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Review

Hydrophobicity estimations by reversed-phase liquid chromatography

Implications for biological partitioning processes

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ABSTRACT

Liquid chromatography has long been used for the estimation of "hydrophobicity" of solutes of biological, environmental and agricultural interest. These measurements have taken the form of octanol–water partition coefficient estimation, or less often the more fundamental processes that the octanol–water partition coefficient is intended to model. Here we review both the chromatographic methods used for these estimations, their successes and failures, and discuss pertinent solution thermodynamics of the partitioning of small molecules between bulk phases, such as octanol and water, and between a bulk phase and an interphase, such as partitioning of solute molecules into lipid layers and biological membranes.

CONTENTS

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1. INTRODUCTION

This review is not intended to be a comprehensive review of all approaches to measuring octanol–water partition coefficients, or any of the processes that the octanol–water coefficient is intended to model. Rather, we have tried to take a critical look at the available literature and discuss approaches, philosophies, and remaining questions regarding the measurement of "hydrophobicity", bioavailability, bioaccumulation and other such interfacial partitioning processes. We have focused on the literature since the comprehensive reviews of Braumann [1] and Kaliszan [2], with only especially relevant earlier literature cited. In addition to these two important reviews, there have been several other reviews published [3–7].

Since the classic work of Overton [8] and Meyer [9] in 1899 showing a strong correlation between anesthetic action and olive oil solubility, scientists have tried to correlate solubility with many biological and environmental processes, such as bioavailability, bioaccumulation, and transport through soils. While the inherent logic in these studies is good —compounds must be soluble in lipid structures to have an effect on biological processes, the measurement of this solubility is an extremely difficult and expensive analytical problem. In 1985, for example, bioconcentration tests were estimated to cost US$ 6000–10 000 for each chemical, and acute toxicity tests were estimated at US$ 2000–3000 for each test [10]. As the exact measurement is difficult at best, there have been many approaches taken to develop methods of *estimation* of this solubility phenomenon. An entire area, generally known as quantitative structure–activity relationships (QSARs), has developed around these estimations, and a news report and a nice review of QSAR techniques have recently appeared [11,12]. While artificial intelligence and chemometric approaches are increasing in sophistication and usage, chemical methods of estimation are still far the most heavily used. Chromatographic techniques have been very heavily used for these studies, and here the technique is known as quantitative structure–retention relationships (QSRRs). However, as we will show below, the thermodynamic driving forces for biological partitioning and for bulk-phase hydrocarbon–water partitioning are generally based on opposite processes. That is, biological partitioning is generally *entropy* driven, and bulk-phase hydrocarbon–water partitioning is generally *enthalpy* driven. Efforts aimed at modelling the octanol–water process are quite possibly not the best approach to the problem.

The process that these estimates are designed to model is a *partitioning* process. The partitioning of solute molecules into lipid bilayers and biological membranes is the basis for drug and metabolite uptake, passive transport across membranes, and bioaccumulation. An excellent tutorial review on the process of bioconcentration has recently appeared [13]. These partitioning processes have most often been characterized with bulk thermodynamic models as though bilayer membranes were identical with bulk phases. Lipid bilayer membranes, however, have high surface to volume ratios, they are *interfacial* phases of matter. In interfacial phases physical properties vary with distance from the interface. In contrast, in bulk phases physical properties are uniform throughout. For example, there is a gradient of chain disorder in the hydrocarbon core of the bilayer; the surfactant chains are most highly aligned near the headgroups, and the order diminishes with distance toward mid-bilayer [14–16]. Moreover, the chain ordering of the bilayer phospholipids increases with surface density. Properties of interfacial phases depend on surface density whereas properties of bulk phases do not.

These structural differences between bilayers and other interfacial phases and bulk phases such as oil or octanol should be manifested as differences in the nature of solute partitioning into them. Recent theory [17,18] has predicted the following: (i) there will be an equilibrium gradient of solute concentration in the bilayer in contrast to the uniform distribution expected in a bulk phase; this prediction is consistent with neutron scattering experiments [19]; (ii) the partial chain ordering should disfavor solute retention in the bilayer relative to amorphous bulk phases; and (iii) solute uptake should decrease significantly with increased surface den-

sity of the chains. There is also evidence in favor of (ii) and (iii). Partition coefficients of many anesthetic agents into membranes are 2–15 times lower than their coefficients of partitioning into olive oil [20]. Entropies of transfer of short-chain hydrocarbons [21,22] and noble gases [23] into bilayers are more negative than that of transfer into amorphous hydrocarbon. More recent experiments [24,25] have measured the membrane–water partition coefficients of benzene into lipid bilayers as a function of the surface density of the phospholipid chains and have shown that partitioning into the bilayer is dependent not only on the partitioning chemistry, but also on the surface density of the bilayer chains. Increasing surface density leads to solute expulsion; benzene partitioning decreases by an order of magnitude as the surface density increases from 50 to 90% of its maximum value, a range readily accessible in bilayers and biomembranes under physiological conditions. This theory then predicts that partitioning models based on bulk phases cannot be an accurate representation of an *interfacial* partitioning process; the most commonly used bulk phase model being the octanol–water partition coefficient.

