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Applications of immobilised artificial membrane chromatography to quaternary alkylammonium sulfobetaines and comparison of chromatographic methods for estimating the octanol–water partition coefficient

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Abstract

The chromatographic behaviour of a series of quaternary alkylammonium sulfobetaines of general formula $RN^{+}(CH_3)_2(CH_2)_nSO_3^{-}$, where n=2–4, has been examined using an immobilised artificial membrane column. The k values have been correlated both with experimentally determined values for the octanol–water partition coefficient (P) and with aquatic toxicity measurements. Other indirect chromatographic methods for measuring log P for these compounds using reversed-phase columns and octanol-coated columns have also been investigated.

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1. Introduction

Quantitative structure–activity relationships (QSARs) based on the octanol–water partition coefficient (P, usually expressed as $\log_{10} P$) are well established and provide reliable mathematical models for predicting aquatic toxicity. Although other physicochemical properties may be involved, hydrophobicity is clearly a key factor [1]. QSARs for predicting aquatic toxicity of nonionic and anionic surfactants have been developed and a log P based QSAR for cationics has recently been reported [2]. We have ourselves developed a log P based QSAR for predicting the aquatic toxicity of zwitterionic sulfobetaines which shows good correlation between log P and log $(1/EC_{50})$ [3]. However, it is recognised that octanol and water may not be the most realistic model for assessing transport phenomena across a cell membrane [4,5]. As a result other models involving the use of chromatography columns coated with phosphatidylcholine (PC) or containing an immobilised artificial membrane (IAM) have been developed [6,7]. We therefore decided to

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compare the efficacy of using log k_{IAM} and log P as parameters for predicting aquatic toxicity.

In addition we have investigated other chromatographic methods for estimating log P. The conventional stir-flask method for determining log P is reliable and the results obtained for short chain sulfobetaines show good reproducibility [3]. However conducting stir-flask experiments is time consuming. It involves achieving mutual saturation of the octanol–water layers, partitioning of the substrate between the two layers, and quantitative analysis of aliquots from each layer. Measuring retention indices by chromatography is more appealing in terms of efficiency. Other advantages include the fact that there is no risk of emulsions being formed, there is precision in the determination of log P values over a wide range (+4 to −4), impurities that can affect partition do not affect retention times, and only small quantities are required compared to the relatively large amounts required for stir-flask experiments [8].

1.1. IAM chromatography

PC is the major phospholipid found in all cell membranes. IAM chromatography phases prepared from PC analogues therefore closely mimic the surface of the biological cell membrane. Such high-performance liquid chromatography (HPLC) columns are commercially available from Regis Technologies [9].

IAM chromatography has proved successful in drug discovery [10,11], and has recently gained acceptance for estimating the membrane permeability of small molecule drugs. For these compounds, the membrane partition coefficient defines the rate-limiting step for drug absorption. The technique has been shown to provide superior correlation with experimentally determined drug permeability compared to other chromatographic methods for determining hydrophobicity parameters, such as k_{C8} and k_{C18} (see later). These alkyl bonded phases retain analytes solely on the basis of hydrophobicity, in contrast to IAM, where a combination of hydrophobic, ion pairing and hydrogen bonding interactions are possible, all of which are expected to be important in membrane transport. This combination of interactions has been described as phospholipophilicity [9].

The IAM PC phase consists of monolayers of amphiphilic phospholipids covalently immobilised on aminopropyl silica particles through an amide linkage. The resulting IAM surface is chemically stable emulating the exterior of a biological cell membrane [12]. The column has found numerous applications, including the prediction of solute transport across human skin [9], predicting amino acid transport across the blood–brain barrier [13], and mimicking the binding of solutes to liposome membranes [9]. Since PC is the major phospholipid of all cell membranes, we decided to test the use of log k_{IAM} as a parameter to model the transport of zwitterionic sulfobetaines across the surface membrane of the water flea, *Daphnia magna*, and hence predict aquatic toxicity.

