Molecular Factors Influencing Retention on Immobilized Artifical Membranes (IAM) Compared to Partitioning in Liposomes and *n*-Octanol

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Received February 5, 2002; accepted March 1, 2002

Purpose. To assess the effect of molecular factors influencing retention on immobilized artificial membrane (IAM) high-performance liquid chromatography columns compared to liposomal partitioning and traditional *n*-octanol/water partition coefficients.

Methods. IAM capacity factors were measured at pH 7.0 on an IAM.PC.DD2 stationary phase. Liposomal partitioning at pH 7.0 and *n*-octanol/water partition coefficients were measured using the pH metric method. Partitioning in egg-phosphatidylcholine (PhC) liposomes was also measured by equilibrium dialysis for a series of β-blockers.

Results. For the ionized β-blockers, potentiometry and equilibrium dialysis yielded consistent partitioning data. For relatively large bases, IAM retention correlated well with PhC liposome partitioning, hydrophobic forces being mainly involved. For more hydrophilic compounds and for heterogeneous solutes, in contrast, the balance between electrostatic and hydrophobic interactions was not the same in the two systems. Hydrogen bonding, an important factor in liposomes partitioning, played only a minor role in IAM retention.

Conclusions. Partitioning in immobilized artificial membranes depends on size, hydrophobicity, and charge. When hydrophobic interactions dominate retention, IAM capacity factors are well correlated with liposomal partitioning. On the contary, for hydrophilic solutes, the two systems do not yield the same information and are not interchangeable.

KEY WORDS: immobilized artificial membrane (IAM); liposome; solute-membrane interactions; partition coefficients.

INTRODUCTION

Pharmacokinetic and pharmacodynamic effects are strongly dependent on the interactions of drugs with biologic membranes. Also, the impact of lipophilicity on the biologic activity of drugs is well recognized. As a consequence, membrane-like systems, for instance liposomes (1,2) and immobilized artificial membrane (IAM) high-performance liquid chromatography (HPLC) stationary phases (3,4), have been developed to obtain lipophilicity parameters of greater biologic relevance, especially for ionized compounds.

Equilibrium dialysis is often regarded as a gold standard for the determination of drug partitioning in liposomes/water systems (1). However, this technique is time-consuming and tedious and therefore is of little use in routine work. Because over 90% of all drug candidates are ionizable (5), potentiometric titration may be a promising tool to measure drug partitioning not only in bulk organic solvents but also in liposomes (6,7). Compared to equilibrium dialysis, the pH metric technique has the advantage of being fast and the ability to take impurities into account (8). However, it is not clear whether the two methods are interchangeable because only a few comparisons on small series of compounds are available to date (6,7). For this reason, we extended the comparison of liposomal partition coefficients measured by equilibrium dialysis and potentiometric titration to a series of ionized β-blockers (Fig. 1B).

IAM stationary phases model the lipid environment of membranes on a solid matrix and have many experimental advantages compared to the liposomes/water partitioning system. The method is very fast, it allows a relatively high throughput, requires only small amounts of solutes, and impurities as well as degradation products seldom interfere with the procedure.

In this context, it is important to compare the chromatographic membrane partition model to liposomal partitioning as well as to the traditional *n*-octanol/water system. For neutral compounds, the partition coefficients are known to be comparable (7,9). In contrast, charged species and particulary cations are known to partition better into anisotropic lipid membranes than into bulk *n*-octanol (10,11).

For some series of ionized and usually homologous solutes, previous studies showed a good correlation between distribution coefficients measured in liposomal systems and capacity factors determined on IAM stationary phases (4,9,12,13). To date, extensive comparative studies, including large heterogeneous series of compounds, are not available. We therefore compared liposomal partitioning and capacity factors measured on an IAM stationary phase at pH 7.0 (log $k^{7.0}_{IAMw}$) using three series of ionizable compounds (Fig. 1A–C). These compounds were selected to cover a fair range in molecular size and acidity or basicity.

MATERIALS AND METHODS

Chemicals

Acebutolol HCl (**2a**), alprenolol HCl (**2b**), metoprolol tartrate (**2e**), oxprenolol HCl (**2f**), propranolol HCl (**2h**), timolol maleate (**2i**), clonidine (**3a**), imipramine HCl (**3b**), S-(−)-nicotine (**3c**), procaine HCl (**3d**), and phenytoin (**3h**) were purchased from Sigma Chemie (Buchs, Switzerland). Diazepam (**3f**) and phenobarbital (**3g**) were obtained from Lipomed (Arlesheim, Switzerland) and carazolol (**2c**), metipranolol (**2d**), as well as bisoprolol hemifumarate (**2j**) were offered by Boehringer Mannheim (Mannheim, Germany). Penbutolol sulfate (**2g**) was kindly offered by Hoechst Pharma (Zürich, Switzerland), and rilmenidine (**3e**) was a gift from UCB (Braine l'Alleud, Belgium). The (p-methylbenzyl)alkylamines (**1a–1g**) were synthesized by known procedures (14,15). The racemate of chiral drugs was used when not specified otherwise.

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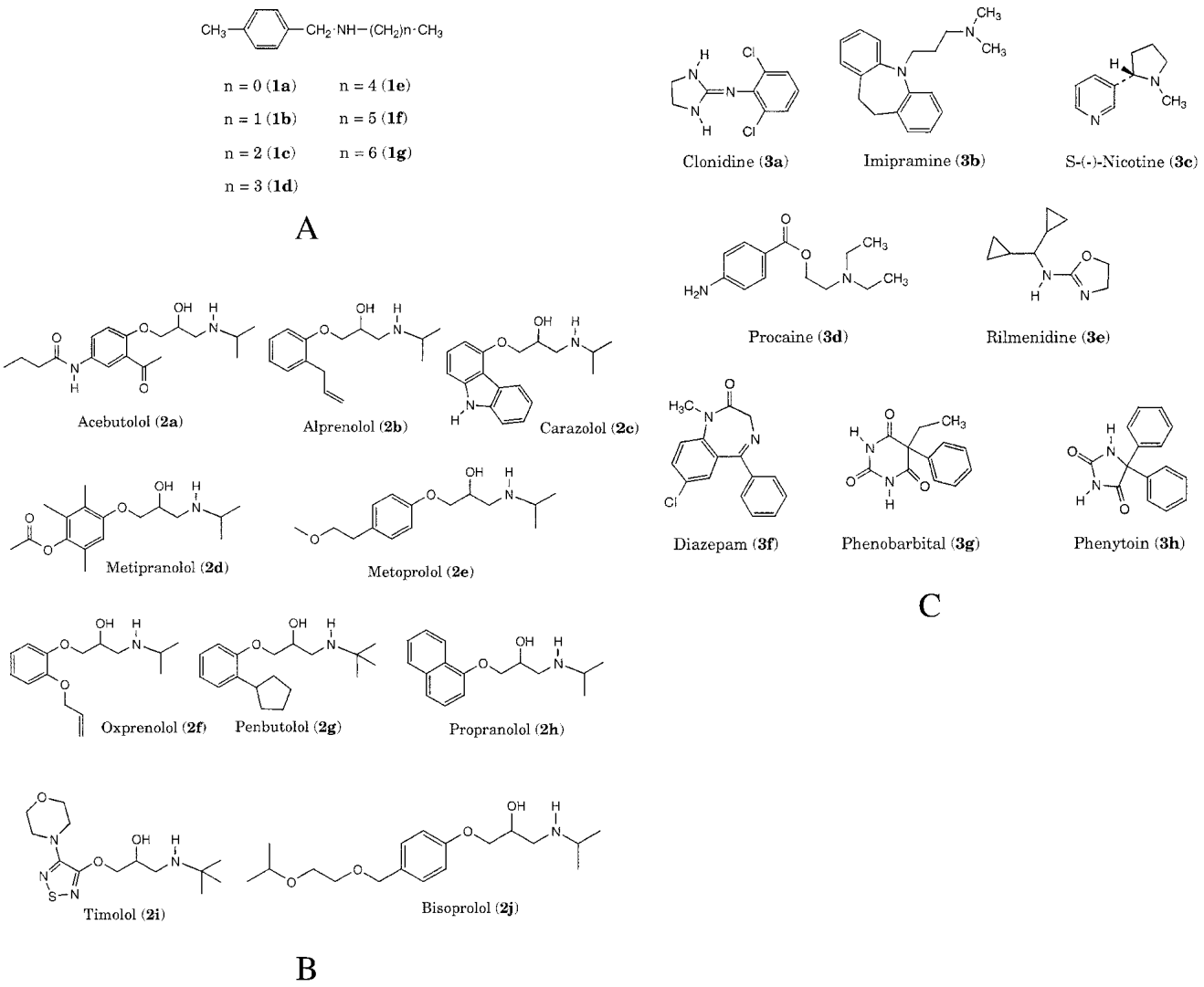

Fig. 1. Chemical structures of compounds under study. (A) Homologous series of (p-methylbenzyl)alkylamines. (B) Congeneric set of β-blockers. (C) Heterogeneous series of basic and acidic drugs (N-acidic) compounds.

The analytical grade solvents *n*-octanol, chloroform, and dimethylsulfoxide (DMSO) were purchased from Fluka Chemie (Buchs, Switzerland) and methanol of superpure quality for HPLC from Romil Chemicals (Cambridge, United Kingdom). Potassium chloride was bought from Merck (Dietikon, Switzerland) and all other chemicals were of analytical grade. Deionized water was used throughout.

Determination of Capacity Factors at pH 7.0 by IAM-HPLC

For the three series of solutes, IAM capacity factors were measured at pH 7.0 by HPLC using a liquid chromatograph consisting of a pump type LC 414-T Kontron Analytica (Kontron Instruments, Zürich, Switzerland) equipped with an Uvikon 730 S LC Kontron UV Spectrophotometer (Kontron) set at 254 or 220 nm. The chromatograms were recorded by an integrator type 3390 A purchased from Hewlett Packard (Avondale, Pennsylvania).

The stainless-steel column was an immobilized artificial membrane S12-300-IAM.PC.DD2 stationary phase (100 × 4.6 mm, 12 μm, 300 Å; Regis Technology, Morton Grove, Illinois). The column temperature was maintained at 25 ± 2°C using a water-bath circulator (Haake, Digitana, Lausanne, Switzerland) and a column-jacket.

The eluents were either phosphate buffer pH 7.0 (0.02 M KH_2PO_4 and 0.15 M KCl, total ionic strength 0.18 M) or mixtures of methanol and phosphate buffer in different percentages at flow rates of 1.5 or 2.0 mL/min. The aqueous portion of the mobile phases were filtered through 0.45-μm HA Millipore filters (Millipore, Milford, Massachusetts) and the eluent mixtures were prepared manually, degassing them prior to use.

Stock solutions (10^{-2} M) of the compounds were prepared in methanol. The solutes were diluted before analysis with the respective mobile phases and end-concentrations ranking from 10^{-5} to 5×10^{-3} M were used. The injection volume was 20 μL.

The chromatographic retention data are means of at least three determinations and are expressed by the logarithm of the capacity factor, log k_{IAM}, defined as:

$$\log k_{IAM} = \log[(t_r - t_0)/t_0] \quad (1)$$

where t_r and t_0 are the retention times of the solute and the non-retained compound (citric acid), respectively.

For the elution of the more lipophilic compounds, mixtures of methanol/phosphate buffer pH 7.0 containing up to 80% organic modifier were used. The log k_{IAMw} values at 100% aqueous phase were extrapolated by linear regression plotting the log k_{IAMw} values vs. the percentage (v/v) of organic modifier in the eluent mixtures.

It was verified that a total buffer ionic strength of 0.18 M (i.e., ionic strength of the buffer used for HPLC measurements) compared to 0.15 M (i.e., ionic strength used in potentiometric titrations) had no influence on the resulting capacity factors.

Potentiometric Determination of Partition Coefficients in the *n*-Octanol/Water System

For some compounds, namely clonidine (**3a**), S-(−)-nicotine (**3c**), procaine (**3d**), and phenytoin (**3h**), the partition coefficients in the *n*-octanol/water system were determined in this work by the potentiometric pH-method using a PCA101 titrator (Sirius Analytical Instruments, Forrest Row, East Sussex, United Kingdom). The principles of the potentiometric method have been explained elsewhere in detail (16) and the procedure as described in (17) was used for all measurements. At least four separate titrations for each compound (ca. 1 mM) were performed in the pH range 1.8 to 12.2 using various volumes of *n*-octanol (volume ratios of *n*-octanol/water ranking from 1 ml/15 ml to 8 ml/8 ml). The titrations were carried out under argon at 25 ± 2°C.

Preparation of Egg-Phosphatidylcholine (PhC) Liposomes

PhC, type Lipoid-E PC, was purchased from Lipoïd (Cham, Switzerland), and large unilamellar liposomes were prepared by the extrusion method (1,18). Approximately 1 g of solid PhC was dissolved in chloroform and the organic solvent was evaporated at about 37°C using a rotary evaporator. The resulting colorless thin film was stored in a desiccator overnight at room temperature. For each gram of PhC, 10 mL 0.02 M phosphate-buffered saline (pH 7.4, 0.15 M KCl) (equilibrium dialysis) or 10 mL of an aqueous solution containing 0.15 M KCl (potentiometry) were added to the dried lipid film to form vesicles. To increase the diameter of the liposomes, five freeze-thaw cycles were performed and the liposomal suspension was finally passed about 10 times through an extruder purchased from Lipex Biomembranes (Vancouver, Canada) equipped with two polycarbonate filters (Osmomics, Livermore, California) having 0.1 μm pores (18,19).

The concentration of the resulting opalescent stock suspension of liposomes was determined using a phospholipase D choline oxidase and peroxidase assay for PhC (MPR 2, Boehringer Mannheim) (20) and the size distribution was controlled by dynamic light scattering using a Malvern Zetasizer 5000 purchased from Malvern Instruments (Worcestershire, United Kingdom). The liposomes were stored at 4°C and for each experiment the pH of the liposomal suspension was decreased to pH 6.0 with HCl. Nitrogen was used to prevent lipid oxidation during the whole procedure (21).

Determination of Partition Coefficients in the PhC Liposomes/Water System

Equilibrium Dialysis

To determine the time necessary to reach concentration equilibrium between the aqueous and the liposomal compartment, an equilibrium dialysis experiment was performed with the most lipophilic compound in the series, penbutolol (**2g**), and a very hydrophilic one, atenolol (structure not shown). The dialysis membranes with a cutoff point of 10,000, the drive unit Type GD 4/90, and the Macro 1 and 1-mL dialysis cells were purchased from Dianorm (Munich, Germany). Drug concentrations in the aqueous compartment were monitored (as described below) after rotation at 3 rpm for 2, 3, 4, and 5 h. Equilibrium was reached after 4 h. For all solutes, 5 h were taken as the time necessary to reach equilibrium.

For each of the β-blockers studied (**2a–2j**), three dialysis cells were filled with 1 mL of drug solution (1 mM in buffer, pH 6.0) in one compartment and 1 mL of liposomal suspension (pH 6.0) in the other. A reference cell was filled with 1 mL of buffer in one compartment and 1 mL of liposomal suspension in the other. After rotation at 3 rpm for 5 h at room temperature, the pH was checked in each compartment. Drug concentrations in the aqueous compartment were determined by UV spectroscopy (Diode Array, HP 8452 A, Hewlett Packard, Avondale, Pennsylvania) at the λ_{max} of each compound by using calibration plots ($r^2 > 0.99$). Equation 2 was then used to calculate the distribution coefficient (D) at pH 6.0, which is in fact equal to the liposomal partition coefficient of the cationic species ($P^C_{lip(EDS)}$) for all β-blockers under study:

$$D = \frac{V_L(C_L - C_B)}{(V_{lip} \cdot C_B)} \quad (2)$$

In this equation C_B and C_L are the molar drug concentrations in the aqueous and the liposomal compartment, respectively; V_L is the volume of the drug compartment (1 mL); and V_{lip} is the volume occupied by the liposomes in the liposomal suspension. The latter parameter was calculated from the lipid concentration assuming a density of 1 g/mL(22).

For the determination of the $P^C_{lip(EDS)}$ values for carazolol (**2c**) and penbutolol sulfate (**2g**) 5% DMSO had to be added to the aqueous buffer solutions to solubilize the compounds. Previous experiments with water-soluble compounds had shown that the addition of 5% DMSO to the aqueous buffer did not affect the $P^C_{lip(EDS)}$ values (results not shown).

All experiments, dialyses as well as calibration plots and determinations of phospholipid concentration, were performed in triplicate.

Potentiometric Titrations

Recently, the potentiometric method (described previously) was also applied successfully to liposomes/water systems (7,23). Although the experimental procedure remains the same, the use of an anisotropic phase requires additional care, as detailed in (23). The general procedure described in (23) was used. Lipid/water ratios ranging from 1.2 to 42 (in mg/mL) and molar lipid/solute ratios from 2.69 to 135 were used.

For the determination of the partition coefficients of

carazolol (**2c**) and penbutolol (**2g**) 5% DMSO had to be added to the aqueous buffer solutions to solubilize the compounds. Control experiments performed with compounds **1g** and **2h** (which have a partition coefficient similar to that of compounds **2c** and **2g**; Table I) but can be solubilized in the aqueous buffer without addition of a co-solvent, had shown that the addition of 5% DMSO to the aqueous buffer did not affect their partition coefficients measured by pH-metric titration (results not shown).

Table I. Physicochemical Parameters of the Compounds under Study

	pK_a[a]	$\log k_{IAMw}^{7.0}$[b]	$\log P_{oct}^{N}$[c]	$\log D_{oct}^{7.0}$[d]	$\log D_{lip}^{7.0}$[d]
1a	9.93	0.88	1.96	–0.97	2.54
1b	10.04	1.08	2.38	–0.44	2.26
1c	9.98	1.33	2.96	0.15	2.11
1d	9.98	1.74[f]	3.49	0.67	1.55
1e	10.08	2.22[f]	4.26	1.32	1.86
1f	10.17[e]	2.61[f]	4.96	1.91	2.45
1g	10.02[e]	3.12[f]	5.12	2.21	2.73
2a	9.52	1.57	2.02	–0.20	1.93
2b	9.59	2.08[f]	3.10	0.70	2.33
2c	9.52[e]	2.62[f]	3.73	1.34	2.41[i]
2d	9.54	1.95[f]	2.81	0.38	2.27
2e	9.63	1.21	1.95	–0.54	1.59
2f	9.57	1.55[f]	2.51	0.21	2.09
2g	9.92[e]	3.11[f]	4.62	1.85	3.39[i]
2h	9.53	2.44[f]	3.48	1.17	2.69
2i	9.53	1.26	2.12	–0.30	1.40
3a	8.11	1.36	1.59	0.47	1.29
3b	9.34[e]	3.30[f]	4.16[g]	1.82[h]	2.83
3c	3.23/8.00	0.78	1.44	0.33	2.30
3d	9.03	1.02	2.03	0.02	1.62
3e	9.22	1.03	1.63[g]	–0.60[h]	2.11
3f	3.45	2.34[f]	2.92	2.92	3.58
3g	7.20	0.81	1.50[g]	1.29[h]	2.15
3h	7.94	1.86[f]	2.68	2.63	3.05

[a] Measured by potentiometry (n = 3, SD < 0.5); or taken from the literature (23,24).

[b] Capacity factors determined on an IAM.PC.DD2 HPLC-column at pH 7.0 (n = 3, SD < 0.1).

[c] Determined by potentiometry (n = 4, SD < 0.05); or taken from the literature (23,24).

[d] Calculated from the partition coefficient of the neutral and ionized form using the following equations:

$$D = P^N \cdot \left(\frac{1}{1 + 10^{pK_a - pH}}\right) + P^I \cdot \left(\frac{10^{pK_a - pH}}{1 + 10^{pK_a - pH}}\right) \text{ for bases and}$$

$$D = P^N \cdot \left(\frac{1}{+ 10^{pH - pK_a}}\right) + P^I \cdot \left(\frac{10^{pH - pK_a}}{1 + 10^{pH - pK_a}}\right) \text{ for acids}$$

[e] Methanol as cosolvent (at least four points; SD < 0.1).

[f] Extrapolation of $\log k_{IAMw}^{7.0}$ using methanol as organic modifier (at least five points; SD < 0.1).

[g] Calculated from the distribution coefficients determined at pH 7.4 by shake-flask (personal communication UCB, Braine l'Alleud, Belgium) using the following equations:

$$\log P = \log D + \log(1 = 10pK_a - pH) \text{ for bases and}$$
$$\log P = \log D + \log(1 + l0pH - pK_a) \text{for acids.}$$

[h] Calculated from [g]) using the equations mentioned under [g]).

[i] pH-metric titration in presence of 5% (v/v) dimethylsulfoxide (DMSO).

RESULTS AND DISCUSSION

Physicochemical Parameters

The set of compounds under (Fig. 1) included a homologous series of (p-methylbenzyl)alkylamines (*N*-methyl to *N*-heptyl) **1a–1g** (Fig. 1A), a set of congeneric β-blockers **2a–2j** (Fig. 1B), and a series of heterogeneous basic and acidic drugs **3a–3h**, among which two N-acids (**3g** and **3h**) (Fig. 1C).

The pK_a and *n*-octanol/water partitioning data of the β-blockers were taken from the literature (24). For the (p-methylbenzyl)alkylamines, the pK_a as well as the partition coefficients in the *n*-octanol/water and PhC liposomes/water system, measured by potentiometry, have been published (23). For all other compounds, partitioning in large unilamellar PhC liposomes was determined potentiometrically, as well as by equilibrium dialysis for the β-blockers (**2a–2j**).

Except for bisoprolol (**2j**), the pK_a, $\log P_{oct}^{N}$, $\log D_{oct}^{7.0}$ values and distribution coefficients determined potentiometrically at pH 7.0 in the PhC-liposomes ($\log D_{oct}^{7.0}$) are reported in Table I. The partitioning data of the ionized β-blockers (**2a–2j**) determined by equilibrium dialysis ($\log P_{lip(EDS)}^{C}$) and pH-metric titration ($\log P_{lip(pot)}^{C}$) are listed in Table II.

To obtain experimental conditions as close as possible to the physiologic pH and compatible with the stability of the stationary phase, the capacity factors on the IAM.PC.DD2 HPLC-column were determined at pH 7.0. At this pH all β-blockers and (p-methylbenzyl)alkylamines were almost fully ionized, whereas the drugs of the heterogeneous series were either neutral, positively or partly negatively charged (see pK_a values in Table I).

Only about half of the compounds eluted in a reasonable time with a fully aqueous eluent. For the other solutes, extrapolation of the $k_{IAMw}^{7.0}$ by linear regression was necessary. At least five different percentages of methanol were used for each compound and good linear relationships (r^2 = 0.991–0.999) between $\log k_{IAM}$ and the percentage organic modifier (v/v) were observed. The logarithms of the capacity factors extrapolated to 100% aqueous phase, $k_{IAMw}^{7.0}$, are also reported in Table I.

Table II. Comparison of Liposomal Partition Coefficients Measured for a Series of Ionized β-Blockers by Equilibrium Dialysis and Potentiometry

	$\log P_{lip(EDS)}^{C}$[a]	$\log P_{lip(pot)}^{C}$[b]
2a	1.15	1.92
2b	2.50	2.32
2c	2.62[c]	2.40[c]
2d	2.10	2.26
2e	1.54	1.60
2f	1.76	2.08
2g	3.88[c]	3.40[c]
2h	2.96	2.68
2i	1.50	1.39
2j	1.10	2.36

[a] Partition coefficient of the cationic species measured at pH 6.0 using the equilibrium dialysis system (EDS) (n = 3, SD < 0.1).

[b] Partition coefficient of the cationic species determined by potentiometric titration (n > 5, SD < 0.2).

[c] Determined in presence of 5% dimethylsulfoxide (DMSO).

Comparison of the Liposomal Partition Coefficients Obtained by Equilibrium Dialysis and Potentiometry

To investigate whether equilibrium dialysis and potentiometric titration gave comparable liposomal partitioning data, we investigated a series of β-blockers (**2a–2j**) covering a wide range of lipophilicity (Table I). However, due to experimental problems polar β-blockers (e.g., atenolol) could not be included. The relationship between the partition coefficients obtained for the ionized species by equilibrium dialysis ($\log P^{C}_{lip(EDS)}$) and potentiometry ($\log P^{C}_{lip(pot)}$), respectively, is shown in Figure 2. Excluding the deviant compound (**2j**), a linear relation is observed (Eq. 3):

$$\log P^{C}_{lip(EDS)} = 1.34(\pm 0.20) \cdot \log P^{C}_{lip(pot)} - 0.77(\pm 0.46) \quad (3)$$
$$n = 9;\ r^2 = 0.87;\ s = 0.34;\ F = 45$$

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

The regression coefficients of Equation 3 clearly indicate that the two methods gave homogeneous partitioning data for this series of β-blockers. However, a closer look at Equation 3 shows that the slope and ordinate differ from 1 and 0, respectively. This may be explained by the fact that the counterions used in equilibrium dialysis and potentiometry were not the same (described previously). The following equation, which was derived from the Nernst equation and describes the apparent partitioning of ionic species (25), supports the argument:

$$\log P_{app} = \log P^{0} + \frac{z_i F}{RT} \cdot \Delta\phi \quad (4)$$

where $\log P^0$ is the intrinsic lipophilicity of the ion; z_i the charge of the ionic species; F the Faraday constant; R the gas constant; T the absolute temperature [K]; and $\Delta\phi$ the Galvani potential difference across the interface between the aqueous and the organic phase.

Equation 4 actually means that the apparent partition coefficient of an ion depends not only on its intrinsic lipophilicity but also on the experimental conditions, as the $\Delta\phi$ term depends on the volume of the aqueous and the organic phase and on all ionic species present in the aqueous solution. Thus, the observed differences can be explained by the absence of phosphate ions in the pH metric titrations and by the fact that different volume ratios were used in equilibrium dialysis and potentiometric experiments.

In order to determine which method caused the deviant behavior of bisoprolol (**2j**) in Figure 2, the partition coefficients obtained by equilibrium dialysis and potentiometry, respectively, were plotted against the partition coefficients obtained for the ionized β-blockers in the *n*-octanol/water system (data not shown). The results indicated that the P^{C}_{lip} value for bisoprolol (**2j**) obtained by potentiometry was too high and it was thus excluded from the comparisons below. The observed differences may be explained by the fact that the same drug/lipid ratio was used in all equilibrium dialysis experiments, whereas this ratio was varied in potentiometry.

Relationship between Retention on IAMs and Partitioning in PhC Liposomes

The comparison for all compounds between $\log k^{7.0}_{IAMw}$ and the distribution coefficients determined at pH 7.0 in the PhC liposomes/water system ($\log D^{7.0}_{lip}$) is illustrated in Figure 3, whereas the relationships obtained for each individual series are represented in Figure 4A–C.

In contrast to Ong *et al.* (4), we observed no correlation including all solutes. These are unexpected results, as the immobilized artificial membranes have been developed as an alternative to liposomes for the modeling of drug partitioning into biologic membranes. However, differences between the two anisotropic partitioning systems are known to exist. Indeed, the immobilized lipids lack lateral mobility, and the density of the polar phospholipid head-groups in the IAM.PC.DD2 stationary phase is half that in PhC-liposomes (26,27). Actually, further results by Ong *et al.* (26) indicated that the polar head-groups are an important factor for drug partitioning in biologic and artificial membranes.

A closer look at Figure 3 and Figure 4B shows that a

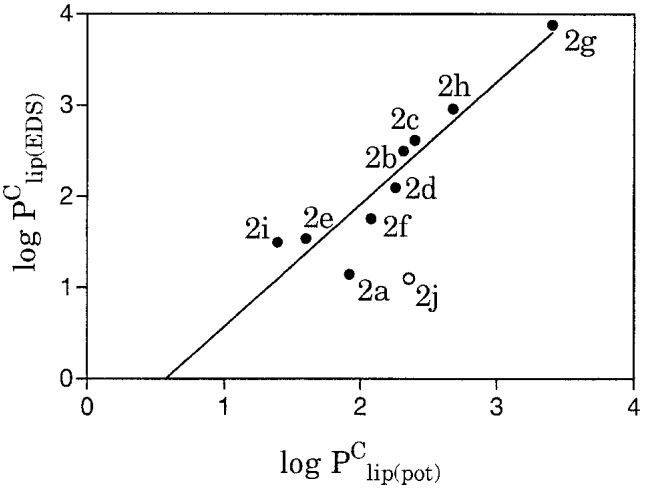

Fig. 2. Comparison of P^{C}_{lip} values of β-blockers measured by equilibrium dialysis and potentiometric titration.

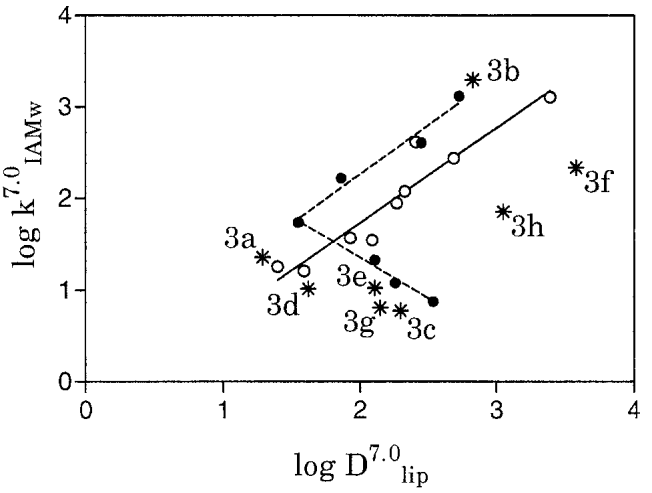

Fig. 3. Comparison between retention on the IAM.PC.DD2 stationary phase and partitioning in the PhC-liposomes/water system. $k^{7.0}_{IAMw}$ vs. $D^{7.0}_{lip}$ for the three investigated series. (●): (p-methylbenzyl)alkylamines, (○): β-blockers and (✱): heterogeneous series of ionizable acidic and basic compounds.

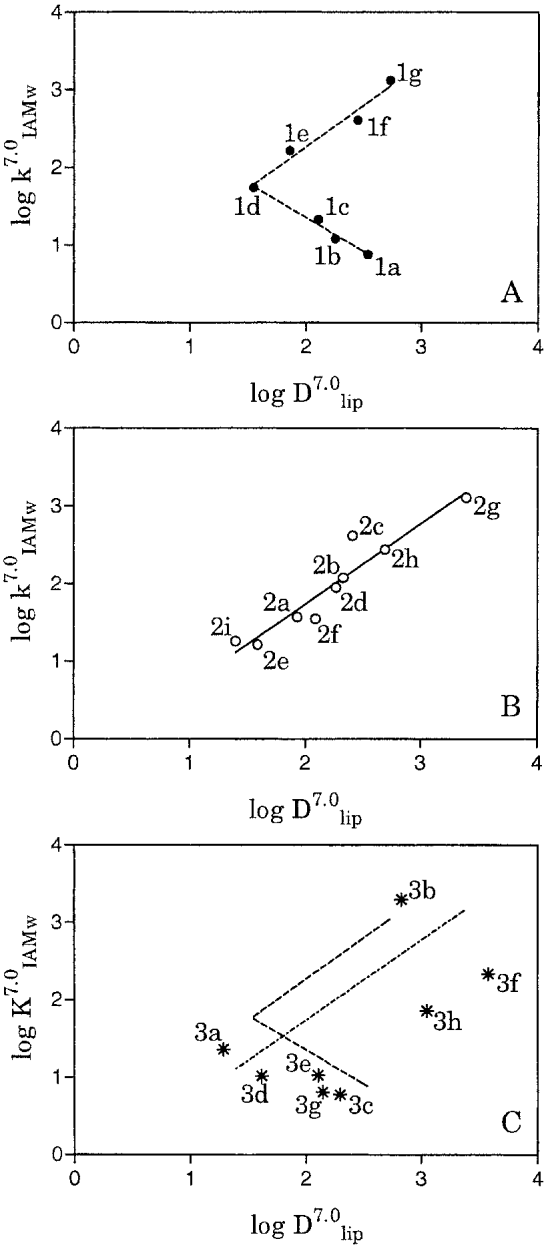

Fig. 4. Comparison between retention on the IAM.PC.DD2 stationary phase and partitioning in the PhC-liposomes/water system. (A) Log $k^{7.0}_{IAMw}$ vs. $D^{7.0}_{lip}$ for the (p-methylbenzyl)alkylamines. (B) log $k^{7.0}_{IAMw}$ vs. $D^{7.0}_{lip}$ for the β-blockers. (C) log $k^{7.0}_{IAMw}$ vs. $D^{7.0}_{lip}$ for the heterogeneous series of ionizable acidic and basic compounds.

linear relationship exists for the congeneric series of β-blockers between $k^{7.0}_{IAMw}$ and log $D^{7.0}_{lip}$:

$$\log D^{7.0}_{lip} = 1.04(\pm 0.31) \cdot \log k^{7.0}_{IAMw} - 0.35(\pm 0.71) \quad (5)$$
$$n = 9;\ r^2 = 0.90;\ s = 0.22;\ F = 63$$

Interestingly, no correlation can be observed for the (p-methylbenzyl)alkylamines (Fig. 4A). Indeed, a bilinear relation between log $k^{7.0}_{IAMw}$ and log $D^{7.0}_{lip}$ was found with a positive slope for compounds **1e–1g** and a negative slope for the smaller solutes **1a–1c**. Fruttero *et al.* (23) observed a similar behavior when comparing their partitioning in *n*-octanol and PhC-liposomes at pH 7.5. They concluded that for the bulkier compounds **1e–1g** hydrophobic interactions dominate the partitioning in *n*-octanol and liposomes. On the contrary, for the shorter solutes **1a–1c** the balance between electrostatic and hydrophobic interactions is not the same towards liposomes and *n*-octanol. For these compounds, Fruttero *et al.* (23) have shown that electrostatic interactions dominate their partitioning in liposomes. According to these findings, Figure 4A thus indicates that electrostatic interactions play a smaller role in the IAM.PC.DD2 stationary phase compared to the liposomes/water system. This may be explained by the smaller density of phospholipids in the IAM.PC.DD2 stationary phase compared to PhC-liposomes.

No correlation between log $k^{7.0}_{IAMw}$ and log log $D^{7.0}_{lip}$ exists for the heterogeneous series of neutral and ionized drugs (Fig. 4C).

Further analysis of Figure 3 shows that the two linear relations obtained for the β-blockers and the long-chain (p-methylbenzyl)alkylamines **1e–1g** do not overlap. The relative position of the β-blockers compared to compounds **1e–1g** suggests that an additional interaction besides hydrophobic and electrostatic forces increases the affinity of β-blockers for liposomes. The formation of a H-bond between the hydroxy group of β-blocking agents and the ester bond of phospholipids seems likely. Thus, the partitioning of β-blockers in liposomes is increased compared to that of the long-chain (p-methylbenzyl)alkylamines. In fact, as the density of polar head-groups is smaller in IAMs they may have less possibility of forming this additional H-bond. By measuring log k_{IAMw} values for some compounds at pH 7.0 and pH 6.0, it could be excluded that the observed difference in Figure 3 between β-blockers and long-chain (p-methylbenzyl)alkylamines was caused by silanophilic interactions.

A closer look to the series of heterogeneous compounds shows that imipramine (**3b**) lies on the linear regression obtained for the long-chain (p-methylbenzyl)alkylamines. Its chemical structure is close to that of compounds **1e–1g** and thus, hydrophobic interactions seem to be mainly responsible for its partitioning in liposomes and retention on the IAM phase.

Clonidine (**3a**), which is almost completely protonated at pH 7.0, can be compared to compound (**1d**), whose electrostatic interactions are counterbalanced by hydrophobic interactions. Indeed, the affinity of clonidine for liposomes is the smallest of all investigated compounds, implying that a different balance of interactions governs its IAM retention.

The small size of S-(−)-nicotine (**3c**) and rilmenidine (**3e**), which are positively charged at pH 7.0, explains that they lie on the regression line for the short-chain (p-methylbenzyl)alkylamines **1a–1c**. Indeed, their well-localized charge facilitates their interactions with the liposomes. In contrast, they are not well retained by the IAM phase, as they are quite polar and less hydrophobic than clonidine (**3a**),.

From a structural point of view procaine (**3d**) is comparable to β-blockers and is logically located on the same regression line.

Finally, it can be seen that neutral diazepam (**3f**) and the two N-acidic compounds phenobarbital (**3g**) and phenytoin (**3h**), whose neutral form predominates at pH 7.0, are located on a separate line. We postulate that in contrast to smaller neutral drugs which partition deep in the hydrophobic core of membranes (7), these rather bulky and non-planar com-

pounds fail to reach the hydrophobic core of the IAM phase due to the limited mobility and high order of the immobilized phospholipids.

Relationship between Retention on IAMs and Partitioning in the *n*-Octanol/Water System

The comparison between log $k^{7.0}_{IAMw}$ and log $D^{7.0}_{oct}$ is shown in Figure 5. No correlation was obtained which included all solutes. However, very good linear correlations exist for β-blockers (Eq. 6) and (p-methylbenzyl)alkylamines (Eq. 7):

$$\log D^{7.0}_{oct} = 0.79(\pm 0.11) \cdot \log k^{7.0}_{IAMw} + 1.57(\pm 0.11) \quad (6)$$
$$n = 9;\ r^2 = 0.97;\ s = 0.11;\ F = 266$$

$$\log D^{7.0}_{oct} = 0.69(\pm 0.14) \cdot \log k^{7.0}_{IAMw} + 1.38(\pm 0.18) \quad (7)$$
$$n = 7;\ r^2 = 0.97;\ s = 0.16;\ F = 166$$

The good quality of Equation 7 demonstrates that for the (p-methylbenzyl)alkylamines, the balance between electrostatic and hydrophobic interactions underlying IAM retention is closer to the balance observed in *n*-octanol than to that observed in PhC-liposomes. When comparing partitioning data determined by immobilized liposomes chromatography to published data for liposomes/water, *n*-octanol/water and IAM partitioning, Oesterberg *et al.* (28) also stressed the similarity between IAM capacity factors and log P_{oct}.

The above relationship suggests that hydrophobic interactions govern the partitioning of β-blockers. Kaliszan *et al.* (29) also found good correlations between log $k^{7.0}_{IAMw}$ and log $D^{7.0}_{oct}$ for a series of β-blockers and hydrophobic phenothiazine neuroleptics.

Comparing the relative position of the two series of bases in Figure 5, we postulate that the β-blockers form an additional H-bond with the ester bond in IAMs, thus accounting for the fact that the two regression lines are not exactly superimposable.

Finally, no correlation was obtained for the heterogeneous series of compounds at pH 7.0, and it clearly appears that the predominantly neutral and thus lipophilic drugs diazepam (**3f**), phenytoin (**3h**) and phenobarbital (**3g**), as well as the partly neutral S-(−)-nicotine (**3c**) (see pK_a Table I) lie on a line separated from that of the charged compounds. The difference between partition coefficients in *n*-octanol/water for neutral and ionized solutes (*diff*(log P^{N-I}) seems to be larger than the one observed between the retention of neutral and ionized compounds on IAMs.

Demare *et al.* (30) also observed modest correlation between log $k^{7.0}_{IAMw}$ and log $D^{7.0}_{oct}$ for a heterogeneous series of 13 compounds with log D values ranking from −2.35 to 1.90 (eight acidic ionized antiinflammatory drugs, three protonated basic molecules and two neutral compounds). They concluded that even ionized acidic compounds partition better in IAMs than in *n*-octanol.

The comparison between log $k^{7.0}_{IAMw}$ and log P^{N}_{oct} for the three series of compounds is shown in Figure 6. Including all compounds, a better relationship is observed between $k^{7.0}_{IAMw}$ and P^{N}_{oct} than between $k^{7.0}_{IAMw}$ and $D^{7.0}_{oct}$ (Fig. 5). Indeed, the neutral compound diazepam (**3f**), and the partially neutral drugs phenobarbital (**3g**), phenytoin (**3h**) and S-(−)-nicotine (**3c**) lie in the same range as the ionized solutes.

In contrast, a closer look at Figure 6 shows that the ordinate of the regression line for the β-blockers (Eq. 8) is higher than the one for the (p-methylbenzyl)alkylamines (Eq. 9):

$$\log P^{N}_{oct} = 0.71(\pm 0.12) \cdot \log k^{7.0}_{IAMw} - 0.10(\pm 0.37) \quad (8)$$
$$n = 9;\ r^2 = 0.97;\ s = 0.13;\ F = 193$$

$$\log P^{N}_{oct} = 0.66(\pm 0.13) \cdot \log k^{7.0}_{IAMw} - 0.52(\pm 0.49) \quad (9)$$
$$n = 7;\ r^2 = 0.97;\ s = 0.15;\ F = 170$$

As seen in Figure 5, only a slight difference exists when comparing the partitioning of ionized forms of the two series in both systems. