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Perspective

Immobilized Artificial Membrane HPLC in Drug Research

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1. Background: Drug Permeation and Lipophilicity

To elicit its pharmacological and therapeutic effects, a drug has to cross various cellular barriers by passive diffusion and/or by carrier-mediated uptake. As a result, drug design and discovery cannot have pharmacodynamic potency as the sole criterion of optimization but must also take pharmacokinetic behavior into account, in particular absorption and distribution. Numerous quantitative structure−permeability relationship (QSPR) studies[1−3] have unambiguously demonstrated that lipophilicity, as related to membrane partitioning and hence passive transcellular diffusion, is a key parameter in predicting and interpreting permeability. Within these studies, lipophilicity is often equated with the *n*-octanol/water partition coefficient (log P_{oct}),[1] since partitioning in this solvent system is traditionally accepted as an informative model of membrane partitioning.[4]

Lipophilicity as a molecular parameter encodes both polar and hydrophobic intermolecular forces (Figure 1). But when expressed by partition coefficients measured in traditional organic solvent/water systems, lipophilicity fails to encode some important recognition forces, most notably ionic bonds, which are of particular importance when modeling the interaction of ionized compounds with membranes.[5] And indeed, a recent count found that 62.9% of drugs are ionizable, of which 14.5% are acids, 67.6% are bases, and 17.9% are ampholytes of various types.[6] Whether these numbers apply also to chemical compounds in drug discovery can only be guessed. However, because the majority of drugs are ionizable, any prediction of their pharmacodynamic and pharmacokinetic properties should take their ionization into account.

RECOGNITION FORCES		LIPOPHILICITY	
		measured in liposomes, IAM or micelles	measured in liquid/liquid biphasic systems or RP-HPLC
• Charge transfer and aryl/aryl stacking interactions			
• Ionic bonds		***Polarity***	
• Ion-dipole bonds (permanent, induced) • Reinforced H-bonds • Normal H-bonds			***Polarity***
• Van der Waals Forces	• Orientation forces (permanent dipole – permanent dipole) • Induction forces (permanent dipole – induced dipole)		
	• Dispersion forces (instantaneous dipole – induced dipole)	***Hydrophobicity***	***Hydrophobicity***
• Hydrophobic interactions			

Figure 1. Comparison between recognition forces governing intermolecular interactions in biochemical and pharmacological processes, and forces encoded in lipophilicity parameters obtained by different methods (adapted from ref 88).

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The traditional view of passive transport is that hydrophilic compounds and particularly ions do not permeate into and across biological membranes. However, recent experimental evidence invalidates this assumption and documents the distribution of ion pairs and the passive transport of ionic species.[7,8] Indeed, it is increasingly clear that the behavior of ionizable drugs in the body is controlled by the interaction of both their neutral and ionized forms with biological membranes. The importance of partitioning of ionic and zwitterionic species in biphasic media has been emphasized in connection with their pharmacokinetic and pharmacodynamic behavior,[9−12] and hydrophobic as well as electrostatic interactions are expected between biological membranes and ionized compounds. In this context, a better understanding of the partitioning of ionized compounds is needed,[13] which itself awaits the development of experimental approaches able to mimic adequately the complexity of biological membranes.[14]

A new approach for measuring partition coefficients is based on artificial membranes, i.e., liposomes,[15−17] micelles,[18−21] and immobilized artificial membrane HPLC.[22,23] Since artificial membranes provide the amphiphilic microenvironment of biological membranes, they should be able to take ionic bonds into account (Figure 1).

This review focuses on immobilized artificial membrane liquid chromatography (IAM-HPLC). The technique is currently receiving marked interest because it presents the additional advantage of being suitable for high-throughput screening.

2. Structural Features of Single- and Double-Chain Immobilized Artificial Membranes

Immobilized artificial membranes (IAMs) are prepared by covalent binding of a monolayer of phospholipids to silica particles,[24] thus mimicking the lipid environment of a fluid cell membrane on a solid matrix. The prototype IAM surface (as well as two of the currently commercially available IAM stationary phases) is made by linking diacylphosphatidylcholine (PC) molecules covalently to silica−propylamine through their ω-carboxylic group on the C2 fatty acid chain. The synthetic immobilized lipid, according to current nomenclature rules, is called 1-myristoyl-2-[(13-carboxyl)-tridecanoyl]-*sn*-3-glycerophosphocholine and is denoted as lecithin-COOH.[24] The surface is referred to as IAM.PC, and to date, all IAM.PC surfaces utilize silica−propylamine containing 300 Å pores as a mechanically stable matrix for lipid immobilization. Because of steric hindrance, some residual free propylamino groups still remain on the silica surface after coupling of the PC molecules. Approximately 2 mol of residual amines per mol of immobilized PC reside at a depth of about 15 Å below the immobilized PC headgroups, resulting in a basic IAM.PC subsurface.[25]

Residual amines decrease the chemical stability of the bonded phase (lipid leaching),[26] increase the retention of acidic compounds, and decrease the retention of basic compounds.[27] To overcome these limitations, the residual amino groups are end-capped. Originally, end-capping was performed with glycidol and methyl glycolate (MG).[25] End-capping IAM.PC surfaces with MG converts the residual amino groups into chemically neutral amides (a highly desirable outcome considering the hydrocarbon environment of artificial bilayers), but 1 mol of hydroxy groups is formed during the process. These "new" hydroxy groups are about 10−12 Å below the immobilized PC headgroups. Other artificial and biological membranes do not contain hydroxy groups near the center of the membrane. Thus, as the hydroxy groups contribute to the selectivity (i.e., surface recognition properties) of the IAM.PC stationary phase, the IAM.PC.MG surface may not have the same chromatographic behavior as newer IAM columns. The IAM.PC surface end-capped with MG is commercially available and is called IAM.PC.MG (Figure 2).

