Drug–Membrane Interaction on Immobilized Liposome Chromatography Compared to Immobilized Artificial Membrane (IAM), Liposome/Water, and Octan-1-ol/Water Systems

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The objective of this study was to investigate drug–membrane interaction by immobilized liposome chromatography (ILC; expressed as lipophilicity index log K_s) and the comparison with lipophilicity indices obtained by liposome/H_2O, octan-1-ol/H_2O, and immobilized artificial membrane (IAM) systems. A set of structurally diverse monofunctional compounds and drugs (nonsteroidal anti-inflammatory drugs and β-blockers) were selected in this study. This set of solutes consists of basic or acidic functionalities which are positively or negatively charged at physiological pH 7.4. No correlation was found between log K_s from ILC and lipophilicity indices from any of the other membrane model systems for the whole set of compounds. For structurally related compounds, significant correlations could be established between log K_s from ILC and lipophilicity indices from IAM chromatography and octan-1-ol/H_2O. However, ILC and liposome/H_2O systems only yield parallel partitioning information for structurally related large molecules. For hydrophilic compounds, the balance between electrostatic and hydrophobic interactions dominating drug partitioning is different in these two systems.

Introduction. – Successful drug development requires not only the optimization of the specific and potent pharmacological activity at the target site, but also efficient delivery of the compounds to that site. Drug design and discovery must take pharmacokinetic behavior into account, in particular absorption and distribution. Numerous quantitative structure–permeability relationship (QSPR) studies have clearly demonstrated that lipophilicity, as related to membrane partitioning and hence passive transcellular diffusion, is a key parameter in predicting and interpreting permeability [1][2]. Lipophilicity is generally expressed by the octan-1-ol/H_2O partition coefficient (log P_{oct}, for a single chemical species) or distribution coefficient (log D_{oct}, for a pH-dependent mixture of ionizable compounds). In some studies, a relationship has been established between log P_{oct} (or log D_{oct}) and absorption or permeability in intestinal models [3][4], blood-brain-barrier models [5], and cell-culture models [6–9], to name a few. However, in many other situations, log P_{oct} (or log D_{oct}) cannot give a good estimate of a drug's absorption or permeation [10–14]. The lipophilicity parameters log P_{oct} or log D_{oct} fail to encode some important recognition forces, most notably ionic bonds, which are of particular importance when modeling the interaction of ionized compounds with biomembranes [15]. Because the

majority of the drugs are ionizable [16], any prediction of their pharmacodynamic and pharmacokinetic properties should take their ionization into account.

Thus, the development of membrane-like systems such as liposome/H_2O partitioning systems [17][18] has been of marked interest to obtain lipophilicity parameters of greater biologic relevance, especially for ionized compounds. However, the determination of drug partitioning in liposome/H_2O systems is time-consuming and tedious, and, therefore, of little use in medium or high-throughput screening in drug discovery. As surrogates, immobilized artificial membrane (IAM) chromatography [18–21] and immobilized liposome chromatography (ILC) [22–24] were recently developed as convenient and rapid methods for the analysis of drug–membrane interactions. However, it was shown for a set of structurally unrelated compounds that IAM retention and liposome/H_2O partitioning are governed by a different balance of intermolecular interactions, and, thus, the lipophilicity index from IAM retention is not exchangeable with that from liposome/H_2O partitioning for structurally unrelated compounds [18][25].

In the present study, we aimed to investigate the lipophilicity index from ILC and its comparison to lipophilicity indices obtained from liposome/H_2O, octan-1-ol/H_2O, and IAM membrane model systems. In ILC, liposomes are entrapped in the pores of gel beads which are packed into HPLC columns. The lipophilicity index from ILC is expressed as the capacity factor $\log K_s$, which is calculated according to *Eqn. 1*,

$$\log K_s = \log [(V_R - V_0)/A] \quad (1)$$

where V_R and V_0 are the retention volumes of the drug and of an unretained compound, respectively, and A is the amount of immobilized phospholipids. Some studies have shown that ILC is a useful method for the study or rapid screening of drug–membrane interactions [22–24]. *Österberg et al.* demonstrated a good correlation between the lipophilicity index determined by ILC ($\log K_s$) and the lipophilicity index from a liposome/H_2O system, while a poor correlation with the index from the octan-1-ol/H_2O system and a moderate correlation with the index from IAM chromatography was observed for a chemically diverse set of drugs [25].

