlled pore glass as a stationary phase for the determination of log $P$ (octanol–water). However, the correlation appears to be dependent on whether the compound has hydrogen bonding substituents or not, suggesting that the system is different from bulk octanol.

### 4.4. Phospholipid-modified supports

Pidgeon and Venkataram [117] are studying di-$C_{14}$-lecithin covalently bound to Nucleosil-300 by the terminal carboxy group on the C-2 chain as a method of modeling biomembranes. This approach suffers from the fact that the structures are less fluid at the tail than at the headgroups, which is the opposite of natural bilayers [58]. Nonetheless, this novel approach may provide new insights into the molecular processes occurring in natural membranes.

### 4.5. Related approaches

A number of related chromatographic approaches have been used to determine oil–water partition coefficients. Since these techniques are only peripherally related to reversed-phase retention, they will only be briefly touched upon in the present review.

Thin layer chromatography represents one of the earlier uses of chromatography to measure lipophilicity, and has been extensively reviewed [25,32,118–120]. For partition chromatography, log $P$ for the system is related to $R_M$ [118], which like log $k'$, has a linear relationship with the chain length of homologous series [121] and shows a reasonable correlation for partition coefficients for reversed-phase systems [122,123]. Typically RP-TLC plates and methanol–water as the mobile phase are utilized, in an analogous manner to the HPLC method. Like log $k'$, there is controversy over whether one should extrapolate to 0% organic when reporting $R_M$ values [124].

Gago *et al.* [125] have proposed that micellar chromatography derived log capacity factors may be correlated with log $P$. These authors also suggest this method may have an advantage in that the use of different surfactants would vary partitioning involving the micelles, but would not affect partitioning between the stationary phase and bulk water. However, this would seem to assume that the surfactants will not adsorb to the stationary phase. It should also be noted that

plots of log $k'$ (from micellar chromatography) *versus* chain length ($n$) for homologous series are not linear. This non-linearity may be due to variation in methylene selectivity as a function of micelle depth [126] and to the fact that one partitioning system is in motion [127]. Since log $P$ is generally linear with $n$ (see eqn. 4), a linear correlation of log $k'$ and log $P$ is not expected. Nonetheless, a good correlation of the inhibition of bacterial growth by phenols and micellar capacity factor has been obtained [128].

Counter-current chromatography (CCC) and centrifugal partition chromatography (CPC) allow the direct determination of partition coefficients between two immiscible phases. Technical advances in equipment over the last decade are bringing these techniques to much broader use. Interested readers are referred to papers by El Tayar *et al.* [129], Berthod and Armstrong [130], and Terada *et al.* [131].

## 5. INSIGHTS INTO RP-HPLC RETENTION MECHANISMS

Much of the content of this volume of the *Journal of Chromatography* deals with the mechanisms of retention on a molecular level. While partitioning plays an important role in the process of retention, it is clear that specific interactions may influence retention, particularly with silica-based stationary phases. The structured nature of the alkyl chains in chromatographic phases also provides an entropic difference from partitioning in bulk liquids.

An interesting question to this reviewer relates to the fact that log $k'$ on ODS columns with methanol–water mobile phases generally has an excellent correlation with octanol–water partition coefficients, but a poor correlation with alkane–water partition coefficients. If the retention mechanism is due to adsorption/partitioning to the $C_{18}$ chains, why is this so? The better correlation with octanol–water rather than with alkane–water partition coefficients would suggest that polar solutes are able to specifically interact with some polar component of the stationary phase. It is not clear if the hydrogen-bonding nature of ODS stationary phases is simply due to unreacted silanol groups, or adsorbed methanol imparting a polar nature to the stationary phase. Since polymer-based reversed-phase columns lack free silanol groups, some insight may be gleaned from $C_{18}$ derivatized polymer columns. A $C_{18}$ derivatized PS–DVB (Act-I) column has been shown to correlate well with alkane–water partition coefficients, and not octanol–water partition coefficients [89]. When the octanol data was analyzed for only the non-H bonders, an excellent correlation was obtained, indicating that polar compounds have positive deviations which are most likely due to solute hydrogen bonding in the octanol phase. The Act-I data would appear to imply that free silanols are responsible for the good octanol–water partition coefficient correlation with ODS columns. However, a good correlation with octanol–water partition coefficients has been obtained with capacity factors using an ODPV column (no attempt was made to correlate log $k'$ with log $P$ for alkane–water partition coefficients) [109]. These data, would seem to suggest that adsorbed methanol imparts an octanol-like quality to the ODS and ODPV stationary phases. However, it is possible that the polar nature of the ODPV column is due to unreacted hydroxyl groups of the polyvinyl alcohol, or an effect of the ester functionalities. The use of alternate polymers may allow these mechanisms to be investigated. It is likely that additional studies like the above, coupled with quantitative structure–retention relationships and temperature dependence studies, should provide additional insight into retention mechanisms in reversed-phase chromatography.

