High-Throughput Chromatographic Approaches to Assess Drug Partition into Biomembranes

Kumulative Habilitationsschrift

vorgelegt von

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Xiangli Liu

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Summary

Modern drug design and discovery not only focus on the pharmacological activity of a compound, but also take its pharmacokinetic behavior into account. The prediction of *in vivo* barrier permeation, particularly intestinal absorption and blood-brain barrier passage, are substantial concerns in the development of new drug compounds. For several decades, lipophilicity expressed as n-octanol/water partition coefficient ($\log P_{oct}$) dominated absorption prediction schemes, since partitioning in this solvent system is traditionally accepted as an informative model of membrane partitioning. Recent advances in automated synthesis and combinatorial chemistry result in a vast number of potential drug candidates, and thus the demand for fast and reliable methods to measure this parameter has grown rapidly in recent years.

However, in many relevant cases especially for the structurally unrelated or ionized compounds, $\log P_{oct}$ can not give a good estimate of a drug´s absorption or permeation. Indeed, the isotropic n-octanol/water system can only roughly mimic natural membrane barriers, which are made of ordered and anisotropic lipid membranes. Therefore bio-mimicking artificial membrane systems have been developed for a better prediction of drug absorption.

The objective of this work is to further develop the present high-throughput techniques to measure relevant lipophilicity indices, and more importantly to understand the structural properties encoded in lipophilicity indices derived from different systems. This is important for designing better-suited drugs for medical therapy by improving the biopharmaceutical properties of the drugs via their chemical structure modifications.

Chapter 1 gives a brief description of drug permeation and lipophilicity, and the relationship between them. Chapter 2 reviews the high-throughput chromatographic approaches to assess drug partition into biomembranes.

The habilitation thesis consists of three parts of work. First, we investigated thoroughly the optimal conditions of the high-throughput reversed-phase high performance liquid chromatography (RP-HPLC) method for the measurement of $\log P_{oct}$ values. Our studies revealed that under optimal mobile phase and stationary phase conditions, RP-HPLC is a powerful method for efficient and accurate determination of $\log P_{oct}$ values, and that partitioning of the compounds in the RP-HPLC system is controlled by the same balance of intermolecular forces (Van der Waals volume, H-bond

acceptor basicity and dipolarity/polarizability) as in n-octanol/water system by quantitative structure-property relationship analysis (Publications 1-4).

Second, a flow ratiometry technique was also developed for the quick determination of the partition coefficient of the volatile compounds (Publication 5).

Third, we investigated drug-membrane interactions by using artificial membrane systems including immobilized artificial membrane (IAM) chromatography and immobilized liposome chromatography (ILC). The lipophilicity indices from these artificial membrane systems were compared to that from n-octanol/water. These studies showed that, electrostatic force plays an important role for the interaction between ionized solutes and phospholipids in the membrane-like systems (Publications 6-7).

These studies contribute significantly to a better understanding of the partitioning mechanisms of drugs in different model membrane systems, and thus make it possible on a scientific basis to develop highly predictive artificial membrane systems for drug membrane permeation *in vivo*.

1 Drug permeation and lipophilicity

1.1 Drug diffusion and transport across biological barriers

Successful drug development requires not only optimization of specific and potent pharmacodynamic activity, but also efficient delivery to the target site. To elicit its pharmacological and therapeutic effects, a drug has to cross various cellular barriers by passive and/or by carrier-mediated uptake. Membrane permeability is a key determinant in the effectiveness of pharmacokinetic behaviour (*a*bsorption, *d*istribution, *m*etabolism and *e*limination, ADME) of drugs especially absorption and distribution. In recent years, the advances in automated synthesis, combinatorial chemistry and innovative high-throughput screening have led to the production of a vast number of potential drug candidates, often making delivery problems the rate-limiting step in drug research [1]. In order to overcome this problem, it is necessary to have a detailed picture of the structures of pharmacokinetic barriers.

1.1.1 Structure of cell membranes

The currently accepted structure of cell membranes is a fluid-like bilayer arrangement of phospholipids [2] (Figure 1). Proteins and other substances such as steroids and glycolipids are either associated with its surface or embedded in it to different degrees. This structure is an intermediate state between the true liquid and solid states, with the lipid and protein molecules having a limited degree of rotational and lateral movement [3]. The polar heads of the phospholipids molecules are orientated to form an almost continuous polar layer on both the inside and outside of the cell membrane. In contrast, the long hydrophobic chains of the phospholipids molecules extend into the central core of the membrane.

The lipid component of the cell membrane of mammals is mainly composed of glycerophospholipids, sphingolipids and cholesterol whose structures are shown in Figure 2 [4-5]. It can be seen from the structures that the lipid molecules are either negatively charged or zwitterionic [5-6] (electrically neutral at all physiological values of pH). These lipids are distributed asymmetrically in the inner and outer leaflets in most biological membranes. The outer leaflet of the bilayer consists mainly of electrically

neutral lipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), while the negatively charged lipids such as phosphatidylserine (PS) are located in the inner layer [7]. These lipid molecules are held together by weak hydrophobic bonding and van der Waals' forces.

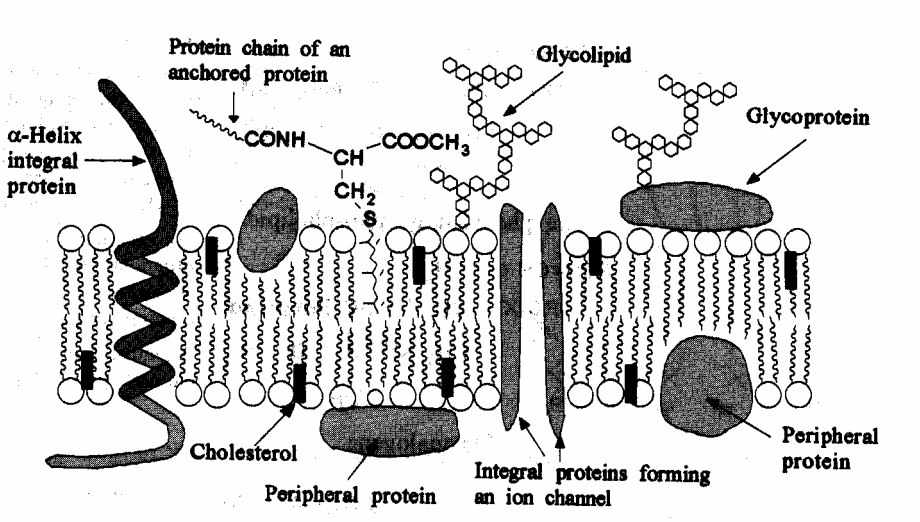
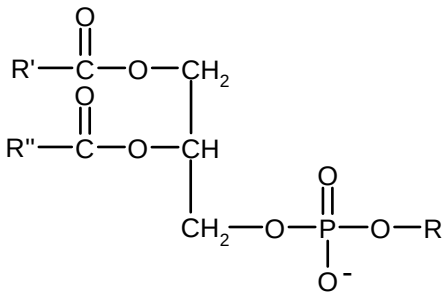


Figure 1. The fluid mosaic model of membranes [8].

The peripheral and integral proteins located in the membranes are responsible for carrying out many of the active functions of membranes, such as acting as receptors and transportation routes for various substances across the membranes. The formation of pores including ion channels are also associated with integral proteins.

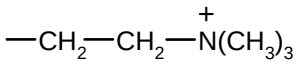
The neighboring cells are linked to each other by a continuous junctional complex referred to as tight junction. It is a region where the outer leaflets of the lipid bilayer comprising the membrane of neighboring cells are fused. The interconnected monolayer of cells in the intestinal epithelium is the principle permeation barrier for oral absorption of drugs. Similarly, a special class of capillary endothelial cells interlinked by exceptionally tight junctions constitute the principle barrier for drug transport from blood to brain [9].

Glycerophospholipids

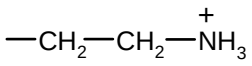


R' and R'' groups are long-chain fatty acid residue that may or may not be the same.

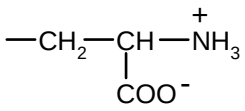
R:



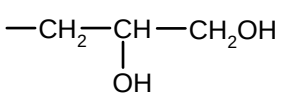
phosphatidylcholines (PC)



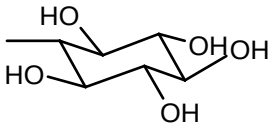
phosphatidylethanolamine (PE)



phosphatidylserine (PS)

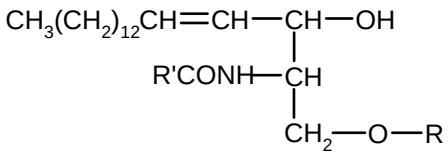


phosphatidylglycerol (PG)



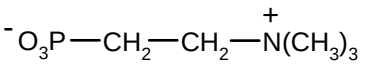
phosphatidylinositol (PI)

Sphingolipids



R' is long-chain fatty acid residues

R:



sphingomyelins (SM)

oligosaccharides ganglioside (GM)

Cholesterol

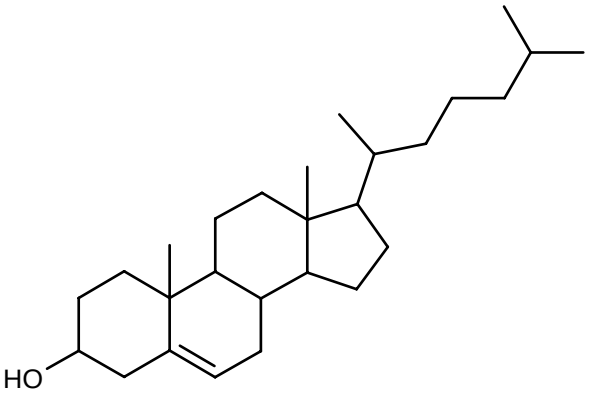


Figure 2. The chemical structures of principle lipids found in membranes.

1.1.2 The transfer of drugs through cell membranes

1.1.2.1 Passive Diffusion

The process, in which a molecule moves down its concentration gradient without the membrane actively participating, is termed passive diffusion. Passive diffusion through a biomembrane may occur through its lipid structures (= transcellular pathway, B in Figure 3), or through its water-filled pores or tight junctions (= paracellular pathway, A in Figure 3) [10]. For the vast majority of drugs, the transport is mediated by passive transcellular diffusion through the apical cell membranes, across the cell proper and across the basolateral membrane [9]. The ease of passive transcellular diffusion depends on the ability of the molecular to partition into the cell membranes. In order to permeate by this route, a compound must have an optimum lipophilicity because if the solute is too lipophilic it will remain trapped in the membrane. A measure of lipophilicity can be obtained by the partition coefficients in different systems such as n-octanol/water partitioning system. The predictive value of the relations between lipophilicity and membrane permeation depends on the relevance of the partitioning systems as models of biomembranes [11].

Intestinal absorption via paracellular way is relevant for hydrophilic compounds with molecular weights lower than 200 [12]. Since the paracellular pathway is an aqueous extracellular route across the intestinal epithelium, sufficient hydrophilicity is the most important prerequisite for a drug to traverse the cell barrier via this pathway [13]. In addition, the size and charge of drug are also the crucial molecular characteristics for this route. It is reported that tight junctions are impermeable to molecules with radii larger than 10-15 Å [14] and that an optimal net charge is very important for the efficient transport through intestinal epithelium via this route [15].

In contrast, paracellular absorption is negligible at the BBB due to the occlusive network of tight junctions of the brain capillary endothelial cells [16].

1.1.2.2 Active transport

Some hydrophilic drugs whose chemical structure mimics various nutrients can be transported across the membranes by carrier-facilitated transport (C in Figure 3). This process usually operates against a concentration gradient and is fairly substrate-

specific. Different carriers and transporters have been described in various types of cells. They have been identified mostly as integral membrane proteins [17].

Contrary to carrier-facilitated transport, efflux systems (D in Figure 3) present in the membranes create a major barrier to the absorption of a wide variety of xenobiotics. Although these efflux systems are most commonly observed in tumor cells, they are also known to be present in normal intestinal epithelia and BBB [18]. These efflux systems are related to P-glycoprotein, the principle component of multidrug resistance in a variety of cell types. P-glycoprotein is a 170-180 kDa membrane glycoprotein that acts as an ATP-dependent efflux pump, thereby reducing the transcellular flux of a wide variety of drugs [19].

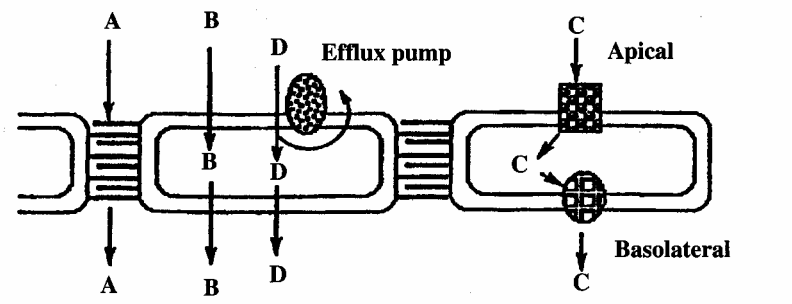


Figure 3. Pathways of transport across the intestinal mucosa. A: Passive diffusion via paracellular route. B: Passive diffusion via transcellular route. C: Transporter-facilitated pathway. D: Transport-restricted pathway (e.g., by efflux transporters) [20].

1.1.3 The main physiological barriers

1.1.3.1 Intestinal epithelium

Because the majority of the marketed drugs (about 90%) are administered orally, the main physiological barrier drugs have to pass to enter the body is the intestinal epithelial membrane. The human small intestine membrane has a fractal-like structure, i.e., the ridges (oriented circumferentially around the lumen), villi and microville [21]. The membrane surface is expanded approximately up to 600-fold by the villi and microvilli. Due to this large surface area, the small intestine is the main

region of absorption for drugs [22]. The intestinal membrane has the mucus layer on the villi, which is thought to maintain the unstirred water layer (UWL). The UWL is also considered a significant barrier to the passive diffusion of lipophilic drugs [23].

The permeation of drugs across the intestinal epithelial membrane can occur via passive transcellular pathway, the paracellular pathway, and active transport, depending on the physicochemical properties and structural characteristics of the compounds.

1.1.3.2 Blood-brain barrier (BBB)

The BBB, which has an estimated surface area of 12 m^2 in humans [24], is formed by the tight endothelial cell layer in the brain capillaries and control the exchange of drugs, nutrients, hormones, metabolites and immunlogical cells between the blood and brain in both directions. BBB endothelium forms a much tighter interface than peripheral endothelia. In the periphery most small solutes can diffuse between the intercellular clefts of 50-200 nm width [25]. In contrast, the gaps between capillary endothelial cells in most part of the brain are tightly sealed by tight junctions (which exclude molecules with a diameter larger than 20Å) and thus have severely limited permeability [26]. The paracellular pathway is negligible for most compounds under physiological conditions. The passive permeation is mainly restricted to the lipophilic compounds which are able to traverse the lipid membranes of the cells [24].

1.1.3.3 The skin permeation barrier

The transdermal route has advantages over other routes for the delivery of drugs with systemic activity. These include the ease of use (and withdrawal in the occurance of side-effects), avoidance of first-pass metabolism, and improved patient compliance. The transdermal permeation rate of most drugs is limited by the stratum corneum (SC) [27]. The SC comprises 10-15 layers of flat keratin-filled cells, closely packed in a non-polar lipid matrix, mainly composed of free fatty acids (10-15%), cholesterol (25%), sterol esters (5%), and ceramides (50%) [28]. Ceramides are categorized into 9 subgroups (structures shown in Figure 2) [29], whose head groups can form lateral intermolecular hydrogen-bonds. The phase behavior of the lammellar lipid is different from that of the lipid bilayer mainly composed of phospholipids. The thickness of the SC is different in each body part, for example about 15 μm in the abdominal skin and

10 μm in the dorsal skin. The primary transport pathway for most drugs passively traversing the SC is the intercellular lipid region [30].

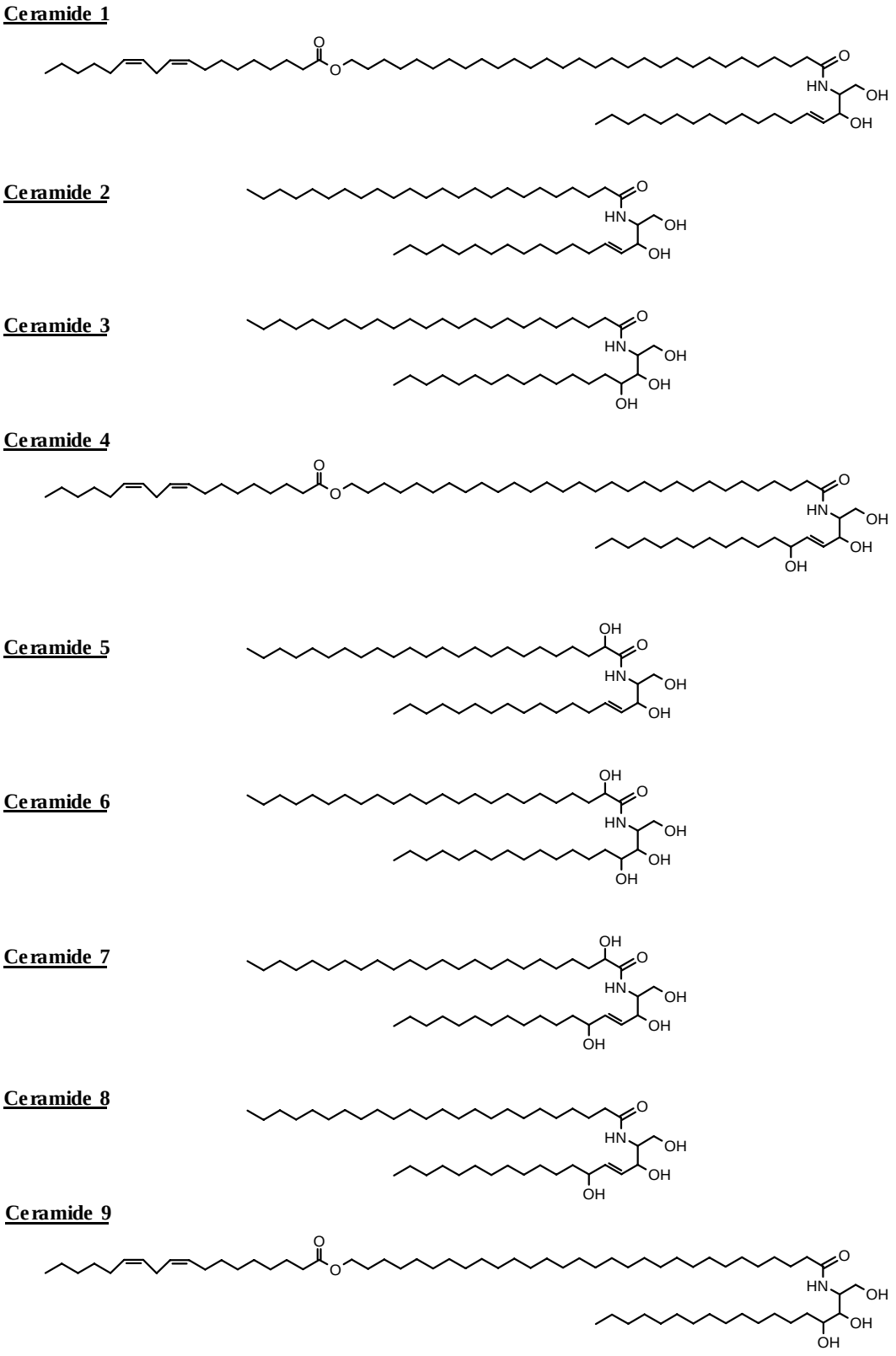


Figure 4. The chemical structures of ceramides.

1.2 The influence of lipophilicity on drug transcellular permeation

The relationship between the physicochemical properties of drugs and the pharmacokinetic process has been extensively studied. Some physicochemical parameters of drugs, such as lipophilicity, hydrogen-bonding capacity, molecular size and polar surface area, have proved useful for predicting passive transfer and permeation across biomembranes in ADME, but none has attracted as much interest in quantitative structure-permeation relationship (QSPeR) studies as lipophilicity.

1.2.1 Lipophilicity from isotropic solvent systems and its relationship with drug transcellular permeation

Lipophilicity is usually expressed by the partition coefficient (log P), a molecular parameter which describes the partition equilibrium of a solute molecule between water and an immiscible organic solvent. Partition coefficients are obtained as the logarithm of the ratio of concentrations at equilibrium:

$$\log P = \log \frac{C_{organic}}{C_{aqueous}} \qquad \text{[Eq. 1.1]}$$

The most commonly used measure of lipophilicity is n-octanol/water partition coefficient ($\log P_{oct}$). The effectiveness of $\log P_{oct}$ in correlating biological properties has been extensively investigated. However, the $\log P_{oct}$ scale of lipophilicity alone is not effective in modelling the crossing of any kind of cell membranes due to the severe differences in their biophysical properties [31-32]. Thus four types of isotropic solvent systems (amphiprotic, inert, hydrogen bond donor and hydrogen-bond acceptor) called 'critical quartet' (e.g., n-octanol/water, alkanes/water, chloroform/water and dibutyl ether/water) are necessary in order to cover adequately the range of biophysical properties of membranes [33-34]. The 'critical quartet' expresses in partly overlapping and partly complementary ways the recognition forces that account for membrane partitioning and biological selectivity [35-36].

For ionizable drugs, the apparent distribution coefficient (log D) at pH 7.4 is also often used. Unlike log P, which is valid only for a single chemical species, log D refers to a pH-dependent mixture of electrical species present at that pH:

$$\log D^{pH} = \log (f_N \bullet P^N + f_I \bullet P^I) \qquad \text{[Eq. 1.2]}$$

where f_N and f_I are the molar fractions of the neutral and ionized forms, and P_N and P_I are their respective partition coefficients.

Lipophilicity is the net result of all intermolecular forces involving a solute and the two phases between which it partitions. A highly informative interpretation of lipophilicity is based on solvatochromic analysis [37-40] (see also Publications 1-3). This method factorizes lipophilicity into a number of parameters:

- molar volume V (or van der Waal volume V_w) which represents hydrophobic and dispersion forces
- the solute's H-bond donor acidity (α) and the solute's H-bond acceptor basicity (β)
- the solute's dipolarity/polarizability (π^*) which accounts for orientation and induction forces

For example, the n-octanol/water and heptane/water partition coefficient ($\log P_{oct}$ and $\log P_{hep}$) can be expressed as [41]:

$$\log P_{oct} = 5.83(\pm 0.53) \bullet V/100 - 0.74(\pm 0.31) \bullet \pi^* - 3.51(\pm 0.38) \bullet \beta - 0.15(\pm 0.23) \bullet \alpha - 0.02(\pm 0.34) \quad \text{[Eq. 1.3]}$$

$N = 78$; $r^2 = 0.92$; $s = 0.30$; $F = 248$

where N is the number of compounds, r^2 the squared correlation coefficient, s the standard deviation and F the Fisher's test.

$$\log P_{hep} = 6.78(\pm 0.69) \bullet V/100 - 1.02(\pm 0.39) \bullet \pi^* - 5.35(\pm 0.50) \bullet \beta - 3.54(\pm 0.30) \bullet \alpha - 0.06(\pm 0.43) \quad \text{[Eq. 1.4]}$$

$N = 75$; $r^2 = 0.96$; $s = 0.36$; $F = 438$

As a result of such equations, lipophilicity can be factorized into two sets of terms, namely hydrophobicity which accounts for hydrophobic and dispersion forces, and polar terms which account for hydrogen-bonds, and orientation and induction forces [42]:

$$\text{Lipophilicity} = \text{Hydrophobicity} - \text{Polarity} \quad \text{[Eq. 1.5]}$$

Eq. 1.3 shows that V and β are the most important structural descriptors contributing to $\log P_{oct}$, while π^* is of secondary importance and α has no statistical significance in n-octanol/water system. A different partitioning mechanism exists in

heptane/water system as shown in Eq. 1.4: V_β and α are the most important structural descriptors contributing to $\log P_{hep}$, while α and π^* are of secondary importance.

Lipophilicity descriptors obtained in different solvent systems can be compared to derive a $\Delta\log P$ when the two log P values do not contain identical structural information [41]. A well-known example is $\Delta\log P_{oct\text{-}alk}$, the difference between n-octanol/water and alkane/water partition coefficients [39]. This parameter is physicochemically meaningful and expresses mainly the H-bond donor acidity of solutes as shown by Eq. 1.6:

$$\log P_{oct\text{-}hep} = 3.54(\pm 0.36) \bullet \alpha - 0.37(\pm 0.15)$$
$$n = 75;\ r^2 = 0.84;\ s = 0.45;\ F = 325 \qquad \text{[Eq. 1.6]}$$

To overcome some experimental problems caused by the low alkane solubility of many compounds, the 1,2-dichloroethane/water (DCE/water) system was suggested to replace the alkane/water system [40]. Therefore, $\Delta\log P_{oct\text{-}dce}$ is now proposed instead of $\Delta\log P_{oct\text{-}alk}$.

The experimental techniques for lipophilicity measurement in isotropic solvent/water systems are shake-flask method [43] and potentiometric titration [44], which have been thoroughly described in the literatures.

In numerous studies on drug permeation through biological membranes (e.g., gut wall, skin, blood-brain barrier, and more recently Caco-2 cell monolayers), relationships between permeation and lipophilicity have been developed with homologous series of compounds of a diverse nature (acidic, alkaline and neutral) to explore the influence of lipophilicity on passive diffusion.

Thus, linear relationships were found between absorption rate constants from rat stomach and a) partition coefficient in CCl_4/water for 16 barbiturates [45], b) partition coefficient in n-octanol/water for 11 carbamates [46]. A bilinear correlation was found between in situ gastric absorption rate constants determined in rats and lipophilicity indices (measured in RP-HPLC) for a series of phenylalkylcarboxylic acids [47]. Sigmoidal relationships were established between permeability coefficients in Caco-2 cell monolayers and $\log D_{oct}$ (pH 7.4) for a set of β-blockers [48] and a series of N-acylated derivatives of 5-fluorouracil [49], respectively. The same type of relationship between permeability in the rat jejunum and $\log P_{oct}$ were also found for

7 steroids [50] and 11 β-blockers [51]. Parabolic correlations were found between human skin permeability coefficients and $\log P_{oct}$ for a homogeneous set of phenols [52], between human epidermis permeability coefficients and $\log P_{oct}$ for 6 non-steroidal anti-inflammatory agents [53], and between corneal permeability coefficients and $\log D_{oct}$ (pH 7.65) for a series of 12 β-blockers [54]. To rationalize the observed relationships, different theoretical models for passive membrane diffusion have been derived and discussed [10, 55-56].

The $\Delta\log P_{oct\text{-}alk}$ parameter, which mainly expresses H-bond donor acidity, also showed its power in the prediction of drug permeation. For a series of H_2-receptor antagonists, brain penetration (logarithm of brain/blood ratio) in the rat was inversely correlated with $\Delta\log P_{oct\text{-}cyc}$ parameter (cyc = cyclohexane) [57]. Similar correlations were found between permeability coefficients across excised rabbit cornea and $\Delta\log P_{oct\text{-}cyc}$ for a set of drugs including β-blockers and steroids [58], between oral absorption and $\Delta\log P_{oct\text{-}cyc}$ for a family of azole endothelin antagonists [59], and between human in vitro permeability coefficients and $\Delta\log P_{oct\text{-}hep}$ for a set of compounds including alcohols and steroids [52]. These examples imply that the H-bond donor acidity is very important in drug design to improve the pharmacokinetic profile of drugs.