The IAM PC phase has progressed since 1995 when it was first developed. The first modification was in the form of methylglycolate end-capping, converting residual amines to neutral amides and introducing a hydroxyl group (IAM PC MG). Secondly, the IAM PC DD material was developed, consisting of end-capping with C_{10}/C_3 alkyl chains. The IAM PC DD column, however, has recently been replaced by the IAM PC DD2 column. Generally excellent correlation is reported between the DD and DD2 columns, but the extra hydrophobicity offered by the ester bonding of the DD2 column provides longer retention times for compounds not well retained on the DD packing. Retention times are typically double for analytes run on the DD2 column compared with the DD column. Generally, the DD and DD2 columns are used in drug membrane permeability studies while the earlier IAM phases (PC and PC MG) are more commonly used for protein purification [9]. Due to the hydrophilic nature of many of the sulfobetaines, it was predicted that the DD2 column would provide the best retention. We were initially worried that the sulfobetaines with lowest log P would not be retained, but this proved not to be the case. They did have short retention times but were satisfactorily retained compared with citric acid, which was used as the t_0 marker.

1.2. Chromatographic methods for measuring log P

HPLC offers a number of methods for estimating log P values, either indirectly or directly. The most

widely used indirect HPLC method involves measuring the retention time of a given analyte on a C_8 or C_{18} reversed-phase (RP) column and then calculating log *k* [14–18]. This method obviously requires calibration to establish a relationship between log *P* and log *k* for a series of similar reference compounds, before using the method to determine log *P* for the analytes themselves. There have been a number of publications confirming excellent correlation between log *k* (C_8 or C_{18}) and log *P* for simple, neutral compounds, but correlations for ionic and more complicated molecules are less common.

The greatest errors between log *P* determined by this method and reported log *P* values (determined by calculation or by conventional methods) are observed for polar compounds that dissociate in water. Here, dissociation of ionisable polar groups is more significant than adsorption interactions. These chemicals therefore elute more rapidly than expected. The OECD guidelines also acknowledge that the method is not applicable to strong acids and bases, metal complexes, substances that react with the eluent, or surface-active agents [18]. However measurements may be performed on ionisable substances in their non-ionised form by using a buffer at a pH below the pK_a of the free acid or above the pK_a of the free base [19,20]. When log *P* is determined for use in environmental risk assessment, the test should clearly be performed in a pH range relevant to the natural environment (i.e., 5.0–8.5).

Finally, we were interested in investigating the possibility of using a more direct chromatographic method for determining log *P* of the sulfobetaines. One such method involves the measurement of log *k* on an octanol-coated column [21–23]. This can be achieved by coating an RP C_8 or C_{18} column with water-saturated octanol and using octanol-saturated water as the mobile phase. Excellent correlation has been reported between log *k* measurements and log *P* for neutral compounds. Whether a good correlation would be observed for the sulfobetaines was expected to depend on whether the octanol could be considered to be mobile or immobile (see below), since this has been reported to affect the performance of this method when studying homologues [21–23].

2. Experimental

The compounds utilised in this study were a series of short chain zwitterionic sulfobetaines **1**–**22** (Fig. 1) [24]. These compounds belong to three sub-series varying in the length of the spacer unit separating the quaternary ammonium centre from the sulfonate group. In addition, compounds **8**–**13** and **17**–**22** contain an aromatic ring which is separated from the quaternary ammonium centre by up to four methylene groups and, in the case of **12**, **13**, **21**, and **22**, the aromatic ring also carries a *para* alkyl substituent.

Retention times were determined using a HP1100 instrument using a UV detector set at 266 nm for analysis of the unsubstituted arylsulfobetaines and at

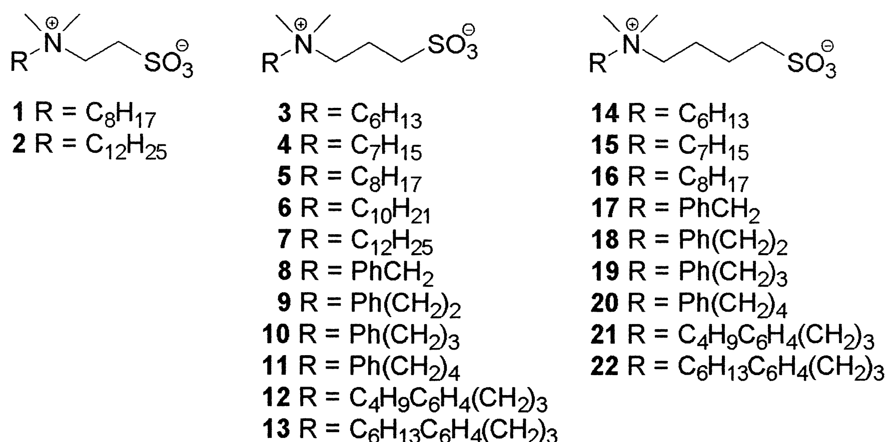

Fig. 1. Structures of zwitterionic sulfobetaines.

220 nm for analysis of the *para*-substituted arylsulfobetaines. Electrospray mass spectrometry (MS) (measuring total ion current) was employed for detection of the alkylsulfobetaines, since they lacked a suitable chromophore for UV detection. All chromatographic retention data were taken as the mean of three determinations.

2.1. Measurement of log k_{IAM}

Samples for HPLC–UV were made up to a concentration ~1 mg/ml and for HPLC–electrospray ionisation (ESI) MS to a concentration of ~10 μg/ml. The mobile phase was 100% water, which was eluted at 1 ml/min. Citric acid was used as the t_0 marker and was co-injected with each analyte. The results obtained are shown in Table 1. Since *N*-[3-(4-hexylphenyl)propyl]-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (**13**) and *N*-[3-(4-hexylphenyl)-propyl]-*N*,*N*-dimethyl-4-ammonio-1-butanesulfonate (**22**) did not elute within 1.5 h, k_{IAM} for these compounds was determined at various percentage compositions using an organic modifier (acetonitrile) and k_{IAM} was then determined by extrapolation [25]. Log *P* values determined by the stir-flask method and aquatic toxicity values, expressed in terms of 48 h EC_{50} to the water flea *Daphnia magna* [3], for the compounds are listed in Table 2.

Table 1
Measurement of log k_{IAM}

Compound	Average t_R (min)	Average t_0 (min)	k_{IAM}	Log k_{IAM}
3	2.310	0.950	1.43	0.156
5	6.420	0.950	5.76	0.760
6	41.17	0.950	42.3	1.63
8	1.941	0.969	1.00	$1.34\cdot10^{-3}$
9	2.267	0.963	1.35	0.132
10	3.132	0.951	2.29	0.562
11	2.801	0.951	1.95	0.289
13	–	–	–	2.04[a]
15	3.970	0.950	3.18	0.502
16	7.750	0.950	7.16	0.855
17	2.120	0.939	1.26	$9.96\cdot10^{-2}$
18	2.506	0.961	1.61	0.206
19	5.076	0.956	4.31	0.634
20	4.421	0.951	3.65	0.360
22	–	–	–	2.03[a]

[a] Log k_{IAM} for compounds **13** and **22** was determined by extrapolation (see Experimental).

2.2. Measurement of log k_{C18}

Retention measurements were performed on a Genesis C_{18} (4 μm, 150×4.6 mm) column. Uracil was used as the t_0 marker and was co-injected with each analyte. Samples were made up to a concentration ~2 mg/ml. The mobile phase was water–acetonitrile (ACN) (80:20). The flow-rate was 1 ml/min. The results obtained are shown in Table 3. k_{C18} values for **13** and **22** were determined at various compositions and then extrapolated to obtain the value in water–ACN (80:20).