## 6. ACKNOWLEDGEMENTS

I would like to thank Corwin Hansch and his colleagues at Pomona College for their assistance with the log $P$ database, Walt Morozowich (The Upjohn Company) for his numerous discussions on partition coefficients and Lloyd Snyder (LC Resources, Inc.) for his suggestions on this manuscript, particularly on the area of extrapolating capacity factors as a function of volume fraction.

## REFERENCES


1 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129–156.
2 H. Terada, *Quant. Struct.-Act. Relat.*, 5 (1986) 81–88.
3 W. Klein, W. Kordel, M. Weib and H.J. Poremski, *Chemosphere*, 17 (1988) 361–386.
4 T. Braumann, *J. Chromatogr.*, 373 (1986) 191–225.
5 C.F. Carney, *J. Liq. Chromatogr.*, 8 (1985) 2781.
6 *Assessment of Reversed-Phase Chromatographic Methods for Determining PCs*, European Chemical Industry Ecology and Technology Centre, Brussels, 1983.
7 T. Fujita, J. Iwasa and C. Hansch, *J. Am. Chem. Soc.*, 86 (1964) 5175–5180.
8 C. Hansch and A. Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology*, Wiley, New York, 1979.
9 A. Leo, C. Hansch and D. Elkins, *Chem. Rev.*, 71 (1971) 525–616.
10 G.G. Nys and R.F. Rekker, *Chim. Ther.*, 8 (1973) 521–535.
11 K.S. Rogers and A. Cammarata, *J. Med. Chem.*, 12 (1969) 692–693.
12 N. Bodor, Z. Gabanyi and C.-K. Wong, *J. Am. Chem. Soc.*, 111 (1989) 3783–3786.
13 C. Hansch and S.M. Anderson, *J. Org. Chem.*, 32 (1967) 2583–2586.
14 J.H. Hildebrand and R.L. Scott, *Regular Solutions*, Prentice Hall, Englewood Cliffs, NJ, 1962.
15 W.J. Lyman, in W.J. Lyman, W.F. Reehl and D.H. Rosenblatt (Editors), *Handbook of Chemical Property Estimation Methods*, American Chemical Society, Washington, DC, 1990, Ch. 2.
16 S.H. Yalkowsky and S.C. Valvani, *J. Pharm. Sci.*, 8 (1980) 912–922.
17 P. Isnard and S. Lambert, *Chemosphere*, 18 (1989) 1837–1853.
18 C. Hansch, J.E. Quinlan and G.L. Lawrence, *J. Org. Chem.*, 33 (1968) 347–350.
19 I. Moriguchi, *Chem. Pharm. Bull.*, 23 (1975) 247–257.
20 A. Opperhuizen, *Toxicol. Environ. Chem.*, 15 (1987) 249.
21 T.L. Hafkenscheid and E. Tomlinson, *J. Chromatogr.*, 218 (1981) 409–425.
22 K.D. Bartle, A.A. Clifford, S.A. Jafar, J.P. Kithinji and G.F. Shilstone, *J. Chromatogr.*, 517 (1990) 459–476.
23 S.H. Neau and G.L. Flynn, *Pharm. Res.*, 7 (1990) 1157.
24 R.G. Hollenbeck, *J. Pharm. Sci.*, 69 (1980) 1241–1242.
25 M.S. Tute, *Adv. Drug Res.*, 6 (1971) 1–77.
26 H. Kubinyi, *Prog. Drug Res.*, 23 (1979) 98.
27 P.J. Goodford, *Adv. Pharmacol. Chemother.*, 11 (1973) 51–97.
28 K.C. James, *Prog. Med. Chem.*, 10 (1974) 205–243.
29 R. Kaliszan, *Anal. Chem.*, 64 (1992) 619A–631A.