1.2.2 Lipophilicity from anisotropic biomembrane-like systems and its relationship with drug transcellular permeation

Although partitioning in n-octanol/water system is accepted as an informative model of membrane partitioning, $\log P_{oct}$ (or $\log D_{oct}$) can not give a good estimate of a drug´s absorption or permeation [32] - in some cases especially for the structurally unrelated ionized compounds. Lipophilicity as a molecular parameter encodes different intermolecular forces (Figure 5). When expressed by log P measured in isotropic organic solvent/water systems, lipophilicity fails to encode some important recognition forces, mostly by ionic bonds, which are of particular importance when modeling the interaction of ionized compounds with biomembranes [41]. Because the majority of the drugs are ionizable [60], any prediction of their pharmacodynamic and pharmacokinetic properties should take their ionization into account. In anisotropic membrane-like systems such as liposome/water partitioning system, lipophilicity can be expressed as:

Lipophilicity = Hydrophobicity – Polarity + Ionic Interactions [Eq. 1.7]

Liposomes have been used for several decades as model membranes to study solute/biological membranes interactions. The partitioning behavior of solutes between liposomal membranes and aqueous phases provides information on their affinity to biological membranes and on their in vivo pharmacokinetic behavior in general. The liposomes/water partitioning system is increasingly employed as an alternative to n-octanol/water for the estimation of pharmacokinetic behavior of drugs [61].

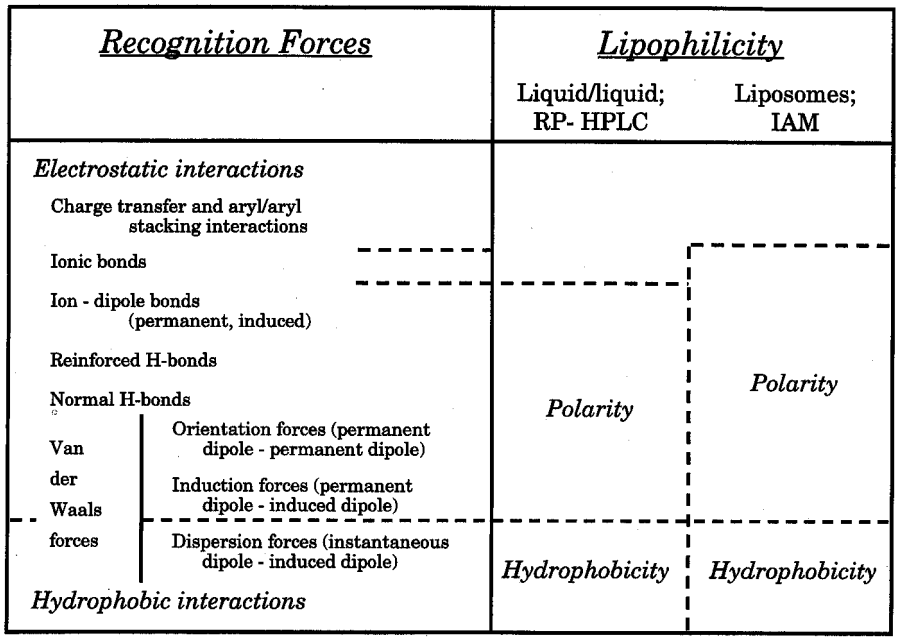


Figure 5. A comparison between the intermolecular forces that govern molecular recognition in biochemical and pharmacological processes, and those expressed in lipophilicity [41].

Liposomes can be prepared from a variety of lipids and mixtures of them. Phospholipids are frequently used to obtain standardized and easily reproducible systems [62]. Liposomes have a spherical shape and are composed of one to several hundred concentric bilayers. The sizes range from 20 nm to several dozens micrometers, whereas the thickness of the bilayer membrane is approximately 4-7 nm [5]. Depending on the number of bilayer sheets, liposomes can be distinguished between large multilamellar vesicles (LMV's), large unilamellar vesicles (LUV's) and

small unilamellar vesicles (SUV's, diameter smaller than 100 nm). Liposomes in partition studies are ideally unilamellar, which have a reproducible size distribution.

Liposomes of various compositions have been shown to provide better correlations of pharmacokinetic behavior, as well as biological activities of certain classes of drugs than the n-octanol/water system [63-66]. Here are the examples to name a few.

For a series of nine nitroimidazole drugs, liposomal partitioning system (LMV's) composed of L-α-Dimyristoylphosphatidylcholine (DMPC) provided a much better prediction of plasma clearance rate than n-octanol/water partitioning system [64]. For a set of structurally diversed ionisable compounds, the phosphatidylcholine liposomes (SUV's)/saline revealed a better correlation between passive intestinal absorption in humans and so called absorption potential (AP) than n-octanol/water partition system. The AP is calculated from logD at pH 6.8 (pH of the fasted small intestine), solubility, mean single oral dose, and intestinal fluid volumes [65]. For a set of 10 imidazolines, liposomal partitioning systems composed of DMPC or DMPC/CHOL(cholesterol)/DCP (dicetylphosphate) showed advantages than n-octanol/water to predict α_2-adrenergic potencies [66].

The most popular methods to investigate lipophilicity in liposome/water systems are potentiometric titration [67] and equilibrium dialysis [68]. However these two methods are very labor-consuming. In recent years, high-throughput chromatographic techniques, including immobilized artificial membrane (IAM) HPLC-columns [69], immobilized liposome chromatography [70], and liposome (or microemulsion) electrokinetic chromatography [71] have been developed for the determination of drug partitioning into biomembranes. These methods are discussed in detail in Chapter 2.

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2 Chromatographic approaches to assess drug partition into biomembranes

As discussed in Chapter 1, log P from n-octanol/water ($\log P_{oct}$) and liposome/water partitioning systems is a widely used measure of lipophilicity to predict pharmacokinetic behaviors of drugs. For many years, the conventional procedures to measure log P is a shake-flask method [1], potentiometric titration [2] and equilibrium dialysis [3]. However, these methods are time-consuming and not suitable for the high-throughput screening in modern drug discovery. In order to overcome the disadvantages of these techniques, the chromatographic approaches have been introduced to measure drug partition into biomembranes.

2.1 Reversed phase HPLC (RP-HPLC) method

The low throughput and resource intensity of the traditional shake-flask method for measurement of $\log P_{oct}$ resulted in the investigation of reversed-phase HPLC chromatography as a surrogate [4-12]. Test compounds partition between the mobile phase and the lipophilic HPLC stationary phase. The degree of partitioning is measured by the analyte's retention expressed by retention factor, which is either isocratic capacity factor (Eq. 2.1) or the capacity factor extrapolated to 100 % water ($\log k_w$) (Eq. 2.2).

$$\log k = \log \frac{t_r - t_0}{t_0} \qquad \text{[Eq. 2.1]}$$

$$\log k = -S\varphi + \log k_w \qquad \text{[Eq. 2.2]}$$

If the proper stationary and mobile phases are chosen, the log k (or $\log k_w$) values are correlated with the $\log P_{oct}$ values as shown by equation 2.3:

$$\log P_{oct} = a \log k \,(\log k_w) + b \qquad \text{[Eq. 2.3]}$$

From this equation, the $\log P_{oct}$ value of a previously unmeasured compound can be estimated. Many investigators have used $\log k_w$ as the RP-HPLC measured lipophilicity parameter to eliminate organic solvent effects [6-7, 13-16]. On the other hand, other reporters use isocratic log k values at appropriate methanol concentration [4, 17-19].

Extensive studies have been done to find optimal stationary and mobile phase conditions in order to obtain $\log k_w$ or $\log k$ values best correlated with $\log P_{oct}$ values. Several types of stationary phases such as silica-based columns and polymer-based columns have been investigated. In the advantage of this method, the most frequently used are octadecylsilane (ODS) stationary phase. Generally, the correlations between $\log k_w$ or $\log k$ obtained on this kind of columns and $\log P_{oct}$ are good only as long as the solutes analyzed are structurally related (congeneric) [20-21]. It was supposed that the decrease in correlation between capacity factors and $\log P_{oct}$ with an increasing structural diversity of solutes results mainly from specific interactions of the compounds with residual silanol groups on this kind of stationary phase [22].

Much has been done to decrease the effects of free silanol groups. A masking agent (n-decylamine) was proposed to be added to the mobile phase [23-24]. Great progress has been achieved in silica-based stationary phases exhibiting high level of silanol deactivation, among which LC-ABZ, ABZ+Plus and Discovery™ RP Amide C16 (Supelco, Bellefonte, PA, USA) are good examples. In these stationary phases, the alkyl chains contain an amide group which electrostatically shields silanols from highly polar analytes. In addition, it is hypothesized that the combination of amide groups and a hydration layer at the silica surface leads to a high degree of orientation of the alkyl chains of the stationary phase. This avoids blocking the solute from reaching the surface as happens in the conventional C18 RP-HPLC stationary phase due to the folding of the alkyl chains [25]. $\log k_w$ values measured on these three columns gave good correlations with $\log P_{oct}$ values for a set of monofunctional compounds which cover a broad property space as shown in [26-27]. using a mobile phase enriched in 1-octanol, highly significant correlations were also built between $\log P_{oct}$ and $\log k_w$ values obtained on LC-ABZ [15-16] and on Discovery™ RP Amide C16 [28-29] for a set of noncongeneric complex drugs.

In spite of the improvement in silanol deactivation of silica-based stationary phases, one big disadvantage of this type of stationary phases is their chemical instability at pH above 8 and below 2. In case of majority of organic bases, lipophilicity can not be determined directly for neutral forms.

Polymer-based stationary phases are void of reactive silanol groups and chemically stable over a wide pH range. Poly(styrene-divinylbenzene) copolymers (PS-DVB) have been studied and reported to provide rather moderate correlations with log

P_{oct} [30]. Instead, they were found to mimic alkane/water partition [31]. The restrictions of PS-DVB columns are that they are characterized by low efficiency and the material suffers from excessive shrinkage and swelling [30].

Octadecyl polyvinyl copolymer (ODP) does not undergo swelling nor shrinkage and offers the possibility of having reasonable flow rate without undesirable pressure increase at the column inlet [30, 32]. Moreover, it has the advantage that one can measure $\log k_w$ values directly, i.e., at 100% water as mobile phase. For a set of monofunctional compounds, correlations between $\log k_w$ obtained on this type of columns and $\log P_{oct}$ are as good as obtained with the ODS stationary phase [30, 33]. However, by using a wide range of noncongeneric compounds including complex drugs, ODP stationary phase yielded lipophilicity index $\log k_w$ significantly lower correlated with $\log P_{oct}$ than that obtained with Discovery™ RP Amide C16 phase [29].

Recently, a novel type of RP-HPLC stationary phase such as XbridgeShield™ RP18 produced by BEH Technology has become available [34]. This support is a hybrid stationary phase with ethylene bridges inside the silica matrix. It affords a wide pH resistance (1-12) to silica-based materials. It has the same alkyl chains as those in LC-ABZ and Discovery™ RP Amide C16, and thus make it possible to measure lipophilicity not only for neutral and acidic compounds, but also for basic ones. The lipophilicity index $\log k_w$ obtained with this stationary phase is highly correlated with $\log P_{oct}$ values for a wide range of structurally diversed neutral, acidic, ampholytic and basic solutes including complex drugs [35]. By using a same type of column (Acquity BEH Shield RP18), it was shown that isocratic log k at 50% methanol could give a precise prediction of $\log P_{oct}$ values for basic compounds [36]. The results of these studies are of potential interest for the high-throughput screening of lipophilicity in drug discovery, where basic compounds predominate.

On most lipophilic HPLC stationary phases, pure water (or aqueous buffer) cannot be used as mobile phase. Therefore, mainly binary mixtures of water (or aqueous buffer) with an organic modifier are used. Methanol is by far the most commonly used organic modifier in the determination of partition coefficients [7, 18, 20]. There are several reasons for this. First, methanol is the most water-like of all commonly used RP-HPLC solvents including also acetonitrile and tetrahydrofuran. It is capable of hydrogen bond acceptance and donation and thus can easily be incorporated in the network of water molecules [8]. Not only the mobile phase, but also the stationary phase, are affected less

with methanol. It has been shown that the adsorption of acetonitrile and tetrahydrofuran to alkyl-bonded silica occurs to a greater degree than with methanol [37].

The second reason to use methanol is that it generally has significantly less curvature than either tetrahydrofuran or acetonitrile in plots of log k versus volume fraction of organic modifier [8, 38]. Thus, the errors are minimized in extrapolations to obtain log k_w.

Phosphate buffers and zwitterionic buffers (such as morpholinopropane sulfonic acid) are often used to adjust the pH of the mobile phase. However, zwitterionic buffers are preferred for charged basic compounds since phosphate buffers are likely to form ion-pairs with them.

For charged organic compounds, it is often tempting to buffer the mobile phase at the pH of interest. For example, pH 7.4 is often used for pharmaceutical applications since this is the physiological pH. However, there are several problems with this approach. First, the pH is only an apparent pH since the mobile phase is not entirely made of water. Second, ionization constants (pK_a) will be affected by the organic modifiers. Thus, it is preferred to buffer the mobile phase in order to ensure that the compound is in its neutral form (if possible), yielding intrinsic log k_w [18]. The log k_w at a particular pH ($\log k_w^{app}$) can then be calculated by the following equations:

For monoprotic acids:

$$\log k_w^{app} = \log k_w - \log (1 + 10^{pH-pK_a}) \qquad \text{[Eq. 2.4]}$$

For monoprotic bases:

$$\log k_w^{app} = \log k_w - \log (1 + 10^{pK_a-pH}) \qquad \text{[Eq. 2.5]}$$

2.2 Immobilized artificial membrane (IAM) chromatography

The main reason to use the chromatographic system to determine lipophilicity is to conveniently model drug transport processes across biological membranes. Hence, the components of the chromatographic and biological systems should be comparable. A HPLC system which is used to model transport of a drug through biological membranes should be composed of an aqueous phase and an organized phospholipids layer.

Immobilized artificial membranes (IAMs) consist of monolayers of phospholipids covalently immobilized to silica surface, thus mimicking the lipid environment of a fluid cell membrane on a solid matrix [39-40]. The structures of the commercially available immobilized artificial membranes are shown in Figure 1.

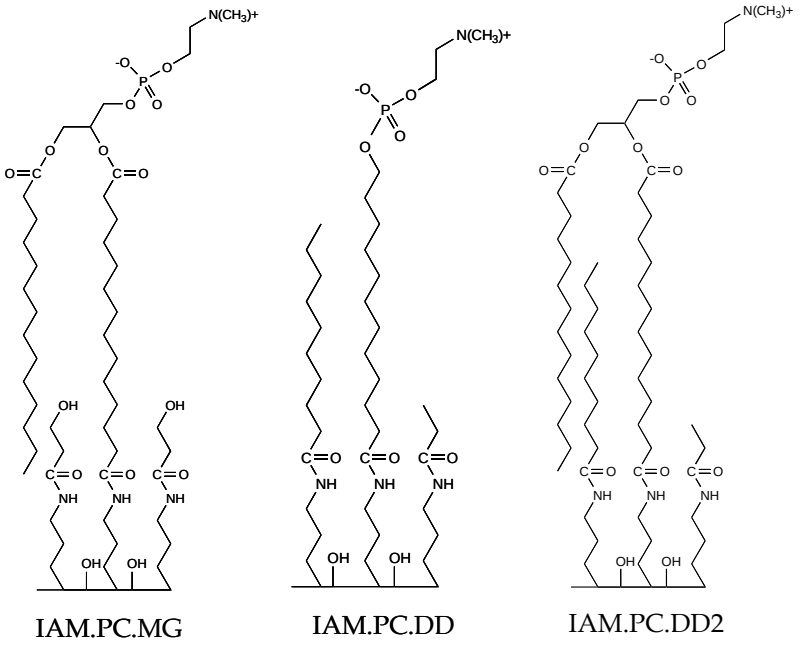

Figure 1. Structures of the commercially available immobilized artificial membranes.

The lipophilicity index from IAM chromatography is the capacity factor log k_{IAMw} at 100 % aqueous phase. For hydrophilic compounds, log k_{IAMw} can be determined directly by using the aqueous mobile phase. For lipophilic drugs, it is necessary to add organic modifier (methanol or acetonitrile) to the mobile phase to accelerate the elution. The log k_{IAMw} value is then extrapolated from isocratic capacity factors using Eq. 2.2. No significant difference was observed for the linear extrapolation when using either methanol or acetonitrile. However, methanol is more appropriate when charged compounds are chromatographed [41]. Furthermore, when acetonitrile is used, mobile phases containing more than 30% (w/w) must be avoided because their microheterogeneity disrupts the structure of water [42]. It was reported the chromatography on IAM stationary phase is deteriorating after 3 months of use [43], thus measures should be taken to check the decrease of the capacity factor over time.

The interaction of drugs with phospholipids has been investigated by IAM-HPLC for different sets of neutral and charged compounds such as β-blockers [44], non-

steroidal anti-inflammatory drugs (NSAID) [45], dihydropyridine (DHPs) calcium channel blockers [46], and for a set of compounds with a wide structural diversity [47]. It was found that log k_{IAMw} values from IAM columns correlate with log P_{oct} only for neutral and structurally related compounds, and that electrostatic interaction between the charged solutes and the polar heads of phospholipid play a vital role in IAMs.

The relationship between IAM retention and partitioning in egg phosphatidylcholine (EPC) liposome/water was also studied [48]. No correlation existed between log $k_{IAMw}^{7.0}$ and log $D^{7.0}$ in EPC liposome/water for noncongeneous set of compounds. One possible reason for the difference between the lipophilicity indices from these two anisotropic systems is the different density of the polar phospholipid head-groups, which were proved an important factor for drug partitioning in biological and artificial membranes [49].

The log k_{IAMw} determined on IAM columns appears to be a better predictor of bioactivity than log P_{oct} for drugs of different chemical nature. The log k_{IAMw} values obtained on this type of columns were reported to be well correlated with human skin permeation for a set of steroids and phenols [50]. The oral absorption of cephalosporin prodrugs in mice could be better predicted by log k_{IAMw} than by analogous parameters determined on traditional ODS reversed phases [49]. The log $k_{IAMw}^{7.0}$ (measured at pH 7.0) values of 12 structurally diverse compounds were determined on an IAM.PC.DD2 phase, and their permeabilities (P_a) were measured through rat everted gut sacs for passively diffused compounds or through non-everted sacs for actively transported molecules. The log $k_{IAMw}^{7.0}$ correlated with P_a better than log P_{oct}. The addition of molar volume (Vx) as a second independent variable slightly improved the correlation [51]. For a series of (NSAIDs), a parabolic relationship was obtained between log k_{IAMw} and the capacity of diffusion across the rat blood-brain barier (BBB) [52], suggesting an optimum lipophilicity of drugs in the transport to brain.

2.3 Immobilized liposome chromatography (ILC)

ILC was a simple and fast tool for analysis of solute-membrane interactions. In ILC, phospholipid-based liposomes are noncovalently immobilized to gel beads (e.g. Superdex 200) as a stationary phase of chromatography [53]. The main advantages of this method are that phases of different chemical composition can be easily and

reversibly immobilized on suitable gel supports. Phosphatidylcholine (PC), mixtures of PC/PS (phosphatidylserine) and PC/PE (phosphatidylethanolamine), egg phosphatidylcholine (EPC), lipids extracted from human red cells have been used for ILC [54-55]. In addition, chromatographic retention on phospholipids is devoid of any effect caused by the presence of a silica matrix.

Various techniques have been developed for immobilization of liposomes. In some studies, liposomes are mixed with dry gel beads and immobilized by gel bead swelling followed by freezing-thawing to induce liposome fusion [53-55]. During the freezing-thawing process, the liposomes grow in size and are entrapped in the gel beads pore. The immobilized liposome has a multiamellar structure [56]. The multiamellar structure would not affect the partitioning of the majority of amphiphilic drugs because they pass the bilayers fast enough during the chromatographic run [54]. In other studies, unilamellar liposomes are immobilized in the gel pores of gel beads by avidin-biotin binding [57-58]. The liposome immobilized gel is packed into a column. The capacity factor of the drugs log K_s (Eq. 2.6.) is measured by HPLC using an aqueous buffer eluent as the lipophilicity index.

$$\log K_s = \log [(V_R - V_0) / A] \qquad \text{[Eq. 2.6]}$$

where V_R and V_0 are the retention volumes of the drug and of an unretained compound, and A is the amount of the immobilized phospholipids.

No significant correlation was found between log K_s and log D from liposome/water partitioning system, log k_{AMw} or log D_{oct} for structurally unrelated compounds [54, 59].

A hyperbolic relationship between oral absorption in human and log K_s from EPC liposomes was established for a set of 12 structurally unrelated drugs [53]. In another study by the same group, it was further observed that drugs with log K_s values (from human red blood extract) between 1.5 and 2.5 are nearly 100% absorbed [55], the results showed that the essential feature of a good biomembrane model for drug partitioning analyses is a bilayer heterogeneity mimicking that of natural membranes. A sigmoidal relationship was found between log K_s values from EPC-PS-PE-Chol (cholesterol) and intestinal absorption for a set of 29 structurally diverse drugs [58].

2.4 Liposome electrokinetic chromatography (LEKC)

LEKC is a capillary electrophoresis method where liposomes are incorporated in the buffer as a pseudostationary phase for the determination of drug-membrane interactions [60-61]. In LEKC, the composition of the lipid bilayer pseudophase can be carefully controlled to nearly mimic the properties of the natural membranes through adjustment of the type and mole fractions of phospholipids as well as incorporating "additives" such as cholesterol and even proteins.

Figure 2 illustrates the mechanism of the migration and separation of two uncharged solutes, S_1 and S_2, in LEKC based on negatively charged liposomes. The electrophoretic migration of the liposomes is toward the anode; but the stronger electroosmotic flow carries the liposomes and solutes toward the cathode where they are detected. Neutral solutes are separated according to their differences in liposome/water partition.

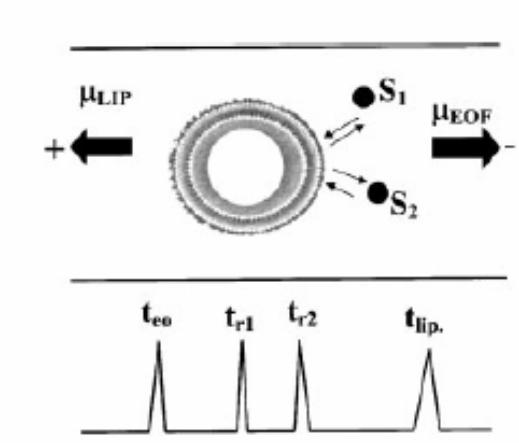

Figure 2. Schematic of the migration pattern in LEKC with a negatively charged liposome. S_1 and S_2 represent two solutes that partition into the liposome, and μ_{Lip} and μ_{EOF} are the mobilities of a liposome and the ElectroOsmotic Flow (EOF), respectively. Finally, t_{eo}, t_{r1}, t_{r2}, and t_{lip} are the retention times of the unretained marker (methanol), two solutes, and liposome marker (n-decanophenone), respectively [60].

The retention factor log k of neutral solutes, as the lipophilicity index from LEKC is calculated by Eq. 2.7, where t_r, t_{eo}, and t_{lsp} are the retention times of the solute, methanol and the marker of the pseudostationary phases (n-decanophenone), respectively. Charged solutes will possess their own electrophoretic mobility in the

aqueous phase in addition to partitioning into the liposomes and migrating at the liposome mobility. As a result, the migration of solutes in the bulk aqueous (t_0) needs to be included in the calculation of retention factor as shown in Eq. 2.8 [62].

$$\log k = \log [(t_R - t_{eo})/t_{eo} (1 - t_R/t_{psp})] \quad \text{[Eq. 2.7]}$$

$$\log k = \log [(t_R - t_0)/t_0 (1 - t_R/t_{psp})] \quad \text{[Eq. 2.8]}$$

There are significant advantages in using LEKC for assessment of drug-membrane interaction over the existing models such as n-octanol/water, IAM or ILC. Liposomes are spherical lipid bilayer microstructures that are made of phospholipids and closely resemble biologic cell membranes, thus they are more suitable models for the dynamic and fluid lipid bilayer environment of cell membranes than other models. Also, using LEKC, it is possible to establish universal and consistent lipophilicity scales for drug-membranes interaction studies for interlaboratory use. On the contrary, RP-HPLC and IAM method lack a universal scale due to the difference of HPLC columns.

Unilamellar liposomes with a narrow size distribution are most often used as pseudostationary phases. For multilamellar liposomes, the size can range from approximately 100 nm to 1 μm due to large number of bilayers. They are not suitable as carriers in LEKC because the background in the electropherogram was very noisy and the sensitivity was low [63].

For nonhomogenous drugs of different chemical natures, the following correlations were found: a good correlation was built between the log k values from LEKC based on POPC-PS liposomes and caco-2 monolayer permeability [64]; A sigmoidal relationship was found between log k values from LEKC based on EPC-PS liposomes and human intestinal drug absorption, where $\log P_{oct}$ could not show a good predicting power [65]; log k from LEKC using PC-PS liposomes gave the better correlation with drug penetration across BBB than $\log D_{7.4}$, Clog P, PSA [66]. Recently it was proved that LEKC is a promising method to predict drug penetration through the skin. Quantitative retention-activity relationships (QRARs) were successfully constructed between the compound skin permeability coefficient (log Kp) and the retention values (log k) as well as some calculated molecular descriptors by the stepwise regression method (R^2=0.902) [67].

As a summary, the major advantage of chromatographic methods is its good throughput. Second, only small amounts of compounds are needed, typically in the order of 1 mg. Third, the methods are generally insensitive to impurities or degradation products which might affect bulk partitioning or analysis.

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3 Publications serving as the basis of this habilitation thesis

1. Xiangli Liu, Hideji Tanaka, Aiko Yamauchi, Bernard Testa and Hiroshi Chuman. Lipophilicity measurement by RP-HPLC: A comparison of two stationary phases based on retention mechanisms, *Helv. Chim. Acta*, 87, 2866-2876 (2004).

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4. Xiangli Liu, Hossam Hefesha, Hideji Tanaka, Gerhard Scriba, Alfred Fahr, Lipophilicity measurement of drugs by reversed phase HPLC over wide pH Range using an alkaline-resistant silica-based stationary phase, XBridge™ Shield RP$_{18}$. *Chem. Pharm. Bull.*, 56, 1417-1422 (2008).

5. Hideji Tanaka, Xiangli Liu, Daichi Kawabata, Hiroshi Chuman, chisako Yamagami. Application of a stepwise flow rate method in phase separation to the determination of the chloroform/water distribution coefficients of volatile diazines, *Chem. Pharm. Bull.*, 53, 374-377 (2005).

6. Xiangli Liu, Hossam Hefesha, Gerhard Scriba, Alfred Fahr. Retention behavior of neutral, positively and negatively charged bases on immobilized artificial membrane (IAM) stationary phases, *Helv. Chim. Acta*, 91, 1505-1512 (2008).

7. Xiangli Liu, Ping Fan, Ming Chen, Gerhard K.E. Scriba, Detlef Gabel, Alfred Fahr, Drug-membrane interaction on immobilized liposome chromatography compared to immobilized artificial membrane (IAM), liposome/water and n-octanol/water systems, *Helv. Chim. Acta*, 93, 203-211 (2010).

Lipophilicity Measurement by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC): A Comparison of Two Stationary Phases Based on Retention Mechanisms

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The mechanisms of retention of two recent stationary phases of interest in lipophilicity measurements, namely of the silica-based *Discovery-RP-Amide-C16* phase and the polymer-based *ODP-50-4B* phase, were assessed and compared. A set of 41 model solutes and drugs with well-defined solvatochromic parameters were selected to allow a broad distribution of property spaces. The chromatographic results showed that, under the conditions of study, the lipophilicity index $\log k_w$ obtained with the former stationary phase was more closely related to experimental $\log P_{oct}$ values than was $\log k_w$ obtained with the *ODP-50-4B* phase. Linear solvation/free-energy relationship (LSER) analyses showed that the retention mechanisms of the two stationary phases are different, retention on the *Discovery-RP-Amide-C16* phase and partitioning in octan-1-ol/H_2O being controlled by the same balance of intermolecular forces (*Van der Waals* volume V_w, H-bond acceptor basicity β, and dipolarity/polarizability π^*).