2.3. Measurement of log $k_{(octanol\text{-}coated\ C8)}$

Retention measurements were performed on a Genesis C_8 (4 μm, 150×4.6 mm) column, previously coated with water-saturated octanol. Water-saturated octanol and octanol-saturated water were prepared by allowing mutual saturation of water (HPLC

Table 2
Comparison of log *P* and log k_{IAM} with aquatic toxicity

Compound	Log $P_{expt.}$	Log k_{IAM}	Log $(1/EC_{50})$
1	0.29	–	2.44
2	1.79	–	4.20
3	−1.22	0.156	2.71
4	−1.97	–	1.74
5	−0.47	0.760	1.97
6	0.57	1.63	2.87
7	1.65	–	3.99
8	−2.27	$1.34\cdot10^{-3}$	1.46
9	−1.87	0.132	1.84
10	−1.57	0.562	–
11	−1.17	0.289	–
12	0.55	–	3.70
13	1.89	2.04	3.82
14	−1.08	–	1.71
15	−1.23	0.502	1.62
16	−0.36	0.855	2.07
17	−2.32	$9.96\cdot10^{-2}$	–
18	−2.06	0.206	1.64
19	−1.70	0.634	–
20	−1.41	0.360	–
21	0.30	–	2.89
22	1.64	2.03	3.46

Table 3
Relationship of log P and log k_{IAM} with log k_{C18}

Compound	Log $P_{expt.}$	Log k_{IAM}	Average t_R (min)	Average t_0 (min)	k_{C18}	Log k_{C18}
8	−2.27	$1.34\cdot10^{-3}$	2.196	1.711	0.283	−0.548
9	−1.87	0.132	2.765	1.715	0.612	−0.213
10	−1.57	0.562	3.859	1.717	1.25	0.0960
11	−1.17	0.289	6.773	1.711	2.96	0.471
12	0.55	–	31.57	1.635	18.3	1.26
13	1.89	2.04[a]	–	–	–	2.06[a]
17	−2.32	$9.96\cdot10^{-2}$	2.213	1.711	0.293	−0.533
18	−2.06	0.206	2.897	1.715	0.689	−0.162
19	−1.70	0.634	3.976	1.717	1.32	0.119
20	−1.41	0.360	7.110	1.711	3.16	0.499
21	0.30	–	32.84	1.673	18.6	1.27
22	1.64	2.03[a]	–	–	–	2.04[a]

[a] Log k_{IAM} and log k_{C18} for compounds **13** and **22** were determined by extrapolation (see Experimental).

grade) and octanol (HPLC grade). Coating was performed by passing water-saturated octanol through the column at a flow-rate of 0.5 ml/min [21]. After approximately 3 h a stable baseline was obtained. The t_0 marker employed was KNO_3 and this was injected on its own prior to the injection of each analyte. Samples were made up to a concentration ~2 mg/ml. The mobile phase employed was octanol-saturated water, which was eluted at 0.5 ml/min or at 3.5 ml/min. The results obtained at the two different flow-rates are shown in Tables 4 and 5. The retention times for *N*-[3-(4-butylphenyl)propyl]-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (**12**) and *N*-[3-(4-butylphenyl)propyl]-*N*,*N*-dimethyl-3-ammonio-1-butanesulfonate (**21**) at 0.5 ml/min were determined using higher flow-rates and then extrapolating to obtain the value at 0.5 ml/min.

3. Results and discussion

3.1. Correlation of log P and log k_{IAM} with aquatic toxicity

The correlation between log P and log k_{IAM} is shown in Fig. 2 and is summarised by Eq. (1):

$$\log k_{IAM} = 0.49(\pm0.08)\log P + 1.13(\pm0.12)$$
$$n = 15, R^2 = 0.9253, s = 0.19, F = 160.9 \quad (1)$$

Table 4
Relationship between log P and log $k_{OC/C8}$ at 0.5 ml/min

Compound	Log P	Average t_R (min)	Average t_0 (min)	$k_{OC/C8}$	Log $k_{OC/C8}$
8	−2.27	1.935	1.653	0.171	−0.768
9	−1.87	2.306	1.653	0.395	−0.403
10	−1.57	2.651	1.653	0.604	−0.219
11	−1.17	4.362	1.653	1.64	−0.215
12	0.55	36.18	1.653	20.9[a]	1.32
17	−2.32	1.910	1.653	0.155	−0.808
18	−2.06	2.268	1.653	0.372	−0.429
19	−1.70	2.595	1.653	0.570	−0.244
20	−1.41	4.195	1.653	1.54	0.187
21	0.30	32.97	1.653	18.9[a]	1.28

[a] t_R for compounds **12** and **21** at 0.5 ml/min was determined by extrapolation (see Experimental).