30 R. Kaliszan, *Quantitative Structure-Chromatographic Retention Relationships*, Wiley, New York, 1987, pp. 232–278.
31 R. Kaliszan, *CRC Crit. Rev. Anal. Chem.*, 16 (1986) 323–383.
32 K. Valko, *Trends Anal. Chem.*, 6 (1987) 214–219.
33 J.M. McCall, *J. Med. Chem.*, 18 (1975) 549–552.
34 A. Leo and C. Hansch, *J. Org. Chem.*, 36 (1971) 1539–1544.
35 Y. Ichikawa, T. Yamano and H. Fujishima, *Biochim. Biophys. Acta*, 171 (1969) 32–46.
36 M. Charton, in E.B. Roche (Editor), *Design of Biopharmaceutical Properties through Prodrugs and Analogs*, American Pharmaceutical Association, Washington, DC, 1977, pp. 228–280.
37 C. Hansch, J.P. Bjorkroth and A. Leo, *J. Pharm. Sci.*, 76 (1987) 663–687.
38 K.S. Rogers, *Proc. Soc. Exptl. Biol. Med.*, 130 (1969) 1140–1142.
39 F. Helmer, K. Kiehs and C. Hansch, *Biochemistry*, 7 (1968) 2858.
40 C. Tanford, *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, Wiley, New York, 1980.
41 A.S. Michaels, S.K. Chandrasekaran and J.E. Shaw, *AIChEJ.*, 21 (1975) 985–996.
42 R.D. Schoenwald and H.S. Huang, *J. Pharm. Sci.*, 72 (1983) 1266–1272.
43 V.A. Levin, *J. Med. Chem.*, 23 (1980) 682–684.
44 R.C. Young, R.C. Mitchel, T.H. Brown, C.R. Ganellin, R. Griffiths, M. Jones, K.K. Rana, D. Saunders, I.R. Smith, N.E. Sore and T.J. Wilks, *J. Med. Chem.*, 31 (1988) 656–671.
45 D.W. Osborne and W.J. Lambert, in K.B. Sloan (Editor), *Prodrugs: Topical and Ocular Drug Delivery*, Marcel Dekker, New York, 1992, pp. 163–178.
46 T. Sakane, M. Akizuki, S. Yamashita, T. Nadai, M. Hashida and H. Sezaki, *Chem. Pharm. Bull.*, 39 (1991) 2456–2458.
47 D.-S. Chien, H. Bundgaard and V.H.L. Lee, *J. Ocular Pharmacol.*, 4 (1988) 137–146.
48 G.M. Grass, E.R. Cooper and J.R. Robinson, *J. Pharm. Sci.*, 77 (1988) 24–26.
49 T. Nook, E. Doelker and P. Buri, *Int. J. Pharm.*, 43 (1988) 119–129.
50 S.H. Yalkowsky and W. Morozowich, in E.J. Ariens (Editor), *Drug Design*, Vol. IX, Academic Press, New York, 1980, pp. 122–185.
51 L.S. Schanker, *J. Med. Pharm. Chem.*, 2 (1960) 343–359.
52 D.C. Corbo, J.-C. Liu and Y.W. Chien, *J. Pharm. Sci.*, 79 (1990) 202.
53 J. Hadgraft and G. Ridout, *Int. J. Pharm.*, 42 (1988) 97–104.
54 E. Sanchez-Moyano, C. Seco, A. Santolaria, S. Fabra-Campos, M. Herraez and A. Martin-Villodre, *J. Pharm. Sci.*, 81 (1992) 720–725.
55 R.H. Guy and R.O. Potts, *J. Pharm. Sci.*, 81 (1992) 603–604.
56 D.C. Taylor, J. Lynch and D.E. Leahy, in J.G. Hardy, S.S. Davis and C.G. Wilson (Editors), *Drug Delivery to the Gastrointestinal Tract*, Ellis Horwood, Chichester, 1989, pp. 133–145.
57 W. Curatolo, *Pharm. Res.*, 4 (1987) 271–277.