To increase the surface density of phospholipids and especially the density of the polar headgroups and to obtain a greater stability under acidic conditions, a single-chain phosphatidylcholine lacking the glycerol backbone ($^{\delta}$IAM.PC, 11-carboxylundecylphosphocholine) was prepared and was attached to the silica−propylamine matrix.[22] To avoid hydroxy groups in the lipophilic core of the immobilized membrane, end-capping of this surface is performed using decanoic and propionic anhydride.[25] Two reaction steps are needed for quantitative end-capping. The first end-capping reaction uses decanoic anhydride and converts about 85% of accessible residual amines into amides. This step increases significantly the hydrocarbon density in the IAM.PC interphase. The second step-capping is done with propionic anhydride in order to convert the remaining amino groups into amides.[25] However, IR analysis has shown that even after the second end-capping reaction, about 30% of residual amino groups remain in small crevices of the silica surface.[28] The $^{\delta}$IAM.PCC10/C3 surface so prepared is called IAM.PC.DD and is also commercially available (Figure 2). The IAM.PC.DD (Drug Discovery) column is particularly recommended when short retention times are needed, since this stationary phase is more hydrophilic than the IAM.PC surface because of the immobilization of monoacylated phosphatidylcholine.

Some authors have observed the premature aging of IAM.PC.DD stationary phases.[29,30] Hence, a third IAM.PC surface has been developed and is known as IAM.PC.DD2. This HPLC column is a combination of the two other commercialized products, insofar as diacylated phosphatidylcholine is immobilized on silica−propylamine and the residual amino groups are end-capped using C_{10} and C_3 alkyl chains (Figure 2).

The double-chain IAM.PC.DD2 and the single-chain IAM.PC.DD stationary phases were compared using 16 compounds from three structurally related series.[31] A good correlation was found between the two surfaces, but the slope of the linear regression indicated that the double-chain IAM.PC.DD2 is more hydrophobic than the single-chain IAM.PC.DD column. Furthermore, the results suggested that drug−membrane interactions on IAMs are controlled by partitioning.[32] Another good correlation was found between the retention on IAM.PC.DD and IAM.PC.MG stationary phases showing that the IAM.PC.MG column is also more hydrophobic than the IAM.PC.DD chromatographic surface.[29] A recent com-

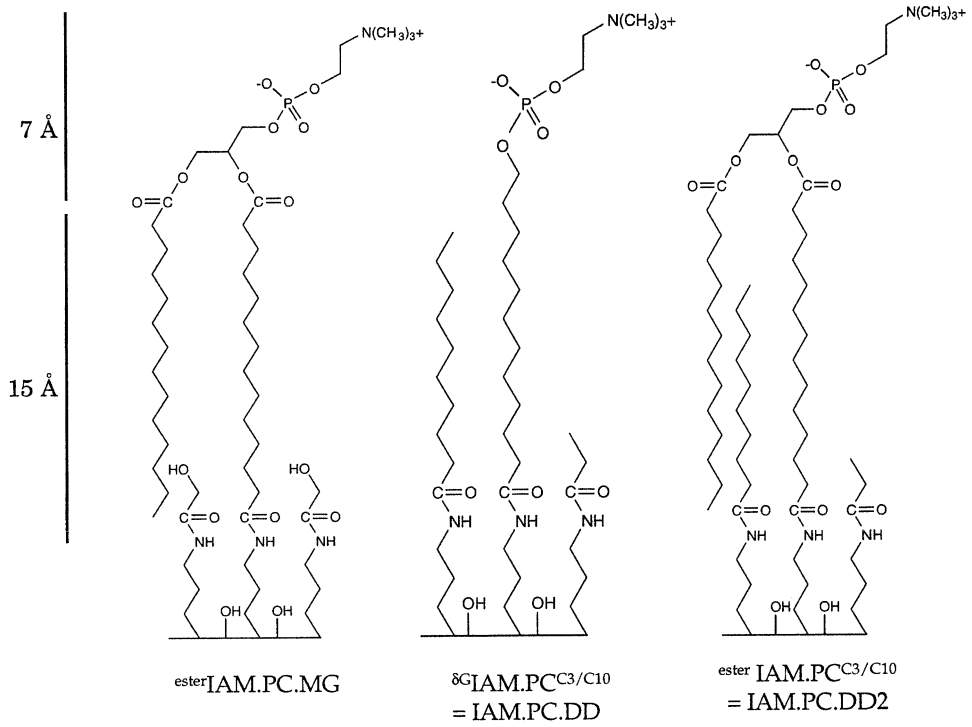


Figure 2. Structures of the commercially available immobilized artificial membranes (IAMs).

parison of IAM capacity factors measured on the three commercially available IAM stationary phases for 68 small and neutral model compounds confirmed the above results and showed that good correlation also exists between the two double-chain columns.[33] The above findings suggest that the hydroxy groups within the hydrocarbon core of the IAM.PC.MG phase do not contribute to retention selectivity.

3. Capacity Factors as a Measure of Partitioning in IAMs

IAM chromatography is clearly less tedious and less time-consuming than liposomes/water or *n*-octanol/water partitioning systems.[31,34] The determination of partition coefficients between the aqueous mobile phase and the IAM bonded phase only requires measurements (in minutes) of the solute's retention time (t_r) and the column dead time or void volume (t_0). The latter can be determined using a nonretained compound, e.g., methanol or better citric acid as recommended for IAM columns by the manufacturer (Regis Technology, Morton Grove, IL). Knowing the retention time of the analyte and the column void volume, the solute's capacity factor k_{IAM} can be calculated using the following equation:

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

The capacity factor k_{IAM} is linearly related to the equilibrium partition coefficient K_{IAM}:

$$K_{IAM} = \frac{V_m}{V_s} k_{IAM} = \phi k_{IAM} \quad (2)$$

where V_m is the total volume of the solvent within the IAM-HPLC column, V_s the volume of the IAM interphase created by the immobilized phospholipids, and ϕ ($=V_m/V_s$) the phase ratio (which is constant for a given column). As shown by eq 2, the solute's partition coefficient in the IAM interphase (K_{IAM}) can easily be calculated from the experimentally determined k_{IAM}.