Table 5
Relationship between log P and log $k_{OC/C8}$ at 3.5 ml/min

Compound	Log P	Average t_R (min)	Average t_0 (min)	$k_{OC/C8}$	Log $k_{OC/C8}$
12	0.55	11.90	0.365	31.6	1.50
13	1.89	53.74	0.365	146	2.17
21	0.30	10.18	0.365	26.9	1.43
22	1.64	42.77	0.365	116	2.07

It is clear that over a reasonably wide range, log P and log k_{IAM} show a good correlation, despite the fact that log P is a measure of hydrophobicity, while log k_{IAM} is measure of phospholipophilicity. In addition, it should be noted that, while log P can be regarded as a three-dimensional model of the lipid membrane, log k_{IAM} is best regarded as a two-dimensional model. However, the direct relationship between log k_{IAM} and log P is less impressive for close homologues, which would be expected to show small, incremental differences in both hydrophobicity and phospholipophilicity. Although the trend in log P (measured by the stir-flask method) appears to be uniform for close members of an homologous series, e.g., compounds **8–11** and **17–20**, this trend is not reflected in the log k_{IAM} values.

The fact that the slope of Fig. 2 is far from unity supports the idea that the two processes being compared are distinctly different. Previous authors have proposed that, in equations correlating log k with log P, the slope is an estimate of how closely the free energies of the processes compare [19,20,26].

The correlations between log k_{IAM} and aquatic toxicity, and between log P and aquatic toxicity, are shown in Figs. 3 and 4, respectively, and are summarised in Eqs. (2) and (3). As can be seen there is a better correlation between log k_{IAM} and aquatic toxicity than between log P and aquatic toxicity.

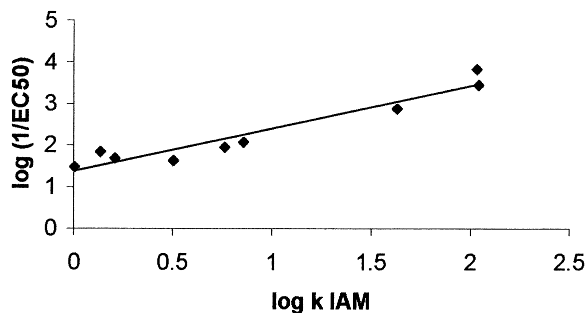

Fig. 3. Relationship between log k_{IAM} and aquatic toxicity.

$$\log(1/\mathrm{EC}_{50}) = 1.03(\pm 0.22)\log k_{IAM} + 1.38(\pm 0.26)$$
$$n = 9, R^2 = 0.9258, s = 0.25, F = 87.3 \quad (2)$$

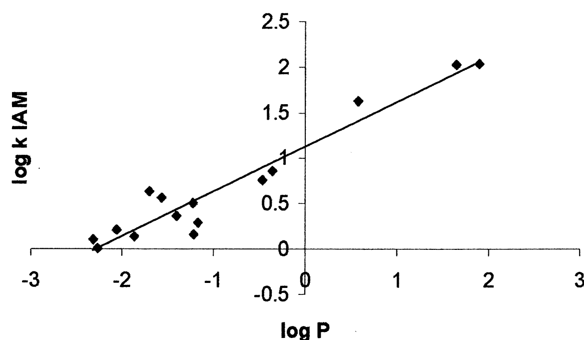

Fig. 2. Relationship between log P determined by stir-flask method and log k_{IAM}.