58 J.M. Diamond and Y. Katz, *J. Membr. Biol.*, 17 (1974) 121.
59 J.H. Rytting, S.S. Davis and T. Higuchi, *J. Pharm. Sci.*, 61 (1972) 816–818.
60 B.D. Anderson, in S.H. Yalkowsky, A.A. Sinkula and S.C. Valvani (Editors), *Physical and Chemical Properties of Drugs*, Marcel Dekker, New York, 1980, pp. 231–266.
61 F.A.P.C. Gobas, J.M. Lahittete, G. Garofalo, W.Y. Shiu and D. MacKay, *J. Pharm. Sci.*, 77 (1988) 265–272.
62 G.V. Betageri and J.A. Rogers, *Pharm. Res.*, 6 (1989) 399–403.
63 J.G. Dorsey and K.A. Dill, *Chem. Rev.*, 89 (1989) 331–346.
64 K.A. Dill, *J. Phys. Chem.*, 91 (1987) 1980–1988.
65 C. Hansch and T. Fujita, *J. Am. Chem. Soc.*, 86 (1964) 1616–1626).
66 G.L. Flynn and S.H. Yalkowsky, *J. Pharm. Sci.*, 61 (1972) 838.
67 R.M. Smith, *J. Chromatogr. A*, 656 (1993) 381.
68 H. Durrheim, G.L. Flynn, W.I. Higuchi and C.R. Behl, *J. Pharm. Sci.*, 69 (1980) 781.
69 W.M. Smith, *Ph.D. Thesis*, University of Michigan, Ann Arbor, MI, 1982.
70 N.A. Gordon, *Ph.D. Thesis*, University of Michigan, Ann Arbor, MI, 1981.
71 K. Knuston, S.L. Krill, W.J. Lambert and W.I. Higuchi, *J. Controlled Release*, 6 (1987) 59–74.
72 S.E. Bysshe, in W.J. Lyman, W.F. Reehl and D.H. Rosenblatt (Editors), *Handbook of Chemical Property Estimation Methods*, American Chemical Society, Washington, 1990, Ch. 5.
73 W.B. Neely, D.R. Branson and G.E. Blau, *Environ. Sci. Tech.*, 8 (1974) 1113–1115.
74 D.S. Brown and E.W. Flagg, *J. Environ. Qual.*, 10 (1981) 382–385.
75 W.J. Lyman, in W.J. Lyman, W.F. Reehl and D.H. Rosenblatt (Editors), *Handbook of Chemical Property Estimation Methods*, American Chemical Society, Washington, DC, 1990, Ch. 4.
76 D.R. Jenke, *Pharm. Res.*, 8 (1991) 782–786.
77 D.R. Jenke, D.S. Hayward and R.A. Kenley, *J. Chromatogr. Sci.*, 28 (1990) 609–612.
78 L.R. Snyder and J.J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979, pp. 22–25.
79 R. Collander, *Acta Chem. Scand.*, 5 (1951) 774.
80 P. Seiler, *Eur. J. Med. Chem.*, 9 (1974) 473–479.
81 J.C. Dearden and G.M. Bresnen, *Quant. Struct.–Act. Relat.*, 7 (1988) 133–144.
82 A.F.M. Barton, *Chem. Rev.*, 75 (1975) 731–753.
83 R.M. McCormick and B.L. Karger, *Anal. Chem.*, 52 (1980) 2249–2257.
84 A. Bechalany, A. Tsantili-Kakoulidou, N. El Tayar and B. Testa, *J. Chromatogr.*, 541 (1991) 221–229.
85 R. Calvino, R. Fruttero and A. Gasco, *J. Chromatogr.*, 547 (1991) 167–173.
86 P.J. Schoenmakers, H.A.H. Billiet and L. de Galan, *J. Chromatogr.*, 185 (1979) 179–185.
87 A.J. Leo, *J. Pharm. Sci.*, 76 (1987) 166–168.
88 R.W. Spencer, L.J. Copp and J.R. Pfister, *J. Med. Chem.*, 28 (1985) 1828–1832.
89 W.J. Lambert, L.A. Wright and J.K. Stevens, *Pharm. Res.*, 7 (1990) 577–586.
90 K. Valko, L.R. Snyder and J.L. Glajch, *J. Chromatogr. A*, 656 (1993) 501.
91 P. Jandera, H. Colin and G. Guiochon, *Anal. Chem.*, 54 (1982) 435–441.
92 P.J. Schoenmakers, H.A.H. Billiet, R. Tijssen and L. de Galan, *J. Chromatogr.*, 149 (1978) 519–537.
93 W.E. Hammers, G.J. Meurs and C.L. De Ligny, *J. Chromatogr.*, 247 (1982) 1–13.
94 W.J. Lambert and L.A. Wright, *J. Chromatogr.*, 464 (1989) 400–404.
95 C. Yamagami and N. Takao, *Chem. Express*, 6 (1991) 113–116.
96 D.J. Minick, J.H. Frenz, M.A. Patrick and D.A. Brent, *J. Med. Chem.*, 31 (1988) 1923–1933.
97 C. Yamagami and N. Takao, *Chem. Express*, 7 (1992) 385–388.
98 S.H. Unger and G.H. Chiang, *J. Med. Chem.*, 24 (1981) 262–270.
99 D.D. Perrin, B. Dempsey and E.P. Serjeant, $pK_a$ *Prediction for Organic Acids and Bases*, Chapman and Hall, London, 1981.
100 J.C. Kraak, H.H. van Rooij and J.L.G. Thus, *J. Chromatogr.*, 352 (1986) 455–463.
101 C.M. Riley, E. Tomlinson and T.M. Jefferies, *J. Chromatogr.*, 185 (1979) 197–224.
102 M. Davies, P. Jones, D. Patraik and E.A. Moelwyn-Hughes, *J. Chem. Soc.*, (1951) 1249–1252.
103 G.E. Beredsen, P.J. Schoenmakers, L. de Galan, G. Bigh, Z. Varga-Puchone and J. Inczedy, *J. Liq. Chromatogr.*, 11 (1980) 1669.
104 T.L. Hafkenscheid and E. Tomlinson, *Int. J. Pharm.*, 16 (1983) 225–239.
105 M.S. Mirrlees, S.J. Moulton, C.T. Murphy and P.J. Taylor, *J. Med. Chem.*, 19 (1976) 615–619.
106 K. Miyake, N. Mizuno and H. Terada, *J. Chromatogr.*, 439 (1988) 227–235.
107 V. De Biasi, W.J. Lough and M.B. Evans, *J. Chromatogr.*, 353 (1986) 279–284.
108 K. Miyake, F. Kitaura, N. Mizuno and H. Terada, *Chem. Pharm. Bull.*, 35 (1987) 377–388.
109 A. Bechalaney, T. Rothlisberger, N. El Tayar and B. Testa, *J. Chromatogr.*, 473 (1989) 115–124.
110 M. Verzele and C. Dewaele, *J. Chromatogr.*, 217 (1981) 399–404.
111 J.R. Benson and D.J. Woo, *J. Chromatogr. Sci.*, 22 (1984) 386–399.
112 G. Thevenon-Emeric, A. Tchapla and M. Martin, *J. Chromatogr.*, 550 (1991) 267–283.
113 A.V. Kiselev, *J. Colloid Interface Sci.*, 28 (1968) 430–442.
114 J. Paleos, *J. Colloid Interface Sci.*, 31 (1969) 7–18.
115 D. Henry, J.H. Block, J.L. Anderson and G.R. Carlson, *J. Med. Chem.*, 19 (1976) 619–626.

116 K. Miyake and H. Terada, *J. Chromatogr.*, 240 (1982) 9–20.
117 C. Pidgeon and U.V. Venkataram, *Anal. Biochem.*, 176 (1989) 36–47.
118 E. Tomlinson, *J. Chromatogr.*, 113 (1975) 1.
119 R.F. Rekker, *J. Chromatogr.*, 300 (1984) 109.
120 R. Kaliszan, *J. Chromatogr.*, 220 (1981) 71.
121 C.B.C. Boyce and B.V. Milborrow, *Nature*, 208 (1965) 537.
122 J. Iwasa, T. Fujita and C. Hansch, *J. Med. Chem.*, 8 (1965) 150–153.
123 A.E. Bird and A.C. Marshall, *J. Chromatogr.*, 63 (1971) 313–319.
124 A.D. Kossoy, D.S. Risley, R.M. Kleyle and D. Nurok, *Anal. Chem.*, 64 (1992) 1345–1349.
125 F. Gago, J. Alvarez-Builla, J. Elguero and J.C. Diez-Masa, *Anal. Chem.*, 59 (1987) 921–923.
126 M.G. Khaledi, E. Peuler and J. Ngeh-Ngwainbi, *Anal. Chem.*, 59 (1987) 2738–2747.
127 W.L. Hinze and S.G. Weber, *Anal. Chem.*, 63 (1991) 1808–1811.
128 E.D. Breyer, J.K. Strasters and M.G. Khaledi, *Anal. Chem.*, 63 (1991) 828–833.
129 N. El Tayar, R.-S. Tsai, P. Vallat, C. Altomare and B. Testa, *J. Chromatogr.*, 556 (1991) 181–194.
130 A. Berthod and D.W. Armstrong, *J. Liq. Chromatogr.*, 11 (1988) 547–566.
131 H. Terada, Y. Kosuge, W. Murayama, N. Nakaya, Y. Nunogaki and K.-I. Nunogaki, *J. Chromatogr.*, 400 (1987) 343–351.