To further understand the relationship between the lipophilicity indices from different membrane model systems, we selected a set of 22 structurally diverse monofunctional compounds (alkyl(4-methylbenzyl)amines and carboxylic acids; **1**–**22**) and drugs (nonsteroidal anti-inflammatory drugs and β-blockers), which are positively charged or negatively charged at the physiological pH 7.4. We determined the lipophilicity index $\log K_s$ by ILC with immobilized large unilamellar egg-phosphatidylcholine (egg PC) liposomes and compared it to published partitioning data from liposome/H_2O, octan-1-ol/H_2O, as well as IAM chromatography.

Results and Discussion. – *Stability of ILC Column.* Two reference compounds (**5** with a $\log P_{oct}$ value of 4.26 and **11** with a $\log P_{oct}$ value of 1.95) were used to determine the stability of the column. The $\log K_s$ values did not change significantly over the time of the measurements (four weeks). This indicates that this immobilized liposome column is stable under the experimental conditions at the flow rate of 0.2 ml/min used in this study.

Physicochemical Parameters. The log K_s values determined by ILC with immobilized large unilamellar egg PC liposomes, as well as published lipophilicity indices from liposome/H_2O (log $D_{lip}^{7.0}$), octan-1-ol/H_2O (log P_{oct}^{N} and log $D_{oct}^{7.0}$) and IAM chromatography (log $k_{IAMw}^{7.0}$) are summarized in the *Table*. The log $D_{lip}^{7.0}$ values (distribution coefficient at pH 7.0) were determined using large unilamellar egg PC liposome/H_2O by potentiometric titration [18].

Table. *Physicochemical Parameters of the Compounds under Study*

	Solutes	log P_{oct} [a])	log $D_{oct}^{7.0}$ [a])	log $k_{IAMw}^{7.0}$ [a])	log $D_{lip}^{7.0}$ [b])	log K_s [c])
1	4-$MeC_6H_4CH_2NHMe$	1.96	– 0.97	0.96	2.54	0.95
2	4-$MeC_6H_4CH_2NHEt$	2.38	– 0.44	1.02	2.26	0.96
3	4-$MeC_6H_4CH_2NHPr$	2.96	0.15	1.30	2.11	1.13
4	4-$MeC_6H_4CH_2NHBu$	3.49	0.67	1.87	1.55	1.41
5	4-$MeC_6H_4CH_2NH(CH_2)_4Me$	4.26	1.32	2.27	1.86	1.85
6	4-$MeC_6H_4CH_2NH(CH_2)_5Me$	4.96	1.91	2.77	2.45	2.34
7	4-$MeC_6H_4CH_2NH(CH_2)_6Me$	5.12	2.21	2.92	2.73	2.88
8	Acebutolol	2.02	– 0.20	1.57	1.93	0.88
9	Alprenolol	3.10	0.70	2.25	2.33	2.19
10	Metipranolol	2.81	0.38	1.78	2.27	1.81
11	Metoprolol	1.95	– 0.54	1.45	1.59	1.02
12	Oxprenolol	2.51	0.21	1.70	2.09	1.48
13	Penbutolol	4.62	1.85	3.70	3.39	3.45
14	Propranolol	3.48	1.17	2.48	2.69	2.64
15	$Ph(CH_2)_2COOH$	1.89	– 0.59	– 0.25		0.37
16	$Ph(CH_2)_4COOH$	2.85	0.40	0.43		0.80
17	$Ph(CH_2)_7COOH$	4.09	2.12	2.02		2.09
18	Flurbiprofen	3.81	0.72	1.78		2.08
19	Indomethacin	4.27	1.77	2.37		2.48
20	Ketoprofen	2.77	0.06	1.26		1.38
21	Mefenamic acid	5.12	2.45	2.35		2.93
22	Naproxen	3.06	0.21	1.35		1.56

[a]) Taken from [21]. [b]) Taken from [18]. [c]) Determined at pH 7.4 in this study, $n = 3$, SD ≤ 0.05.

The log $k_{IAMw}^{7.0}$ values (capacity factor at pH 7.0) were obtained from experiments using an IAM.PC.DD2 HPLC column [21]. The octan-1-ol/H_2O partitioning data (log P_{oct}^{N} and log $D_{oct}^{7.0}$) were taken from the literature [21]. According to the pK_a values of the compounds [21], the alkyl(4-methylbenzyl)amines and β-blockers (compounds **1**–**14**) are fully positively charged, whereas the monofunctional carboxylic acids and NSAIDs (compounds **15**–**22**) are fully negatively charged at both pH 7.4 and 7.0.