The often observed correlation of the octanol–water partition coefficient with biological partitioning processes is a fortuitous coincidence, which unfortunately sometimes fails. A serious problem with reliance on this measure, is the inability to *a priori* predict failure. The failure of the octanol–water modelling for bioaccumulation processes has been elegantly demonstrated by Opperhuizen *et al.* [26]. They investigated the thermodynamic properties of the partitioning of chlorobenzenes between fish lipids and water, and showed that bioconcentration is accompanied by *positive* enthalpy and entropy changes. The free energy of this transfer process at room temperature is dominated strongly by the favorable entropy contribution. In contrast, the partitioning of these compounds between octanol and water is accompanied by *negative* enthalpy and by small negative or positive entropy changes. They conclude that the differences in the thermodynamic properties of these processes arise from the different structures of fish lipids and octanol, and that only under very specific conditions and only for structurally similar compounds can a relationship between octanol–water partitioning and bioaccumulation be expected. Even the more comprehensive fugacity based models of bioavailability are generally based on an octanol–water relationship [27,28].

Cabani et al. have recently published a thorough study of the thermodynamics of transfer of small molecules between water and octanol [29]. By using gas phase transfers for both water and octanol, they were able to calculate enthalpies and entropies for *pure* octanol–water mixtures, as well as measure these values for mutually saturated solutions. They found that the values of the enthalpy of transfer from water to pure octanol are almost always positive, and more importantly, the entropic term always favors the transfer towards the octanol phase. These results are in disagreement with the results of Opperhuizen *et al.* [26], although this may be explained by the choice of compounds studied. Opperhuizen et al. studied chlorinated benzenes [26], while Cabani *et al.* [29] investigated alkanes and monofunctional saturated organics. They also found little effect on either enthalpy or entropy when comparing pure and water saturated octanol.

1.1. Log $P_{o/w}$

Fujita *et al.* [30] first proposed the octanol–water partition coefficient ($P_{o/w}$) as a measure of "hydrophobicity" in the early 1960s. Since that time it has become the *de facto* standard for measuring hydrophobicity, but the direct measurement of this partition coefficient is not trivial. Values of log $P_{o/w}$ which are in excess of about 7 are very difficult to measure, due to the very low concentration of solute which would be in one phase or the other, and the solute must be inherently pure and available in reasonable quantities. Liquid chromatographic approaches to $P_{o/w}$ values have been proposed since the very beginnings of modern liquid chromatography [31–33], and offer a number of advantages over the static methods. These include speed of determination, better reproducibility, ease of automation, the requirement of only a very small amount of the sample to be determined and the

capability of separating impurities in the sample simultaneously with the determination.

One of the greatest advantages of the log P scale is the possibility of calculating partition coefficient values from substitutent constants [34]. Such a system can be very beneficial in an area such as drug design as it obviates the need for synthesizing compounds. The development of a chromatographic based substituent scale has received some interest [2,5]. This, however, is a far more difficult task (as compared to the octanol–water partition system) due to the complexity of the retention process in RPLC.

There are now literally hundreds of papers reporting the use of RPLC for the estimation of octanol–water partition coefficients, with correlation coefficients reported from approximately 0.5 to 0.999, depending on the particular column and compounds tested. After years of study, however, there is still no universally accepted method of performing these estimations. The reasons are twofold. First, as we have argued above, octanol–water partitioning is an invalid thermodynamic model for many physiological and environmental events of interest. This leads to the conclusion that the RPLC experiments to date have been attempting an estimate of an estimate (or a model of a model). Secondly, the partitioning process of RPLC has only recently come to be understood on a microscopic level, and different chromatographic columns will behave differently when used for these estimates. Illustrative of this problem is the comparison of two recent reports correlating chromatographic retention with octanol–water partition coefficients. Thus and Kraak [35] reported that a phenyl bonded column gave significantly better correlations than an octadecyl (C_{18}) bonded phase. Minick *et al.* (36), using the *same* type of phenyl column, but a different octadecyl column, reported significantly better correlations with the C_{18} column.

2. APPROACH TO $P_{o/w}$ ESTIMATIONS

The application of linear free energy relationships in QSARs, QSRRs and quantitative retention–activity relationships (QRARs) can be expressed in terms of eqns. 1–3, respectively:

$$\log BA = S_1 \log P + I_1 \quad \text{(QSAR)} \tag{1}$$

$$\log P = S_2 \log k' + I_2 \quad \text{(QSRR)} \tag{2}$$

$$\log BA = S_3 \log k' + I_3 \quad \text{(QRAR)} \tag{3}$$

where BA represents biological activity, P is the octanol–water partition coefficient, and S and I represent the slope and intercept, respectively.

Collander described the extra-thermodynamic relationship between the partition coefficients in different solvent systems with similar underlying molecular interactions as [37]:

$$\log P_2 = S \log P_1 + I \tag{4}$$

A common underlying driving force for aqueous–organic media partitioning is the hydrophobic effect, that is, the repulsion of hydrophobic solutes from an aqueous medium into an organic environment. In other words, linear relationships in the form of eqn. 4 are observed for any two-phase aqueous–organic system as long as hydrophobic interactions play a predominant role. It may be useful here to carefully describe the hydrophobic effect. Dill [38] has described three common interpretations of the term: (i) any transfer of a non-polar solute to any aqueous solution; (ii) transfers of non-polar solutes into aqueous solution when a particular characteristic temperature dependence is observed, and (iii) particular molecular models, generally involving the ordering of water molecules around the non-polar solute. He also comments that definition (ii) seems to be the most popular, but in RPLC definition (i) is probably more common. There have been other recent descriptions of this phenomenon [39–41]. It is also important to point out that many usages of "hydrophobicity" in RPLC are incorrect! Typical RPLC mobile phases are mixtures of organic solvent and water, and in many common mobile phases (especially non-hydrogen bonding mobile phases) the hydrophobic effect is not observed [42]! Alvarez-Zepeda *et al.* [43] have described an elegant study of thermodynamic differences between methanol–water and acetonitrile–water mobile phases which is relevant to this problem. It is clear that much is left to be understood about the thermodynamics (and especially the entropies) of the transfer process of

solutes between the mobile and stationary phases in RPLC.

While a thorough review of reversed-phase retention mechanisms is not appropriate here, other papers in this issue are thoroughly covering these aspects, one important point is worth discussing. There are still many references in the current literature which cite the "solvophobic" theory of retention as our current level of understanding. The solvophobic theory is very important in the history of RPLC retention mechanisms, as it was the first quantitative approach to describing retention based on physical chemistry principles. There are two major shortcomings of this theory, however, which have negated its ability to correctly describe RPLC retention. As noted above, the hydrophobic effect is often *not* a driving force for retention in mixed solvent mobile phases, especially those using acetonitrile as the organic modifier. Secondly, the solvophobic theory treats the retention process as a one-phase transfer, and does not account for the interactions in the stationary phase. A thorough review of approaches to correctly describing RPLC retention was published by Dorsey and Dill [44].

Linear correlations in the form of eqns. 1–4 should not be misinterpreted as indications of identical phase properties or interaction mechanisms between various media as different as octanol, alkyl bonded phases, and biological membranes. The existence of other interaction forces and/or mechanisms of bonding would simply cause specific behavior for different groups of solutes. For example, using different organic solvents and octanol as a reference solvent, Leo *et al.* [45] observed different slopes and intercepts for Collander relationships for various classes of compounds with differing affinities for hydrogen bonding interactions. The partitioning of "congener" groups of solutes which belong to different classes but show similar interactions with different solvents could then be explained by a single equation.

The congenerity phenomenon has been observed to an even larger extent in the relationships between octanol–water partition coefficients and retention in RPLC [1–7]. This is due to the complex nature of the factors that contribute to retention in RPLC which is drastically different from the transfer of solutes between two isotropic aqueous and organic solvents. These problems have been discussed extensively in the past [1–7]. The focus of several papers since 1986 has been to solve the "congenerity" problem in the relationships between retention in RPLC and octanol–water partition coefficients; that is to find a single equation that relates log k' in RPLC to log P for a wide range of compounds that differ in size, shape, functional groups, and type of interactions. The primary approach has been to adjust the chromatographic conditions (*i.e.*, composition of mobile and stationary phases) such that the transfer of solutes from the aqueous media to the non-polar bonded phases of RPLC resembles closely the partitioning process in an octanol-water system. Subsequently workers have continued their search for the effect of mobile phase and stationary phase compositions on the log k' *vs.* log P relationships.

A more complex approach is to describe the retention behavior of solutes by a multiparameter equation that, in addition to hydrophobicity, includes other descriptor terms for different structural properties (*e.g.* hydrogen bonding parameters, size, etc). This approach, which falls in the category of QSRRs is mainly useful for the prediction of retention behavior and is directly relevant to structure–activity and retention–activity studies [1–7,46].

2.1. Mobile phase effects

An initial question which must be addressed in the chromatographic modelling of other partitioning processes is both the choice of organic modifier and its concentration. It has been generally agreed that the best measure of chromatographic retention is the capacity factor (k') with a mobile phase of 100% water (k'_w). The advantages of using k'_w are that it is independent of any specific organic modifier effects, it reflects polar–non-polar partitioning in a manner similar to shake-flask measurements, and it is dependent on the solute's structure and polar functionalities [47–49]. The k'_w parameter is difficult to measure directly, however, because of prohibitively long

retention times. The value is most often estimated by the extrapolated intercept of a plot of log k' *vs.* volume percent organic modifier. While these plots are linear for a narrow range of organic modifier, it is well known that deviation from linearity occurs if a wide range of organic modifier is examined [50,51]. Even more troubling is that for a given solute–column pair, extrapolation of log k' *vs.* volume percent organic modifier gives significantly different intercepts for different organic modifiers [52–55].

Johnson *et al.* [56] have shown that an empirical measure of mobile phase polarity based on the $E_T(30)$ solvatochromic solvent polarity scale gives a linear relationship with log k' and is generally a better descriptor of retention than commonly used plots of log k' *vs.* volume percent organic modifier. Michels and Dorsey [52] further found that extrapolation of retention plots for a solute–column pair using aqueous mobile phases of methanol, ethanol and acetonitrile lead to a common intersection point at approximately the $E_T(30)$ value of pure water. Using over 200 sets of chromatographic retention data, they compared the estimation of log k'_w by the volume percent approach and by the $E_T(30)$ approach and found that the $E_T(30)$ approach gave a more reliable estimation of this lipophilicity parameter [53]. Kuchar *et al.* [57] also recently reported that the use of the $E_T(30)$ parameter improved the goodness of fit of plots of slope (of k' *vs.* organic modifier concentration) *vs.* log P. Hsieh and Dorsey [58] have described a thorough comparison of measured *vs.* extrapolated k'_w values, and have found that while no extrapolated value gives a true estimate of the measured value for every solute, the $E_T(30)$ extrapolated value gives the most useful and reliable measure.

As well as these studies of the measurement or prediction of k'_w, the influence of mobile phase composition on the relationship between retention (log k') and octanol-water partition coefficient was examined in several papers. Hafkensheid [59] reported the influences of mobile phase methanol content and solute character on the log k' relationship with hydrophobic–lipophilic parameters for aromatic compounds. Good predictions were reported for either using single system isocratic retention factors or isocratic retention factors determined at any desired mobile phase methanol content. In either case, better correlations were observed by separate treatment of non-ionizable, acidic and basic solutes. It was also shown that the accuracy of predictions strongly depends on the "degree of mobile phase modification" (volume percent organic modifier).

Miyake *et al.* [60] also reported that the accuracy of predictions of log k' *vs.* log P was increased with a decrease in percent organic modifier in the RPLC mobile phase. It was shown that the retention behavior of a wide range of solutes which included non-H-bonders, H acceptors, and amphiprotic compounds can be explained by a single equation provided that the H bonding property of the compounds is considered.

In an effort to quantitatively define the concept of structural similarities (*i.e.*, congenerity), Valko [61] studied the effect of concentration of two organic modifiers (methanol and acetonitrile) on retention behavior of 59 compounds of different classes. The correlation coefficients for the linear fit of slope *vs.* intercept of log $k' = -S\varphi_{org} + \ln k_w'$ (*i.e.*, S *vs.* $\ln k_w'$), where φ is volume fraction organic modifier, was suggested as a quantitative measure of structural similarities with regard to their partition behavior in a RPLC system. Schoenmakers *et al.* [50] have previously observed a direct correlation between S and $\ln k'_w$ for a large group of compounds in methanol–water eluents.

Recanatini [62] studied the log k' *vs.* log P relationships for a group of chromons and flavones at different concentrations of methanol. Good linearity was observed for all compounds except ones with phenolic groups, at four concentrations and for the purely aqueous medium. Sabatka *et al.* [63] found a systematic deviation of log k' *vs.* log P relationships for a group of biphenyl acids as two separate lines were observed for the hydrogen bonding acceptors and non-hydrogen bonding substituents. They attributed this to the influence of steric effects on the solute–stationary phase interaction.

The influence of steric effects on chromatographic retention is a highly studied topic. San-

der and Wise [64] have published a thorough review of shape selectivity in RPLC, and have shown that different commercially available columns can differ dramatically in selectivity, being largely dependent on whether the stationary phase was prepared by the "monomeric" or "polymeric" bonding scheme. Sentell and Dorsey [65] have shown that shape selectivity of reversed-phase stationary phases depends on the bonding density of the grafted ligand, and have attributed this to entropy effects associated with the partitioning of the solute to the stationary phase. While this effect may be useful in more closely modelling biological partitioning processes, it is yet another variable that can complicate the LC determination of octanol–water or other partition coefficients. This is a good example of the "tunability" of chromatographic approaches to modeling other partition processes. While octanol–water, or any other bulk phase measure, would show a constant value for any solute of particular size or shape, different RPLC stationary phases would show different values.

Due to the dependence of log k' on the chromatographic phase composition, attempts have been made to find an alternative chromatographic parameter that is less dependent on the conditions and can be used as a continuous and universal scale. For example, instead of log k', Kaibara and co-workers [66–68] defined r-values, the slope of log k' *vs.* log $(1/\varphi_{org})$ as a good indicator for hydrophobicity and compared the effects of polar functional groups of solutes on the r values and log P. Both Diaz-Marot *et al.* [69] and Nieves *et al.* [70] used an RPLC retention index as the physicochemical parameter to characterize lipophilicity. As noted earlier, Kuchar *et al.* [57] used the slope of log k' *vs.* $E_T(30)$ values as the dependent variable against log P.

In a series of papers, Yamagami and co-workers [71–74] studied the relationship between RPLC retention factors and log P for various groups of heteroaromatic compounds and their ester derivatives as well as groups of pyrazines and pyridines at pH 9.2 on a Capcellpack C_{18} column [71–74]. They reported good linear correlations with eluents containing 50–70% of methanol. However, deviations from linearity were observed with water-rich eluents. This was attributed to the electronic and other specific effects involving the ester and amide groups. They even suggested that the log k'_w value is not suitable for the determination of log P of polar solutes. This is in contradiction with most reports which suggest that log k'_w is the best RPLC hydrophobic parameter in QSAR studies. However, the contradiction may not be so severe. As we have argued above, log P should not be well modelled by typical RPLC experiments. We will also show below that many biological partitioning processes *are* well modelled by the log k'_w concept. It has been suggested previously that log k'_w may be a better descriptor of the relevant partitioning process than log P [1].

The usefulness of micellar liquid chromatography (MLC) for the determination of hydrophobicity was also reported by several workers. In principal, MLC should be viewed as a mode of RPLC where micelles are used as the modifier of the aqueous mobile phase with a hydrocarbonaceous stationary phase. Retention is influenced by two competing equilibria of solute interactions with micelles in the mobile phase and their partitioning into the stationary phase. Hydrophobicity of solutes is the predominant influence on retention and their interactions with micelles. Gago *et al.* [75] reported good correlations (r^2 range 0.944–0.994) between log k' in MLC with different types of surfactants and log P for eleven monosubstituted benzenes. A similar result was observed for a methanol–water mobile phase. Lavine *et al.* [76] also observed similar correlations for 22 aromatic compounds using an MLC system with a liquid crystal stationary phase. Khaledi and Breyer [77] observed a much better correlation in MLC as compared to hydro–organic RPLC for a group of 35 compounds with various functional groups. Interestingly they reported excellent correlations for k' (instead of log k') *vs.* log P for both anionic and cationic surfactants on alkyl and phenyl bonded stationary phases. The log k' *vs.* log P plots consistently showed a curvature and had poorer linear correlation coefficients. To our knowledge, there are no reports of the correlation of micelle–water partitioning (K_{MW}) in

MLC with biological partitioning processes. The K_{MW} value is easily derived from retention measurements in MLC, and this partitioning should well mimic the relevant bio-partitioning process.

2.2. Ionizable solutes

The influence of solute ionization on RPLC determination of log P has been addressed in previous reviews [1–7]. In general, for the determination of hydrophobicity, the unionized form of solutes is taken as the reference state. This can be problematic in the RPLC determination of hydrophobicity of many acids and bases due to the limited pH operating range of silica bonded phases. In other words, it is not possible to obtain retention data for many acids and bases in their uncharged state. A number of solutions have been proposed in the past, some of which have been discussed in the previous reviews. These include estimation of the retention factor of the unionized form using a retention model which was originally developed by Horváth *et al.* [78].

Recently, Rittich and Pirochtova [79] used this methodology in a QSAR study of a group of aromatic acids. They observed improvements in correlations between the RPLC retention and log P at lower pH values (*i.e.*, unionized solutes). Interestingly both the logarithm of retention of the fully protonated and fully dissociated acids correlated well with their fungicidal activities in a direct QRAR study.

In addition to the limited pH range problem, organic bases can also interact with the unreacted silanol groups of the silica particles, thus complicating the description of retention in terms of hydrophobicity. The usefulness of ion pair RPLC in QSAR studies was originally demonstrated by Riley *et al.* [80]. Kraak *et al.* [81] also used methanol-water eluents, containing sodium dodecyl sulphate (SDS) as pairing-ion for the determination of hydrophobicity of organic bases as well as neutral and acidic solutes. Good correlations were observed for log k' *vs.* log P relationships for neutral and basic compounds. The correlations for bases improved at lower pH values where they exist in a fully protonated form which ensures a similar degree of electrostatic interaction in the ion-pairing system for all solutes. Due to the long retention times of some of the organic bases, 0.10 M sodium perchlorate was added to the mobile phase which reduced the overall retention of the solutes without an adverse affect on the relationship between log k' and log P. It was concluded that an ion-pairing system is also suitable for neutral and acidic solutes. They also compared an octadecyl with a phenyl bonded silica stationary phase and reported better correlations for the latter. Taylor *et al.* [82] studied both ion-pairing and non-ion-pairing conditions for the determination of the hydrophobicity of a set of 29 basic antimalarial drugs. For ion-pairing conditions they used the k'_{max} value to correlate with oil–water partition coefficients.

With advances in column technology, new possibilities have emerged in recent years for the analysis and determination of hydrophobicity of ionizable solutes. Polymeric resin stationary phases and alumina bonded phases have become attractive alternatives to silica bonded phases due to their wider pH operating range and lack of residual silanol groups.

De Biasi *et al.* [83] reported a correlation coefficient of 0.906 for log k' *vs.* log P for a group of eleven non-ionized bases using a polystyrene–divinylbenzene (PS–DVB) copolymer stationary phase (PRP-1) using a methanol–0.5 M NaOH (90:10) mobile phase. Apparently, the retentive nature of these stationary phases requires the use of an organic-rich mobile phase which is not a suitable condition for hydrophobicity determination of molecules. They observed no apparent correlation ($r = 0.2$) using a graphitized carbon stationary phase [83]. Lambert and Wright [84] also studied the PS–DVB reversed-phase materials, and correlated retention at 60% methanol with alkane–water partition coefficients. Bechalany and co-workers [85,86] compared an octadecylpolyvinyl (ODP), a PS–DVB and an octadecylsilane stationary phase for the assesment of lipophilicity, and found that the ODP and ODS stationary phase both gave good correlations. However, the ODS phase required a masking agent for the silanol sites. The PS–DVB stationary phases suffer from

lower efficiency and higher retentivity (especially for aromatic solutes) compared to the ODS phases.