For rather lipophilic drugs, prohibitively long retention times are obtained using purely aqueous mobile phases. To circumvent this problem, it is necessary to adjust the column length or add an organic modifier to the mobile phase. Adding an organic modifier then necessitates extrapolation to 100% aqueous phase (yielding log k_{IAMw}) in order to compare capacity factors independently of the amount and type of organic modifier and to avoid fictitious interaction scales due to differences in the elution order.[35−37]

Methanol and acetonitrile are the most popular cosolvents in liquid chromatography.[38−41] Their use in the extrapolation of log $k_{IAM}^{7.0}$ values was studied with an IAM.PC.DD2 column using a congeneric series of five β-blockers, all ionized at pH 7.0.[42] Plotting the log $k_{IAM}^{7.0}$ values against the percentage (v/v) of the organic modifier when extrapolating log $k_{IAM}^{7.0}$ values gave more reliable results than using the mole fraction of cosolvent. This result contradicts other findings.[34]

No significant difference was observed when comparing the log $k_{IAMw}^{7.0}$ values obtained by linear extrapolation using either methanol or acetonitrile.[42] Nevertheless, it was concluded that methanol is more appropriate for obtaining the log $k_{IAMw}^{7.0}$ values of charged compounds, since its solvent properties are closer to the properties of water. Furthermore, when acetonitrile is used, mobile phases containing more than 30% (w/w) must be avoided because their microheterogeneity disrupts the structure of water.[43] The use of methanol, however, is not recommended for the IAM.PC.MG stationary phase because of the danger of methanolysis.[28]

4. Column Stability and Silanophilic Interactions

Several sites susceptible to hydrolytic cleavage exist on immobilized artificial membrane chromatographic surfaces and may lead to accelerated degradation of the stationary phase.[27] Such hydrolysis-sensitive sites are the siloxane bonds (Si−O−Si) linking the silylating moiety to the silica surface, the amide bonds formed between the propylamino groups and immobilized phospholipids, and (in double-chain IAM stationary phases) the ester bonds between fatty acids and the glycerol backbone of phosphatidylcholine (Figure 2). Hydrolysis of the siloxane and amide bonds is essentially due to the basic characteristics of the silica−propylamine, and end-capping of the residual amines is thus crucial for increased stability.

Despite such precautions, lipid leaching is reduced but not totally suppressed on end-capped IAM surfaces, and capacity factors may decrease during a column's lifetime (aging).[25,27−29] This is not very surprising, knowing that about 30% of residual amino groups persist in small crevices after end-capping.[28] Furthermore, column stability also depends on the type and purity of silica support[44] and on eluent composition (e.g., methanol as cosolvent causes more lipid leaching than acetonitrile[26]). Thus, it is recommended that capacity factors be corrected for aging.[29,42,45]

Additionally, a recent investigation of four different IAM.PC.DD stationary phases from two different batches showed premature failure of one column, different rates of aging of the other columns, and a column-to-column variability sometimes exceeding 10%.[29] Similar results have recently been reported in a study of the intra- and interbatch variability of IAM.PC.DD2 phases using four columns and six neutral model compounds.[42] Such findings make it clear that the inter- and/or intrabatch variability of IAM HPLC columns must be checked regularly when capacity factors obtained with different columns are to be compared.

Free acidic silanol groups, which exist normally on silica-based chromatographic surfaces, elicit silanophilic interactions with basic and polar compounds. The proportion of unreacted silanol groups (up to one-half[46]) can be reduced by end-capping. One of the most promising approaches to avoid silanophilic interactions is to introduce a functional group on the modified silica surface able to react with the silanol groups through electrostatic and/or hydrogen-bonding interactions (electrostatic shielding). The amido group is of particular interest in this context because it possesses useful properties such as stability to bases, a strong H-bonding capacity, and inertness toward different functional groups in the analytes. The most convenient method to introduce the amido moiety during end-capping is acylation of a preformed propylamine−silica matrix. Indeed, this is now the standard end-capping process for IAM stationary phases.[24] The efficiency of electrostatic shielding was deduced from the improved peak shape (symmetric peaks, no tailing) observed for basic compounds on reversed-phase (RP) C_{18} HPLC columns.[47]

As repeatedly mentioned,[48] an end-capped silica still bears free silanol groups that may affect solute retention.[49] Indeed, the pH-dependent retention behavior of ionizable compounds on a single-chain IAM.PC.DD stationary phase was found to differ from their pH-dependent partitioning in liposomes/water, the deviations being ascribed to silanophilic interactions occurring on the IAM phase.[34] Silanophilic and silanophobic interactions also exist in double-chain IAM.PC.DD2 columns.[42] Unfortunately, masking agents such as *n*-decylamine or *N*,*N*-dimethyloctylamine, which are common in RP chromatography, cannot be used with IAM stationary phases because they will interact not only with free acidic silanol groups but also with negatively charged phosphates in the immobilized lipids.

5. Structural Comparison between IAMs and Liposomes

Liposomes are thought to be good models of biological membranes because they exhibit structural similarities with phospholipid bilayers. Liposomal partition coefficients have indeed been used extensively in the study of solute−membrane interactions[17,50−56] and in a number of quantitative structure−activity relationship (QSAR) studies.[16,57,58]

Although liposomes appear to be well suited for modeling solute partitioning into physiological membranes,[59] their wide-scale development as an in vitro model for predicting drug−membrane interactions is limited. The preparation and validation of liposomes make their use in partitioning studies time-consuming and tedious.

The use of IAMs, in contrast, combines the speed of HPLC technology (ideal for high-throughput screening) with a model of phospholipid partitioning. Capacity factors measured on IAMs are indeed expected to reflect the partitioning of compounds in the liposomes/water system. Furthermore, IAM-HPLC requires only minute amounts of analytes, and impurities as well as degradation products do not interfere.