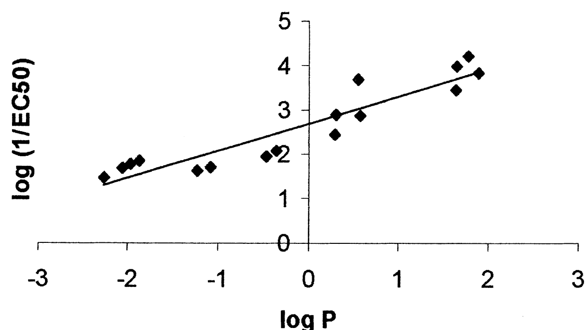

Fig. 4. Relationship between log P and aquatic toxicity.

$$\log(1/EC_{50}) = 0.61(\pm 0.13)\log P + 2.69(\pm 0.18)$$
$$n = 16, R^2 = 0.8705, s = 0.36, F = 94.1 \quad (3)$$

Compound **3** has been omitted from Figs. 3 and 4 and from Eqs. (2) and (3) since in both cases it is a significant outlier. Its inclusion changes the correlation equation dramatically and lowers the correlation coefficient. It therefore seems possible that this sulfobetaine is displaying a different mode of toxic action. Since it is the sulfobetaine with the lowest molecular mass it may be small enough to penetrate the membrane directly and this may override its dependence on hydrophobicity (or phospholipophilicity). This conclusion is supported by the fact that compounds **3** and **15** have almost identical log P values, but totally different aquatic toxicities.

The fact that log k_{IAM} correlates better with aquatic toxicity than log P suggests that the interactions involved in membrane transport and in aquatic toxicity are more complex than are revealed by the measurement of log P. It is expected that two-dimensional models of the lipid membrane are likely to be more realistic, since in two dimensions ionic interactions are most significant.

3.2. Correlation of log P and log k_{IAM} with log k_{C18}

The results on the C_{18} column (Table 3) show a good correlation between log P and log $k_{C_{18}}$ (Eq. (4)) and, more surprisingly, also between log k_{IAM} and log k_{C18} (Eq. (5)):

$$\log k_{C18} = 0.60(\pm 0.06)\log P + 1.03(\pm 0.11)$$
$$n = 12, R^2 = 0.9735, s = 0.16, F = 366.7 \quad (4)$$

$$\log k_{C18} = 1.21(\pm 0.21)\log k_{IAM} - 0.38(\pm 0.20)n$$
$$= 10, R^2 = 0.9418, s = 0.24, F = 129.5 \quad (5)$$

A good correlation exists between log P and log k_{C18} despite one parameter being a three-dimensional and the other a two-dimensional model, since both are models of hydrophobicity. It seems likely that the close correlation found between log k_{IAM} and log k_{C18}, even for close homologues, is due to the fact that these parameters are both measured using immobilised HPLC-bonded phases and involve two-dimensional models of the lipid membrane. It is significant that the equation correlating log P with log k_{C18} (Eq. (4)) has a slope of 0.60 while that correlating log k_{C18} with log k_{IAM} (Eq. (5)) has a slope somewhat closer to unity.

3.3. Correlation between log $k_{(octanol\text{-}coated\ C8)}$ and log P

The correlation between log k on the octanol-coated C_8 column at 0.5 ml/min (Table 4) and log P is shown in Eq. (6). An excellent correlation is observed between these parameters. Log k was also determined at a higher flow-rate for four compounds (Table 5) and once again, although only a small number of compounds are involved, an excellent correlation was observed (Eq. (7)):

$$\log k_{OC/C8(0.5)} = 0.75(\pm 0.07)\log P + 1.02(\pm 0.12)$$
$$n = 10, R^2 = 0.9811, s = 0.11, F = 415.3 \quad (6)$$

$$\log k_{OC/C8(3.5)} = 0.48(\pm 0.04)\log P + 1.26(\pm 0.05)$$
$$n = 4, R^2 = 0.9964, s = 0.03, F = 556.7 \quad (7)$$