Introduction. – The lipophilicity of solutes, traditionally expressed by their partition coefficients in the octan-1-ol/H_2O system (noted $\log P_{oct}$), is of high significance from both a physicochemical and a pharmacological viewpoint [1][2]. The partitioning process expresses the combined effects of a number of intermolecular forces between a solute and its environment, here the solvents between which the solute partitions. These intermolecular forces are of particular importance in pharmacology since they also control the partitioning of solutes into biomembranes. Numerous studies have reported a relationship between $\log P_{oct}$ and absorption or permeability in cell cultures and tissue preparations used as models of, *e.g.*, the gastrointestinal tract or the blood-brain barrier [3–7].

The reference procedure to measure $\log P_{oct}$ is the shake-flask method which, however, is time-consuming and limited in range (*ca.* $-3 < \log P < 4$). Beyond these limits, $\log P_{oct}$ values measured by the shake-flask method become unreliable.

The reversed-phase HPLC method is a promising alternative to the shake-flask method, having such advantages as a higher throughput, an insensitivity to impurities or degradation products, and a broader lipophilicity range. In reversed-phase HPLC, lipophilicity indices are derived from the capacity factor $\log k$, which is calculated by *Eqn. 1*, where t_R and t_0 are the retention times of the solute and of an unretained compound, respectively. Some workers have used isocratic $\log k$ values measured in an appropriate mobile phase as a lipophilicity parameter [8–10]. However, many more investigators use capacity factors extrapolated to 100% H_2O ($\log k_w$) to eliminate organic-solvent effects [11–15], and they have indeed demonstrated the usefulness of

the log k_w parameter when investigating series of solutes covering a broad lipophilicity range. Generally, the extrapolation to 100% H_2O is based on a quadratic relationship between the isocratic capacity factor log k and the volume fraction of organic solvent in the mobile phase, ϕ [16]. When MeOH is used as the organic modifier, a linear relationship, *Eqn. 2*, is often obtained for neutral solutes [17][18], where S is the slope and log k_w the intercept of the regression curve.

$$k = (t_R - t_0)/t_0 \tag{1}$$

$$\log k = -S\phi + \log k_w \tag{2}$$

Until recently, most lipophilicity studies were based on reversed-phase HPLC octadecyl silica (ODS) stationary phases. The correlations between log P_{oct} and log k_w or log k so obtained are good mostly for structurally related solutes [19][20]. The decrease in correlation between capacity factors and log P_{oct} with increasing structural diversity of solutes is believed to result from specific interactions of the compounds with the residual silanol groups in such stationary phases [21].

Measures have been taken to decrease the effects of free silanol groups. A masking agent such as decylamine was added to the mobile phase [22][23]. Great progress has been achieved with silica-based stationary phases exhibiting a high level of silanol deactivation, of which the *Discovery-RP-Amide-C16* phase is a good example. In this stationary phase, the alkyl chains contain an amide group that electrostatically shields silanols from highly polar analytes.

The polymer-based octadecylpolyvinyl (ODP) stationary phase is devoid of reactive silanol groups and is regarded as promising for assessing lipophilicity. Unlike other polymer-based stationary phases, it does not undergo swelling or shrinkage and offers the possibility of having reasonable flow rates without undesirable pressure increases at the column inlet [24–26].

A highly informative interpretation of retention mechanisms on reversed-phase HPLC stationary phases can be obtained by linear solvation/free-energy relationships (LSERs) based on the solvatochromic parameters [27–32]. This method has also been used to evaluate partitioning mechanisms of solutes in various organic/aqueous biphasic systems [33–36]. LSERs can be expressed by *Eqn. 3*, where S_p is a given molecular property of a neutral organic solute, here log k_w or log P_{oct}. The four structural parameters are the *Van der Waals* volume V_w, which accounts for hydrophobic and dispersive forces, and polar terms known as solvatochromic parameters (dipolarity/polarizability π^*, H-bond donor acidity α, and H-bond acceptor basicity β), which account for polar interactions between solute and solvents. The regression coefficients v, p, a, and b reflect the relative contribution of each solute parameter to S_p.

$$S_p = v \cdot V_w + p \cdot \pi^* + a \cdot \alpha + b \cdot \beta + c \tag{3}$$

The objective of this study was to assess and compare lipophilicity values measured with the silica-based *Discovery-RP-Amide-C16* and the polymer-based *ODP-50-4B* stationary phases. A set of solutes with well-defined structural parameters (V_w, π^*, β, and α) were selected (*Table 1*). This set of solutes included simple monofunctional

Table 1. *Investigated Solutes*[a]) *and Their Physicochemical Parameters*

						Discovery RP Amide C16		*ODP-50-4B*	
	V_w [b])[c])	π^* [b])[d])	β [b])[e])	α [b])[f])	log P_{oct} [b])	log k_w [g])	S [h])	log k_w [i])	S [j])
Model solutes									
Bases:									
Acridine	174.9	1.57	0.52	0.00	3.40	2.74	4.40	3.29	4.00
$PhNH_2$	98.0	0.94	0.41	0.06	0.90	0.11	0.96	1.41	2.04
PhNHEt	133.0	0.78	0.45	0.03	2.16	1.52	2.70	2.46	3.00
2-Cl-C_6H_4-NH_2	111.8	1.06	0.41	0.06	1.91	1.48	2.80	2.55	3.00
2-NH_2-C_6H_4-Ph	173.9	1.55	0.41	0.18	2.84	2.53	4.00	3.49	3.90
Neutrals:									
$PhCH_2CN$	121.5	1.22	0.45	0.00	1.56	1.16	2.70	2.28	2.90
PhCOMe	122.3	1.12	0.51	0.00	1.58	1.11	2.50	1.91	2.60
MeCOOBu	123.0	0.55	0.45	0.00	1.82	1.33	2.50	2.10	3.20
$PhNO_2$	107.6	1.01	0.28	0.00	1.85	1.50	2.80	2.51	2.90
2-Cl-C_6H_4-NO_2	122.0	1.13	0.28	0.00	2.24	2.10	3.50	3.04	3.50
$Ph(CH_2)_2Ph$	196.9	0.99	0.20	0.00	4.80	4.27	5.40	4.70	4.90
$PhCH_2OH$	111.6	0.84	0.58	0.33	1.08	0.70	2.10	1.37	2.50
4-Cl-C_6H_4-CH_2OH	126.3	0.96	0.58	0.33	1.96	1.62	3.00	2.10	3.00
Acids:									
3-Cl-C_6H_4-OH	107.8	0.84	0.16	0.69	2.49	2.36	3.80	2.88	3.60
3-NO_2-C_6H_4-OH	112.9	1.54	0.23	0.79	2.00	1.75	3.10	2.78	3.50
$Ph(CH_2)_2COOH$	146.0	1.12	0.45	0.60	1.89	1.59	2.90	2.18	3.10
$Ph(CH_2)_3COOH$	162.4	1.12	0.45	0.60	2.42	2.08	3.40	2.64	3.60
$Ph(CH_2)_4COOH$	179.8	1.12	0.45	0.60	2.85	2.53	3.80	3.06	4.00
$Ph(CH_2)_7COOH$	230.6	1.12	0.45	0.60	4.09	4.43	5.80	4.34	5.10
$C_9H_{19}COOH$	188.7	0.55	0.45	0.60	4.09	4.37	5.70	3.99	4.90
PhCOOH	111.8	0.74	0.40	0.59	1.96	1.50	3.00	1.83	2.70
4-Br-C_6H_4-COOH	133.8	0.94	0.40	0.59	2.86	2.48	3.70	2.94	3.60
3-Cl-C_6H_4-COOH	126.2	0.86	0.30	0.59	2.71	2.29	3.50	2.75	3.50
4-Cl-C_6H_4-COOH	126.5	0.86	0.27	0.59	2.06	2.28	3.50	2.76	3.50
4-I-C_6H_4-COOH	141.6	0.96	0.42	0.59	3.13	2.51	3.60	2.92	3.40
1-Naphthoic acid	158.5	1.05	0.40	0.59	3.10	2.47	3.80	2.95	3.50
Drugs:									
Flurbiprofen	223.1	1.78	0.49	0.60	3.81	3.54	4.90	3.93	4.60
Ibuprofen	197.0	1.14	0.49	0.60	3.87	3.62	4.90	3.84	4.80
Indomethacin	283.5	1.86	1.29	0.60	3.18	3.67	5.00	4.41	4.70
Ketoprofen	239.1	2.12	0.99	0.60	2.77	2.54	4.00	3.23	4.10
Naproxen	216.5	1.64	0.79	0.60	3.06	2.72	4.10	3.39	4.00
Phenobarbital	204.5	0.59	1.26	0.20	1.44	0.96	2.40	2.04	3.00
Phenytoin	228.3	1.45	1.02	0.60	2.68	1.83	3.40	2.87	4.00
Sulfabenzamide	233.6	2.48	1.25	0.33	1.46	0.88	2.40	2.62	3.60
Sulfacetamide	174.8	2.58	1.25	0.33	–0.16	–0.66	0.93	0.54	1.45
Sulfamethazine	237.5	2.72	1.90	0.33	0.25	–0.32	1.34	0.76	1.40
Sulfamethizole	211.7	2.25	1.46	0.36	0.55	0.07	1.85	1.37	2.20
Sulfamethoxazole	207.5	2.59	1.64	0.36	0.72	0.30	1.78	2.04	2.85
Sulfamethoxypyridazine	229.6	2.93	2.38	0.33	0.35	–0.18	1.40	1.17	1.80
Sulfanilamide	139.1	1.89	1.26	0.60	–0.69	–1.30	0.00	0.47	1.25
Sulfapyridine	209.4	2.76	1.78	0.33	0.02	–0.33	2.04	0.81	1.50

[a]) The structures of the complex compounds are shown in *Fig. 1*. [b]) Taken from [36]. [c]) *Van der Waals* volume. [d]) Dipolarity/polarizability. [e]) H-Bond acceptor basicity. [f]) H-Bond donor acidity. [g]) $0.01 \leq$ s.d. ≤ 0.15. [h]) $0.01 \leq$ s.d. ≤ 0.29. [i]) $0.01 \leq$ s.d. ≤ 0.26. [j]) $0.01 \leq$ s.d. ≤ 0.35.

compounds and complex drugs (*Fig. 1*) covering a broad range of structural parameters (*Fig. 2*) and log P_{oct} values. The relationship between log k_w and log P_{oct}, as well as the relationship between log k_w and the slope S, were investigated. The LSERs approach was applied to unravel the retention mechanisms of the solutes on the two stationary phases and to compare them with the partitioning mechanism in octan-1-ol/H_2O.

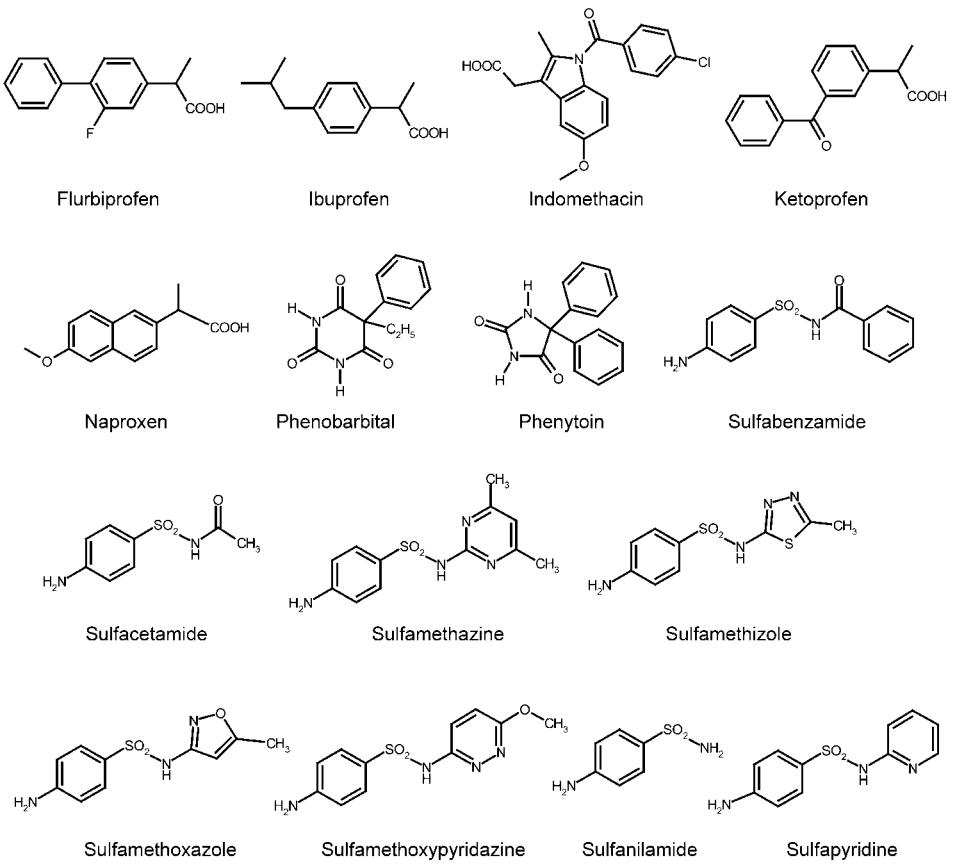

Fig. 1. *Structures of the drugs under study*

Results and Discussion. – *Relationship between log* k *and* ϕ. With both the *Discovery-RP-Amide-C16* and *ODP-50-4B* phases, a linear relationship between log k and ϕ was found for all compounds. In all cases, the squared correlation coefficient was higher than 0.99, except for aniline and sulfapyridine ($r^2 = 0.98$) on the *Discovery-RP-Amide-C16* phase and sulfamethazine ($r^2 = 0.98$) on the *ODP-50-4B* phase. The log k_w and S values of the 41 compounds on both phases were calculated by *Eqn. 2* and are presented in *Table 1*.

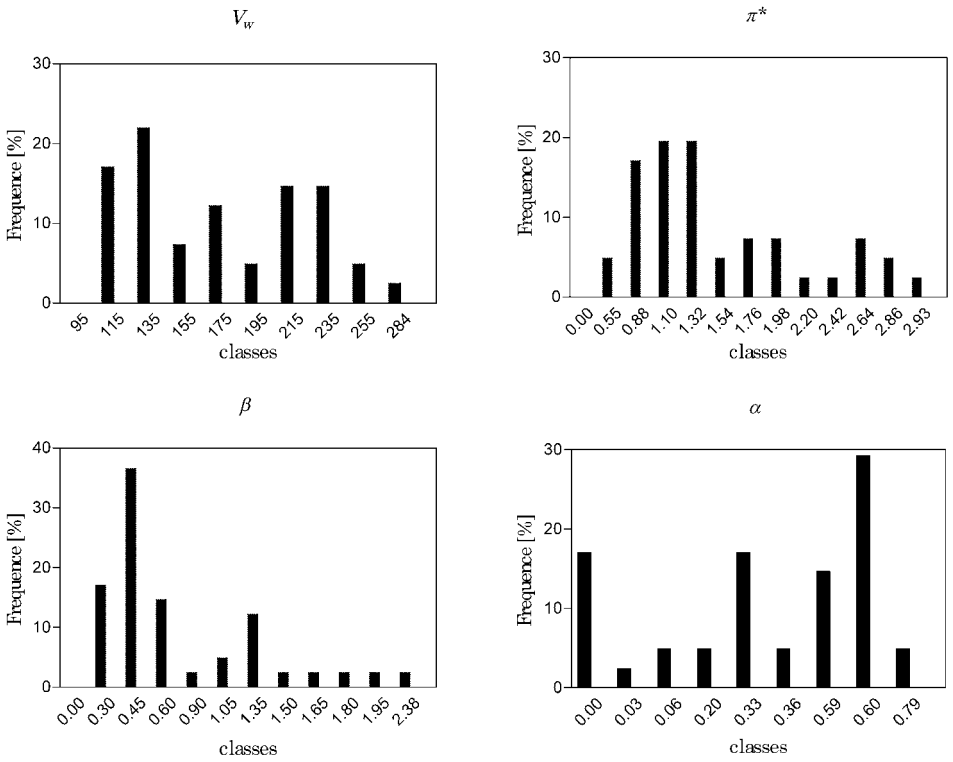

Fig. 2. *Distribution of the investigated compounds in the parameter space of the* Van der Waals *volume* V_w, *dipolarity/polarizability* π^*, *H-bond acceptor basicity* β *and the H-bond donor acidity* α

Correlation between log k_w *and Slope* S. The correlations between log k_w and slope S are described by *Eqns. 4* and *5* and are shown in *Fig. 3*, *i.e.* by *Eqn. 4* for the *Discovery-RP-Amide-C16* phase and by *Eqn. 5* for the *ODP-50-4B* phase.

$$S = 0.94\ (\pm 0.05)\ \log k_w + 1.50\ (\pm 0.11) \tag{4}$$

$$n = 41,\ q^2 = 0.97,\ r^2 = 0.97,\ s = 0.22,\ F = 1407$$

$$S = 0.93\ (\pm 0.07)\ \log k_w + 0.90\ (\pm 0.18) \tag{5}$$

$$n = 41,\ q^2 = 0.95,\ r^2 = 0.96,\ s = 0.21,\ F = 839$$

In these and the following equations, 95% confidence limits are in parentheses, n is the number of the compounds, q^2 the cross-validated correlation coefficient, r^2 the squared correlation coefficient, s the standard deviation, and F the *Fisher's* test.

The linear correlations between log k_w and S are significant for both phases and for the complete set of compounds. The correlation is slightly better for the *Discovery-RP-Amide-C16* than for the *ODP-50-4B* phase. However, and as shown in *Fig. 3a*, there are two outliers on the *Discovery-RP-Amide-C16* phase, namely sulfapyridine and aniline

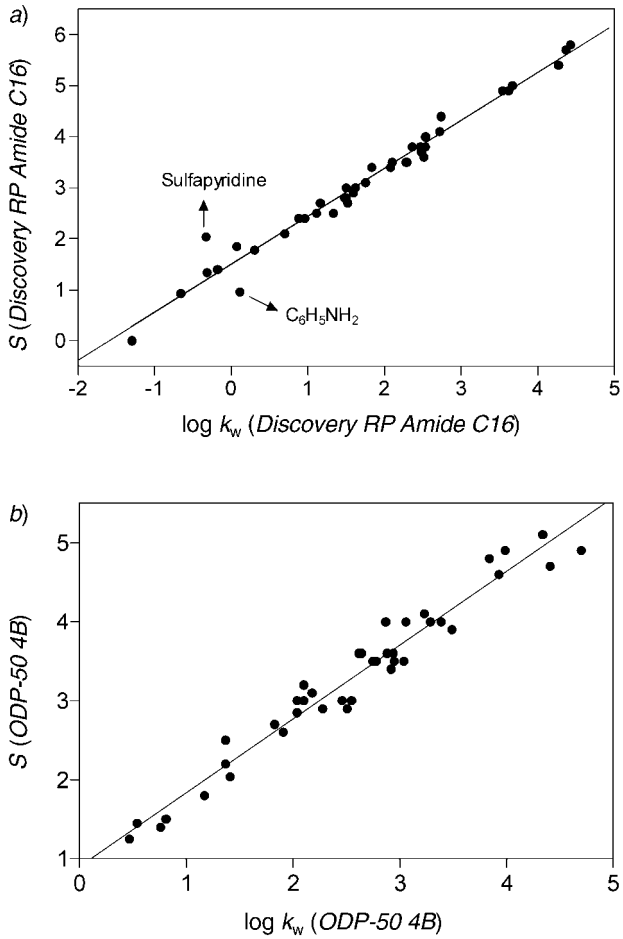

Fig. 3. *Relationship between log* k_w *and slope* S: a*) on the* Discovery-RP-Amide-C16 *phase and* b*) on the* ODP-50-4B *phase*

probably due to the lower quality of the linear regression between log k *vs.* ϕ. After omission of the two outliers, the correlation between log k_w and S becomes excellent ($r^2 = 0.99$).

In previous studies, good correlations between log k_w and S were obtained only for simple or closely related compounds [13][16][17]. Here, significant correlations were obtained for a structurally diverse set of analytes including model compounds and drugs. These significant correlations imply that log k_w and S are controlled by the same factors under the present conditions. In agreement with previous results [20], the slope of the correlation between log k_w and S is nearly 1 on each of the two stationary phases.

Correlation between log P_{oct} *and log* k_w. *Eqns. 6* and *7* and *Fig. 4* show the correlations between log P_{oct} and log k_w values, *i.e.*, *Eqn. 6* for the *Discovery-RP-Amide-C16* phase and *Eqn. 7* for the *ODP-50-4B* phase.

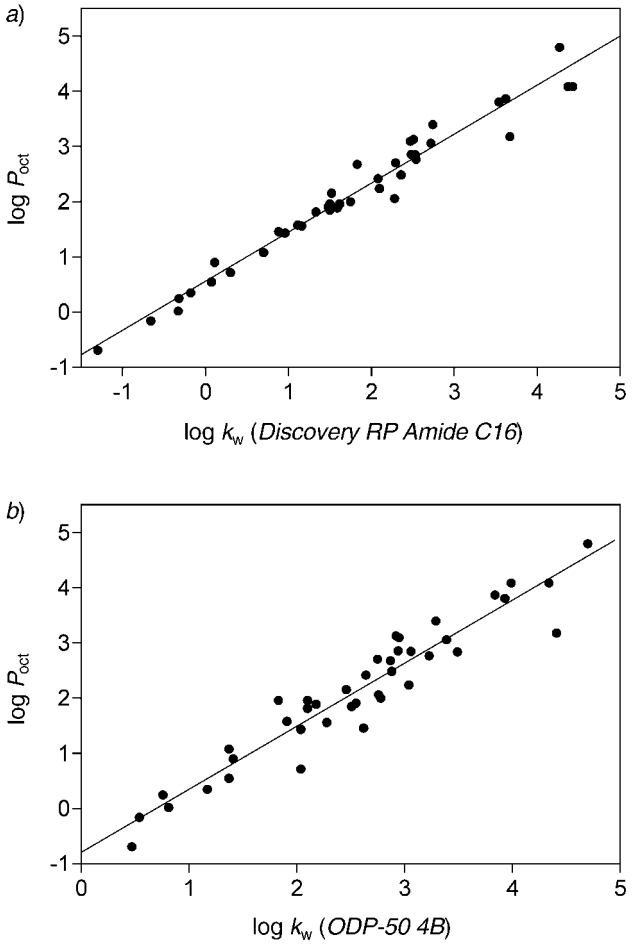

Fig. 4. *Relationship between log* P_{oct} *and log* k_w*:* a) *on the* Discovery-RP-Amide-C16 *phase and* b) *on the* ODP-50-4B *phase*

$$\log P_{oct} = 0.89\ (\pm 0.06)\ \log k_w + 0.56\ (\pm 0.12) \tag{6}$$

$$n = 41,\ q^2 = 0.96,\ r^2 = 0.96,\ s = 0.24,\ F = 1054$$

$$\log P_{oct} = 1.14\ (\pm 0.12)\ \log k_w - 0.80\ (\pm 0.32) \tag{7}$$

They clearly show that log k_w obtained with the *Discovery-RP-Amide-C16* phase is better correlated with log P_{oct} than log k_w obtained with the *ODP-50-4B* phase. In other words, there is a greater similarity between the partitioning mechanism in octan-1-ol/H_2O and the chromatographic retention mechanism on the *Discovery-RP-Amide-C16* phase than on the *ODP-50-4B* phase. This is probably due to the polar amido groups embedded in the former phase. Also, the throughput of the *Discovery-RP-Amide-C16*

phase to derive $\log k_w$ is higher than that of the *ODP-50-4B* phase due to a more-limited flow rate of the latter and its larger lipophilicity scale.

The correlation between $\log P_{oct}$ and S was found to be of lower quality than that between $\log P_{oct}$ and $\log k_w$, implying that the $\log k_w$ parameter is better suited than S for $\log P_{oct}$ approximations by reversed-phase HPLC.

We conclude from the above results that the silica-based *Discovery-RP-Amide-C16* phase is a better choice than the polymer-based *ODP-50-4B* phase to derive a lipophilicity index ($\log k_w$) correlated with $\log P_{oct}$ under the mobile-phase conditions used here.

Comparison between Retention Mechanisms on the Two Stationary Phases and Partitioning Mechanism in Octan-1-ol/H_2O by LSERs Analysis. The $\log k_w$ values obtained with the two stationary phases were analyzed by linear solvation/free-energy relationships (LSERs), yielding statistically significant equations describing the structural properties governing retention mechanisms, *i.e.* for the *Discovery-RP-Amide-C16* phase, *Eqn. 8* and, after removal of the insignificant variable, *Eqn. 8a*, and for the *ODP-50-4B* phase, *Eqn. 9* and, after removal of the insignificant variables, *Eqn. 9a*. *Eqn. 8a* shows that the main factors governing retention on the *Discovery-RP-Amide-C16* phase are the solute's molecular volume (V_w) and H-bond acceptor basicity (β), while the importance of dipolarity/polarizability (π^*) is smaller and the H-bond donor acidity (α) is not significant. *Eqn. 9a* reflects the different balance of structural parameters controlling $\log k_w$ on the *ODP-50-4B* phase, for which V_w and β are important parameters, whereas π^* and α are not significant.

$$\log k_w = 2.65 \cdot 10^{-2}(\pm 0.46 \cdot 10^{-2}) \cdot V_w - 0.49(\pm 0.45) \cdot \pi^* - 2.58(\pm 0.58) \cdot \beta + 0.30(\pm 0.64) \cdot \alpha - 0.28(\pm 0.64) \quad (8)$$

$$n = 41,\ q^2 = 0.86,\ r^2 = 0.88,\ s = 0.50,\ F = 66$$

$$\log k_w = 2.72 \cdot 10^{-2}(\pm 0.44 \cdot 10^{-2}) \cdot V_w - 0.48(\pm 0.44) \cdot \pi^* - 2.62(\pm 0.57) \cdot \beta - 0.24(\pm 0.63) \quad (8a)$$

$$n = 41,\ q^2 = 0.87,\ r^2 = 0.88,\ s = 0.50,\ F = 87$$

$$\log k_w = 2.18 \cdot 10^{-2}(\pm 0.38 \cdot 10^{-2}) \cdot V_w - 0.12(\pm 0.36) \cdot \pi^* - 2.18(\pm 0.47) \cdot \beta - 0.16(\pm 0.55)\ \cdot \alpha + 0.69(\pm 0.52) \quad (9)$$

$$n = 41,\ q^2 = 0.84,\ r^2 = 0.86,\ s = 0.52,\ F = 56$$

$$\log k_w = 2.12 \cdot 10^{-2}(\pm 0.34 \cdot 10^{-2}) \cdot V_w - 2.27(\pm 0.32) \cdot \beta + 0.63(\pm 0.46) \quad (9a)$$

$$n = 41,\ q^2 = 0.85,\ r^2 = 0.86,\ s = 0.40,\ F = 114$$

To allow a better comparison, the $\log P_{oct}$ values were also analyzed by LSERs, yielding *Eqn. 10*. After removal of the insignificant variable, *Eqn. 10a* is obtained.

$$\log P_{oct} = 2.40 \cdot 10^{-2}(\pm 0.42 \cdot 10^{-2}) \cdot V_w - 0.42(\pm 0.40) \cdot \pi^* - 2.41(\pm 0.52) \cdot \beta + 0.01(\pm 0.64) \cdot \alpha + 0.41(\pm 0.57) \quad (10)$$
$$n = 41,\ q^2 = 0.87,\ r^2 = 0.88,\ s = 0.45,\ F = 67$$

$$\log P_{oct} = 2.41 \cdot 10^{-2}(\pm 0.38 \cdot 10^{-2}) \cdot V_w - 0.42(\pm 0.40) \cdot \pi^* - 2.41(\pm 0.51) \cdot \beta + 0.41(\pm 0.56) \quad (10a)$$
$$n = 41,\ q^2 = 0.87,\ r^2 = 0.88,\ s = 0.45,\ F = 92$$

One can see from *Eqns. 10* and *10a* that V_w and β are the two main structural properties governing the partitioning mechanism in octan-1-ol/H_2O, whereas π^* is of lesser significance and α is devoid of any significance. The ratios of the normalized regression coefficients in *Eqns. 8a* and *10a* are nearly identical (details not shown), meaning that the same balance of intermolecular forces is encoded by $\log P_{oct}$ and $\log k_w$ measured on the *Discovery-RP-Amide-C16* phase. This finding confirms the highly significant correlation between these two parameters as shown in *Eqn. 6*.