Kaliszan and co-workers [87,88] reported the use of polybutadiene phases coated on alumina support particles. These stationary phases also have a wide operating pH range without the interfering silanol groups. They observed high correlation ($r = 0.96$) for a group of 24 acidic, basic, and neutral solutes. They further demonstrated the usefulness of these phases in QRAR studies of a group of pharmacologically active azole derivatives that possessed different circulatory activity. They observed correlation coefficients of 0.915 and 0.944 at pH values of 7.3 (physiological pH) and 11.5 (at which solutes are unionized) between log k' and log P. Correlation coefficients of 0.784 and 0.856 were observed between the anti-aggregatory activity of the solutes and their log k' at the two pH values respectively. Haky and Vemulapalli [89] also studied an octadecyl-bonded alumina for lipophilicity estimation. For a wide range of compounds, including phenolics, they found better correlation of retention with log P for the alumina column compared to an ODS, octadecyl–PS–DVB, or a polybutadiene-coated alumina stationary phase.

2.3. Stationary phases

The question of the stationary phase in RPLC measurements of hydrophobicity is an enigma. On the one hand, when modelling octanol–water coefficients, the octanol phase is fixed and unchanging. This means that the variability of the commercial columns adds great complications to the universal measurement of $P_{o/w}$ values. On the other hand, the stationary phase provides an *adjustable* parameter for the tuning of hydrogen bonding effects in the stationary phase, for the control of the chemistry of the interaction, whether phenyl or alkyl chains or other groups are bonded to the silica, and, if the chain density of the bonded alkyl chains is high enough, it provides a true *interphasic* partitioning site for the better modelling of bio-partitioning processes.

The influence of the stationary phase in the determination of partition coefficients by RPLC has been the focus of several studies. First from the standpoint of finding stationary phases whose interactions with solutes closely resemble those of octanol or biomembranes. It is expected that most solutes will have a congener retention behavior on phases which correlate well with the partition process. The second area of study has been the problem of pH stability and residual silanols, discussed above.

In a review, Braumann [1] collected log k' *vs.* log P relationships from the literature until 1986 for a wide range of compounds under different chromatographic conditions. Not surprisingly, the slopes and the intercepts of the equations varied greatly with the nature of solute group, the mobile phase composition and the type of alkyl bonded stationary phase. This variation in chromatographic results is considered a major drawback especially as compared to water–octanol, which at least in principle, should provide a continuous log P scale. In practice, however, the difficulties in accurate and precise measurement of log P values have created a similar situation; that is, one can find widely different log P values for a given compound. Chemometric approaches to the *calculation* of log P values have received much interest for this reason [34].

Braumann *et al.* [90] later studied the retention behavior of neutral benzene derivatives of six different alkyl bonded stationary phases in methanol–water eluents. The goal of the study was to evaluate the suitability of RPLC hydrophobic parameters. The stationary phases varied in terms of the functionality of the silane reagent, endcapping, the surface area of the bonded phase, the carbon content and the concentration of residual silanol groups. They observed retention factor variations of 20–30% on different phases. The strong dependence of retention on column properties showed the difficulty of using log k' as a hydrophobic parameter in place of log P. Interestingly, however, they observed that log k'_w values were basically independent from the column specifications. As a result they have recommended that log k'_w can be used directly as a hydrophobic parameter in QSAR studies.

The results presented by Sherblom and

Eganhouse [54], however, were somewhat different. They compared published log k'_w values for a group of chlorobiphenyls and alkylbenzenes with values determined in their laboratory. They observed a dependence of the log k'_w values on both the mobile phase and the chromatographic system. Separate regressions were performed on log k'_w *vs.* log P for individual and combined compound classes. Similar slopes but different intercepts were observed for the two compound classes.

While alkyl bonded phases (mainly C_{18}) are the most widely used phases, the suitability of other functional groups has also been examined. In 1985, Thus and Kraak [35] observed significantly better correlations between log k'_w determined on a phenyl bonded phase and log P for a group of 29 aromatic compounds. However, as noted earlier, other workers found the opposite [36]. Pietogrande *et al.* [91] also described a similar study of the comparison of log k'_w *vs.* log P correlations on octadecyl, phenyl, and cyano phases for a series of benzodiazepines. It was concluded that the phenyl column is best for log P predictions, cyano the most sensitive to the particular solutes moieties, and ODS "allows to obtain the most sensible measure of of solute hydrophobicity". Khaledi and Breyer [77], using a micellar mobile phase also observed better correlations on a phenyl column as compared to a C_{18} stationary phase.

Miyake *et al.* [92] used a phospholipid-coated silica gel in the determination of log P. High correlation ($r = 0.983$) was observed for log P *vs.* log k' for a group of 52 non-hydrogen bonders, hydrogen bond acceptors and amphiphilic solutes using a dipalmitoyl phosphatidylcholine stationary phase and an acetonitrile–water (20:80) mobile phase.