Comparison between log K_s *and log* $D_{lip}^{7.0}$ *Values.* For the basic compounds; **1**–**14**, of which the log $D_{lip}^{7.0}$ values are available, the correlation between log K_s and log $D_{lip}^{7.0}$ values is shown in *Fig. 1*. In contrast to the result from the study of *Österberg et al.* [25], no correlation could be found for the whole set of basic compounds between these two lipophilicity indices, although the same type of phospholipid (egg PC) liposome was used in both systems. Good correlation was found for the β-blockers, *i.e.*, **8**–**14**. The correlation *Eqn.* is:

$$\log K_s = 1.52\ (\pm 0.38)\ \log D_{lip}^{7.0} - 1.62\ (\pm 0.90) \qquad (2)$$

$$n = 7,\ r^2 = 0.93,\ s = 0.26,\ F = 66$$

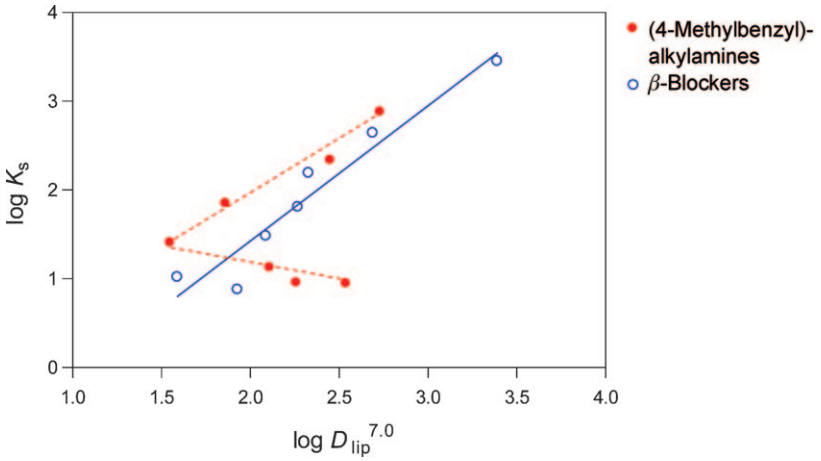

Fig. 1. *Correlation between log* K_s *and log* $D_{lip}^{7.0}$ *values for the 14 basic compounds investigated*

In this and the following *Eqns.*, 95% confidence limits are in parentheses, n is the number of compounds, r^2 the squared correlation coefficient, s the standard deviation, and F *Fisher*'s test. The significant correlation implies that the retention on the ILC column with immobilized egg PC liposome and partitioning in egg PC liposome/H_2O are controlled by the same balance of intermolecular interactions for the seven β-blockers.

No correlation was observed for the alkyl(4-methylbenzyl)amines **1**–**7**. Instead, a bilinear relationship between log K_s and log $D_{lip}^{7.0}$ values was found with a positive slope for compounds **5**–**7** and a negative slope for compounds **1**–**3**, which indicates that different balances of intermolecular interactions dominate the retention in the ILC and partitioning in liposome/H_2O for this set of compounds. As pointed out in one study [26], hydrophobic interactions dominate the partitioning for bulkier compounds **5**–**7**, whereas electrostatic interactions dominate the partitioning for the solutes with shorter alkyl chains, *i.e.*, compound **1**–**3** in liposome/H_2O systems. The present results indicate that electrostatic interactions play a smaller role in ILC than in liposome/H_2O partitioning. Therefore, the lipophilicity index from ILC is not exchangeable with that from the liposome/H_2O partitioning for this set of compounds, as well as for structurally unrelated compounds. The reasons for this observation are not understood and need further investigation.

Comparison between log K_s *and log* $k_{IAMw}^{7.0}$ *Values.* No correlation was observed between log K_s and log $k_{IAMw}^{7.0}$ values for the present set of 22 compounds (*Fig. 2*), indicating that the two systems do not yield comparable lipophilicity parameters for structurally unrelated drugs. This result is in agreement with a previous study [25], where only a weak correlation was found between log K_s values on ILC with immobilized phosphatidylcholine (PC) liposome and log $k_{IAMw}^{7.0}$ values on IAM.PC.MG

HPLC column for a set of NSAIDs, local anaesthetics and β-blockers. One possible reason for the difference between the lipophilicity indices from these two anisotropic chromatographic systems is the different density of the polar phospholipid headgroups, which was established as an important factor for drug partitioning in biological and artificial membranes [20].

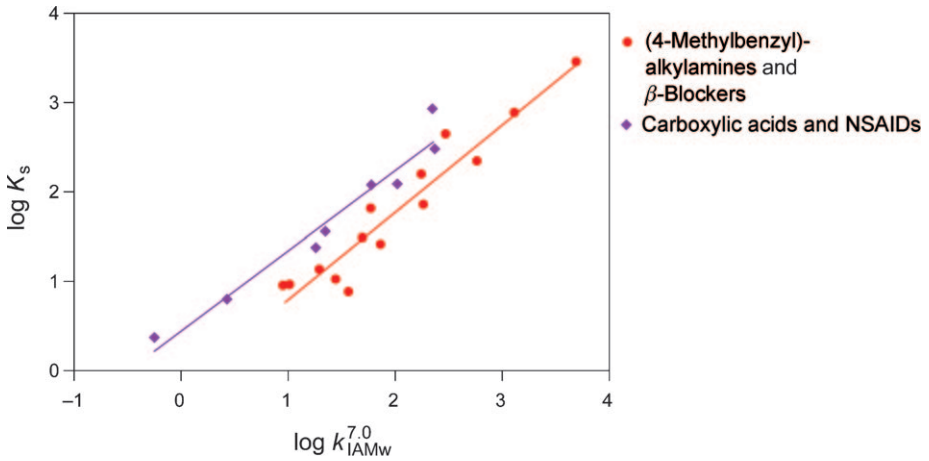

Fig. 2. *Correlation between log* K_s *and log* $k^{7.0}_{IAMw}$ *values for the compounds investigated*

However, significant correlations were found for the basic compounds, **1**–**14**, as shown in *Eqn. 3*, and for the acidic compounds, **15**–**22**, as shown in *Eqn. 4*.

For basic compounds **1**–**14**:

$$\log K_s = 0.98\ (\pm 0.16)\ \log k^{7.0}_{IAMw} - 0.19\ (\pm 0.36) \qquad (3)$$

$$n = 14,\ r^2 = 0.92,\ s = 0.24,\ F = 134$$

For carboxylic acids **15**–**22**:

$$\log K_s = 0.90\ (\pm 0.16)\ \log k^{7.0}_{IAMw} + 0.44\ (\pm 0.28) \qquad (4)$$

$$n = 8,\ r^2 = 0.95,\ s = 0.20,\ F = 117$$

These significant correlations indicate that the balance of intermolecular interactions (mainly hydrophobic and electrostatic) in ILC and IAM chromatography is similar for the positively charged basic compounds or negatively charged acidic compounds investigated. In other words, the lipophilicity index by ILC is interchangeable with that by IAM chromatography for structurally related compounds. For the basic compounds investigated, ILC system is more similar to IAM chromatography than to liposome/H_2O partitioning system.

The similar slopes and different intercepts of *Eqns. 3* and *4* are probably caused by the different balance of electrostatic interactions of different ion types in these two

membrane systems. However, more investigations are necessary to reach a sound conclusion.

Comparison between log K_s *and log* P_{oct} *or log* $D_{oct}^{7.0}$ *Values.* The comparison between the log K_s and log P_{oct} values is shown in *Fig. 3,a*, for compounds **1**–**22**. No correlation exists for the complete set of compounds, but good correlations are detected for structurally related sets of alkyl(4-methylbenzyl)amines, β-blockers, and carboxylic acids as shown in *Eqns. 5–7.*

For alkyl(4-methylbenzyl)amines **1**–**7**:

$$\log K_s = 0.58\ (\pm 0.16)\ \log P_{oct} - 0.42\ (\pm 0.58) \tag{5}$$

$$n = 7,\ r^2 = 0.92,\ s = 0.24,\ F = 54$$

For β-blockers **8**–**14**:

$$\log K_s = 0.98\ (\pm 0.12)\ \log P_{oct} - 0.94\ (\pm 0.36) \tag{6}$$

$$n = 7,\ r^2 = 0.98,\ s = 0.13,\ F = 289$$

For carboxylic acids **15**–**22**:

$$\log K_s = 0.81\ (\pm 0.16)\ \log P_{oct} - 1.10\ (\pm 0.60) \tag{7}$$

$$n = 8,\ r^2 = 0.94,\ s = 0.23,\ F = 93$$

The good quality of *Eqn. 5* demonstrates that for the alkyl(4-methylbenzyl)amines series, the intermolecular interactions underlying the retention in ILC are closer to those in octan-1-ol/H_2O compared to those in egg PC liposome/H_2O. The significant correlations shown in *Eqns. 5–7* indicate that log K_s values obtained by the anisotropic ILC system can be predicted by the traditional log P_{oct} values for the three sets of structurally related compounds.

Fig. 3,a, shows that β-blockers which are positively charged at the experimental conditions are retained strongest on the ILC column among the three sets of solutes. The same result was also found for IAM chromatography [21]. This suggests that the interaction of ionized drugs with immobilized liposomes depends not only on their lipophilicity expressed as log P_{oct}, but also on additional interactions including electrostatic interactions and their ability to form H-bonds with the polar head groups of the phospholipids (β-blockers can form more H-bonds than alkyl(4-methylbenzyl)-amines). The strength of these additional interactions depends on the structural characteristics of the drugs, as clearly indicated in *Fig. 3*.

It was found that positively charged solutes are more retained than negatively charged solutes on an IAM.PC.DD2 stationary phase [21], which was explained as the result of a larger affinity of positively charged drugs to phospholipid membranes compared to the negatively charged compounds. However, we did not obtain the same results by ILC with immobilized unilamellar egg PC liposomes in this study. As shown in *Fig. 3*, some carboxylic acids which are negatively charged at the experimental

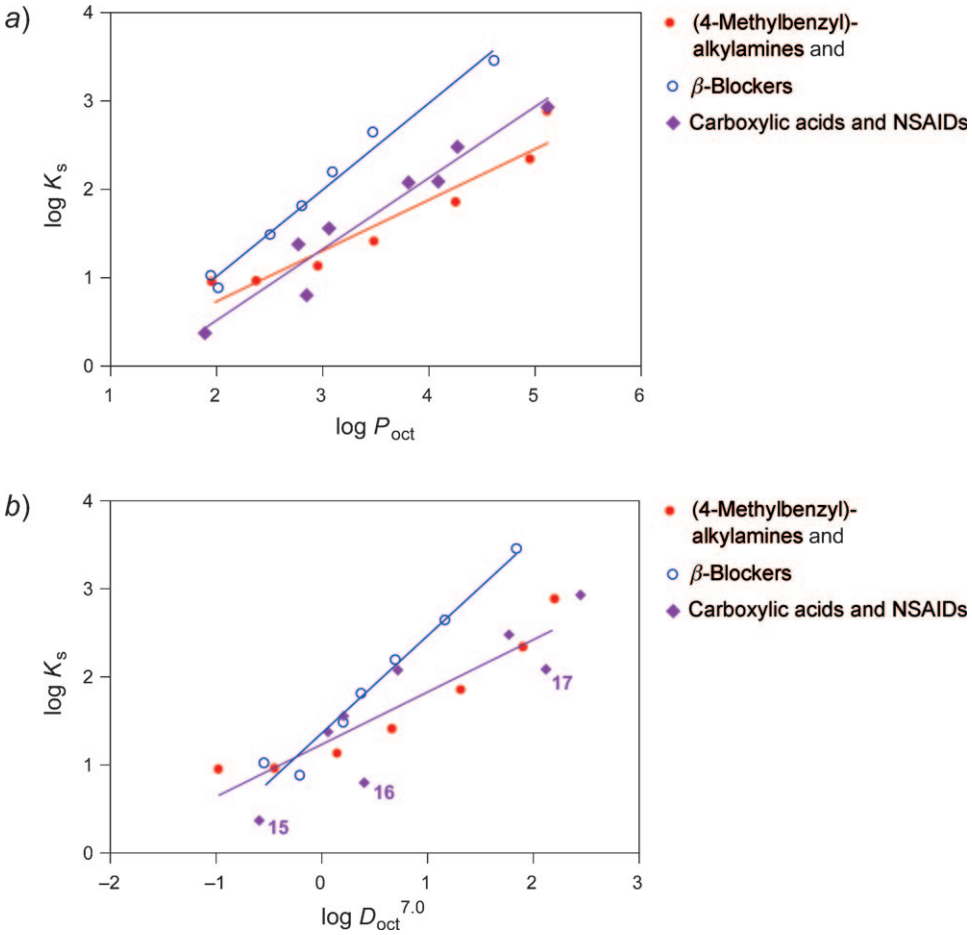

Fig. 3. *Correlation between log* K_s *and log* P_{oct} (*a*) *or log* $D_{oct}^{7.0}$ (*b*) *values for the compounds investigated*

conditions are retained stronger than alkyl(4-methylbenzyl)amines which are positively charged at pH 7.4. This implies that the drugs do not interact with phospholipid membranes in ILC in the same way as in IAM chromatography. Thus, further investigations on the factors which influence drug–membrane interactions in different membrane model systems are required.

The log P_{oct} and log $D_{oct}^{7.0}$ values are highly interrelated for the alkyl(4-methylbenzyl)amines and β-blockers. Therefore, the correlations between log K_s and log $D_{oct}^{7.0}$ values are also significant for these two sets of solutes (r^2 0.91 and 0.97, resp.). However, no correlation exists for the whole set of acidic compounds between these two parameters. Good correlations were established only for NSAIDs (r^2 0.97) and monofunctional carboxylic acids (r^2 0.98) as shown in *Fig. 3*, *b*.

Conclusions. – In this work, we investigated drug–membrane interactions by ILC with immobilized large unilamellar egg PC liposomes, and compared them with

lipophilicity indices from liposome/H_2O, octan-1-ol/H_2O, and IAM chromatography, by using a set of monofunctional compounds and drugs which are positively or negatively charged under the experimental conditions. For the whole set of solutes, the lipophilicity index log K_s from ILC was not exchangeable with those from any of the other membrane model systems. For subsets of structurally related compounds, significant correlations were found between log K_s values and the lipophilicity indices obtained by IAM chromatography and octan-1-ol/H_2O, indicating that a comparable balance of intermolecular forces dominate the drug–model membrane interactions for structurally related solutes. However, for the basic compounds investigated, **1**–**14**, good correlations were only found between lipophilicity indices from ILC and egg PC liposome/H_2O for large molecules (β-blockers and long-chain alkyl(4-methylbenzyl)-amines, **5**–**7**), implying that different balances of hydrophobic and electrostatic interactions dominate the partitioning of drugs in these two systems. Electrostatic interactions play a smaller role in ILC than in liposome/H_2O.

Experimental Part

General. The alkyl(4-methylbenzyl)amines (**1**–**7** in the *Table*) were synthesized according to known procedures [27]. All other test compounds were obtained from commercial sources (*Sigma-Aldrich*, D-Steinheim; *Carl Roth*, D-Karlsruhe; *VWR*, B-Leuven) in the highest available purity. Dist. H_2O was used throughout. *Superdex 200* prep. grade and glass column (*HR 5/5*) were bought from *Amersham* (S-Uppsala). Egg phosphatidylcholine (egg PC) was purchased from *Lipoid* (D-Ludwigshafen).

Measurement of Capacity Factors. The capacity factors were measured with a liquid chromatograph *Merck L-6200 A* separation module equipped with a UV/VIS detector *L-4250* (*Merck-Hitachi Ltd.*, Tokyo, Japan).

Liposomes were prepared by rehydration of lipid films in 150 mM NaCl, 1 mM Na_2EDTA and 10 mM *Tris*·HCl, pH 7.4 (to obtain an egg PC concentration of 100 mmol/l). The liposome suspension was extruded 15 times through a polycarbonate filter having 100 nm pores (*Avestin Europe*, D-Mannheim). The size distribution was controlled by dynamic light scattering using a *Zetasizer Nano ZES3600* (*Malvern Instruments Inc.*, UK). The developed large unilamellar liposomes were mixed with thoroughly dried *Superdex 200* prep. grade gel beads (1.5 ml of suspension to 110 mg of beads) and immobilized by gel bead swelling for 5 h at r.t. The material was packed into a 5-mm (I. D.) glass column to a 1.2 ml gel bed. The amount of phospholipid (*A* in *Eqn. 1*) was determined as the phosphorus amount in the gel suspension [28]. Lipid content in the liposome column after the retention analyses was 18.7 mmol.

The mobile phase was 10 mM *Tris*·HCl buffer with 150 mM NaCl and 1 mM Na_2EDTA, pH 7.4, filtered under vacuum through a 0.45 μm *HA Millipore* filter (*Millipore*, Milford, MA, USA) and degassed before analysis. The flow rate was 0.2 ml/min. The retention times were measured at r.t. by the UV/VIS detector at 250 nm.

The analyte solns. (10^{-4} to 10^{-3} M) were prepared by dissolving the solutes in the mobile phase or in EtOH, and diluted with the mobile phase (> 5% EtOH) in the case of lipophilic compounds. The injection volume was 10 μl. $K_2Cr_2O_7$ was used as the unretained compound to obtain V_0.

The capacity factor log K_s was calculated according to *Eqn. 1*. All log K_s values are the averages of three measurements.

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