A comparison between *Eqns. 9a* and *10a* indicates that the balance of forces encoded by $\log k_w$ measured on the *ODP-50-4B* phase is different from that encoded by $\log P_{oct}$, explaining the lower correlation between these two parameters (*Eqn. 7*). When the structural descriptors were included in *Eqn. 7*, the correlation quality becomes higher as shown by *Eqn. 11* and, after removal of the insignificant term, *Eqn. 11a*. The correlation coefficient in *Eqn. 11a* is better than that in *Eqn. 7*, but it remains lower than that between $\log P_{oct}$ and $\log k_w$ on the *Discovery-RP-Amide-C16* phase (*Eqn. 6*).

$$\log P_{oct} = 0.80(\pm 0.26)\ \log k_w + 0.65 \cdot 10^{-2}(\pm 0.64 \cdot 10^{-2}) \cdot V_w - 0.32(\pm 0.28) \cdot \pi^* - 0.66(\pm 0.67) \cdot \beta + 0.14(\pm 0.43) \cdot \alpha - 0.14(\pm 0.44) \quad (11)$$
$$n = 41,\ q^2 = 0.94,\ r^2 = 0.94,\ s = 0.32,\ F = 118$$

$$\log P_{oct} = 0.80(\pm 0.26)\ \log k_w + 0.70 \cdot 10^{-2}(\pm 0.62 \cdot 10^{-2}) \cdot V_w - 0.32(\pm 0.28) \cdot \pi^* - 0.70(\pm 0.66) \cdot \beta - 0.12(\pm 0.42) \quad (11a)$$
$$n = 41,\ q^2 = 0.94,\ r^2 = 0.94,\ s = 0.31,\ F = 150$$

Conclusions. – For the two stationary phases investigated, linear relationships were found between isocratic $\log k$ and the volume fraction of MeOH in the eluent. The significant correlation between the derived parameters $\log k_w$ and slope S (*Eqn. 2*) implies that these two parameters encode the same information under the experimental conditions of the present study.

By using a wide range of solutes (including drugs) and eluents enriched in octan-1-ol, the silica-based *Discovery-RP-Amide-C16* phase yielded a lipophilicity index $\log k_w$ which was better correlated with $\log P_{oct}$ than the $\log k_w$ index obtained with the

polymer-based *ODP-50-4B* phase. A LSER analysis showed that retention on the *Discovery-RP-Amide-C16* phase and partitioning in octan-1-ol/H_2O are controlled by the same balance of structural properties, namely *Van der Waals* volume (V_w), H-bond acceptor basicity (β), and dipolarity/polarizability (π^*). In contrast, the retention mechanism on the *ODP-50-4B* phase is governed by a different balance of structural properties.

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Experimental Part

Solutes and Reagents. All compounds were obtained from commercial sources (*Wako*, Osaka, Japan; *TCI*, Tokyo, Japan; *Sigma-Aldrich*, Tokyo, Japan, and *Steinheim*, Germany; *ICN*, Aurora, USA; *Merck*, *Schuchardt*, Germany) and in the highest available purity. Dist. H_2O, HPLC-grade MeOH, and octan-1-ol (*Sigma-Aldrich*, Steinheim, Germany) were used throughout.

Selection of the solutes. A set of 41 compounds with exper. log P_{oct} values ranging from -0.69 to 4.80 were selected. This set consists of model compounds and drugs having a relatively rigid structure and well-defined parameters (V_w, π^*, β, and α) [36]. The investigated compounds and their physicochemical parameters are shown in *Table 1*. The broad range of parameter spaces (V_w, π^*, β, and α) is demonstrated in *Fig. 2*.

Measurement of Capacity Factors. The capacity factors were measured with a liquid chromatograph equipped with a *880-PU*-HPLC pump, a *875*-UV/Vs detector (both from *Jasco*, Tokyo, Japan), a *655A-40* autosampler, and a *D-2000* chromato-integrator (both from *Hitachi*, Tokyo, Japan). The *Supelcosil Discovery-RP-Amide-C16* column (5 cm × 4.6 mm i.d., 5 μm) was from *Supelco* (Bellefonte, PA, USA) and the *Asahipak ODP-50-4B* column (5 cm × 4.6 mm i.d., 5 μm) from *Asahi Chemicals* (Kawasaki, Japan). The mobile phase consisted of 0.02M phosphate buffer and MeOH in varying proportions (80 → 10% *v*/*v*). The phosphate buffer was adjusted to pH 7 for all nonionizable compounds and to a pH value (pH 3, 4, or 7) where the neutral form was in large excess for the ionizable compounds. To increase the similarity with octan-1-ol/buffer partitioning [15][22], a 0.25% (*v*/*v*) amount of octan-1-ol was added to MeOH, and octan-1-ol-sat. H_2O was used to prepare the buffer. The phosphate buffer was filtered under vacuum through a 0.45-μm *HA-Millipore* filter (*Millipore*, Milford, MA, USA) before being mixed with MeOH. The retention times t_R were measured at r.t. by the UV/Vs detector at wavelength λ_{max} of the analytes. The solns. to be injected (10^{-4} M to 10^{-3} M) were prepared by dissolving the solutes in the mobile phase; the injection volume was 10 μl. Uracil was used as the unretained compound. On the *Discovery-RP-Amide-C16* phase, the measurements were carried out at a flow rate of 1.0 ml/min for compounds with a log P_{oct} value higher than 1, and 0.5 ml/min for compounds with log P_{oct} below 1. Since the highest pressure limit of the *ODP-50-4B* column is much lower (*ca.* 730 psi) than that of silica-based columns (4000 psi), a low flow rate (0.5 ml/min) was used for the *ODP-50-4B* phase to prolong its life. In all cases, three different MeOH concentrations were used for extrapolation to log k_w. MeOH concentrations were adapted to the log P_{oct} values of the solutes as described in *Table 2*. The capacity factor log *k* was calculated by *Eqn. 1*. All log *k* values were the average of three measurements. The log *k* values were then extrapolated to 100% H_2O by using *Eqn. 2*.

Table 2. *Concentrations of Organic Modifier (MeOH) Used with the Two Stationary Phases*

log P_{oct} of solutes	% MeOH	
	Discovery-RP-Amide-C16	*ODP-50-4B*
> 3	60, 65, 70	70, 75, 80
1 – 3	40, 45, 50	60, 65, 70
< 1	10, 20, 25	20, 30, 40

Statistical Analysis. All regression analyses were performed *via* the JMP statistical software package (version 5.1.1, Japanese Edition, *SAS Institute Inc*).

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Determination of lipophilicity by reversed-phase high-performance liquid chromatography
Influence of 1-octanol in the mobile phase

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Abstract

Lipophilicity was evaluated using a novel RP-HPLC stationary phase (Discovery-RP-Amide-C16) with and without 1-octanol added to the mobile phase. A set of 46 drugs and flavonoids characterized by a broad structural diversity and a wide $\log P_{oct}$ range (−0.69 to 5.70) was selected for this study. This set consists of neutral solutes and solutes with acidic or ampholytic functionalities which were maintained neutral at pH 2.5 or 4. In our conditions, the addition of 1-octanol in the mobile phase proved a key factor to derive a lipophilicity index $\log k_w$ highly correlated with $\log P_{oct}$ for all investigated solutes. 1-Octanol improved the correlation between $\log P_{oct}$ and $\log k_w$ mainly by influencing the retention behavior of the solutes with $\log P_{oct}$ values below +3. This study brings additional evidence that under proper experimental conditions of stationary and mobile phases, RP-HPLC is a very useful method to obtain $\log P_{oct}$ values.

Keywords: Lipophilicity; $\log P_{oct}$; $\log k_w$; Mobile phase; Stationary phase; 1-Octanol; LC

1. Introduction

Since many years, lipophilicity is recognized as a meaningful parameter in structure–activity and structure–ADME relationships. It is also the single most informative and successful physicochemical property in medicinal chemistry. Not only has lipophilicity found innumerable applications in quantitative structure–activity relationships (QSARs) and quantitative structure-pharmacokinetic relationships (QSPkRs), but its study has revealed a wealth of information on intermolecular forces, intramolecular interactions, and molecular structure in the broadest sense [1–4].

The most widely used measure of lipophilicity is the partition coefficient in the 1-octanol/water system (noted $\log P_{oct}$). The reference procedure to measure $\log P_{oct}$ is the shake-flask method, which however is time-consuming and limited in range (ca. $-3 < \log P < 4$). Beyond these limits, $\log P_{oct}$ values measured by the shake-flask method become unreliable.

The reversed-phase HPLC (RP-HPLC) method is a promising alternative to the shake-flask method, having such advantages as a higher throughput, an insensitivity to impurities or degradation products, and a broader lipophilicity range. In RP-HPLC, lipophilicity indices are derived from the capacity factor $\log k$, which is calculated by Eq. (1):

$$k = \frac{t_r - t_0}{t_0} \quad (1)$$

where t_r and t_0 are the retention times of the solute and of an unretained compound, respectively. Some workers have used isocratic $\log k$ values measured in an appropriate mobile phase as a lipophilicity parameter [5–7]. However, many more investigators have used capacity factors extrapolated

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to 100% water ($\log k_w$) to eliminate organic solvent effects [8–13], and they have indeed demonstrated the usefulness of the $\log k_w$ parameter when investigating series of solutes covering a broad lipophilicity range. Generally, the extrapolation to 100% water is based on a quadratic relationship between the isocratic capacity factor $\log k$ and the volume fraction of organic solvent in the mobile phase, φ [14]. When methanol is used as the organic modifier, a linear relationship (Eq. (2)) is often obtained for neutral solutes [15,16]:

$$\log k = -S\varphi + \log k_w \quad (2)$$

where S is the slope and $\log k_w$, the intercept of the regression curve.

Until recently, most lipophilicity studies were based on RP-HPLC octadecyl silica (ODS) stationary phases. The correlations between $\log P_{oct}$ and $\log k_w$ or $\log k$ values so obtained are usually good for structurally related solutes [15,17,18]. The decrease in correlation between capacity factors and $\log P_{oct}$ with increasing structural diversity of solutes is believed to result from specific interactions of the compounds with the residual silanol groups in such stationary phases [19]. Therefore it is a very big challenge in this method to find the optimal stationary and mobile phase conditions in order to obtain $\log k_w$ values highly correlated with $\log P_{oct}$ for a broad range of noncongeneric compounds.

Measures have been taken to decrease the effects of free silanol groups. Great progress has been achieved with silica-based stationary phases exhibiting a high level of silanol deactivation, of which LC-ABZ and Discovery-RP-Amide-C16 phases are good examples. In these stationary phases, the alkyl chains contain an amido group which electrostatically shields silanols from highly polar analytes. In addition, it is hypothesized that the combination of amido groups and a hydration layer at the silica surface leads to a high degree of orientation of the alkyl chains of the stationary phase [20], which facilitates their hydrophobic interaction with the solutes, in contrast to what happens in conventional ODS stationary phases where the alkyl chains are mostly folded. The advantage of LC-ABZ over the conventional ODS stationary phases in $\log P_{oct}$ measurement has been verified and discussed [18,21–23]. A highly significant correlation was found between $\log P_{oct}$ and $\log k_w$ values obtained with the Discovery-RP-Amide-C16 phase for a wide range of compounds including model solutes and drugs [24].

The influence of the mobile phase on the $\log k_w$ versus $\log P_{oct}$ correlation has also been investigated using different organic modifiers [25,26] and/or adding low levels of *n*-decylamine or 1-octanol [12,13,24,27,28]. Lombardo et al. [12] investigated the influence of 1-octanol in the mobile phase on the $\log P_{oct}$ measurement for a set of noncongeneric neutral drugs on LC-ABZ stationary phase. A highly significant correlation between $\log k_w$ and $\log P_{oct}$ was obtained in the presence of 1-octanol in the mobile phase. The authors also expanded the applicability of the method to the determination of the 1-octanol/water distribution coefficients ($\log D_{oct}$) at pH 7.4 for neutral and basic drugs [13].

In order to further investigate the optimal conditions and the applicable range for obtaining $\log P_{oct}$ values from RP-HPLC measurements, we selected here a set of 46 neutral solutes and solutes with acidic and ampholytic functionalities, which were maintained neutral at pH 2.5 or 4. We determined their $\log k_w$ values on the Discovery-RP-Amide-C16 stationary phase using a methanol/phosphate buffer eluent with and without 1-octanol. The compounds in this set are all biologically active and cover a broad structural diversity as well as a wide $\log P_{oct}$ range (−0.69 to 5.70). They are nonsteroidal anti-inflammatory drugs (NSAIDs), steroids, 4-phenyldihydropyridine (DHPs) calcium-channel blockers, antibacterials and flavonoids, as shown in Fig. 1. The correlation between $\log k_w$ and $\log P_{oct}$ values, and the relation between $\log k_w$ and S (see Eq. (2)), were explored. In addition, the mechanism of the influence of 1-octanol in the mobile phase on the $\log k_w$ versus $\log P_{oct}$ correlation was investigated.

2. Experimental

2.1. Solutes and reagents

All compounds were obtained from commercial sources (Wako, Osaka, Japan; TCI, Tokyo, Japan; Sigma–Aldrich, Tokyo, Japan and Steinheim, Germany; ICN, Aurora, USA; Merck, Schuchardt, Germany; TRC, North York, Canada; LKT laboratories Inc. Tokyo, Japan) and in the highest available purity. Distilled water, HPLC grade methanol, and 1-octanol (Sigma–Aldrich, Steinheim, Germany) were used throughout.

2.2. Measurement of capacity factors

The capacity factors were measured with a liquid chromatograph equipped with a *880-PU*-HPLC pump, a *875*-UV–vis detector (both from Jasco, Tokyo, Japan), a *655A-40* autosampler and a *D-2000* chromato-integrator (both from Hitachi, Tokyo, Japan).

The column was a Supelcosil Discovery-RP-Amide-C16 (5 cm × 4.6 mm I.D., 5 μm) from Supelco (Bellefonte, PA, USA). The mobile phase consisted of 0.02 M phosphate buffer and methanol in proportions varying from 70 to 10% (v/v). The phosphate buffer was adjusted to pH 3 for all neutral drugs (steroids 16–23, DHPs calcium-channel blockers 24–29 in Table 2) and flavonoids (42–46 in Table 2), and for the ionizable drugs to a pH value where the neutral form was in large excess (pH 2.5 for NSAIDs 1–15 and pH 4 for antibacterials 30–41 in Table 2). Two sets of measurements were conducted for all compounds. In one set, a 0.25% (v/v) amount of 1-octanol was added to methanol [27,28], and 1-

octanol saturated water was used to prepare the buffer. In the other set, the mobile phase condition was the same as that used in the first set except for the absence of 1-octanol in the eluents. The phosphate buffer was filtered under vacuum through a 0.45 μm HA Millipore filter (Millipore, Milford, MA, USA) before being mixed with methanol. The retention times were measured at ambient temperature by the UV–vis detector at the λ_{max} of the analytes.

The solutions to be injected (10^{-4} M to 10^{-3} M) were prepared by dissolving the solutes in the mobile phase; the injection volume was 10 μL. Uracil was used as the unretained compound. The measurements were carried out at a

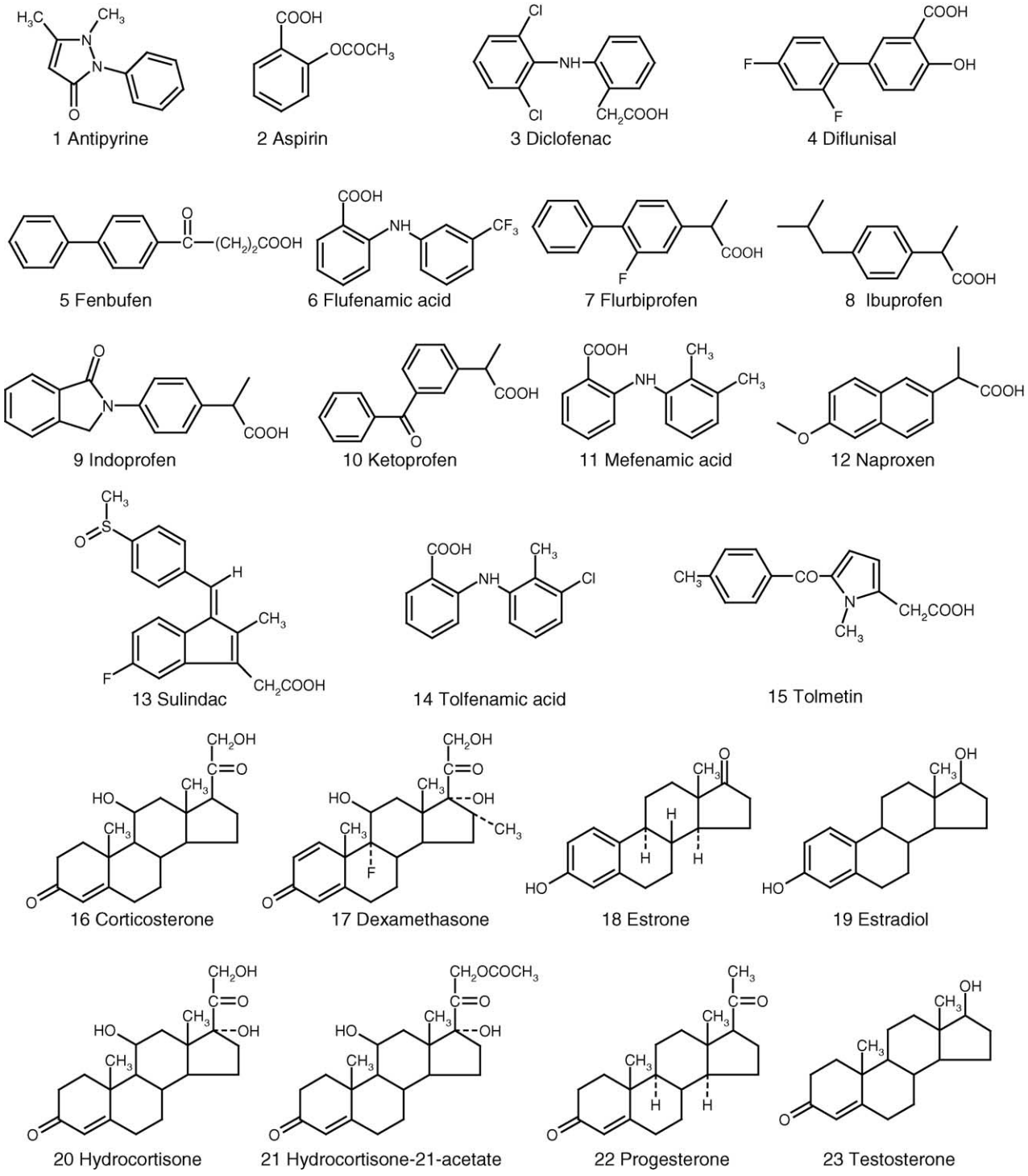

Fig. 1. Structures of the drugs and flavonoids under study.

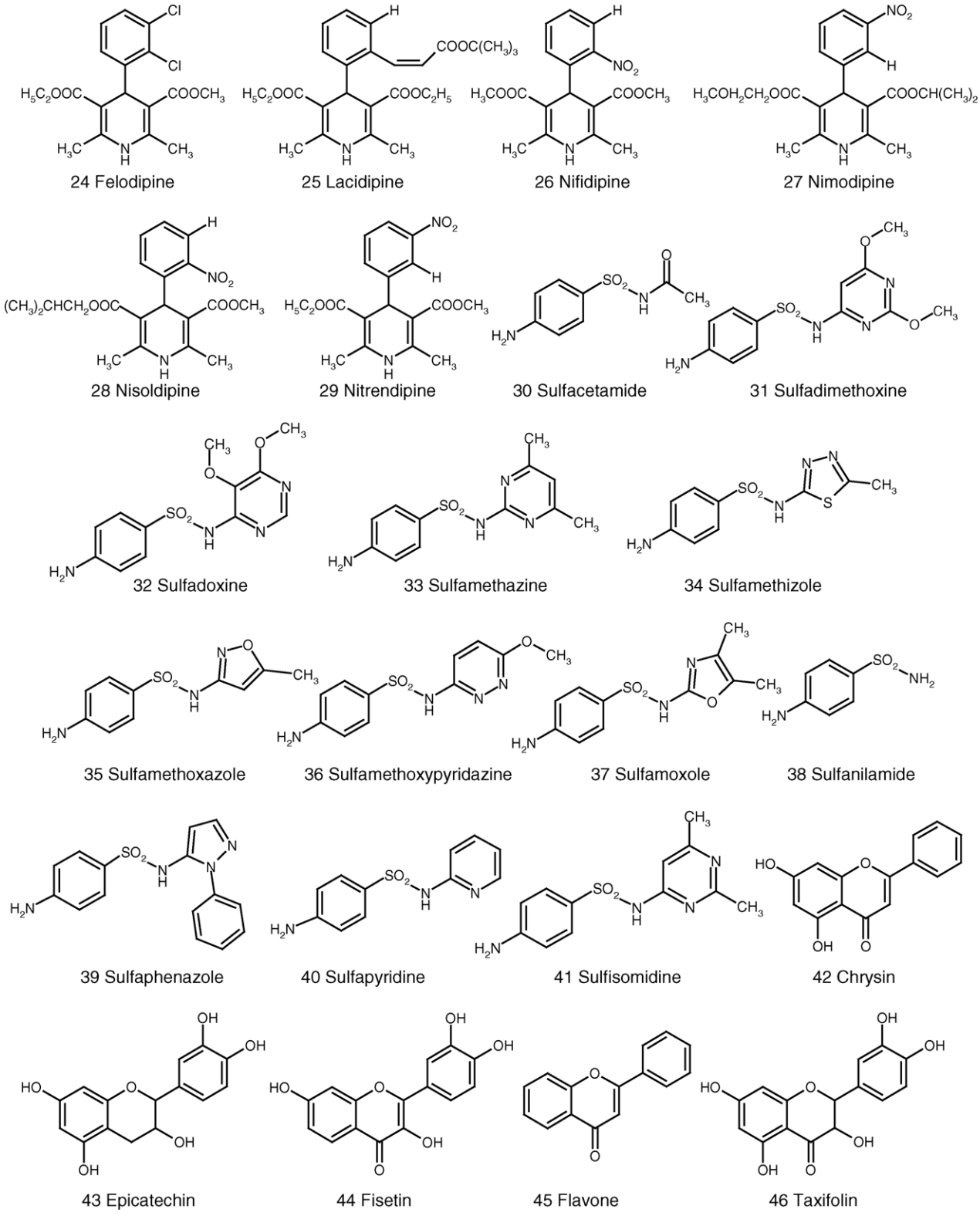

Fig. 1. (*Continued*).

flow rate 1.0 mL/min for compounds with a $\log P_{oct}$ value higher than +1, and 0.5 mL/min for compounds with $\log P_{oct}$ below +1. In all cases, three different methanol concentrations were used for extrapolation to $\log k_w$. Methanol concentrations were adapted to the $\log P_{oct}$ values of the solutes as described in Table 1.

The capacity factor $\log k$ was calculated by Eq. (1). All $\log k$ values were the average of three measurements. The

Table 1
Concentrations of organic modifier (methanol) used in the two sets of experiments

log P_{oct} of the solutes	%MeOH
>3	60, 65, 70
1–3	40, 45, 50
<1	10, 20, 25

log k values were then extrapolated to 100% water using Eq. (2).

2.3. Statistical analysis

All regression analyses were performed via the JMP statistical software package (Version 5.1.1, Japanese Edition, SAS Institute Inc.).

3. Results and discussion

3.1. Relationship between log k and φ

A linear relationship between log k and φ (the volume fraction of organic solvent in the eluent) was found for all compounds under both eluent conditions. In all cases, the squared correlation coefficient was higher than 0.99, excepting sulfamoxole and sulfapyridine ($r^2 = 0.98$) under the mobile phase with the presence of 1-octanol. The log k_w and S (slope) values of the 46 solutes were calculated by Eq. (2) and are presented in Table 2.

3.2. Correlation between log k_w and S

The correlation between log k_w and S was investigated under the two mobile phase conditions. Fig. 2 shows a large difference in statistical quality in the presence or absence of 1-octanol. The correlation was highly significant in the presence of 1-octanol (Eq. (3A) and Fig. 2A), while a poor correlation was established in the absence of 1-octanol (Eq. (3B) and Fig. 2B).

$$S = 0.86(\pm 0.04)\ \log k_w + 1.70(\pm 0.11)$$
$$n = 46;\ q^2 = 0.97;\ r^2 = 0.97;\ s = 0.24;\ F = 1549 \quad (3A)$$

$$S = 0.36(\pm 0.13)\ \log k_w + 3.54(\pm 0.36)$$
$$n = 46;\ q^2 = 0.41;\ r^2 = 0.41;\ s = 0.50;\ F = 31 \quad (3B)$$

In this and the following equations, 95% confidence limits are in parentheses; n, the number of the compounds; q^2, the cross-validated correlation coefficient; r^2, the squared correlation coefficient; s, the standard deviation and F the Fisher's test.

In spite of the significant correlation between log k_w and S for the complete set of solutes in Eq. (3A), there are three outliers as shown in Fig. 2A, namely sulfanilamide (38), sulfapyridine (40) and epicatechin (43). The reason for sulfapyridine being an outlier is probably the lower quality of the linear regression between log k versus φ. For the other two outliers, no reason is apparent and further investigation is needed. After omission of the three outliers, the correlation between log k_w and S becomes even better ($r^2 = 0.98$).

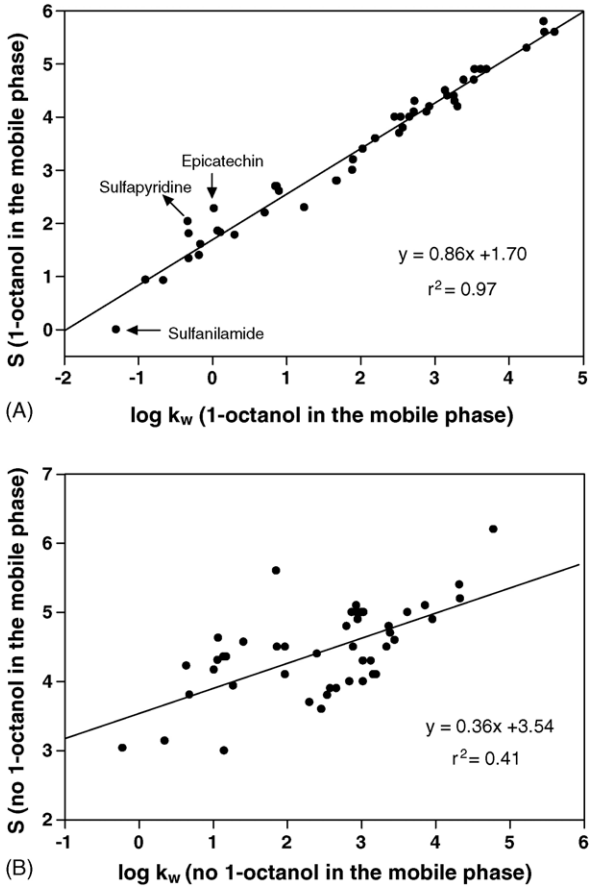

Fig. 2. Relationship between log k_w and slope S (A) in the presence of 1-octanol in the mobile phase and (B) in the absence of 1-octanol in the mobile phase.