Szabo *et al.* [93] described a unique approach for the prediction of the adsorption coefficient of solutes to soil by chemically bonding humic acid to silica. They compared the correlation of adsorption coefficients to log k'_w values determined on this column and on an ethyl-silica phase, and adsorption coefficients correlated to $K_{o/w}$. They found the humic acid phase gave significantly improved correlations over the other methods.

Interestingly, to our knowledge, there have been no descriptions of correlation of biologically relevant events with k'_w values as a function of alkyl chain bonding density. While it can be argued that the bonded alkyl chains provide an environment similar to a membrane, the chain density of most commercial columns is much too low. Sentell and Dorsey [94] and Cole and Dorsey [95] have shown that as the alkyl chain density increases, the retention process changes from an enthalphically driven to an entropically driven process. Hsieh and Dorsey [96] have just completed a study of the correlation of biological partitioning processes with k'_w values for a high and a low bonding density C_{18} stationary phase, and have found that the high density phase gives equivalent or better correlations than the low density phase. The ability to tune the enthalpic and entropic contributions gives great promise for the design of stationary phases which will better model the relevant bio-partitioning processes.

3. QSRRs

The goal of QSRR studies is to predict retention behavior based on structural properties of solutes. The linear relationship between log k' and log P is the simplest example of QSRR, however, in a majority of papers multiparameter equations are developed in order to describe the behavior of a wide range of non-congener compounds. This review is not intended to be a comprehensive listing of all reports of RPLC approaches to log P determination. The papers cited are intended to give a survey of the approaches taken to this problem.

Funasaki *et al.* [97] showed linear correlations between log k' and log P as well as with other structural descriptors such as molecular cavity surface area and molecular connectivity indices for alcohols and ethers at different mobile phase compositions. The QSRR study was conducted for the prediction of log P as well as for the retention prediction of other compounds. Opperhuizen *et al.* [98] conducted an extensive study of retention behavior of alkyl benzenes, chlorotoluenes, chloronaphthalenes and chlorobiphenyls on an ODS column at different methanol compositions and temperatures. Based on the evaluation of the thermodynamic data, they

concluded that the distribution process for benzene, naphthalene and biphenyl are different. Interestingly, based on similar slopes for plots of group selectivity vs. enthalpy change in retention caused by different functional groups it was suggested that chlorine and methylene groups made comparable contributions to retention of the different aromatic hydrocarbon parent compounds. Based on the differences in retention behavior of different classes it was then concluded that the possibility of predicting log P from isocratic RPLC retention data would be limited.

Noel and Vangheluwe [99] predicted the retention behavior of a group of diols from the combination of log P, the Wiener index and the first order valence molecular connectivity index. Miyake *et al.* [100] reported that the retention behavior of a group of non-congeneric compounds (that included non H-bonders, H-acceptors and amphiprotic solutes) on both an ODS column and a glycerol-coated controlled pore glass stationary phase could be described by a single equation that combined log P with hydrogen bonding parameters. On both columns, the influence of H-bonding became smaller with a decrease in φ_{org}.

Kuchar *et al.* [101] used the enthalpy–entropy compensation effect in RPLC for a group of aromatic acids to gain information about the effect of intramolecular interactions on retention and its relationship with hydrophobicity. Tipker *et al.* [102] compared the log P values predicted by RPLC with those calculated from substituent constants for 49 aryl sulphoxides. An improvement in correlations was observed upon the introduction of Hammett sigma values as a measure of electronic effects.

The use of multiparameter equations of the description of retention behavior in terms of hydrophobicity, resonance and inductive effects was reported by Siwek and Sliwiok [103] for isomeric methylquinolines. Gago *et al.* [104] used RPLC for the determination of log P values of a group of pyridines, based on which they calculated the substituent values for different functional groups and compared with those obtained using benzene as the parent compound. They observed similar values only for halogen and methoxy groups in 3- and 4-positions.

Yamagami *et al.* [105] observed good linear relationships between log k' and log P for a group of pyrazines and pyridines, excluding hydrogen bond donors, with eluents containing 50–70% methanol. Interestingly, as the methanol content decreased (*i.e.*, a more water rich mobile phase) the linearity decreased which required the addition of correction terms for electronic and specific effects attributed to ester and amide groups. In all cases, shorter retention than that predicted based on log k' *vs.* log P relationships was observed for amphiphilic substituents which suggested that they act as hydrogen donors.

Woodburn *et al.* [106] observed two different linear relationships between log k' with log P (as well as a connectivity index and hydrophobic surface area), one for polycyclic aromatic compounds and halobenzenes and one for alkylbenzenes. This confirmed the previous observations that in RPLC, in addition to solute hydrophobicity, molecular size, shape, and conformation can play an important role. This conclusion, along with the fact that hydrogen bonding can also be a major retention contributor, shows the large differences that exist between an RPLC and an octanol–water system. Using multiple linear regression analysis, Wells and Clark [107] evaluated functional group retention increments of substituted benzanilides and benzamides. The magnitude of the substituent constants were dependent on the mobile phase and stationary phase compositions. Using a bilogarithmic analysis of the data, standardized retention increments were developed that seemed to be independent of the chromatographic conditions.