Liposomes are artificial lipid bilayer vesicles of known composition and controlled size that can be classified according to various criteria. They are often classified into multilamellar or unilamellar vesicles, and the latter, according to their size, can be subdivided into small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs).[60] Large unilamellar vesicles prepared by a standardized method from egg phosphatidylcholines (PhC)[17] are preferably used to measure the partitioning of drugs into liposomes (phosphatidyl- stands for 1,2-diacyl-*sn*-glycero-3-phospho-).

Liposomes are a fluid-phase system, and each vesicle is formed by a bilayer of phospholipids that separates the external from the inner aqueous phase. IAM chromatographic surfaces, in contrast, are solid-phase systems where a monolayer of synthetic phospholipids is bound to silica particles. Thus, the hydrophobic core is twice as large in liposomes as in IAMs (Figure 3).

The naturally occurring phospholipids in egg-PhC have an unsaturated acyl chain in the *sn*-2 position, and the double bonds have normally *Z* or cis configuration.[61,62] The synthetic phospholipids linked to silica−propylamine to form IAMs are, in contrast, fully saturated and hence more ordered. Furthermore, egg-PhC is composed of phospholipids with acyl chains having 16−20 carbon atoms,[62] whereas synthetic phospholipids bound to silica in IAMs have acyl chains of usually 16 carbon atoms (propylamine included).[31]

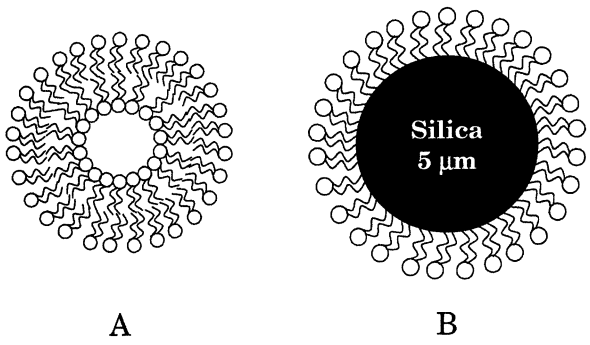


Figure 3. Schematic representation of (A) a unilamellar liposome and (B) a phospholipid monolayer bound to a silica particle in immobilized artificial membranes (IAMs). Both systems are used to model membrane partitioning.

Furthermore, the density of phospholipid headgroups is different in liposomes and IAMs. The surface area per lipid headgroup is around 62 Å^2 in liposomes,[63] whereas for the single-chain IAM surface it amounts to 85 Å^2 and for the double-chain stationary phases it amounts to 105 Å^2.[31]

Another important difference between liposomes and IAMs is that lipid molecular dynamics (i.e., lateral diffusion, flip-flop, and axial displacement) are absent in IAMs because each lipid molecule is covalently linked to the silica surface.[25,64] This factor again implies that phospholipids on IAMs are more ordered.[65] On the other hand, the mean position of atoms and the extent of fluctuations of the phosphate, choline, and glycerol groups are virtually identical in IAMs and fluid POPC (1-palmitoyl-2-oleyl-*sn*-3-glycerophosphocholine) liposomes. Furthermore, in both systems the choline groups exhibit large motional fluctuations compared to the phosphate groups, and the distribution of water near the polar headgroups in both IAMs and POPC liposomes is the same.[65]

Overall, the results of Sheng et al.[65] indicate that the polar interfacial region of the IAM surface mimics well that of fluid membranes, the noteworthy difference being that the density of the phospholipid headgroups in IAMs is smaller than in liposomes. However, these results also indicate that the hydrocarbon part of the IAM surface has unique physicochemical properties compared to fluid membranes. As stated by Pidgeon et al.,[25] in IAM technology the "inability" to exactly emulate lipid dynamics must be traded for the increased stability gained by covalently linking lipids to solid surfaces.

6. Predictive Value of IAM Capacity Factors

During the past decade, many efforts have been devoted to evaluate the predictive power of IAM capacity factors toward drug permeation. Whereas some authors examined correlations between IAM capacity factors and partition coefficients determined in *n*-octanol/water[35,36,66−69] or liposomes/water,[34,51,70−74] others investigated the relationships between IAM retention and gastrointestinal absorption,[70,75,76] permeation across Caco-2 cells,[77] transdermal transport,[37,78,79] blood−brain barrier permeation[45,80,81] and biological activity.[29,82,83] Readers are referred to the review of Stewart et al.[30] for earlier studies.

It must be noted, however, that these studies were often restricted to a comparison of log k_{IAM} values with conventional log *P* parameters. We will review below recent studies, paying special attention to the significance of ionized compounds.

6.1. Relations between IAM Capacity Factors and Other Lipophilicity Parameters. Demare et al.[66] investigated the relationship between log $k^{7.0}_{IAMw}$ values measured on an IAM.PC.DD2 column and partitioning in *n*-octanol. They studied a series of 13 antiinflammatory and analgesic drugs (6 acidic, 4 basic, and 3 amphoteric compounds) and observed a slightly better correlation between log $k^{7.0}_{IAMw}$ and log $D^{7.0}_{oct}$ than with log P^{N}_{oct} ($r^2 = 0.70$ and $r^2 = 0.62$, respectively). These results contrast with others,[35] showing that for a set of 16 nonsteroidal antiinflammatory drugs the correlation between log $k^{7.0}_{IAMw}$ and log P^{N}_{oct} was better than between log $k^{7.0}_{IAMw}$ and the ionization-corrected log $D^{7.4}_{oct}$ ($r^2 = 0.91$ and $r^2 = 0.68$, respectively). The authors of the latter study considered that all drugs were fully ionized in this pH range. Kaliszan et al.,[83] using a series of β-blockers, also found a good correlation of IAM capacity factors with log $D^{7.0}_{oct}$. However, this study does not confirm that of Demare et al.,[66] since log P^{N}_{oct} and log $D^{7.0}_{oct}$ are strongly intercorrelated in the series considered ($r^2 = 0.97$). It must be stressed that the set of compounds investigated by Demare et al. spanned a broader pK_a range and chemical diversity than the compounds used in previous studies,[35] thus allowing a better assessment of the relations between IAM retention and *n*-octanol/water partitioning.