Since the only difference between the two sets of experimental conditions was the presence or absence of 1-octanol in the mobile phase, the factor producing the much better correlation between log k_w and S is clearly the addition of 1-octanol. The significant correlation in Eq. (3A) implies that S is controlled by the same intermolecular forces as those in log k_w, which are van der Waals volume, H-bond acceptor basicity and dipolarity/polarizability as unraveled by a solvatochromic analysis in our previous study [24]. On the contrary, the non-significant correlation in the absence of 1-octanol (Eq. (3B) and Fig. 2B) means that these two parameters encode different structural information. A clear interpretation of S in this condition needs further quantitative structure-property (here S) relationship analysis.

In previous studies, good correlations between log k_w and S were obtained mostly for simple or closely related

Table 2
Investigated compounds and their physicochemical parameters

Number	Solutes	log P_{oct} [a]	1-Octanol in the mobile phase		No 1-octanol in the mobile phase	
			log k_w [b]	S [c]	log k_w [d]	S [e]
	NSAIDs					
1	Antipyrine	0.17	−0.16	1.61	1.06	4.31
2	Aspirin	1.13	0.71	2.20	1.15	3.00
3	Diclofenac	4.40	3.53	4.70	3.45	4.60
4	Diflunisal	4.44	3.31	4.20	3.20	4.10
5	Fenbufen	3.39	2.66	4.00	2.54	3.80
6	Flufenamic acid	5.25	4.48	5.60	4.32	5.40
7	Flurbiprofen	3.81	3.54	4.90	3.39	4.70
8	Ibuprofen	3.87	3.62	4.90	3.34	4.50
9	Indoprofen	2.77	1.90	3.20	2.93	5.10
10	Ketoprofen	2.77	2.54	4.00	3.03	5.00
11	Mefenamic acid	5.12	4.24	5.30	3.96	4.90
12	Naproxen	3.06	2.72	4.10	2.58	3.90
13	Sulindac	3.42	2.73	4.30	2.89	4.50
14	Tolfenamic acid	5.70	4.62	5.60	4.33	5.20
15	Tolmetin	2.79	2.46	4.00	2.95	4.90
	Steroids					
16	Corticosterone	2.20	1.68	2.80	2.80	4.80
17	Dexamethasone	1.83	1.67	2.80	2.87	5.00
18	Estrone	3.13	2.89	4.10	2.84	4.00
19	Estradiol	4.01	3.26	4.40	3.02	4.00
20	Hydrocortisone	1.55	1.24	2.30	2.40	4.40
21	Hydrocortisone-21-acetate	2.19	1.89	3.00	3.00	5.00
22	Progesterone	3.57	3.17	4.40	3.13	4.30
23	Testosterone	3.29	2.57	3.80	2.66	3.90
	DHPs caicium-channel blockers					
24	Felodipine	4.80	3.70	4.90	3.86	5.10
25	Lacidipine	5.56	4.47	5.80	4.78	6.20
26	Nifedipine	3.22	2.20	3.60	2.30	3.70
27	Nimodipine	4.18	3.14	4.50	3.37	4.80
28	Nisoldipine	4.53	3.39	4.70	3.62	5.00
29	Nitrendipine	4.15	2.93	4.20	3.02	4.30
	Antibacterials					
30	Sulfacetamide	−0.16	−0.66	0.93	0.35	3.14
31	Sulfadimethoxine	1.40	0.87	2.70	1.97	4.50
32	Sulfadoxine	0.56	0.11	1.83	1.41	4.57
33	Sulfamethazine	0.25	−0.32	1.34	1.01	4.17
34	Sulfamethizole	0.55	0.07	1.85	1.18	4.36
35	Sulfamethoxazole	0.72	0.30	1.78	1.27	3.94
36	Sulfamethoxypyridazine	0.35	−0.18	1.40	1.14	4.36
37	Sulfamoxole	−0.14	−0.32	1.81	1.07	4.63
38	Sulfanilamide	−0.69	−1.30	0.00	−0.22	3.04
39	Sulfaphenazole	1.27	0.85	2.70	1.86	4.50
40	Sulfapyridine	0.02	−0.33	2.04	0.68	3.81
41	Sulfisomidine	−0.37	−0.90	0.94	0.64	4.23
	Flavonoids					
42	Chrysin	3.52	3.27	4.30	3.16	4.10
43	Epicatechin	0.56	0.02	2.28	1.85	5.60
44	Fisetin	2.53	2.03	3.40	2.93	5.00
45	Flavone	3.56	2.52	3.70	2.46	3.60
46	Taxifolin	0.95	0.90	2.61	1.97	4.10

[a] The values of drugs 1–41 are taken from [12,29–32], those of flavonoids 42–46 are MlogP from the Bio-loom software [33].
[b] $0.01 \leq SD \leq 0.18$.
[c] $0.01 \leq SD \leq 0.30$.
[d] $0.01 \leq SD \leq 0.19$.
[e] $0.01 \leq SD \leq 0.30$.

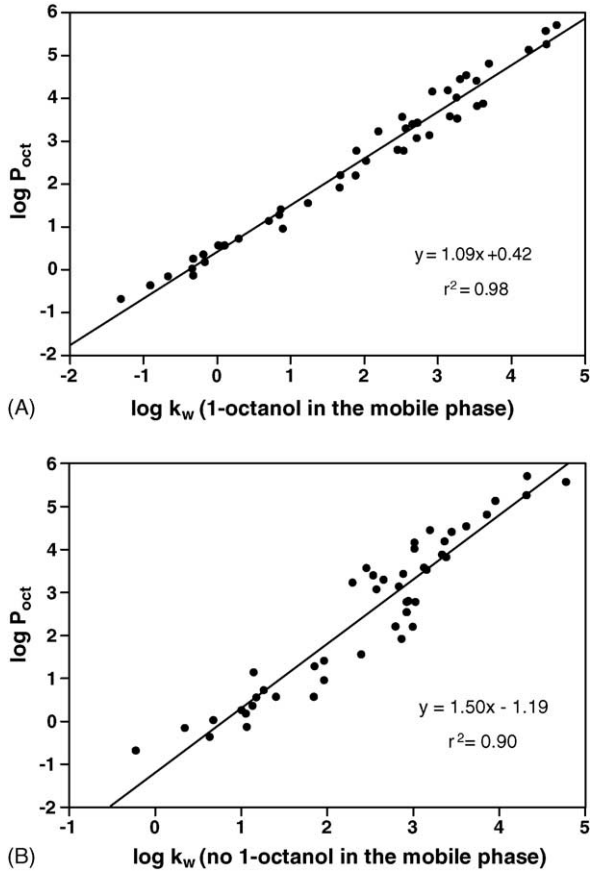

Fig. 3. Relationship between $\log P_{oct}$ and $\log k_w$ (A) in the presence of 1-octanol in the mobile phase and (B) in the absence of 1-octanol in the mobile phase.

compounds [10,14,15]. Here, significant correlations were obtained for a structurally diverse set of chemically complex drugs and flavonoids by using a 1-octanol enriched eluent. In agreement with previous results [18], the slope of the correlation between $\log k_w$ and S in Eq. (3A) is close to unity.

3.3. Correlation between $\log P_{oct}$ and $\log k_w$

The correlations between $\log P_{oct}$ and $\log k_w$ values obtained with the two sets of eluents are also markedly different, with 1-octanol producing a significant improvement. Eq. (4A) and Fig. 3A show that, in the presence of 1-octanol, the correlation between $\log P_{oct}$ and $\log k_w$ was highly significant for the whole set of solutes investigated. In contrast, the correlation was good but of lower quality when 1-octanol was absent (Eq. (4B) and Fig. 3B). This result is in agreement with the work of Lombardo et al. [12], who compared lipophilicity measurement in the presence or absence of 1-octanol using a set of 27 structurally diverse neutral solutes on a Supelcosil LC-ABZ column.

$$\log P_{oct} = 1.09(\pm 0.05)\log k_w + 0.42(\pm 0.13)$$
$$n = 46;\ q^2 = 0.97;\ r^2 = 0.98;\ s = 0.28;\ F = 1785 \quad (4A)$$

$$\log P_{oct} = 1.50(\pm 0.15)\log k_w - 1.19(\pm 0.42)$$
$$n = 46;\ q^2 = 0.89;\ r^2 = 0.90;\ s = 0.58;\ F = 387 \quad (4B)$$

In addition to the higher squared correlation coefficient and smaller standard deviation of Eq. (4A) compared to Eq. (4B), the slopes of these two equations are very different. As stated by Minick et al. [27], the slope of an equation correlating $\log k_w$ and $\log P_{oct}$ is an estimate of how closely the free energies of the processes compare. A unit slope in such a plot indicates that the two processes are homoenergetic. In Eq. (4A), the slope is very close to unity, meaning that the chromatographic retention process on the Discovery-RP-Amide-C16 stationary phase with 1-octanol in the eluent is very energetically similar to the partitioning process in 1-octanol/water.

On the contrary, the large deviation from unity in the slope in Eq. (4B) implies that RP-HPLC retention in the absence of 1-octanol and 1-octanol/water partitioning are governed by dissimilar processes.

The above results show that, with a set of highly diverse and functionally complex solutes (including neutral compounds and ionizable compounds which were maintained neutral at pH 2.5 or 4) and using the Discovery-RP-Amide-C16 stationary phase, 1-octanol in the eluent is a key factor to obtain a lipophilicity index $\log k_w$ highly correlated with $\log P_{oct}$. In other words, RP-HPLC with proper stationary and mobile phases is a very promising alternative to the traditional shake-flask method to derive $\log P_{oct}$ values not only for neutral drugs, as verified by Lombardo et al. [12], but also for drugs with acidic and ampholytic functionalities, although, neutral at the conditions studied.

As for the majority of basic drugs, $\log P_{oct}$ values cannot be determined with most silica-based stationary phases due to the pH limitation of these stationary phases. Instead, their distribution coefficient $\log D_{oct}$ at pH 7.4 was successfully determined by this method [13].

The wide applicable range of the Discovery-RP-Amide-C16 stationary phase in lipophilicity measurement demonstrated and confirmed the advantage of the amide embedded stationary phases over the conventional ODS stationary phases which could only be successful in $\log P_{oct}$ measurement of structurally related compounds. The reasons why this kind of stationary phases is a better model for the 1-octanol/water partition system are possibly (1) the high level of silanol deactivation on this stationary phase due to the electrostatic coating and (2) the selective solvation of the silica surface by water attracted into the bonded phase by the amide group as discussed by Dias et al. [22].

By comparing the $\log k_w$ values obtained with the two sets of experiments, Fig. 4 shows how the addition of 1-octanol to the eluent differently affects the chromatographic retention of solutes with $\log P_{oct}$ values below and above a value of 3. Indeed, all compounds with $\log P_{oct}$ values greater than 3 (open circles in Fig. 4) are close to the unity line, meaning that their $\log k_w$ values are not influenced by the addition of 1-octanol to the eluent. In contrast, the solutes with $\log P_{oct}$

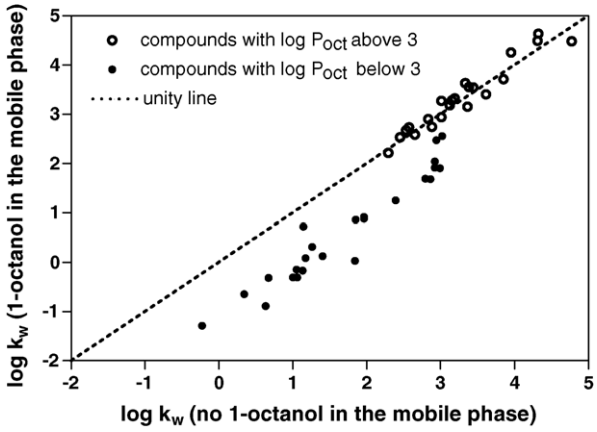

Fig. 4. Relationship between log k_w values derived from the two set of experiments.

values lower than 3 (closed circles in Fig. 4) deviate clearly from the unity line, implying that the addition of 1-octanol markedly decreases their log k_w values. This is interpreted to mean that the improved log P_{oct} versus log k_w correlation resulting from the addition of 1-octanol is due mainly to a modification of the retention behavior of the less lipophilic solutes.

As shown in Table 1, the log k_w values of the compounds with log P_{oct} values greater than 3 were extrapolated from higher methanol concentrations (60, 65, 70%) in the eluent. The negligible influence of 1-octanol on the retention behavior of these solutes may be related to their specific properties and/or to the higher methanol concentrations used.

4. Conclusion

Using the Discovery-RP-Amide-C16 stationary phase, linear relationships were found between isocratic log k values and the volume fraction of MeOH in the eluent in the presence and absence of 1-octanol. The correlation between the derived log k_w and S (Eq. (2)) is highly significant when a 1-octanol-enriched eluent was used, implying that under such conditions the two parameters encode the same intermolecular forces. In contrast, no significant correlation between these two parameters was seen in the absence of 1-octanol.

The addition of 1-octanol to the mobile phase is a key factor to obtain a lipophilicity index log k_w highly correlated with log P_{oct} values for a set of structurally complex and diverse solutes (log P_{oct} ranging from −0.69 to 5.70). This implies that the RP-HPLC method with proper stationary and mobile phases is of value to derive log P_{oct} values for neutral drugs, as found by Lombardo et al. [12], and for drugs with acidic and ampholytic functionalities which were maintained neutral at the experimental conditions.

Our study also unravels the mechanism by which 1-octanol improves the log k_w versus log P_{oct} correlation. By comparing the log k_w values obtained in the presence or absence of 1-octanol, it can be concluded that the influence of 1-octanol on the chromatographic retention is smaller for the more lipophilic compounds (log $P_{oct} > 3$) than for the less lipophilic ones (log $P_{oct} < 3$).

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Novel RPLC stationary phases for lipophilicity measurement: Solvatochromic analysis of retention mechanisms for neutral and basic compounds

An RPLC was developed to rapidly determine lipophilicity of neutral and basic compounds using three base deactivated RPLC stationary phases particularly designed for the analysis of basic compounds, namely, Supelcosil ABZ$^+$Plus, Discovery RP Amide C_{16}, and Zorbax Extend C_{18}. The work consisted of three sets of experiments. In the first log k_w values of neutral compounds were extrapolated using hydroorganic mobile phases at different compositions. Good correlation between log k_w and log P_{oct} indicated that the method was appropriate for these supports, without adding a silanol masking agent. In the second set of experiments, isocratic log k values of neutral and basic compounds were measured with three different mobile phases. The best estimation of lipophilicity was obtained for neutral and basic compounds when the secondary interactions were strongly reduced (*i.e.*, when basic compounds were under their neutral form). In the third set of experiments, isocratic retention factors of basic compounds (in their neutral form) were measured with a high-pH mobile phase, on a chemically stable support (Zorbax Extend C_{18}). Under these chromatographic conditions, correlation between the isocratic retention factors and log P_{oct} (log $D^{10.5}$) for basic compounds was similar to that for neutral compounds.

Key Words: Basic compounds; Neutral compounds; Quantitative structure–property relationships; RPLC

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1 Introduction

1.1 Lipophilicity determined by RP-HPLC

Lipophilicity is a well-known physicochemical descriptor of xenobiotics and plays an essential role in the control of a number of pharmacokinetic (regulation of transport to site of action, toxicity, and metabolism) and pharmacodynamic properties (*e.g.*, interactions with targets or enzyme induction). Lipophilicity is traditionally measured using an *n*-octanol/water system because these partition coefficients (log P_{oct}) were in good agreement with model lipophilic interactions for biological membranes.

Recent advances in automated synthesis and combinatorial chemistry offer medicinal chemists access to a vast number of potential drug candidates. The key role of lipophilicity in absorption, distribution, metabolism, elimination, and toxicity (ADMET) predictions has been widely demonstrated by the early identification of potential pharmacokinetic problems. The demand for fast methods to measure lipophilicity parameters has grown rapidly in the last few years.

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For many years, the conventional and time-consuming procedure to measure log P_{oct} was a shake-flask method, which was limited to a narrow range of log P_{oct} values, namely, $-3 < \log P_{oct} < 3$ [1, 2]. In order to overcome the disadvantages of this technique, various chromatographic methods such as TLC [3], centrifugal partition chromatography (CPC) [4], and RP-HPLC have been extensively studied. In particular, the benefits of chromatographic methods to give access to suitable lipophilicity parameters were underlined in recent reviews [5, 6].

To derive the most suitable experimental conditions, retention on several RP-HPLC stationary phases was determined for a set of varying model compounds. Indeed, in RP-HPLC methods, the lipophilicity index is derived from the retention factor log k, where k is given by Eq. (1)

$$k = (t_r - t_0)/t_0 \quad (1)$$

where t_r and t_0 are the retention time of the solute and of an unretained compound, respectively. Both isocratic log k values and extrapolated log k_w (retention factor at 100% of water) values are used. The former being obtained at a specific mobile phase concentration, and the latter by extrapolation at 100% water plotting isocratic log k values as a function of the mobile phase composi-

tion. Thus, the relationships between different retention factors and partition coefficients in *n*-octanol/water system were carefully examined. The balance of intermolecular interactions revealed by these lipophilicity indices was then analyzed in depth by quantitative structure–property relationships (QSPR).

The intermolecular forces responsible for the retention on RP-HPLC phases can be determined by solvatochromic analysis, a method based on linear solvation-energy relationships (LSERs). This method was first used to evaluate and identify the intermolecular interaction forces underlying the partitioning mechanisms of solutes in various organic/aqueous biphasic systems [7–10]. This approach was introduced into chromatography and extensively developed by various authors [11–13] in order to characterize RPLC stationary phases [12, 14–18]. LSERs can be expressed by the multilinear Eq. (2)

$$S_p = \nu \cdot V_w + p \cdot \pi^* + a \cdot \alpha + b \cdot \beta + c \qquad (2)$$

In this equation, S_p is a given molecular property of a neutral organic solute. In this study, this parameter corresponds to log P_{oct} or retention factors log k_w determined on RPLC stationary phases. The structural parameters represent a steric term (van der Waals volume V_w) and polar terms known as solvatochromic parameters (dipolarity/polarizability π^*, hydrogen-bond donor acidity α, and hydrogen-bond acceptor basicity β). The steric term accounts for solvophobic/hydrophobic and dispersive forces, and the polar terms account for polar interactions between solutes and solvents. The regression coefficients ν, p, a, and b reflect the absolute contribution of each solute parameter to S_p, and c is a constant.

1.2 Stationary phases used in lipophilicity measurements

Octadecyl silica (ODS) columns were commonly used for lipophilicity measurements [19–21]. When used with an aqueous methanol mobile phase, important correlations can be obtained between measured retention factors (log k and log k_w) and literature log P_{oct} values. However, it is known that conventional ODS stationary phases contain free acidic silanol groups which can interact with strong hydrogen-bonding compounds and can cause peak tailing and incorrect estimation of lipophilicity [22]. Compared to *n*-octanol/water system, where only the partitioning mechanism exists, silanophilic interactions of the solute-stationary phase result in a dual mechanism of retention (*i.e.*, ion exchange and partition) [23, 24].

Silanophilic interactions can be strongly reduced, or even suppressed, by the addition of a masking agent (*e.g.*, *n*-decylamine, triethylamine) to the mobile phase. Lombardo *et al.* [11] used *n*-decylamine for lipophilicity determination of neutral and basic compounds and found that its presence was necessary for the reliability of the method. A different approach explored to reduce silanol activity was the use of new stationary phases possessing functional groups introduced to mask free silanol groups. These supports were specially developed for the analysis of basic compounds and are named *base deactivated* stationary phases [25]. Another important factor to obtain precise results was the column-to-column reproducibility [11]. In this reported study, different base deactivated supports known for possessing good batch-to-batch reproducibility were selected and used to determine the lipophilicity of neutral and basic compounds.

1.3 Retention factors as lipophilicity indices

In general, for lipophilicity determination, the retention factor extrapolated at 100% of water (k_w) was preferred to isocratic retention factors k (measured at a given organic solvent concentration) [26]. Hence, the correlation between log k_w values of neutral compounds (measured on base deactivated supports) and their log P_{oct} values were examined using a set of model compounds. Encouraging results were obtained with this series of compounds; thus, the performance of these stationary phases for the lipophilicity determination of basic compounds was examined further.

With basic solutes, many factors must be taken into account when working with buffered mobile phases in the presence of organic solvents. Organic solvents can affect the pK_a of several components: the buffer compounds, leading to pH changes in the mobile phase, the acidic silanol groups on the surface of stationary phase (leading to subtle changes in the intensity of ion-exchange mechanisms), and the solute itself. In general, the pK_a of acids increases as the organic solvent concentration increases, whereas the pK_a of bases decreases. Substantial structure-dependent differences in pK_a shifts for bases from their aqueous values at a given solvent composition were reported in the literature [27], implying a variable correction of pK_a values of basic compounds for each organic solvent concentration. Thus, even if the effect of organic solvent on pK_a of model compounds can be roughly estimated, this prediction is much more complicated for new compounds with a high structural diversity.

In order to minimize effects from the organic modifier, an alternative approach for basic compounds would be to directly measure the retention factor of basic compounds in a 100% aqueous mobile phase. The effect of pH can then be better controlled but, under these chromatographic conditions, lipophilic compounds would be highly retained on RP support. Due to these problems, for basic compounds a fixed organic solvent concentration was used in order to derive isocratic retention factors as lipophilicity indices, designed to correlate with the distribution coefficients, log D^{pH}, of ionized compounds. The hydroor-

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ganic mobile phases were selected because they are commonly used to evaluate performances of RP-HPLC stationary phases, by determining silanol activity at different pH values with different organic modifiers [28, 29].

Finally, in order to improve estimation of log P_{oct} for this class of substances, isocratic retention factors were also measured in a high-pH mobile phase; therefore, basic compounds existed mostly in unionized form and secondary interactions with ionized silanol groups were strongly reduced. Analyses were carried out on a chemically stable support: Zorbax Extend C_{18}, which, even if silica based, is stable for a wide range of pH values (2.0–11.5).

2 Materials and method

2.1 Solutes

All model neutral compounds were obtained from Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland), Janssen (Beerse, Belgium), and Aldrich (Steinheim, Germany) at the highest available purity. ACN and methanol with superpure quality for HPLC were purchased from Romil Chemicals (Cambridge, UK) and SDS (Peypin, France).

Water ($R = 18\ M\Omega M \times cm$) was obtained with the Milli-Q Water Purification System from Millipore (Milford, MA, USA). Aqueous buffers were prepared with dipotassium hydrogen phosphate anhydrous and potassium dihydrogen phosphate from Fluka by measurement of the pH with a Metrohm pH-meter (Herisau, Switzerland). Ammonia buffer was prepared with ammonia chloride supplied from Fluka. The pH measured corresponded to the pH of the aqueous buffer solution (without methanol or ACN).

The basic compounds used were selected to possess a range of physicochemical properties, on the basis of the literature [30] and previous works [31]. Basic compounds were of analytical reagent grade and obtained from several sources. Procainamide hydrochloride, nicotine, pyridine, and benzylamine were provided by Fluka. Nortriptyline hydrochloride was obtained from Sigma (Buchs, Switzerland). Diphenhydramine hydrochloride, morphine hydrochloride, and codeine were supplied by Siegfried (Zofingen Switzerland). Amphetamine was obtained from Socolabo Alltech (Wallisellen, Switzerland). Methadone hydrochloride and quinine hydrochloride were from Hänseler AG (Herisau). Fentanyl citrate was from Macfarlan Smith (Edinburgh, Scotland). Mepivacaine hydrochloride and chloroprocaine hydrochloride were provided by Orgamol (Evionnaz, Switzerland).

2.2 Multivariate statistical analysis

The LSER models were generated by multivariate regression using both the TSAR program (Oxford Molecular, Oxford, GB) running on windows PC and the QSAR module in the Sybyl software (Tripos Associates, St. Louis, MO, USA), running on Silicon Graphics Indy R4400 175 MHz, O_2 R5000 180 MHz, or Origin 2000 R10000 195 MHz workstations. Van der Waals volumes (V_w) were calculated with the standard software MOLSV (QCPE No. 509) and the atomic radii of Gavezzotti. The relative contributions of each variable to the LSER models were obtained by Mager's standardization [32].

2.3 Selection of an optimal set of neutral compounds using cluster analysis

An optimal set of 80 compounds was selected by cluster analysis from 253 organic compounds with known solvatochromic parameters and known *n*-octanol/water partition coefficients determined by Pagliara *et al.* [33]. Compounds of the optimal set, together with their parameters (V_w, π^*, β, α) and partition coefficients (log P_{oct}), are given in [34]. It should be noted that this set of neutral compounds contains some ionizable compounds. These compounds are measured at a pH where their neutral form is dominant.

Different reduced sets of model compounds were investigated because of problems associated with compound availability and poor UV detection. Selections were performed to ensure that each set of compounds had a structural diversity (in terms of V_w, α, β, π^*, and log P_{oct}) comparable to the diversity of the optimal set of 80 solutes [9, 10, 33]. To extrapolate retentions factors, a reduced set of 45 UV active compounds was used while the number of test compounds used in each set of experiments was variable, when different experimental conditions (mobile phase/stationary phases, pH of mobile phases) were used to measure isocratic retention factors.

2.4 Measurement of retention factors

The selected columns were a Supelcosil ABZ$^+$Plus (15 cm × 4.6 mm ID, 5 μm, 100 Å), a Discovery™ RP Amide C_{16} (15 cm × 4.6 mm ID, 5 μm, 100 Å), both from Supelco (Bellefonte, PA, USA), and a Zorbax Extend C_{18} (15 cm × 4.6 mm ID, 5 μm, 100 Å) from Agilent Technologies (Palo Alto, CA, USA).

The retention factors of the different sets of neutral and basic compounds were measured on Supelcosil ABZ$^+$ Plus and Discovery RP Amide C_{16} stationary phases with a liquid chromatograph (Kontron MT1) equipped with an HPLC pump model 420, an autosampler 460, a column oven 480, an oven controller 480, and a UV-VIS detector model 430 (Kontron, Zurich, Switzerland). For UV inactive compounds, a refractive index (RI) detector (Erma refractometer, Tokyo, Japan) was employed.

Retention factors for the 45 UV active compounds were measured on the Zorbax Extend C_{18} with a Merck-Hitachi LaChrom system (Darmstadt, Germany) composed of an L-7100 pump, an L-7200 autosampler, and an L-7400 UV-

VIS detector. Data acquisition and evaluation were performed by the D-7000 HPLC System Manager Software V 4.0.

The retention factors of basic compounds at pH 7.0 on Zorbax Extend C_{18} stationary phase were measured with a Merck-Hitachi LiChrograph, consisting of an L-6200 programmable pump, an AS-2000 automatic injector with 100 μL loop, and an L-4250 UV-VIS programmable detector operating at 215 nm. Data acquisition and evaluation were performed by the D-7000 HPLC System Manager Software.

For the measurements of retention factors (of neutral and basic compounds) at high-pH values, a Dionex GP50 (Sunnyvale, CA, USA) system was used. Pump heads were made of PEEK (chemically resistant from pH 1 to 14). The HPLC system was equipped with an HP 1050 UV-VIS programmable detector operating at 215 nm.

To determine the extrapolated retention factors, several log k values were measured for each neutral compound using methanol/pH 7.0 phosphate buffer and ACN/pH 7.0 phosphate (results not reported) buffer mixtures containing 10:65 v/v of organic modifier. Higher concentrations of organic modifier were not possible due to phosphate buffer precipitation under these conditions (0.0225 M in all mixtures).

To measure isocratic retention factors, three mobile phases were used: ACN/0.0375 M phosphate buffer pH 7.0 (40:60, v/v); methanol/0.0643 M phosphate buffer pH 7.0 (65:35, v/v); and ACN/0.0265 M phosphate buffer pH 3.0 (15:85, v/v). The phosphate buffer was filtered under vacuum through a 0.45 μm HA Millipore filter (Millipore) before being mixed with an organic modifier.