Kamlet et al. combined the linear solvation energy relationship in RPLC with a corresponding equation that describes the relationship between log P and the solvatochromic parameters. This generated new equations that demonstrated the exact relationship between log k' in RPLC and log P [108].

4. QRARs

We will not discuss the myriad of papers which correlated log P with biological activity. Rather, we will focus on those papers that used a *chromatographic* parameter, often log k'_w, to

correlate with other relevant bio-partitioning processes. It is interesting to note that there are many discrepancies in the literature about whether log P or log k'_w best predict the relevant bio-partitioning event. There does not appear to be one answer to this question. Certainly log k'_w cannot perfectly model every other partitioning process. It is also likely that the wide variety of stationary phases which are used play a large role in the success (or failure) of some of the correlations. These are questions which will take many well planned experiments to answer.

Braumann [1] was one of the first to recommend the log k'_w value as a replacement for log P. In a 1983 publication of the study of some herbicides, Braumann *et al.* [109] noted that log P and log k'_w are not completely interchangeable. Their results suggested that log k'_w might be a better model for the assessment of the hydrophobicity of drugs in biological systems.

There are several other studies which have compared log P and log k'_w as descriptors of hydrophobicity. Perhaps the most comprehensive study was by Mailhot [110] who compared ten physicochemical properties for the prediction of algal bioaccumulation and uptake rate of nine organic compounds. She found that when all nine compounds were used in the regression, k' (in 65% methanol) and log P predicted bioconcentration equally. However, if the hydrocarbons alone were considered, k' was the most effective. Minick *et al.* [111] studied bovine serum albumin binding constants and found that the two descriptors were statistically indistinguishable. Hodson and Williams [112] studied the adsorption coefficient of soils, and found using a cyanopropyl column that correlations of log k' with log $K_{o/c}$ (ratio of adsorbed chemical per unit mass of organic carbon to the concentration of the chemical in the aqueous phase) were better than that between log $K_{o/c}$ and log P.

Rittich *et al.* [113,114] studied the antifungal activity of some aliphatic and aromatic acids and compared correlations with log P, with log k'_w and with the first order molecular connectivity index, with no clear consensus. De Voogt *et al.* [115] studied bioconcentration of neutral heteroaromatic hydrocarbons in guppies and compared the utility of log k'_w for predicting bioconcentration and for *predicting* log P, but did not correlate log P with the bioconcentration factor.

Other studies using either k' or log k'_w values as the lipophilicity index included protein binding of xanthine derivatives to guinea pig serum albumin [116], biological activity of cardiac glycosides and steroid hormones [117], micellar cholesterol-solubilizing capacities of bile salts [118], antituberculous activity of dipyridylsulphides [119], 8-substituted xanthines as phosphodiesterase inhibitors [120], mosquito repellent amides [121] and cholesterol-solubilizing capacity and membrane disruption of bile acids [122].

Yvon *et al.* [123] studied the solubility of peptides in trichloroacetic acid solutions, and found that the best correlation was with the retention time of the peptide. The assessment of proteolysis levels is often achieved by global quantification of the peptides soluble at different trichloroacetic acid concentrations, but little information is available on the features of this precipitation mechanism.

Breyer *et al.* [124] reported the first successful application of micellar liquid chromatography to quantitative-retention activity relationships. They observed high correlations between the bioactivity of a group of 26 substituted phenols and an isocratic MLC retention factor. The QSAR required the use of three descriptors of solute properties (log P, pK_a and R, a resonance parameter) to achieve a similar correlation. This was attributed to the fact that the information on hydrophobic and electrostatic interactions is already incorporated in retention data in MLC.

5. CONCLUSIONS

Several statements can be made about the current status of hydrophobicity estimations. First, while octanol–water partition coefficients are still the most popular descriptor of bio-partitioning processes, other estimations of bioavailability, bioaccumulation and soil transport are becoming more popular. This is being driven both by theory, which has shown that log P has little thermodynamic basis for the prediction of

interphasic partitioning, and also by the success of others. As more reports appear showing the ability of chromatographic retention to model these processes, more workers will become aware of these approaches. The choice of stationary phase for these estimations is far from clear. There is much left to understand about hydrogen-bonding effects, the effects of residual hydroxyl sites, the effects of alkyl-chain surface density, and many others. For example, can the phospholipid structure of membranes be mimicked by stationary phases such as those developed by Markovich *et al.* [125]. Also, to our knowledge there have been no studies of these correlations as a function of temperature. Yet there are many reports in the literature showing that reversed phase retention is dramatically affected by temperature. Temperature effects can result in elution order reversal and in dramatic changes in selectivity, yet this has yet to be addressed here. Thermodynamic studies of bio-partitioning processes, such as the one by Opperhuizen *et al.* [26] are needed to understand how RPLC can be best used for these estimations. Is the biological temperature the best to use, or would very low temperature be best, where chain ordering is increased and the entropic contribution to retention is enhanced?

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