Considering the importance of cationic amphiphilic compounds in medicinal chemistry, Barbato et al. investigated the behavior of 23 basic compounds on an IAM.PC.MG phase as influenced by the nature of the amino group (primary, secondary, or tertiary), lipophilicity ($0.56 < \log P_{oct} < 4.96$), and the degree of ionization ($1.10 < pK_a < 10.15$).[67] The log k_{IAMw} measurements were performed at three different pH values (7.0, 5.5, and 3.0) in order to study the combined effects of analyte ionization and the changes in conformation and/or charge distribution occurring in phospholipids.

At pH 7.0, a good correlation was found for all compounds between log P_{oct} and log $k^{7.0}_{IAMw}$ ($r^2 = 0.83$), but the relation between log $D^{7.0}_{oct}$ and log $k^{7.0}_{IAMw}$ was poor ($r^2 = 0.50$), in agreement with earlier findings.[35,36,69] However, a closer look at the results revealed two distinct subsets. One subset (13 analytes) yielded a regression line between log P_{oct} and log $k^{7.0}_{IAMw}$ that matched exactly a previous correlation for 10 neutral compounds,[35] whereas 10 solutes considered as "outliers" were located above this regression line. This second subset consisted of compounds having either an endocyclic or a primary amino function, the latter being only partly ionized at pH 7.0. It was concluded that at pH 7.0 phospholipids are able (a) to cancel the effect of a positive charge on the amino function and to interact with fully protonated amines to the same extent as with neutral isolipophilic compounds (subset of 13 analytes) and (b) to interact more strongly than *n*-octanol with neutral hydrophilic primary (or endocyclic) amines (subset of 10 analytes). The effect observed for the subset of 13 compounds is consistent with the relatively high affinity of positively charged bases for phospho-

lipids in liposomal partition systems as observed by several authors.[82,84,85]

For amines fully ionized at pH 7.0 and 5.5, log k_{IAMw} values were smaller at pH 5.5 than at pH 7.0. In contrast, analytes neutral at both pH values had similar log k_{IAMw} values. When IAM retention was compared with *n*-octanol/water partitioning, better correlations were found between log P_{oct} and log $k^{5.5}_{IAMw}$ than between log $D^{5.5}_{oct}$ and log $k^{5.5}_{IAMw}$.

Compared to pH 7.0 and pH 5.5, retention at pH 3.0 was further decreased, and only neutral compounds were appreciably retained at this pH. This suggested that the retention capacity of protonated forms on the IAM.PC.MG phase decreases with pH and disappears at pH 3.0. This behavior has been explained by probable changes in conformation and/or charge distribution occurring in phospholipids as the pH of the eluent approaches their pK_a of ~2.0.[17] In addition, the occurrence of secondary retention effects due to free silanol groups cannot be excluded.[34,42]

From their studies, Barbato et al.[67] concluded (a) that the retention behavior of amines on the IAM.PC.MG stationary phase cannot be predicted a priori from their *n*-octanol/water partitioning and (b) that the unexpectedly strong interaction of some hydrophilic amines with IAMs at physiological pH implies a better capacity to interact with biomembranes than expected from their log *P* values.

IAM retention was also compared to capacity factors measured in immobilized liposome chromatography (ILC).[51,72,73] This technique was developed to provide a simple and fast tool to perform medium-pressure chromatography on phospholipid-based stationary phases. Its main advantages are that phases of different chemical composition can be easily and reversibly immobilized on suitable gel supports and that chromatographic retention on phospholipids is devoid of any effect caused by the presence of a silica matrix. However, the possible influence of polymeric support on retention and reproducibility of column performance remain to be fully understood. A comparison between ILC and IAM retention was done, for example, for a series of 19 oligopeptides at pH 7.4.[73] An IAM.PC.DD phase was used for the determination of the IAM capacity factors, and liposomes made of egg yolk phospholipids were immobilized in a gel bed for the ILC experiments. Despite the structural differences between the two stationary phases (monolayer of immobilized phospholipids for the IAMs versus bi- and multilayered liposomes for ILC), the authors observed a high correlation between the resulting capacity factors ($r^2 = 0.93$). This was explained by the similarity of the headgroups (phosphatidylcholine in both cases) and the hydrophilicity of the analyzed peptides. In fact, it was postulated that the hydrophilic peptides under study partitioned predominantly in the vicinity of the polar headgroup region, thus canceling the influence of the thickness and number of phospholipid layers.

The correlation between IAM capacity factors and drug retention on ILC was also investigated for a series of compounds spanning a broad lipophilicity range and comprising both positively and negatively charged molecules.[72] The analytes comprised 17 nonsteroidal antiinflammatory drugs, 5 local anaesthetics, and 2 β-blockers, which were investigated at pH 7.4 in immobilized liposomes made from (a) purified egg phospholipids (EPL), (b) synthetic phosphatidylcholine (PC), and (c) PC/synthetic phosphatidylethanolamine (PE) 80:20 (mol/mol). The results were compared to published log $k^{7.0}_{IAMw}$ values measured on an IAM.PC.MG column.[35,69] Surprisingly, the ILC capacity factors determined with PC liposomes showed the weakest relationship with the log $k^{7.0}_{IAMw}$ values ($r^2 = 0.79$), despite having similar headgroups. It thus seems that for this series of rather lipophilic drugs and in contrast to the hydrophilic series investigated by Alifrangis et al.,[73] the structural differences between the hydrophobic cores strongly influence partitioning.

This conclusion is in agreement with that of Beigi et al.[51] who studied 16 basic compounds (7 β-blockers, 5 benzodiazepines, 3 phenothiazines, and 1 antihistamine). The analytes were investigated at pH 7.4 in immobilized liposomes made from (a) phosphatidilcholine (PC), (b) egg phospholipids (EPL), (c) lipids extracted from human red cell membranes (membrane lipids), and (d) cytoskeleton-depleted human red cell membranes (vesicles). The authors emphasized that the composition of liposomes strongly differed as a function of material used. In fact, EPL contain phosphatidylethanolamine (PE) besides PC, their main component. Membrane lipids contained PE, sphyngomyelin, cholesterol, glycolipids, and phosphatidylserine (PS) besides PC, their main component. Vesicles were similar to membrane lipids but also contained an anion transporter, glycophorins, and the glucose transporter Glut1.

The results were compared to log $k^{7.4}_{IAMw}$ values measured on an IAM.PC.DD column. Fair rectilinear correlations were found between log $k^{7.4}_{IAMw}$ and log K_s (K_s is the capacity factor on liposome columns normalized for its liposome content) for the four systems under investigation: (a) PC ($r^2 = 0.83$); (b) EPL ($r^2 = 0.83$); (c) membrane lipids ($r^2 = 0.79$); (d) vesicles ($r^2 = 0.81$). Since these moderate correlations were found for all liposome systems despite their different composition, the authors concluded that there is a marked difference between monolayer (e.g., IAM) and bilayer systems, independent of the bilayer composition.

The relationship between IAM retention and partitioning in suspended phosphatidylcholine (PhC) liposomes was recently studied using three series of ionizable compounds,[74] namely, small homologous basic compounds ((*p*-methylbenzyl)alkylamines), congeneric β-blockers, and a heterogeneous set comprising both basic and acidic drugs. No correlation was observed between log $k^{7.0}_{IAMw}$ and log $D^{7.0}_{lip}$ when all analytes were taken together. When each series was examined separately, however, some trends became apparent. A good relationship was found for the β-blockers. For the (*p*-methylbenzyl)alkylamines, in contrast, a bilinear trend was observed, indicating that the balance between electrostatic and hydrophobic interactions was not the same in the two partitioning systems. Comparing the log $k^{7.0}_{IAMw}$ values to partitioning in *n*-octanol (log $D^{7.0}_{oct}$) showed that hydrophobic interactions were mainly responsible for IAM retention of all (*p*-methylbenzyl)-alkylamines, whereas electrostatic interaction forces were important in the liposomal partitioning of the shorter homologues in this series (Figure 4). No cor-

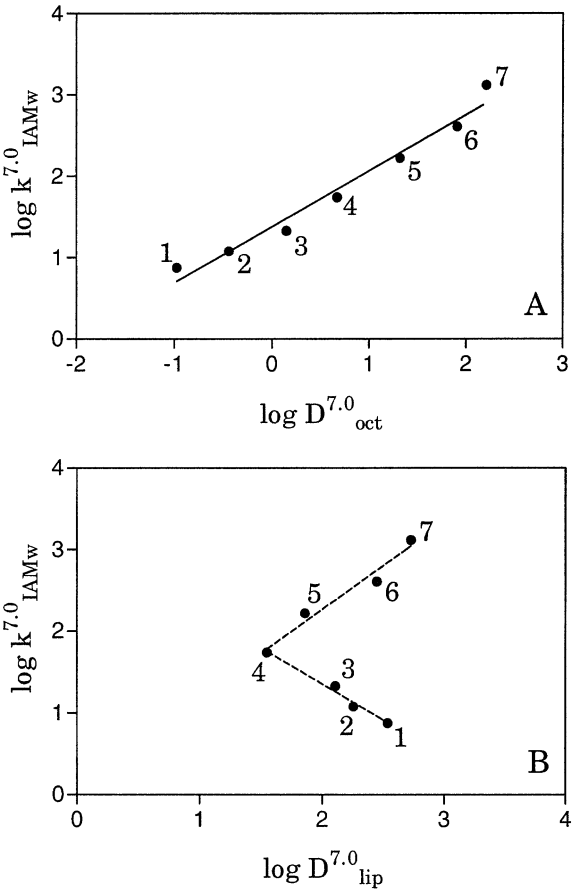


Figure 4. Comparison between retention on the IAM.PC.DD2 stationary phase and partitioning in the (top) *n*-octanol/water and (bottom) PhC-liposomes/water system for a homologus series of (*p*-methylbenzyl)alkylamines (*N*-methyl (point 1) to *N*-heptyl (point 7)) (adapted from ref 74).

relation existed between log $k_{IAMw}^{7.0}$ and log $D_{lip}^{7.0}$ for the set of heterogeneous compounds, but ionized compounds had a higher affinity for the IAM phase than for *n*-octanol, in agreement with the results of Demare et al.[66]

6.2. Relations of IAM Capacity Factors with Drug Permeation and Pharmacokinetic Behavior. The relationship between IAM retention (log $k_{IAMw}^{7.0}$) and *permeability across Caco-2 cells* of six β-blockers was investigated.[72] The predictive power of the IAM capacity factors (IAM.PC.MG stationary phase) was evaluated with respect to other lipophilicity parameters such as log P_{oct}, log $D_{oct}^{7.4}$, and partitioning in immobilized liposomes at pH 7.4. Hyperbolic relationships between the different lipophilicity indices and Caco-2 permeation data were observed, and all parameters showed about the same predictive power. However, the investigated set comprised only six compounds with similar chemical structures, thus seriously restricting any extrapolation and forbidding the calculation of any correlation except linear ones.

Stewart et al.[77] correlated uptake rates into Caco-2 cell monolayers with lipophilicity for a set of nine HIV protease inhibitors. The compounds investigated were either acidic or neutral pyrone derivatives (three compounds) or dihydropyrone derivatives (six compounds). Lipophilicity parameters considered were (i) log $k_{IAMw}^{7.4}$ measured on an IAM.PC.DD column, (ii) log k' measured on a C18 column with eluent at pH 3.0, and (iii) log $D_{oct}^{7.4}$. The correlation between Caco-2 uptake and log $k_{IAMw}^{7.4}$ was improved when molecular weight and hydrogen-bonding capacity were included in the regression (r^2 improving from 0.39 to 0.91). When either log $D_{oct}^{7.4}$ or log k' was used as the lipophilicity parameter, molecular weight was again significant ($r^2 = 0.55-0.90$ or $0.43-0.87$ for log $D_{oct}^{7.4}$ or log k', respectively) but hydrogen-bonding was rejected. The authors concluded that the three lipophilicity parameters, namely, log $k_{IAMw}^{7.4}$, log $D_{oct}^{7.4}$, and log k', provided useful and comparable correlates of HIV protease inhibitor uptake by Caco-2 cell monolayers. However, log $k_{IAMw}^{7.4}$ values were easier to obtain, and this method was therefore recommended. We note that the study on only nine quite similar compounds implies that the three lipophilicity parameters were markedly intercorrelated ($r = 0.847-0.862$), a fact that limits an assessment of their differences in correlating with permeation.

The ability of IAM retention coupled to physicochemical descriptors to model the *passive intestinal absorption of drugs* was also evaluated.[76] The log $k_{IAMw}^{7.0}$ values of 12 structurally diverse compounds were determined on an IAM.PC.DD2 column, and their passive permeabilities (P_a) were measured through rat everted gut sacs or through noneverted sacs for actively transported molecules. The log $k_{IAMw}^{7.0}$ parameter was better correlated with P_a ($r^2 = 0.77$) than with other physicochemical descriptors such as molecular weight, molar volume, water solubility, pK_a, melting point, log P_{oct}, and hydrogen-bonding capacity. The addition of the molar volume (V_x) as a second independent variable slightly improved the correlation ($r^2 = 0.83$). This was explained by the fact that membrane permeation depends not only on the membrane partition coefficient (modeled by log $k_{IAMw}^{7.0}$) but also on the solute's diffusion coefficient, itself a function of molecular size and thus of V_x.

The ability of IAM capacity factors to describe *drug permeation across human skin* was also examined.[37] Twelve structurally unrelated compounds comprising acidic, basic, amphoteric, and neutral drugs were used. Capacity factors (log $k_{IAMw}^{5.5}$) were measured on an IAM.PC.MG column at pH 5.5 in order to be as close as possible to the physiological pH of human skin. A comparison of log $k_{IAMw}^{5.5}$ values with log P_{oct} showed that the two lipophilicity descriptors were poorly related ($r^2 = 0.54$). In addition, neither log P_{oct} nor log $k_{IAMw}^{5.5}$, alone or combined with molecular weight, correlated with the permeability coefficients through human skin (K_p).

Thus, and in analogy with the Λ parameter derived from partition coefficients,[86,87] a new parameter (Δ log k_{IAMw}) was calculated as the difference between the experimental log $k_{IAMw}^{5.5}$ value and the capacity factor estimated from the good relationship between log P_{oct} and log k_{IAMw} previously obtained for compounds interacting with the IAM phase by a purely hydrophobic mechanism[35,36] (Figure 5). The Δ log k_{IAMw} parameter is assumed to quantify polar interactions with phospholipids (hydrogen bond and electrostatic interactions).

For 10 previously investigated compounds,[37] a fair relation was uncovered between K_p and Δ log k_{IAMw}

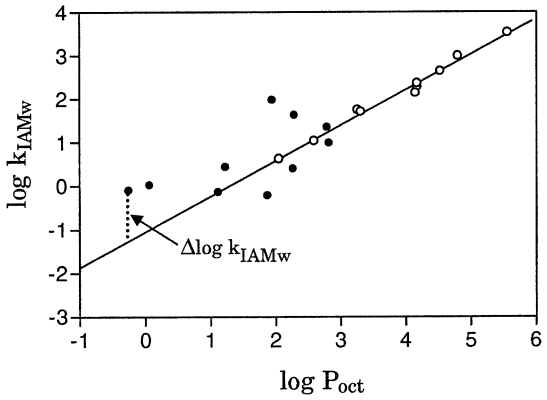


Figure 5. Derivation of the Δ log k_{IAMw} parameter for a series of drugs (●, ref 37) using derivatives retained by pure hydrophobic interactions (○, ref 35).

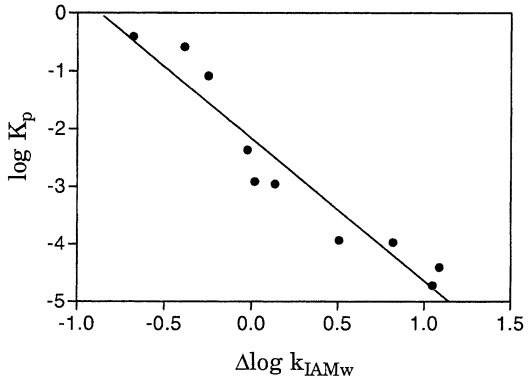


Figure 6. Relationship between the transdermal permeation (K_p) and (log k_{IAMw} of 10 drugs (adapted from ref 37).

($r^2 = 0.91$; Figure 6). The neutral drugs griseofulvin and hydrocortisone were strong outliers and had to be excluded. A preliminary conclusion was that compounds eliciting the lowest polar interactions with phospholipids had the highest ability to cross the skin barrier. However, we note that some anionic solutes may have negative Δ log k_{IAMw} values when they elicit repulsive ionic interactions with phospholipids. Such anionic solutes have good permeability, leading to the hypothesis that *attractive* ionic interactions decrease permeability whereas the *repulsive* ones facilitate it.

Potential relations were also examined between IAM capacity factors and pharmacokinetic parameters of eight β-blockers.[29] The capacity factors were measured on an IAM.PC.MG and an IAM.PC.DD stationary phase at pH 7.0 using a mobile phase containing 10% (v/v) acetonitrile (log $k_{IAM}^{7.0}$). The pharmacokinetic parameters considered were the tissue-bound fraction, the plasma-bound fraction, the albumin-bound fraction, the true red blood cell partition coefficient (log K_{BC}), the volume of distribution at steady state, the plasma protein/plasma water partition coefficient, and the ratio of nonrenal versus renal excretion. Correlation coefficients ranking from $r^2 = 0.45-0.91$ were obtained, and in all cases except log K_{BC}, the log $k_{IAM}^{7.0}$ values from the IAM.PC.MG phase gave better correlations than those from the IAM.PC.DD phase. The authors interpreted these findings to suggest that the double-chain IAM.PC.MG phase might be a better model than the IAM.PC.DD phase.

7. Conclusion

Commercially available IAM HPLC columns provide an efficient and simple method to determine membrane interaction parameters when used with appropriate controls and reference compounds. The primary feature that distinguishes anisotropic partitioning systems such as IAMs and liposomes from organic solvent/water partitioning systems is the presence of the ordered phospholipids able to elicit ionic interactions. This implies that IAMs and liposomes are in principle better suited to predict membrane permeation than the traditional *n*-octanol/water system. Whereas IAM chromatography is fast and reproducible, the determination of liposomal partitioning is time-consuming and tedious.

The IAM capacity factors of *neutral solutes* often correlate with other lipophilicity parameters such as ClogP, log *P*, log *D*, capacity factors measured on reversed-phase HPLC columns, and liposomes/water partitioning. For *ionized solutes*, in contrast, IAM chromatography, liposomes/water, and *n*-octanol/water yield distinct lipophilicity scales, particularly for hydrophilic compounds. In fact, several studies have shown that the balance of recognition forces governing partitioning in liposomes and IAMs is not identical. This may be explained by the marked structural differences existing between the two systems.

With respect to pharmacokinetic properties, the greatest potential for IAM chromatography is the prediction of passive transport. The ability of IAM-HPLC to correlate with membrane permeation is often improved when used together with other physicochemical parameters such as molecular size or hydrogen-bonding capacity. However, no study has yet been able to show which lipophilicity descriptor (IAM capacity factors, *n*-octanol/water, or liposomes/water partitioning) is best suited to predict the membrane permeation of a large series of compounds. The answer, it seems, differs according to the set of compounds under investigation.

In drug discovery and optimization, IAM chromatography provides a fast and simple tool to quantify drug−membrane interactions separately from other relevant factors such as molecular weight or hydrogen-bonding capacity. This may help the early identification of promising candidates.

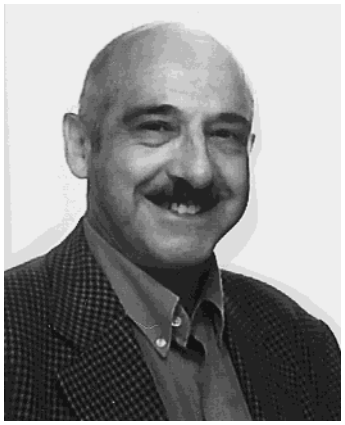
Biographies

Agnes Taillardat-Bertschinger studied pharmacy at the Swiss Federal Institute of Technology (ETH) in Zurich, obtaining her Diploma in 1997. She then undertook doctoral studies

at the University of Lausanne under the supervision of Bernard Testa and Pierre-Alain Carrupt. She received a Ph.D. in Pharmaceutical Sciences in 2001 and was awarded a research grant by the Swiss National Science Foundation, which has allowed her to pursue postdoctoral research in the group of Prof. Charman at the Victorian College of Pharmacy in Melbourne. There, she is currently investigating the biopharmaceutics of poor water-soluble drugs and the digestibility of lipid-based formulations.

Pierre-Alain Carrupt graduated as a chemist and obtained a Ph.D. from the University of Lausanne under the supervision of Prof. Pierre Vogel. After early work in synthetic chemistry, he became interested in computational chemistry and organic physical chemistry with applications in reaction mechanism, the structure of reaction intermediates, and transition states. A tenured Reader in Medicinal Chemistry collaborating with Prof. Bernard Testa, he now specializes in molecular modeling techniques and physicochemical properties as applied to drug design and molecular pharmacology. He is the author or coauthor of over 200 research papers and 35 chapters in scientific books.

Francesco Barbato graduated with a degree in pharmacy from the University of Naples where he also specialized in microbiology. In 1980 he began teaching medicinal chemistry in the Department of Medicinal and Toxicological Chemistry of the University of Naples, and in 1998 he was visiting professor at University of Lausanne. He has recently been appointed as Associate Professor of Pharmaceutics by the Faculty of Pharmacy of the Università degli Studi di Napoli Federico II. He also teaches biopharmaceutics and drug metabolism at the Specialized School of Hospital Pharmacy and teaches quality control at the Specialized School of Phytochemical Science and Technology. His main areas of scientific interest include pharmacokinetics, the interaction of drugs with biological membranes and serum proteins, controlled drug release systems, and cyclodextrin formulation.



Bernard Testa graduated with a degree in pharmacy and obtained a Ph.D. His Ph.D. thesis was on the physicochemistry of drug−macromolecule interactions. He then went for 2 years to Chelsea College, University of London, for postdoctoral research under the supervision of Prof. Arnold H. Beckett. He was appointed as Assistant Professor at the University of Lausanne and became Full Professor and Head of Medicinal Chemistry in 1978. He has served as Dean of the Faculty of Sciences (1984−1986), Director of the Geneva-Lausanne School of Pharmacy (1994−1996 and 1999−2001), and President of the University Senate (1998−2000). He has written 3 books and edited 27 others (2 others are in press) and has coauthored well over 400 research and review articles in the fields of drug design and drug metabolism. He is a member of the Editorial Board of several leading journals: Biochemical Pharmacology, Drug Metabolism Reviews, Helvetica Chimica Acta, Journal of Medicinal Chemistry, Medicinal Research Reviews, and Pharmaceutcial Research. He holds honorary doctorates from the Universities of Milan and Montpellier and Parma and is the latest recipient of the Nauta Award on Pharmacochemistry given by the European Federation for Medicinal Chemistry. He is also a Fellow of the Royal Society of Chemistry and of the American Association of Pharmaceutical Scientists and is a member of several scientific societies, including the French Academy of Pharmacy, the Royal Academy of Medicine of Belgium, and American Chemical Society. His hobbies, interests, and passions include jogging, science fiction, epistemology, teaching, and scientific exploration.

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