The close correlation between log k on the octanol-coated C_8 column and log P for close homologues can be attributed to both being measures of hydrophobicity. More importantly the column in this case, can be considered to be a "partial three-dimensional model" since even though the C_8 backbone is immobilised the octanol itself is effectively mobile and simply held in place by Van der Waals interactions. The fact that the slopes of Eqs. (6) and (7) are different suggests that the partition coefficient is flow-rate dependent. Furthermore, the fact that it differs from unity, even at low flow-rates when mass transfer should be efficient, suggests that for zwitterionic compounds the octanol-coated column does not exactly replicate the process occurring in the stir-flask experiment. It is interesting to note that when the data points corresponding to compounds **12** and **21**, whose k values were determined by extrapolation from higher flow-rates, are omitted from Eq. (6) the gradient increases to 0.90 (Eq. (8)):

$$\log k_{OC/C8(0.5)} = 0.90(\pm 0.17)\log P + 1.32(\pm 0.32)$$
$$n = 8, R^2 = 0.9475, s = 0.09, F = 108.3 \quad (8)$$

Mirrlees et al. [22] and Terada [23] have reported slopes close to unity for non-ionic compounds. Clearly, if the retention time is governed ultimately by partitioning between water and octanol, the slope should be unity since $k=P(V_s/V_m)$, where V_s and V_m are the volumes of the stationary phase and the mobile phase, respectively. The deviation from unity when compounds **12** and **21** are included in Eq. (6), and for compounds **12**, **13**, **21**, and **22** in Eq. (7), may be related to difficulty in reaching equilibrium due to the hydrophilic nature of these compounds. The interaction between the solutes and the octanol-coated column must be considerably less than that between the solutes and octanol in a stir-flask experiment. Certainly it would appear that the relationship between log *k* and flow-rate is not linear for these compounds.

4. Conclusions

In summary, it appears that the value of the correlation coefficient (R^2), especially for close homologues, is affected by whether the parameters that are correlated are two- or three-dimensional models of the lipid membrane. It also depends on whether the correlated parameters are models of hydrophobicity or phospholipophilicity. Good correlations will only be obtained if the degree of mobility of the hydrophobic phases is the same, and the same types of interaction are involved.

The results of this study suggest that for zwitterionic sulfobetaines two-dimensional parameters such as k_{IAM} correlate better with aquatic toxicity than three-dimensional parameters such as log *P* despite the fact that the latter has been used as the sole physicochemical property in QSARs for aquatic toxicity for many years. It would appear that three-dimensional parameters of the lipid membrane take into consideration both ionic and non-ionic interactions, however two-dimensional analogues find ionic interactions of greater significance. It is therefore probable that correlations between two-dimensional models of the lipid membrane and aquatic toxicity provide more useful QSARs for zwitterionics and indeed for other ionic compounds. However, three-dimensional models of the lipid membrane are more useful for non-ionic compounds.

Liu et al. proposed that in an octanol–water system, water-saturated octanol is tied up in a tetrahedral hydrogen-bonded arrangement that retains a high degree of hydrophobicity because of the four eight-carbon non-polar chains surrounding the polar water molecule, whereas octanol molecules in the water-saturated octanol phase are virtually randomly orientated [12]. HPLC-bonded phases cannot accurately mimic this arrangement.

Another direct method for determining log *P* involves the use of counter-current chromatography (CCC). This is a liquid chromatography technique in which both the stationary and mobile phases are liquids. The stationary phase, e.g., octanol, is held in place by centrifugation, while the mobile phase, e.g., water, is passed through the system [27]. Literature generally reports excellent correlation between published log *P* values obtained by the stir-flask method and log *k* obtained using this technique for simple, neutral compounds. The aim of future work is to correlate retention indices on an octanol–water CCC system with log *P* for sulfobetaines. Since both experiments involve three-dimensional models of the lipid membrane, we would expect an excellent correlation between these parameters, even for closely related homologues. Another piece of evidence in support of this proposal is that when investigating zwitterionic amino acids by CCC (using an octanol–aqueous buffer system), Tsai et al. highlighted the influence of intercharge distance on lipophilicity [28]. For homologous piperidinyl carboxylic acids, a decrease in log *D* was seen with an increase in distance between the charges. This is similar to the trend observed for log *P* for the sulfobetaines measured by a stir-flask method, i.e., for homologous sulfobetaines, a decrease in log *P* is observed with increasing length of the methylene spacer unit between N^+ and SO_3^-.

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