For the analyses carried out at high-pH, the mobile phase was ACN/0.315 M ammonia buffer adjusted to pH 10.5 (40:60, v/v).

For all mobile phases, the given pH is the pH of the buffer before the addition of organic modifier. The pH of the mobile phase was not corrected, since this did not improve extrapolation [34].

3 Results and discussion

3.1 Extrapolated log k_w values on ABZ$^+$Plus and Discovery RP Amide C_{16} stationary phases

In the first set of experiments, isocratic retention factors of neutral compounds were measured on two embedded polar group stationary phases, namely, Supelcosil ABZ$^+$-Plus and Discovery RP Amide C_{16}. Retention factors obtained at various concentrations of methanol were extrapolated to 0% of organic solvent using a conventional least square procedure and reported as log k_w in Tables 1, 2. For the 45 investigated compounds, linear relationships between log k and volume fraction of methanol were observed over the range of eluent composition studied (10–65%, v/v) on the two stationary phases. Correlations between log k_w and log P_{oct} values obtained with both stationary phases are described by Eqs. (3), (4) and reported in Fig. 1.

Supelcosil ABZ$^+$Plus:

$$\log P_{oct} = 1.08\,(\pm 0.05) \cdot \log_{w} + 0.02\,(\pm 0.11)$$

$$n = 45,\ r^2 = 0.98,\ s = 0.17,\ F = 2170 \qquad (3)$$

Discovery RP Amide C_{16}:

$$\log P_{oct} = 1.13\,(\pm 0.06) \cdot \log_{w} + 0.28\,(\pm 0.12)$$

$$n = 45,\ r^2 = 0.97,\ s = 0.21,\ F = 1506 \qquad (4)$$

In these and subsequent equations 95% confidence limits are in parentheses, n is the number of compounds, r^2 is the coefficient of determination (squared correlation coefficient), s is the SD, and F is the Fisher value.

On both base deactivated supports, the log k_w values gave good correlation to log P_{oct} as the slopes were very close to unity and had a small value for the intercept. These results underline that the two embedded polar group stationary phases examined offer lipophilicity indices (extrapolated retention factors, log k_w), which allow fast estimation of the partition coefficients in an n-octanol/water system in the range of $-0.8 < \log P_{oct} < 4.8$. Moreover, the good correlations obtained suggest that log k_w (measured on the two stationary phases) and log P_{oct} are governed by similar structural parameters.

In order to unravel the structural properties governing retention mechanisms and to compare them with partitioning in the n-octanol/water system, linear solvation free-energy relationships (LSERs) were applied to the two sets of log k_w values and log P_{oct} values. These results are reported in Table 3.

The relative contributions of each variable to log k_w values obtained by Mager's standardization [32] can be seen in Table 4. Results show that the principal factors governing log k_w values are the van der Waals volume (V_w) and hydrogen-bond acceptor basicity (β), while dipolarity/polarizability (π^*) and hydrogen-bond donor acidity (α) are of less importance.

Partition coefficients measured in the n-octanol/water system (log P_{oct}) were analyzed by LSERs using the same set of neutral compounds (see Tables 3, 4). The good agreement of the calculated parameters confirmed that partitioning in the n-octanol/water system and the extrapolated retention factor (in the situation of 0% of organic modifier) for the two stationary phases are governed by similar structural parameters.

For basic compounds, extrapolation of the retention factors to 100% water was not performed due to the influence

Table 1. Retention factors measured on Supelcosil ABZ$^+$Plus stationary phase

Number[a)]	Solute	log P_{oct}^{N} [b)]	log $k_{w,MeOH}$ [c)]	log k_1 [d)]	log k_2 [e)]	log k_3 [f)]
2	CH_2Cl_2	1.15	g	0.14	−0.27	0.48
3	$CHCl_3$	1.94	g	0.44	−0.03	1.02
4	CCl_4	2.63	g	0.79	0.3	h
5	CH_2ClCH_2Cl	1.48	g	0.23	−0.2	0.7
6	$CHCl_2CHCl_2$	2.39	g	0.63	0.12	h
7	$CH_3(CH_2)_3Cl$	2.64	g	0.71	0.25	h
8	$(C_2H_5)_2O$	0.89	g	−0.14	−0.5	0.13
9	$(n\text{-}C_3H_7)_2O$	2.03	g	0.50	0.04	1.16
10	CH_3COOCH_3	0.18	−0.09	−0.44	−0.89	−0.27
11	CH_3COOC_2H	0.73	0.46	−0.22	−0.68	0.13
12	$n\text{-}CH_3COOC_4H_9$	1.82	1.67	0.31	−0.18	1.04
14	CH_3CH_2CN	0.10	g	−0.39	−0.95	−0.29
15	$CH_3\text{-}CO\text{-}N(CH_3)_2$	−0.77	−0.36	−0.94	*i*	−0.72
16	$CH_3\text{-}CO\text{-}N(C_2H_5)_2$	0.34	0.58	−0.53	−0.92	−0.01
18	$n\text{-}C_3H_7OH$	0.28	g	−0.58	−0.80	−0.36
19	$(CH_3)_3COH$	0.36	g	−0.51	−0.72	−0.23
20	$n\text{-}C_5H_{11}OH$	1.40	g	−0.01	−0.32	0.60
21	$CH_3CH_2C(CH_3)_2OH$	0.93	g	−0.25	−0.49	0.18
22	$1\text{-}C_6H_{13}OH$	2.03	g	0.27	−0.08	1.09
23	HCOOH	−0.54	g	i	j	−0.51
24	CH_3COOH	−0.24	g	i	i	−0.67
25	$n\text{-}C_3H_7COOH$	0.79	0.58	i	i	0.19
26	$n\text{-}C_4H_9COOH$	1.39	1.24	j	j	0.65
27	$n\text{-}C_4H_9NO_2$	1.47	g	0.26	−0.31	0.82
28	THF	0.46	g	−0.37	−0.69	−0.16
29	$C_6H_5CH_3$	2.69	2.23	0.72	0.29	1.56
30	$C_6H_5\text{-}CO\text{-}CH_3$	1.58	1.46	0.16	−0.32	0.86
31	$C_6H_5NO_2$	1.85	1.62	0.38	−0.08	1.09
32	$C_6H_5OCH_3$	2.11	1.76	0.45	0.02	1.19
33	$C_6H_5COOC_2H_5$	2.64	2.45	0.61	0.10	1.62
34	$C_6H_5\text{-}CO\text{-}C_2H_5$	2.20	1.95	0.44	−0.09	1.29
35	$C_6H_5COOCH_2C_6H_5$	3.97	3.63	1.09	0.54	k
36	$2\text{-}Cl\text{-}C_6H_4NO_2$	2.24	2.18	0.56	0.06	1.48
37	$C_6H_5CH_2CN$	1.56	1.48	0.22	−0.41	0.95
38	$C_6H_5CH_2\text{-}CO\text{-}CH_3$	1.44	1.41	0.17	−0.37	0.88
39	$C_6H_5(CH_2)_2\text{-}O\text{-}CO\text{-}CH_3$	2.30	2.23	0.48	−0.04	1.48
40	Pyridine	0.65	0.54	−0.41	−0.78	i
41	Acridine	3.40	2.89	0.63	0.21	j
42	1-Naphthoic acid	3.10	2.82	j	j	1.90
43	2-Naphthylamine	2.28	2.19	0.48	0.04	j
44	$C_6H_5NH_2$	0.90	0.75	−0.05	−0.52	j
45	$C_6H_5NHC_2H_5$	2.16	1.75	0.51	−0.01	j

Table 1. Continued

Number[a)]	Solute	log P_{oct}^{N} [b)]	log $k_{w,MeOH}$ [c)]	log k_1 [d)]	log k_2 [e)]	log k_3 [f)]
46	2-Cl-$C_6H_4NH_2$	1.91	1.64	0.38	−0.07	j
47	2-NH_2-C_6H_4-C_6H_5	2.84	2.67	0.71	0.20	j
48	4,4′-$(NH_2)_2$-Biphenyl	1.53	1.56	0.03	−0.57	j
49	4-NO_2-C_6H_4-NH_2	1.39	1.44	0.13	−0.31	0.85
50	C_6H_5OH	1.49	1.25	0.11	−0.29	0.71
51	3-Cl-C_6H_4OH	2.49	2.32	0.58	0.17	1.51
52	3-CH_3-C_6H_4COOH	2.37	2.22	j	j	1.37
53	$C_6H_5CH_2COOH$	1.46	1.42	j	i	0.83
54	3-Cl-$C_6H_4CH_2COOH$	2.09	2.21	j	j	1.46
55	$C_6H_5(CH_2)_3COOH$	2.42	2.39	j	j	1.51
56	$C_6H_5CH_2OH$	1.08	1.01	−0.11	−0.47	0.45
57	4-Cl-$C_6H_4CH_2OH$	1.96	1.82	0.24	−0.11	1.10
58	4-NO_2-C_6H_4-OH	1.92	1.81	j	j	1.14
59	1,3-$C_6H_4Cl_2$	3.48	3.15	1.06	0.62	k
60	Biphenyl	3.90	3.69	1.20	0.76	k
71	Naphthalene	3.35	3.06	0.97	0.54	k
72	1,3,5-$C_6H_3(CH_3)_3$	3.84	3.40	1.19	0.74	k
73	*n*-$C_9H_{19}COOH$	4.09	4.26	g	g	g
75	1,2,4,5-$C_6H_2Cl_4$	4.51	4.16	1.55	1.12	k
77	$C_6H_5(CH_2)_2C_6H_5$	4.80	4.38	1.48	0.99	k
78	$C_6H(CH_3)_5$	4.56	4.04	1.49	1.08	k

a) Same numbering as in [9, 34].
b) Values taken from [34].
c) Extrapolated retention factors using methanol as organic modifier.
d) log *k* measured using mobile phase 1 (MeCN/phosphate buffer pH 7.0 0.0375 M, 40 : 60 v/v).
e) log *k* measured using mobile phase 2 (MeOH/phosphate buffer pH 7.0 0.0643 M, 65 : 35 v/v).
f) log *k* measured using mobile phase 3 (MeCN/phosphate buffer pH 3.0 0.0265 M, 15 : 85 v/v).
g) Not measured.
h) Not measured due to problems of solubility into the mobile phases.
i) Too hydrophilic compounds under the experimental conditions.
j) Compounds charged under the experimental conditions.
k) Too lipophilic compounds under the experimental conditions.

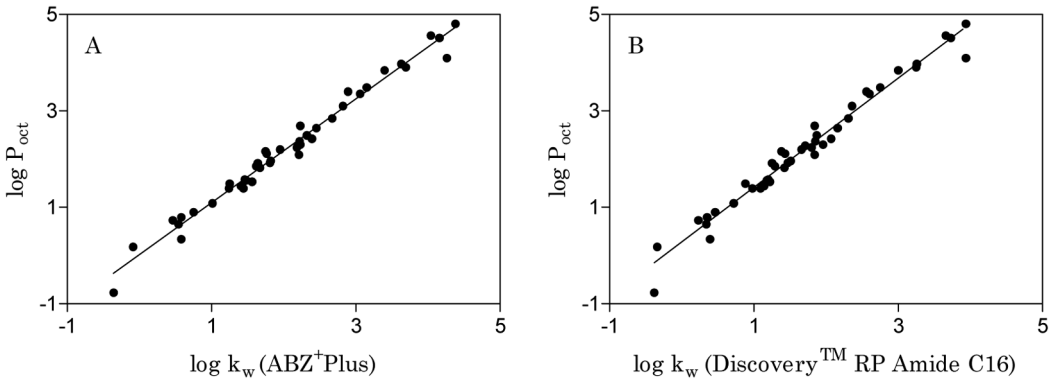

Figure 1. Relationships between extrapolated log k_w and log P_{oct} for (A) the Supelcosil ABZ⁺Plus and (B) the Discovery RP Amide C_{16} stationary phases.

Table 2. Retention factors measured on Discovery RP Amide C_{16} stationary phase

Number[a]	log $k_{w,MeOH}$[b]	log k_1[c]	log k_2[d]	log k_3[e]
2	f	0.02	−0.50	0.28
3	f	0.30	−0.25	0.78
4	f	0.61	0.07	g
5	f	0.12	−0.40	0.49
6	f	0.51	−0.10	1.25
7	f	0.57	0.04	g
8	f	−0.25	−0.65	−0.05
9	f	0.36	−0.15	0.96
10	−0.34	−0.53	−1.02	−0.38
11	0.23	−0.28	−0.78	0.00
12	1.42	0.22	−0.31	0.88
14	f	−0.47	−1.06	−0.44
15	−0.38	−0.99	−1.36	−0.79
16	0.39	−0.59	−0.95	−0.09
18	f	−0.69	−0.94	−0.56
19	f	−0.64	−0.84	−0.39
20	f	−0.17	−0.48	0.40
21	f	−0.39	−0.62	0.00
22	f	0.09	−0.24	0.88
23	f	h	h	−0.64
24	f	i	h	−0.87
25	0.35	h	i	−0.01
26	0.98	h	i	0.44
27	f	0.18	−0.47	0.65
28	f	−0.48	−0.82	−0.33
29	1.84	0.59	0.06	1.31
30	1.20	0.10	−0.45	0.71
31	1.29	0.31	−0.26	0.90
32	1.43	0.35	−0.18	0.98
33	2.16	0.53	−0.05	1.45
34	1.66	0.36	−0.23	1.12
35	3.26	1.01	0.36	j
36	1.80	0.48	−0.12	1.30
37	1.18	0.17	−0.53	0.80
38	1.14	0.12	−0.49	0.75
39	1.96	0.42	−0.17	1.33
40	0.34	−0.47	−0.88	i
41	2.56	0.46	0.05	h
42	2.36	h	i	1.53
43	1.71	0.33	−0.25	h
44	0.46	−0.15	−0.72	h
45	1.38	0.41	−0.20	h
46	1.25	0.27	−0.30	h
47	2.31	0.62	0.00	h
48	1.22	−0.03	−0.74	h
49	1.09	0.07	−0.48	0.68
50	0.88	−0.01	−0.50	0.47
51	1.87	0.42	−0.08	1.23
52	1.85	h	i	1.07
53	1.12	i	i	0.60
54	1.84	h	h	1.12
55	2.07	h	h	1.30
56	0.72	−0.22	−0.62	0.25
57	1.51	0.09	−0.29	0.86
58	1.47	h	h	0.95
59	2.75	0.89	0.36	j
60	3.25	1.04	0.48	j
71	2.60	0.81	0.26	j
72	3.00	1.02	0.48	j
73	3.94	f	f	f
75	3.73	1.34	0.82	j
77	3.94	1.34	0.72	j
78	3.66	1.31	0.80	j

a) Same numbering as in [9, 34].
b) Extrapolated retention factors using methanol as organic modifier.
c) log k measured using mobile phase 1 (MeCN/phosphate buffer pH 7.0 0.0375 M, 40 : 60 v/v).
d) log k measured using mobile phase 2 (MeOH/phosphate buffer pH 7.0 0.0643 M, 65 : 35 v/v).
e) log k measured using mobile phase 3 (MeCN/phosphate buffer pH 3.0 0.0265 M, 15 : 85 v/v).
f) Not measured.
g) Not measured due to problems of solubility into the mobile phases.
h) Too hydrophilic compounds under the experimental conditions.
i) Compounds charged under the experimental conditions.
j) Too lipophilic compounds under the experimental conditions.

of organic solvent on both the pK_a of basic compounds and buffer solution.

3.2 Isocratic log k values determined on Supelcosil ABZ$^+$Plus and Discovery RP Amide C_{16} stationary phases

For the reasons given above, in a second set of experiments, the retention values of neutral and basic compounds were also measured on Supelcosil ABZ$^+$Plus and Discovery RP Amide C_{16} using three isocratic mobile phases, specially selected for the analysis of basic compounds in RPLC [25].

Isocratic retention factors of neutral compounds, measured with the three mobile phases, were compared between the two supports, with k_1 measured with ACN/pH 7.0 phosphate buffer (40 : 60, v/v), k_2 measured with methanol/pH 7.0 phosphate buffer (65 : 35, v/v), and k_3 with ACN/pH 3.0 phosphate buffer (15 : 85, v/v).

Table 3. Solvatochromic analyses of estimated retention factors and partition coefficients

S_p	$v(\times 10^{-2})$	p	a	b	c	n	q^2	r^2	s	F
$\log k^{ABZ^+_{plus}}_{w,MeOH}$	3.05 (±0.26)	−0.33 (±0.29)	0.32 (±0.20)	−3.65 (±0.30)	−0.26 (±0.26)	45	0.98	0.99	0.14	713
$\log k^{Discovery}_{w,MeOH}$	2.92 (±0.30)	−0.38 (±0.34)	0.21 (±0.22)	−3.30 (±0.34)	−0.51 (±0.27)	45	0.97	0.98	0.15	561
$\log k^{Zorbax}_{w,MeOH}$	3.18 (±0.36)	−0.49 (±0.36)	−0.31 (±0.28)	−3.60 (±0.49)	0.23 (±0.31)	40	0.95	0.96	0.20	216
$\log P_{oct}$	3.18 (±0.15)	−0.43 (±0.15)	0.21 (±0.16)	−4.23 (±0.34)	0.10 (±0.27)	45	0.99	0.99	0.13	1031

Table 4. Relative contributions of each variable to the LSER models obtained by Mager's standardization

	n[a)]	% V_W	% π^*	% α	% β
$\log k^{ABZ^+_{plus}}_{w,MeOH}$	45	52.8	5.1	4.7	37.4
$\log k^{Discovery}_{w,MeOH}$	45	54.2	6.2	3.4	36.2
$\log k^{Zorbax}_{w,MeOH}$	40	52.8	7.3	4.7	35.2
$\log P_{oct}$	45	50.9	6.1	2.9	40.1

a) Number of compounds included in the LSER model.

$$\log k_1\,(ABZ^+Plus) = 1.04\,(\pm 0.02)\cdot \log k_1\,(Discovery) + 0.10\,(\pm 0.01)$$
$$n = 52,\ r = 0.996,\ s = 0.04,\ F = 11590 \qquad (5)$$

$$\log k_2\,(ABZ^+Plus) = 1.10\,(\pm 0.02)\cdot \log k_2\,(Discovery) + 0.21\,(\pm 0.01)$$
$$n = 51,\ r = 0.995,\ s = 0.04,\ F = 9240 \qquad (6)$$

$$\log k_3\,(ABZ^+Plus) = 1.05\,(\pm 0.02)\cdot \log k_3\,(Discovery) + 0.10\,(\pm 0.02)$$
$$n = 43,\ r = 0.995,\ s = 0.05,\ F = 7810 \qquad (5)$$

This high correlation (slopes close to the unity and their intercept very small for Eqs. (5)–(7)) suggests that the retention mechanisms on the two stationary phases are similar. Equations (5)–(7) also indicate that the effects of the organic modifier and pH on the structure, ionization, and solvation of the stationary phase are similar for the two phases examined.

According to these results, isocratic retention factors measured on both stationary phases were expected to correlate in a similar way with the partition coefficients ($\log P_{oct}$) of analyzed compounds. Consequently, relationships between isocratic retention factors and $\log P_{oct}$ were then calculated for each mobile phase and chromatographic support (Eqs. (8)–(15)).

With the set of neutral compounds tested in mobile phase at pH 3.0 (Eqs. (8) and (9) for Supelcosil ABZ$^+$Plus and Discovery RP Amide, respectively), slopes differed from unity (and intercepts differ from zero) more than for the extrapolated isocratic factors (Eqs. (3), (4)) on both base deactivated stationary phases.

$$\log P_{oct} = 1.29\,(\pm 0.06)\cdot \log k_3 + 0.50\,(\pm 0.06)$$
$$n = 43,\ r^2 = 0.97,\ s = 0.15,\ F = 1522 \qquad (8)$$

$$\log P_{oct} = 1.34\,(\pm 0.08)\cdot \log k_3 + 0.72\,(\pm 0.07)$$
$$n = 44,\ r^2 = 0.96,\ s = 0.18,\ F = 1020 \qquad (9)$$

These differences were associated with free-energy changes due to the presence of 15% organic solvent (ACN) in pH 3.0 mobile phase. This hypothesis was confirmed as the equations (Eqs. (10), (11)) obtained for neutral compounds in a pH 3.0 mobile phase with a higher amount of ACN (40% instead of 15%) have, as expected, higher slopes and intercepts on both stationary phases and different solvatochromic equations.

Supelcosil ABZ$^+$Plus:

$$\log P_{oct} = 2.18\,(\pm 0.10)\cdot \log k_{40\%\,MeCN} + 1.90\,(\pm 0.08)$$
$$n = 35,\ r^2 = 0.98,\ s = 0.17,\ F = 1730 \qquad (10)$$

Discovery RP Amide C_{16}:

$$\log P_{oct} = 2.29\,(\pm 0.16)\cdot \log k_{40\%\,MeCN} + 1.65\,(\pm 0.09)$$
$$n = 34,\ r^2 = 0.96,\ s = 0.22,\ F = 874 \qquad (11)$$

By increasing the organic modifier quantity, a decrease of the volume contribution in LSER equations is obtained (data not shown). This demonstrates that the presence of an organic solvent facilitates the formation of cavities, which are needed to dissolve solutes in aqueous mobile phases.

Additional correlations between $\log P_{oct}$ and isocratic retention factors measured in pH 7.0 mobile phases were also explored.

On Supelcosil ABZ$^+$Plus stationary phase:

$$\log P_{oct} = 2.16\,(\pm 0.11)\cdot \log k_1 + 1.23\,(\pm 0.07)$$
$$n = 52,\ r^2 = 0.97,\ s = 0.22,\ F = 1531 \qquad (12)$$

$$\log P_{oct} = 2.27\,(\pm 0.12)\cdot \log k_2 + 2.19\,(\pm 0.06)$$
$$n = 51,\ r^2 = 0.97,\ s = 0.22,\ F = 1418 \qquad (13)$$

On the Discovery RP Amide C_{16} stationary phase:

$$\log P_{oct} = 2.24\,(\pm 0.13)\cdot \log k_1 + 2.68\,(\pm 0.06)$$
$$n = 52,\ r^2 = 0.96,\ s = 0.25,\ F = 1189 \qquad (14)$$

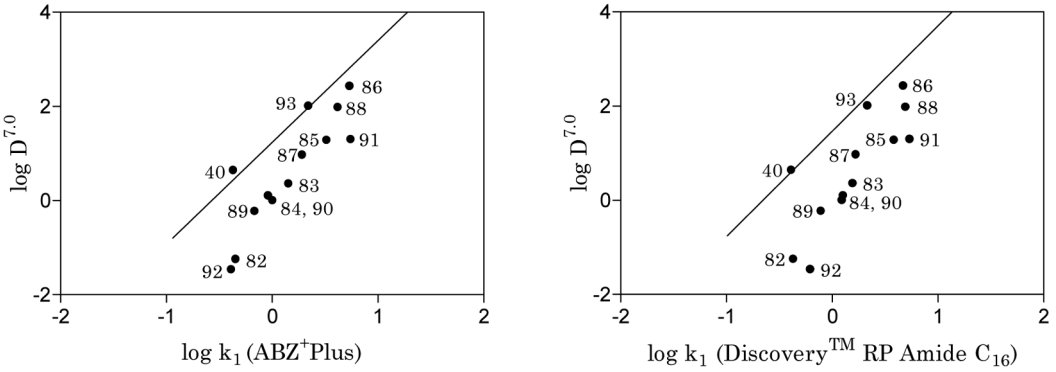

Figure 2. Relationships between isocratic log k_1 and log $D^{7.0}$ on ABZ+Plus and Discovery RP Amide C_{16}. (●) Set of 14 basic solutes, (−) set of neutral compounds.

$$\log P_{oct} = 2.52\,(\pm 0.11) \cdot \log k_2 + 2.68\,(\pm 0.06)$$

$$n = 52,\ r^2 = 0.98,\ s = 0.18,\ F = 2278 \qquad (15)$$

It can be seen that an excellent correlation was found for neutral compounds in all chromatographic conditions (r^2 >0.96). However, the slopes and intercepts were significantly different from 1 and 0, respectively, indicating that the free-energy changes were not the same. If the final goal was not the determination of log P_{oct}, but a self-consistent scale of lipophilicity, then isocratic log k would be of major interest for the determination of the relative lipophilicity, especially in a set of compounds characterized by high structural diversity.

In the same way, isocratic retention factors of basic compounds were measured, plotted against their log $D^{7.0}$ values (Fig. 2), and compared with the relationships between log P_{oct} and log k_1 previously obtained for neutral compounds under the same chromatographic conditions (Eqs. (8), (10)). No clear correlation between log k_1 and log D_{oct} for basic compounds was found. This would suggest that, at pH 7.0, basic compounds and free silanols are, respectively, partially and completely charged inducing strong ion-exchange interactions.

As described above, the effect of the organic modifier on the ionization state of solutes, stationary phases, and on the pH of mobile phases are difficult to distinguish. This suggests that chromatographic supports and mobile phases suitable for the analyses of basic compounds are not suitably optimized for the estimation of lipophilicity.

Obtained results indicate that lipophilicity estimation with an RP-HPLC method should be performed either with a low amount of organic solvent (to remain closer to the partitioning in *n*-octanol/water system) or when basic compounds are in their unionized form. However, with a low amount of organic solvent in the mobile phase, basic compounds can be strongly retained on the stationary phase. For this reason, the use of a higher amount of organic solvent was required in order to estimate the lipophilicity of basic compounds in their unionized form.

3.3 Relationship between isocratic retention factors of basic compounds and log P_{oct}

On the basis of earlier findings, a reliable estimation of the lipophilicity in the case of basic compounds calls for a direct measurement of the retention of basic compounds in their neutral form in order to estimate their log P_{oct}. For this purpose, retention factors have to be measured in a high-pH mobile phase. Since classical silica-based stationary phases are only chemically stable only within a limited range of pH values (pH 2−8), this part of the study was carried out on a more stable silica-based chromatographic support: Zorbax Extend C_{18}. This stationary phase incorporates a bidentate silane, combined with a double-endcapping process that protects silica from dissolution at high pH, up to pH 11.5.

The correlation between the extrapolated log k_w of neutral compounds and their log P_{oct} was investigated and compared with the results previously obtained on Discovery RP Amide C_{16} and Supelcosil ABZ+Plus.

Retention factors of only 40 compounds among the set of 45 UV active compounds were measured on the Zorbax Extend C_{18} (Table 5) stationary phase because this phase is slightly more lipophilic.

Equation (16) is quite similar to Eqs. (3), (4) obtained with the two other stationary phases

$$\log P_{oct} = 1.01\,(\pm 0.09) \cdot \log k_w - 0.25\,(\pm 0.21)$$

$$n = 40,\ r^2 = 0.93,\ s = 0.27,\ F = 524 \qquad (16)$$

As reported for Discovery RP Amide C_{16} and Supelcosil ABZ+Plus, LSERs analyses (Tables 3, 4) were carried out on results obtained on Zorbax Extend C_{18}. Results showed that the principal factor governing log k_w values on Zorbax Extend C_{18} was again the van der Waals volume.

Table 5. Retention factors obtained on Zorbax Extend C_{18} stationary phase

Number[a)]	Solute	log P_{oct}^{N} [b)]	log $k_{w,MeOH}$ [c)]	log k_1 [d)]	log k_2 [e)]
6	$CHCl_2CHCl_2$	2.39	f	f	0.95
10	CH_3COOCH_3	0.18	0.42	f	f
11	CH_3COOC_2H	0.73	1.04	f	f
12	*n*-$CH_3COOC_4H_9$	1.82	2.33	f	f
15	CH_3-CO-$N(CH_3)_2$	−0.77	0.07	−0.74	−0.78
16	CH_3-CO-$N(C_2H_5)_2$	0.34	1.18	−0.37	−0.41
25	*n*-C_3H_7COOH	0.79	1.01	f	f
26	*n*-C_4H_9COOH	1.39	1.69	f	f
27	*n*-$C_4H_9NO_2$	1.47	f	0.48	f
29	$C_6H_5CH_3$	2.69	2.91	f	f
30	C_6H_5-CO-CH_3	1.58	1.91	0.54	0.33
31	$C_6H_5NO_2$	1.85	1.93	0.52	0.54
32	$C_6H_5OCH_3$	2.11	2.22	0.91	f
33	$C_6H_5COOC_2H_5$	2.64	2.99	0.86	0.86
34	C_6H_5-CO-C_2H_5	2.20	2.43	0.65	0.65
35	$C_6H_5COOCH_2C_6H_5$	3.97	4.45	f	f
36	2-Cl-$C_6H_4NO_2$	2.24	2.47	0.71	0.73
37	$C_6H_5CH_2CN$	1.56	1.88	0.38	0.38
38	$C_6H_5CH_2$-CO-CH_3	1.44	1.93	0.37	0.37
39	$C_6H_5(CH_2)_2$-O-CO-CH_3	2.30	2.82	0.73	0.75
40	Pyridine	0.65	0.99	−0.24	−0.37
41	Acridine	3.40	3.19	0.67	0.67
42	1-Naphthoic acid	3.10	2.69	f	f
43	2-Naphthylamine	2.28	2.33	0.76	0.48
44	$C_6H_5NH_2$	0.90	0.98	0.18	0.03
45	$C_6H_5NHC_2H_5$	2.16	2.19	0.71	0.70
46	2-Cl-$C_6H_4NH_2$	1.91	1.88	0.47	0.45
47	2-NH_2-C_6H_4-C_6H_5	2.84	3.11	0.87	0.89
48	4,4′-$(NH_2)_2$-Biphenyl	1.53	1.70	−0.03	−0.02
49	4-NO_2-C_6H_4-NH_2	1.39	1.23	0.07	0.03
50	C_6H_5OH	1.49	1.27	0.37	g
51	3-Cl-C_6H_4OH	2.49	2.32	f	f
52	3-CH_3-C_6H_4COOH	2.37	2.65	f	f
53	$C_6H_5CH_2COOH$	1.46	1.81	f	f
54	3-Cl-$C_6H_4CH_2COOH$	2.09	2.48	f	f
55	$C_6H_5(CH_2)_3COOH$	2.42	2.63	f	f
56	$C_6H_5CH_2OH$	1.08	1.33	−0.11	−0.13
57	4-Cl-$C_6H_4CH_2OH$	1.96	2.17	0.19	0.17
58	4-NO_2-C_6H_4-OH	1.92	1.66	f	f
59	1,3-$C_6H_4Cl_2$	3.48	3.88	f	1.33
60	Biphenyl	3.90	4.44	f	f
65	*N,N*-Dimethylbenzylamine	1.91	f	0.73	f

Table 5. Continued

Number[a)]	Solute	log P_{oct}^{N} [b)]	log $k_{w,MeOH}$ [c)]	log k_1 [d)]	log k_2 [e)]
71	Naphthalene	3.35	3.69	*f*	1.20
72	1,3,5-$C_6H_3(CH_3)_3$	3.84	h	f	f
73	*n*-$C_9H_{19}COOH$	4.09	h	f	f
75	1,2,4,5-$C_6H_2Cl_4$	4.51	h	f	f
77	$C_6H_5(CH_2)_2C_6H_5$	4.80	h	f	f
78	$C_6H(CH_3)_5$	4.56	h	f	f

a) Same numbering as in [9, 34].
b) Taken from [34].
c) Extrapolated retention factors using methanol as organic modifier.
d) log *k* measured in mobile phase 1 (MeCN/phosphate buffer pH 7.0 0.0375 M, 40:60 v/v).
e) log *k* measured in mobile phase 4 (MeCN/ammonia buffer pH 10.5 0.0315 M, 40:60 v/v).
f) Not measured.
g) Compound charged under the experimental conditions.
h) Too lipophilic compounds under the experimental conditions.

Table 6. Retention factors of the 14 basic compounds

Number	Solute	pK_{a1} [a)]	pK_{a2} [a)]	log P_{oct}^{N} [a)]	log k_1 [b)]		log k_4 [c)]	
					ABZ$^+$Plus	Discovery	Zorbax	Zorbax
40	Pyridine	5.25		0.65	−0.37	−0.39	−0.31	−0.34
81	d-Amphetamine	9.91		1.76	−0.13	−0.37	−0.54	0.11
82	Benzylamine	9.33		1.09	−0.35	−0.37	−0.62	−0.17
83	Chloroprocaine	9.49		2.86	0.15	0.19	−0.05	0.55
84	Codeine	8.10		1.14	0.00	0.09	−0.43	−0.09
85	Diphenydramine	8.98		3.27	0.51	0.58	0.51	1.19
86	Fentanyle	8.43		3.89	0.73	0.67	0.95	1.29
87	Mepivacaine	7.92		1.95	0.28	0.22	0.29	0.44
88	Methadone	8.94		3.93	0.62	0.69	0.60	1.83
89	Morphine	7.93	10.00	0.76	−0.1	−0.11	−0.96	−0.66
90	Nicotine	3.12	8.02	1.17	−0.04	0.10	−0.14	−0.05
91	Nortryptiline	9.73		4.04	0.74	0.73	0.48	1.32
92	Procainamide	9.34		0.88	−0.39	−0.21	−1.40	−0.38
93	Quinine	4.13	8.52	3.55	0.34	0.33	−0.16	0.34

N = pH.
a) Values taken from Medchem [35].
b) log *k* measured in mobile phase 1 (MeCN/phosphate buffer pH 7.0 0.0375 M, 40:60 v/v).
c) log *k* measured in mobile phase 4 (MeCN/ammonia buffer pH 10.5 0.0315 M, 40:60 v/v).

Second, retention factors of both neutral and basic compounds were measured on Zorbax Extend C_{18} support with pH 7.0 and 10.5 mobile phases (Table 6, log k_1 and log k_4, respectively). Correlations with their log P_{oct} (pH 7.0) and log *D* (pH 10.5) were investigated. As both mobile phases were composed of 40% organic solvent, slopes and intercepts obtained for neutral compounds (Eqs. (17), (18)) were similar to those previously obtained for the two other stationary phases (Eqs. (8)–(11)).

With the pH 7.0 mobile phase:

$$\log P_{oct} = 1.71\,(\pm 0.31) \cdot \log k_1 + 1.00\,(\pm 0.18)$$
$$n = 23,\ r^2 = 0.86,\ s = 0.31,\ F = 128 \qquad (17)$$

With the pH 10.5 mobile phase:

$$\log P_{oct} = 1.80\,(\pm 0.30) \cdot \log k_4 + 1.13\,(\pm 0.19)$$
$$n = 24,\ r^2 = 0.88,\ s = 0.35,\ F = 1598 \qquad (18)$$

The majority of retention factors measured at pH 7.0 for the basic compounds tested on Zorbax Extend C_{18} support (Table 6), did not have a good correlation to log $D_{oct}^{7.0}$.

Similar to results obtained on Supelcosil ABZ$^+$Plus and Discovery RP Amide C_{16} in the same chromatographic conditions, compounds possessing low pK_a value gave better correlation between log k_1 and log D_{oct}.

With ammonia buffered mobile phase at pH 10.5, the tested basic compounds were nearly or totally under their neutral form and secondary interactions with the stationary phase greatly reduced. For this reason, the correlation between log k_4 and log D_{oct} (Table 6) of basic compounds was closer to the one obtained for neutral compounds under the same chromatographic conditions. These results underline that isocratic retention factors of basic compounds measured in a high-pH mobile phase can give a suitable estimate of their *n*-octanol/water partition coefficients.

4 Concluding remarks

Important correlations were obtained between log P_{oct} and extrapolated log k_w derived from Supelcosil ABZ$^+$Plus, Discovery RP Amide C_{16}, and Zorbax Extend C_{18} stationary phases. This meant that novel RP-HPLC stationary phases offer a promising alternative to the shake-flask method for log P_{oct} determination of compounds in their neutral form. Good correlations were also established between log P_{oct} and the three isocratic retention factors (log k_1, log k_2, and log k_3) for neutral compounds measured at pH 7.0 and 3.0. However, slopes and intercepts were not equal to 1 and 0, respectively, showing that isocratic retention factors are not the best estimators of lipophilicity.

The results from solvatochromic analyses showed that the principal factors which govern retention were the van der Waals volume (V_w) and the hydrogen-bond acceptor basicity (β) of the solutes, which are also the governing factors of partitioning in the *n*-octanol/water system. The effect of an organic modifier on the free-energy changes was mostly due to the influence of the organic modifier on the cavitation energy term, as clearly demonstrated by volume contribution in LSER analysis.

Finally, results obtained on Zorbax Extend C_{18} stationary phase, at high pH and without the addition of a masking agent, suggested that retention factors of basic compounds could accurately estimate *n*-octanol/water partition coefficients, if measured in a mobile phase with high pH.

The well-known advantages of a chromatographic method, in particular the small amount of solutes needed, and automatization suggest that the methods tested here should be used as a method of primary choice in the physicochemical screening of large libraries of neutral and basic compounds.

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Application of a Stepwise Flow Ratiometry without Phase Separation to the Determination of the Chloroform/Water Distribution Coefficients of Volatile Diazines

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The chloroform/water distribution coefficients (K_D) of sixteen diazine compounds were determined by a stepwise flow ratiometry. An aqueous solution of analyte was delivered and merged with chloroform. The flow rate ratio of both the phases was varied stepwise under a constant total (chloroform+aqueous) flow rate. The analyte was extracted to chloroform while both the phases, which were segmented by each other, were passing through an extraction coil. The segmented stream was then led to a UV/Vis detector directly without phase-separation. The absorbance of the chloroform and aqueous phases (A_o and A_a, respectively) was each measured at the maximum absorption wavelength of the analyte. The plots of A^{-1} against R_f, $(AR_f)^{-1}$ against R_f^{-1}, and AR_f against A gave straight lines, where A was A_o, A_a or the sum of them (A_S). The K_D of the analyte was calculated from the slopes and intercepts of the plots. The log K_D values obtained for the analytes (−0.5—1.4) were agreed well with the values measured by a shake-flask method. The present method is simple, rapid (5 min/determination) and applicable to the volatile compounds with reasonable precision (standard deviation of log K_D<0.07).

Key words stepwise flow ratiometry; distribution coefficient; volatile compound; diazine; partition coefficient

Distribution coefficient (K_D), also referred as partition coefficient (P), is an equilibrium constant that is closely relating to the hydrophobicity (lipophilicity) of a substance. It is widely used for the estimation of the activity of drug and pesticide candidates, and of the toxicity of pollutants. Many experimental approaches, such as shake-flask method, reversed phase HPLC, flow injection extraction, micellar electrokinetic chromatography and so forth, have been developed for the determination of K_D; each of them has its intrinsic advantages and disadvantages, as reviewed by Danielsson and Zhang.[1] For example, shake-flask method is accurate in principle but is laborious and time-consuming; HPLC is rapid and simple to operate but requires suitable standards whose K_D values are well established.

Flow ratiometry is a variation of continuous flow analysis, where two independently delivered solutions are merged at various flow ratios (R_f) and the analytical signals are measured at a downstream position.[2] The information of interest is obtained by analyzing the relationship between the R_f and the signals. We reported a system for the K_D determination based on a flow ratiometry.[3,4] The method was applied to the determination of chloroform/water K_D of phenol, benzoic acid and their derivatives. Satisfactory results were obtained for these compounds and the efficiency of the measurement was fairly good (10 min/determination).

In the previous study,[5] the system was further improved by introducing a UV-detection method that required no phase-separation. The phase-separator, which had made the system complicated and limited the efficiency of the measurement, was removed. The absorbance of both the phases was measured almost simultaneously with one detector, which contributed to the ruggedness and reliability of the system. A series of experimental operations can be carried out automatically in a semi-closed flow system. There is no need to determine precisely the initial and final concentration of the analyte so long as the analytical signal of either of the phases at least is proportional to the concentration of the analyte in the corresponding phase.

The features described above are considered to be advantageous to the measurement of volatile compounds, whose concentrations are liable to change during the operations involved in conventional approaches. In the present work, therefore, the improved method with no phase-separation process was further investigated by applying it to volatile compounds. Mono- and di-substituted diazines were selected as the compounds to be analyzed because their K_D values in various solvents systems have been extensively explored in the studies of quantitative structure–activity relationships.[6—11]

Experimental

Apparatus Figure 1 shows the flow system. Chloroform (O) and an aqueous solution of analyte (A) were each delivered and merged with in a Teflon tee-union. The ratio of chloroform/aqueous flow rate (R_f: F_o/F_a) was automatically varied stepwise (each duration time: 1 min) while the total flow rate (F_o+F_a) was kept constant at 1 cm^3 min^{-1}. Both the phases formed small segments and passed through an extraction coil (EC) that was kept at 25 °C with a thermostat (T). The effluent from the coil came into a wide bore Teflon tubing (WT), in which the coalescence between neighboring chloroform segments and that between aqueous segments occurred.[5] Both

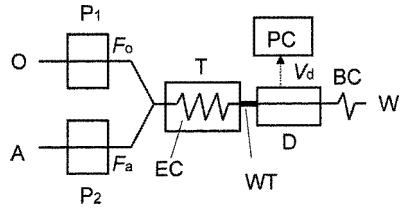

Fig. 1. Flow Diagram

O, chloroform; A, aqueous solution of analyte; P_1 and P_2, Shimadzu LC-10AD$_{VP}$ double-plungers pumps; T, Sanuki R-3000C thermostat; EC, extraction coil (0.5 mm i.d., 3 m long); WT, wide bore tubing (2 mm, i.d., 20 cm long); D, Shimadzu SPD-6AV UV/Vis detector; PC, Toshiba Dynabook Satellite SA70C/5 notebook computer with a Measurement Computing PC-CARD-DAS16/12-AO card; BC, back pressure coil (0.25 mm i.d., 1 m long); W, waste; F_a and F_o, flow rate of aqueous and organic phases, respectively; V_d, detector output voltage (relative absorbance).

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the phases were directly introduced to a handmade optical flow cell[4] set in a commercial UV/Vis detector (D). The relative absorbance was measured at the absorption maximum wavelength of the analyte and acquired in a computer (PC) as a detector output voltage (V_d) at the frequency of 20 Hz.

Materials Sixteen diazines were used in the present study: pyrazine, pyridazine, pyriazineamide (Pyrazine–$CONH_2$) and aminopyrazine (Pyrazine–NH_2) purchased from Nacalai Tesque; methylpyrazine (Pyrazine–Me), 2-chloropyrimidine (Pyrimidine–Cl) and 3-methylpyridazine (Pyridazine–3Me) from Aldrich; 2,6-dimethylpyrazine (Pyrazine–2,6diMe), cyanopyrazine (Pyrazine–CN), acetylpyrazine (Pyrazine–Ac) and 5-methylpyrimizine (Pyrimizine–5Me) from Tokyo Kasei Kogyo; 2-amminopyridazine (Pyridazine–$2NH_2$) from Wako Pure Chemical Industries; 4-methylpyrimizine (Pyrimizine–4Me) from Sigma. Methyl pyrazinecarboxylate (Pyrazine–CO_2Me), *N*,*N*-dimethylpyrazineamide (Pyrazine–$CONMe_2$) and acetylaminopyrazine (Pyrazine–NHCOMe) were synthesized in one of the authors' (C.Y.) laboratory. Chloroform was purchased from Kanto Chemicals. Water is a Milli-Q SP deionized water.

Principles The principle of the K_D determination by flow ratiometry[3,4] and its extension to the method without phase-separation[5] were described before. Briefly, when an aqueous solution of analyte (initial concentration: C_{ai}) is merged with an organic solvent at the flow rate ratio of R_f ($=F_o/F_a$) to reach the distribution equilibrium, the K_D of the analyte is expressed as Eq. 1:

$$K_D=\varepsilon_o^{-1}A_o(C_{ai}-\varepsilon_o^{-1}A_oR_f)^{-1}=(C_{ai}-\varepsilon_a^{-1}A_a)(\varepsilon_a^{-1}A_aR_f)^{-1} \quad (1)$$

where A and ε are the absorbance and the molar absorptivity, respectively, of the analyte in the phase denoted by the subscript (o: organic phase, a: aqueous phase). From Eq. 1, the following three equations are derived for linear plots:

$$A^{-1}=(BC_{ai})^{-1}K_DR_f+(BC_{ai})^{-1} \quad (2)$$

$$(AR_f)^{-1}=(BC_{ai}R_f)^{-1}+(BC_{ai})^{-1}K_D \quad (3)$$

$$AR_f=-K_D^{-1}A+BC_{ai}K_D^{-1} \quad (4)$$

where, A is A_o, A_a or the sum of them (A_S); B is a constant that depends on the species of A, as summarized in Table 1A. The K_D can be calculated from the slopes and intercepts of these linear plots without the information on the values of C_{ai} and B. As for the Eq. 2, for example, A^{-1} is plotted against R_f and K_D is obtained only by dividing the value of the slope by that of intercept. Such the information is summarized in Table 1B.

Table 1. Three Kinds of Linear Plots Based on Eqs. 2—4

(A) A and B

	Plot for A_o	Plot for A_a	Plot for A_S
A	A_o	A_a	A_o+A_a
B	ε_oK_D	ε_a	$\varepsilon_oK_D+\varepsilon_a$

(B) Abscissa, ordinate and K_D

Eq.	Abscissa	Ordinate	K_D
2	R_f	A^{-1}	SI^{-1}
3	R_f^{-1}	$(AR_f)^{-1}$	$S^{-1}I$
4	A	AR_f	$-S^{-1}$

S: slope of linear regression. I: intercept at the ordinate.

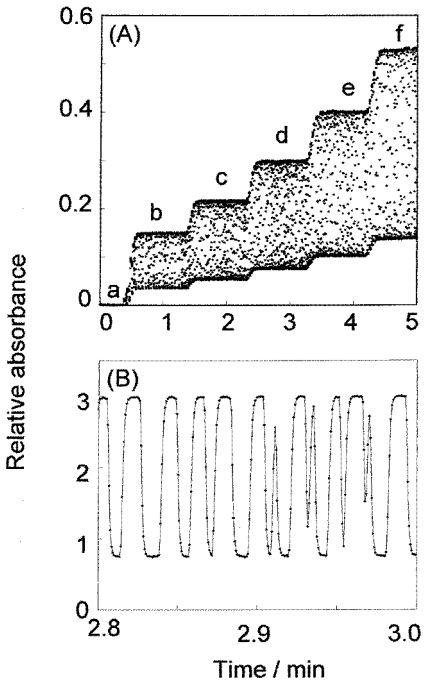

Fig. 2. Typical Example of the Analytical Signals (A) and Its Enlargement in Abscissa in the Range of 2.8—3.0 min (B)

Analyte: pyrazine (*ca.* 1 mmol dm^{-3}). Analytical wavelength: 260 nm. Flow rate ratio (R_f): a, ∞ (chloroform alone); b, 2.333; c, 1.500; d, 1.000; e, 0.667; f, 0.429. The R_f was corrected by taking the mutual solubility of both phases[12] and densities of chloroform[13] and water[14] into account by assuming that there is an additive property of the volumes of both the phases.

Results and Discussion

Selection of the Flow Rate Ratios A set of five R_f was employed for the K_D determination by taking the efficiency of the measurement into account. In the present approach, the K_D is determined not from the ratio of the analyte concentrations in both the phases but from the slopes and intercepts of the linear plots based on Eqs. 2—4. The error in A at each R_f affected, therefore, the calculated value of K_D in a complex way. The K_D is expressed as Eq. 5 for the plot based on Eq. 2 for A_o ($i=1,2,\ldots,5$), for example, when the method of least squares is applied.

$$K_D=\frac{n\sum - \sum \sum A_{o,i}^{-1}}{\sum \sum - \sum \sum K_{f,i}A_{o,i}^{-1}} \quad (5)$$

The extent of the propagation of the error in each A_o ($\partial A_{o,j}$, $j=1,2,\ldots,5$) to that of the final outcome (∂K_D) is, therefore, expressed by the following equation.

$$\frac{\partial K_D}{\partial A_{o,j}}=\frac{\left\{n\sum R_{f,i}^2-\left(\sum R_{f,i}\right)^2\right\}\left\{\sum R_{f,i}A_{o,i}^{-1}-K_{f,j}\sum A_{o,i}^{-1}\right\}}{A_{o,j}^2\left(\sum R_{f,i}^2\sum A_{o,i}^{-1}-\sum K_{f,i}\sum K_{f,i}A_{o,i}^{-1}\right)^2} \quad (6)$$

The higher the $\partial K_D/\partial A_{o,j}$ value becomes, the larger the extent of the propagation is. These equations and those similarly obtained for the other two plots (*i.e.*, the plots based on Eqs. 3 and 4) were used for the selection of R_f values. From the extensive simulations, it was concluded that the R_f values are not so critical for the compounds having the $\log K_D$ below 1.5. On the other hand, for more hydrophobic compounds, even subtle error in A, especially that at high R_f, greatly affects the final result (K_D). Consequently, the R_f set of 2.333, 1.500, 1.000, 0.667 and 0.429 was employed for the diazines except for more lipophilic Pyrazine–2,6diMe and Pyrazine–Ac, to which the set of lower R_f (0.667, 0.429, 0.250, 0.190 and 0.111) was applied.

Flow Signals and Three Linear Plots The raw analytical signals acquired at the frequency of 20 Hz are shown for pyrazine, for example, as a function of time in Figs. 2A. The signals seem scatter in a range of absorbance, whose upper and lower limits increased with a decrease in R_f. However, by

Table 2. The log K_D of Diazines Determinend by a Stepwise Flow Ratiometry

Analyte	λ_{anal} /nm	A	n	log K_D±S.D.				Reference values
				Eq. 2–based plot	Eq. 3–based plot	Eq. 3–based plot	Mean	
Pyrazine	260	A_S	3	$0.53\pm0.00_4$	$0.53\pm0.00_8$	$0.53\pm0.00_7$	0.53	0.59[9]
Pyrazine–Me	264	A_o	5	0.99±0.01	0.96±0.01	0.97±0.01	0.97	1.04[9]
Pyrazine–2,6diMe	271	A_o	8	1.39±0.03	1.35±0.01	1.36±0.01	1.37	1.54[9]
Pyrazine–CN	268	A_o	2	$1.07\pm0.00_1$	$1.06\pm0.00_0$	$1.06\pm0.00_0$	1.07	1.03[9]
Pyrazien–CO_2Me	268	A_o	5	1.41±0.06	1.41±0.03	1.41±0.03	1.41	1.36[9]
Pyrazine–$CONMe_2$	268	A_S	5	0.95±0.04	0.95±0.03	0.95±0.03	0.95	0.71[10]
Pyrazine–$CONH_2$	268	A_S	4	−0.34±0.01	−0.34±0.01	−0.34±0.01	−0.34	−0.34[11]
Pyrazine–Ac	268	A_o	6	1.26±0.02	1.24±0.04	1.24±0.03	1.24	1.42[9]
Pyrazine–NHCOMe	290	A_S	4	$0.07\pm0.00_3$	$0.07\pm0.00_3$	$0.07\pm0.00_3$	0.07	0.05[11]
Pyrazine–NH_2	317	A_S	4	−0.49±0.05	−0.49±0.06	−0.49±0.05	−0.49	−0.56[11]
Pyrimidine–2Cl	250	A_o	7	1.08±0.05	1.08±0.03	1.08±0.03	1.08	1.16[9]
Pyrimidine–4Me	250	A_S	3	$0.71\pm0.00_5$	$0.70\pm0.00_7$	$0.70\pm0.00_6$	0.70	0.74[9]
Pyrimidine–5Me	250	A_S	4	1.01±0.06	0.97±0.02	0.97±0.03	0.98	0.95[9]
Pyrimidine–$2NH_2$	290	A_S	4	−0.26±0.02	−0.26±0.02	−0.26±0.02	−0.26	−0.31[11]
Pyridazine	250	A_S	4	−0.11±0.01	−0.12±0.01	−0.11±0.01	−0.11	−0.15[11]
Pyridazine–3Me	250	A_S	4	0.35±0.04	0.35±0.03	0.35±0.03	0.35	0.29[11]

The log K_D for each plot was the log($\Sigma K_D/n$); standard deviation of log K_D=0.43429 s_{KD}/K_D, where s_{KD} is the standard deviation of K_D.

enlarging the abscissa (time) scale as shown in Fig. 2B, it can clearly be seen that the signals did not scatter randomly but oscillating almost regularly between upper and lower plateaus. The upper and lower plateaus correspond to the absorbance of chloroform and aqueous phases, respectively, because the width of the former plateaus was decreased whereas that of the latter increased with the decrease of R_f (chloroform/aqueous flow rate ratio). The signals between the both plateaus are transition signals corresponding to the interfacial regions between the organic and aqueous phases.

Figures 3A—C show the linear plots based on Eqs. 2—4, respectively, for pyrazine. The linearity of the plots was sufficiently. The coefficients of determination (r^2) of the plots are ranged from 0.9977 to 1.0000. Similar results were obtained for the other diazines. The K_D values obtained from these three plots were 3.390, 3.322 and 3.342 from A_o, 3.305, 3.425 and 3.399 from A_a, and 3.372, 3.343 and 3.353 from A_S, respectively.

Applications to Diverse Diazines Table 2 is the summary of the results obtained for the diazines. The log K_D values determined by a shake flask method[9,11,15] are also listed, as reference. The log K_D obtained from A_S ($=A_o+A_a$) were listed for less lipophilic compounds (log $K_D<1$), because the addition of A_o and A_a is considered to give more reliable results.[5] For more lipophilic compounds, A_o was used instead of A_S, because A_a was too low to be precisely measured.

The log K_D of the present study (Y) agreed well with the reference values (X) listed in the rightmost column in Table 2; the relationship between them is $Y=0.9373X+0.0435$ ($r^2=0.9822$); $Y=0.9281X+0.0390$ ($r^2=0.9802$) and $Y=0.9287X+0.0041$ ($r^2=0.9806$) for the plots based on Eqs. 2, 3 and 4, respectively. The weight of each data for the linear regression depends on the type of plot. This effect seems, however, not so significant because similar values were obtained irrespective of the plot type.

In conclusion, the present approach could realize the simple and rapid (5 min/sample) determination of K_D of diazines with a semi-closed flow system. The method has distinct advantages over conventional methods in the respect that it needs neither standard materials for K_D calibration nor information on the initial and final concentration of analyte: the information needed is only the flow rate (R_f) and the absorbance (A). This approach would be applicable to other volatile compounds whose K_D values are difficult to measure by conventional methods.

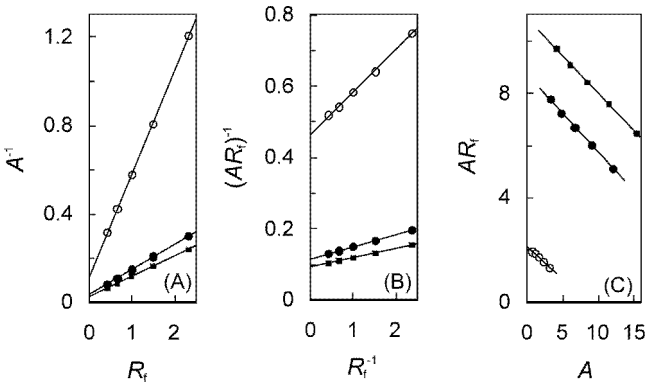

Fig. 3. Typical Example of Three Kinds of Linear Plots Based on Eqs. 2 (A), 3 (B) and 4 (C)

The data shown in Fig. 2 are used for these plots. ●, A_o; ○, A_a; ■, A_S ($=A_o+A_a$). The absorbance was relative absorbance in arbitrary unit.

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Retention Behavior of Neutral and Positively and Negatively Charged Solutes on an Immobilized-Artificial-Membrane (IAM) Stationary Phase

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The retention behavior of neutral, positively charged, and negatively charged solutes on the IAM.PC.DD2 stationary phase was investigated and compared. A set of monofunctional compounds and complex drugs (steroids, nonsteroidal anti-inflammatory drugs, and β-blockers) were selected for this study, *i.e.*, neutral solutes and solutes with acidic or basic functionalities which are positively charged or negatively charged at pH 7.0. The correlation between the retention factor $\log k_w$ at pH 7.0 on the IAM.PC.DD2 stationary phase and the partition coefficient $\log P_{oct}$ or the distribution coefficient $\log D_{7.0}$ showed that the retention mechanism depends on the charge state and structural characteristics of the compounds. The neutrals were least retained on the IAM.PC.DD2 stationary phase, and positively charged solutes were more retained than negatively charged ones. This implies that the retention of the charged solutes is controlled not only by lipophilicity but also by the electrostatic interaction with the phospholipid, with which positively charged solutes interact more strongly than negatively charged ones.

Introduction. – Successful drug development requires not only optimization of specific and potent pharmacological activity at the target site, but also efficient delivery 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 the 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.

[1]) The second author has the same contribution to this paper as the first author.

Thus the development of membrane-like systems such as immobilized-artificial-membrane chromatography has been of marked interest in the understanding of partitioning of ionized compounds [17][18]. Immobilized artificial membranes (IAMs) are solid-phase-membrane mimetics prepared by covalently bonding a monolayer of phospholipids to silica gel particles, thus mimicking the lipid environment of a fluid cell membrane on a solid matrix. Since IAMs provide the amphiphilic microenvironment of biological membranes, they should be able to take ionic bonds into account. In addition, IAM chromatography is a convenient process to measure partitioning of drugs because it involves the fast HPLC methodology.

The lipophilicity index from a IAM-HPLC stationary phase is derived from the capacity factor log k, which is calculated by *Eqn. 1*, where t_R and t_0 are the retention times of the solute and of an unretained compound, respectively. For lipophilic compounds, the retention times would be too long by using a purely aqueous mobile phase. Thus, log k values are determined at different concentrations of an organic modifier and extrapolated to pure aqueous mobile phase (log k_w) by *Eqn. 2*, where φ is the volume fraction of MeOH in the mobile phase, S the slope, and log k_w the intercept of the regression curve. For hydrophilic compounds, log k_w can be determined directly by using the aqueous mobile phase.

$$\log k = \log(t_R - t_0)/t_0 \quad (1)$$

$$\log k = -S\varphi + \log k_w \quad (2)$$

The interaction of drugs with phospholipids has been investigated by IAM-HPLC for different sets of neutral and ionized compounds such as β-blockers [19][20], nonsteroidal anti-inflammatory drugs [21], and dihydropyridine (DHP) calcium-channel blockers [22]. In these studies, the log k_w values obtained from IAM-HPLC were compared with the octan-1-ol/H_2O partitioning, and the occurrence of electrostatic interactions with phospholipids was found for ionized compounds.

To further understand the retention mechanism of solutes on a IAM stationary phase, we selected a set of monofunctional compounds and complex drugs (steroids, nonsteroidal anti-inflammatory drugs, and β-blockers). This set consists of neutral solutes and solutes with acidic or basic functionalities which are positively charged or negatively charged at pH 7.0, as shown in the *Table*. The retention behavior of different sets of solutes was investigated in terms of the influence of different functionalities, lipophilicity, and the charged state of the solutes.

Results and Discussion. – To obtain experimental conditions as close as possible to the physiologic pH and compatible with the stability of the stationary phase (highest pH limit is 7.5), log k values were determined at pH 7.0 on the IAM.PC.DD2 stationary phase. According to the pK_a values of the compounds shown in the *Table*, the monofunctional carboxylic acids **22**–**30** and the NSAIDs **31**–**36** are fully negatively charged at pH 7.0, whereas the (4-methylbenzyl)alkylamines **37**–**43** and β-blockers **44**–**49** are fully positively charged, and the very weak bases and acids **1**–**5**, **12**, and **13**) are fully neutral at this pH. The partition coefficient log P_{oct} and distribution coefficient

Table. *The Physicochemical Parameters of the Investigated Compounds* **1**–**49**. N = Neutral.

		log P_{oct} [a])	pK_a [a])	log $D_{7.0}$ [b])	Charge state	log k_w [c])
1	Acridine	3.40	5.58	3.40	N	2.42
2	$PhNH_2$	0.90	4.60	0.90	N	0.26
3	Ph_2NHEt	2.16	5.12	2.16	N	1.04
4	2-$ClC_6H_4NH_2$	1.91	2.64	1.91	N	1.14
5	2-$H_2NC_6H_4Ph$	2.84	3.82	2.84	N	2.02
6	$PhCH_2CN$	1.56	N	1.56	N	0.94
7	PhC(O)Me	1.58	N	1.58	N	0.86
8	$PhNO_2$	1.85	N	1.85	N	0.99
9	2-$ClC_6H_4NO_2$	2.24	N	2.24	N	1.58
10	$PhCH_2OH$	1.08	N	1.08	N	0.58
11	4-$ClC_6H_4CH_2OH$	1.96	N	1.96	N	1.21
12	3-ClC_6H_4OH	2.49	9.11	2.48	N	1.77
13	3-$O_2NC_6H_4OH$	2.00	8.40	1.96	N	1.38
14	Corticosterone	1.94	N	1.94	N	1.67
15	Dexamethasone	1.83	N	1.83	N	1.79
16	Estradiol	4.01	N	4.01	N	2.65
17	Estrone	3.13	N	3.13	N	1.92
18	Hydrocortisone	1.55	N	1.55	N	1,35
19	Hydrocortisone-21-acetate	2.19	N	2.19	N	1.78
20	Progestrone	3.87	N	3.87	N	3.01
21	Testosterone	3.29	N	3.29	N	2.51
22	$Ph(CH_2)_2COOH$	1.89	4.52	− 0.59	−	− 0.25
23	$Ph(CH_2)_3COOH$	2.42	4.72	0.14	−	0.06
24	$Ph(CH_2)_4COOH$	2.85	4.55	0.40	−	0.43
25	$Ph(CH_2)_7COOH$	4.09	5.03	2.12	−	2.02
26	PhCOOH	1.96	4.20	− 0.84	−	− 0.62
27	4-BrC_6H_4COOH	2.86	3.97	− 0.17	−	0.32
28	3-ClC_6H_4COOH	2.71	3.83	− 0.46	−	0.06
29	4-IC_6H_4COOH	3.13	3.96	0.09	−	0.51
30	1-Naphthoic acid	3.10	3.69	− 0.21	−	0.13
31	Aspirin	1.13	3.48	− 2.39	−	− 0.15
32	Flurbiprofen	3.81	3.91	0.72	−	1.78
33	Ketoprofen	2.77	4.29	0.06	−	1.26
34	Naproxen	3.06	4.15	0.21	−	1.35
35	Indomethacin	4.27	4.50	1.77	−	2.37
36	Mefenamic acid	5.12	4.33	2.45	−	2.35
37	4-$MeC_6H_4CH_2NHMe$	1.96	9.93	− 0.97	+	0.96
38	4-$MeC_6H_4CH_2NHEt$	2.38	10.04	− 0.66	+	1.02
39	4-$MeC_6H_4CH_2NHPr$	2.96	9.98	− 0.02	+	1.30
40	4-$MeC_6H_4CH_2NHBu$	3.49	9.98	0.51	+	1.87
41	4-$MeC_6H_4CH_2NH(CH_2)_4Me$	4.26	10.08	1.18	+	2.27
42	4-$MeC_6H_4CH_2NH(CH_2)_5Me$	4.96	10.17	1.79	+	2.77
43	4-$MeC_6H_4CH_2NH(CH_2)_6Me$	5.12	10.02	2.10	+	2.92
44	Metoprolol	1.95	9.63	− 0.68	+	1.45
45	Metipranolol	2.81	9.54	0.27	+	1.78
46	Oxprenolol	2.51	9.57	− 0.06	+	1.70
47	Penbutolol	4.62	9.92	1.70	+	3.70
48	Pindolol	1.75	9.54	− 0.79	+	1.31
49	Propranolol	3.48	9.53	0.95	+	2.48

[a]) Taken from [23–26]. [b]) Calculated according to $\log D = \log P_{oct} - \log(1 + 10^{pK_a - pH})$ for bases and $\log D = \log P_{oct} - \log(1 + 10^{pH - pK_a})$ for acids. [c]) $n = 3$, s.d. ≤ 0.05.

at pH 7.0, namely log $D_{7.0}$ calculated from pK_a and log P_{oct} values, are also summarized in the *Table*, together with the pK_a values and charge state of the compounds.

Relationship between log k *and* φ. The compounds **22**–**24** and **26**–**31** were eluted with a purely aqueous mobile phase. For the other solutes, four or five different MeOH concentrations in aqueous solutions were used as mobile phase for the extrapolation to log k_w values. Good linear relationships between log k and φ were found in the range of the eluent composition studied. The squared correlation coefficient was higher than 0.99, except for the log k_w of **3**, **4**, **10**, and **46** ($r^2 = 0.98$). The log k_w values are presented in the *Table* together with other physicochemical parameters.

Relationship between log k_w *and log* $P_{oct.}$ The correlation between log k_w and log P_{oct} is shown in *Fig. 1*. No correlation exists for the whole set of compounds. However, the correlations are good for neutral compounds or structurally related compounds. The corresponding correlation equations are shown as follows below (*Eqns. 3*–*7*), wherein 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.

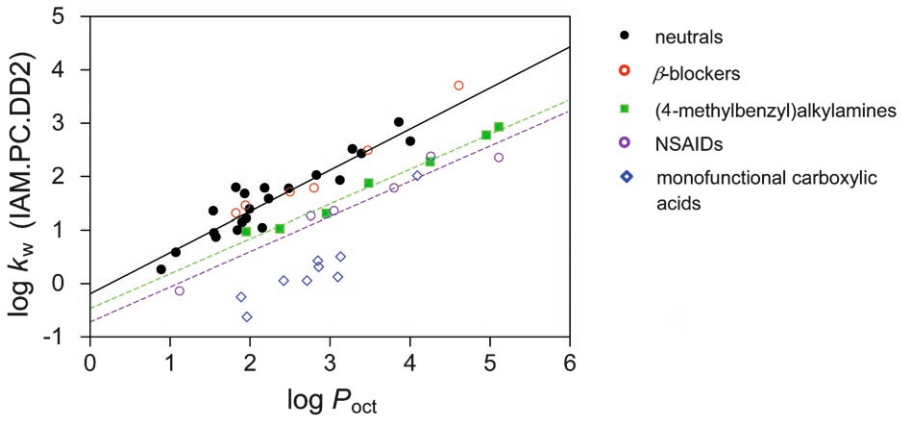

Fig. 1. *Correlation between log* k_w *values from the IAM.PC.DD2 stationary phase at pH 7 and log* P_{oct} *values for the compounds investigated*

For the neutral compounds **1**–**21**, *Eqn. 3* holds ($n = 21$, $r^2 = 0.87$, $s = 0.26$, and $F = 130$). The correlation coefficient for the 13 monofunctional solutes **1**–**13** became much more significant ($r^2 = 0.95$) if the steroids **14**–**21** (see *Table*) were excluded, implying that the correlation quality for neutral compounds is decreased by the increasing structural diversity of the complex drugs.

$$\log k_w = 0.77\ (\pm 0.13)\ \log P_{oct} - 0.19\ (\pm 0.33) \qquad (3)$$

For the β-blockers **44**–**49**, *Eqn. 4* holds ($n = 6$, $r^2 = 0.97$, $s = 0.18$, and $F = 115$). As shown in *Eqn. 4* and *Fig. 1*, the six β-blockers fit the correlation line for neutral compounds. This agrees with the study of *Barbato et al.* [19]. It means that the β-blockers under study can interact with phospholipids as strongly as neutral compounds with the same log P_{oct} values, although they are fully positively charged under the

experimental conditions. It suggests that the retention of the six β-blockers on the IAM.PC.DD2 stationary phase is governed not only by lipophilicity but also by extra interactions of which an electrostatic interaction between positively charged amines and negatively charged phosphates of the phospholipids plays a key role, as discussed by *Avdeef et al.* [27] and *Barbato et al.* [19][28] in their studies.

$$\log k_w = 0.84\ (\pm 0.16)\ \log P_{oct} - 0.34\ (\pm 0.48) \quad (4)$$

For the (4-methylbenzyl)alkylamines **37**–**43**, *Eqn. 5* holds ($n = 7$, $r^2 = 0.98$, $s = 0.11$, and $F = 320$). The retention of these seven positively charged (4-methylbenzyl)alkylamines on the IAM.PC.DD2 stationary phase is weaker than that of the β-blockers and neutral compounds with same log P_{oct} values, as shown by *Eqn. 5* and *Fig. 1*, implying that the strength of extra interactions between charged amines and the phospholipid membrane depends on the structural characteristics of the solutes.

$$\log k_w = 0.65\ (\pm 0.07)\ \log P_{oct} - 0.46\ (\pm 0.27) \quad (5)$$

For the NSAIDs **31**–**36**, *Eqn. 6* holds ($n = 6$, $r^2 = 0.95$, $s = 0.24$, and $F = 73$). The retention of the negatively charged NSAIDs and monofunctional carboxylic acids investigated is weaker than that of positively charged compounds. Contrary to the result from the study of *Barbato et al.* on the IAM.PC.MG stationary phase [21], where the correlation between log k_w and log P_{oct} results in a unique regression line for NSAIDs (with carboxylic function not directly linked to the aromatic ring) and neutral compounds, our study on the IAM.PC.DD2 stationary phase showed two separate regression lines for NSAIDs and neutral compounds, implying that the NSAIDs investigated are less retained than neutral compounds with same log P_{oct} values. It should be noted that the retention behavior of the negatively charged NSAIDs, especially of **32**–**35**, is very similar to that of the positively charged (4-methylbenzyl)-alkylamines.

$$\log k_w = 0.66\ (\pm 0.16)\ \log P_{oct} - 0.72\ (\pm 0.55) \quad (6)$$

For the monofunctional carboxylic acids **22**–**30**, *Eqn. 7* holds ($n = 9$, $r^2 = 0.86$, $s = 0.29$, and $F = 43$). These negatively charged carboxylic acids, except for **25**, are less retained than negatively charged NSAIDs, resulting in a different regression line between log k_w and log P_{oct}. The correlation coefficient of *Eqn. 7* is low, meaning that the retention behavior of this set of compounds cannot be well predicted by their log P_{oct} values.

$$\log k_w = 1.02\ (\pm 0.31)\ \log P_{oct} - 2.54\ (\pm 0.89) \quad (7)$$

Relationship between log k_w *on the IAM.PC.DD2 and log* $D_{7.0}$. The log P_{oct} and log $D_{7.0}$ values are highly interrelated for (4-methylbenzyl)alkylamines and NSAIDs investigated in this study; therefore, the relationship between log k_w and log $D_{7.0}$ values is not reported anymore here. The correlation between log k_w and log $D_{7.0}$ values for β-blockers is less significant than that between log k_w and log P_{oct}. However, for the

monofunctional carboxylic acids, a much more significant correlation equation is established between log k_w and log $D_{7.0}$ values (see *Eqn. 8*, $n = 9$, $r^2 = 0.94$, $s = 0.19$, and $F = 107$), as compared to *Eqn. 7*. It indicates that the retention can be much better predicted by the distribution coefficient log $D_{7.0}$ values for this set of compounds.

$$\log k_w = 0.82\ (\pm 0.16)\ \log D_{7.0} + 0.25\ (\pm 0.13) \quad (8)$$

The extra interaction between ionized solutes and the IAM.PC.DD2 stationary phase can be more clearly and logically shown in *Fig. 2*, the correlation between log k_w and the distribution coefficient log $D_{7.0}$ of the compounds investigated. From *Fig. 2*, it can be seen that the neutral compounds are the least retained on the IAM.PC.DD2 stationary phase. All the ionized solutes are more strongly retained than neutral ones with the same log $D_{7.0}$ values, to a different extent, depending on their charge and structural characteristics. The retention of positively charged solutes is stronger than that of negatively charged ones. Indeed, as discussed by *Avdeef et al.* in the liposomal membrane/water partitioning of ionized drugs [27], the charge distribution in the phospholipid membrane is anisotropic; as the ionized species moves in the direction of the aqueous exterior of the membrane, the first charges it experiences are those of the negatively charged phosphates. Further movement would bring the ionized drug substance in the vicinity of the positively charged trimethylammonium groups. Electrostatic pairing of charges would require a greater movement for weak acids, compared to weak bases. Therefore, the negatively charged solutes have lesser affinity for phosphatidylcholine-based membranes than positively charged solutes. The results from our study with the IAM.PC.DD2 stationary phase verified this point. Further, *Fig. 2* shows that the retention of the ionized solutes on the IAM.PC.DD2 phase also depends on their structural characteristics. For the positively charged amines investigated, β-blockers are slightly more retained than (4-methylbenzyl)alkylamines, which is also shown by *Taillardat–Bertschinger et al.* in their study [24]. For the negatively charged solutes, NSAIDs (except for mefenamic acid (**36**)) are more

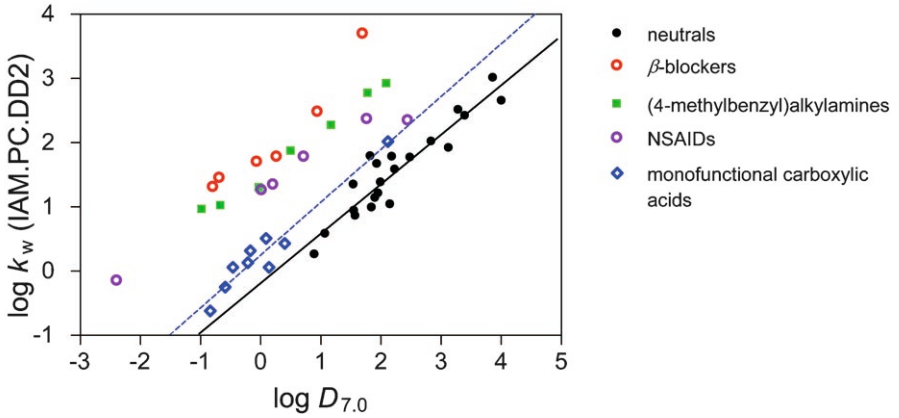

Fig. 2. *Correlation between log* k_w *values from the IAM.PC.DD2 stationary phase at pH 7 and log* $D_{7.0}$ *values for the compounds investigated*

retained than monofunctional carboxylic acids, confirming that the strength of the electrostatic interactions is influenced by different structural characteristics of the solutes.

Conclusion. – In this work, we compared the retention behavior of a set of neutral and positively or negatively charged solutes on the IAM.PC.DD2 stationary phase. Significant correlations were found between the retention factor $\log k_w$ on this stationary phase and $\log P_{oct}$ or $\log D_{7.0}$ for neutral or structurally related compounds, implying that the retention mechanisms are the same for neutral or structurally related compounds. The retention of the ionized compounds on the IAM.PC.DD2 is controlled not only by lipophilicity but also by extra interactions, mainly electrostatic interactions between charged solutes and phospholipids. For the solutes investigated in this study, positively charged compounds are more retained than negatively charged solutes. The ranking order of retention strength is: β-blockers > (4-methylbenzyl)alkylamines > NSAIDs > monofunctional carboxylic acids. This implies that the interaction between positively charged solutes and the phosphatidylcholine-based IAM stationary phase is larger than that between negatively charged solutes and the membrane, and that the electrostatic interaction depends on the structural characteristics of the solutes investigated.

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Experimental part

General. The (4-methylbenzyl)alkylamines **37**–**43** (*Table*) were synthesized by known procedures [29]. All other compounds were obtained from commercial sources (*Sigma–Aldrich*, Steinheim, Germany; *Carl Roth*, Karlsruhe, Germany; *VWR*, Leuven, Belgium) in the highest available purity. Distilled H_2O, HPLC-grade MeOH (*Alfa Aesar*, Karlsruhe, Germany) were used throughout.

Capacity Factors. The capacity factors were measured with a liquid chromatograph equipped with a HPLC pump *System-Gold-125* solvent module, a *System-Gold-507e* autosampler, and a *System-Gold-UV/VIS-168* detector (all from *Beckmann Coulter, Inc.,* Fuerton, CA, USA). The column was an IAM.PC.DD2 (100 mm × 4.6 mm i.d., 10 μm) from *Regis Technology* (Morton Grove, IL, USA). The mobile phases were either 0.02M phosphate buffer pH 7.0 or mixtures of 0.02M phosphate buffer pH 7.0 and MeOH in proportions varying from 70 to 10% (*v*/*v*) for all other compounds. The phosphate buffer was filtered under vacuum through a *HA-Millipore* filter (0.45 μm; *Millipore*, Milford, MA, USA) before being mixed with MeOH. The retention times were measured at r.t. by the UV/VIS detector at the λ_{max} of the analytes. The solns. to be injected (10^{-4} M to 10^{-3} M) were prepared by dissolving the solutes in the mobile phase; the injection volume was 10 μl. Citric acid was used as the unretained compound. The measurements were carried out at a flow rate of 1.0 ml/min for all compounds. For compounds **22**–**24** and **26**–**31**, the $\log k_w$ values were determined directly in the aq. mobile phase. For the other compounds, four or five different MeOH concentrations in aq. soln. were used as mobile phase for the extrapolation to $\log k_w$. The capacity factor $\log k$ was calculated by *Eqn. 1*. All $\log k$ values were the average of three measurements. The $\log k$ values were then extrapolated to 100% H_2O with *Eqn. 2* ($\rightarrow \log k_w$).

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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:

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

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

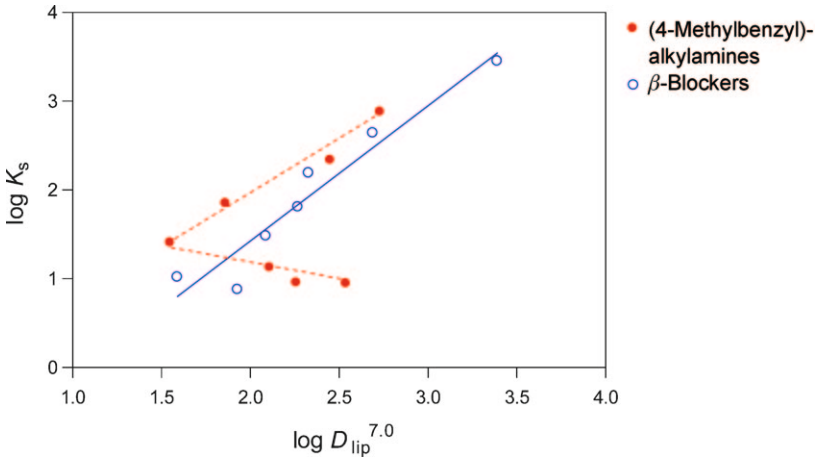

Fig. 1. *Correlation between log* K_s *and log* $D_{lip}^{7.0}$ *values for the 14 basic compounds investigated*

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 head-groups, which was established as an important factor for drug partitioning in biological and artificial membranes [20].

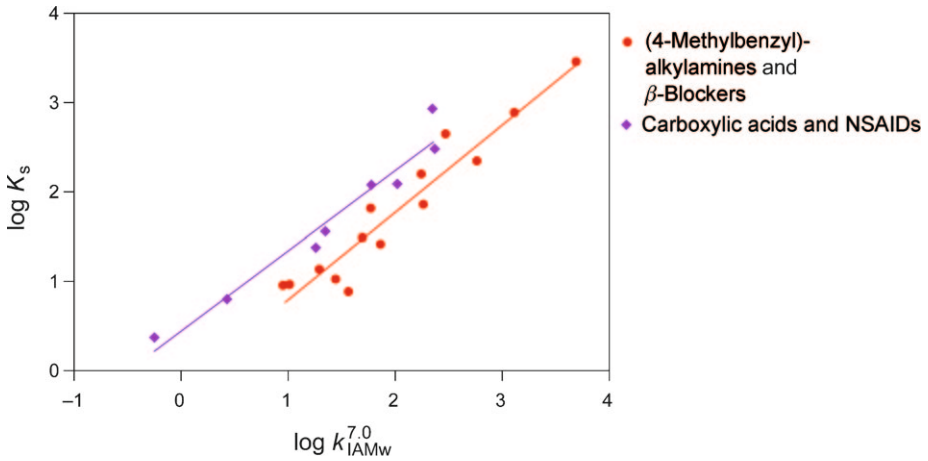

Fig. 2. *Correlation between log* K_s *and log* $k^{7.0}_{IAMw}$ *values for the compounds investigated*

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**:

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

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

For carboxylic acids **15**–**22**:

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

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

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

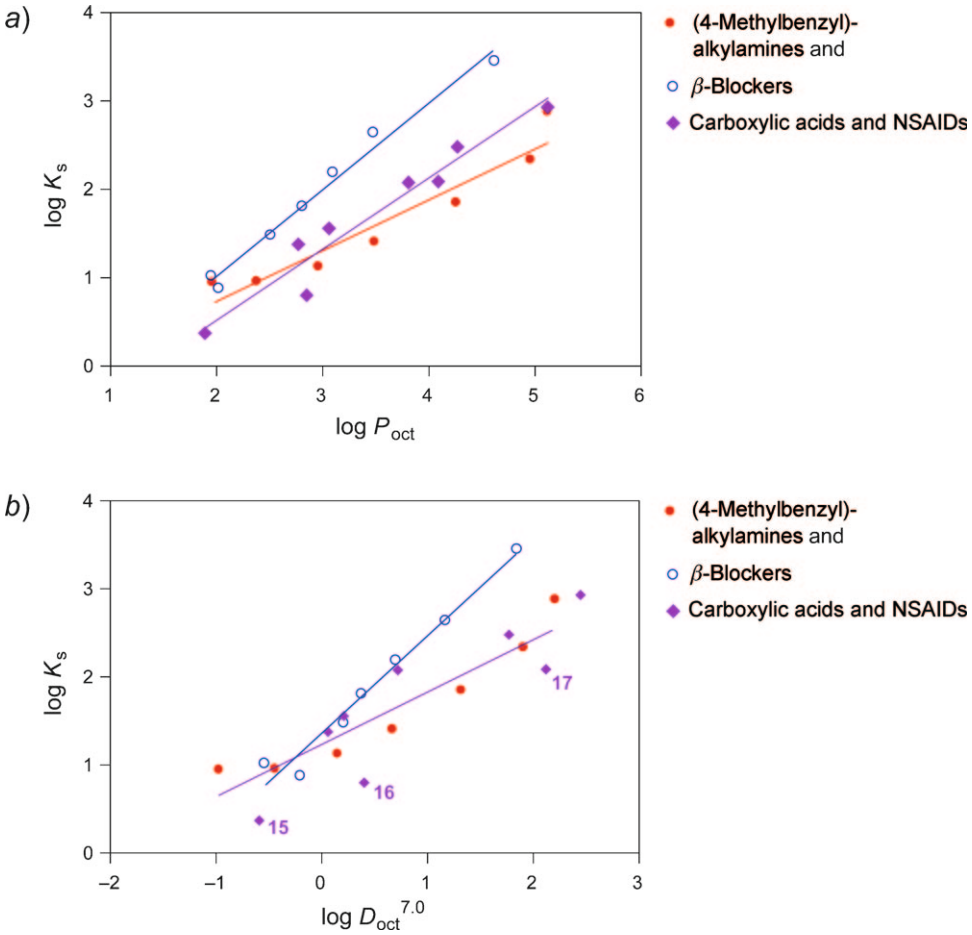

Fig. 3. *Correlation between log* K_s *and log* P_{oct} (*a*) *or log* $D_{oct}^{7.0}$ (*b*) *values for the compounds investigated*

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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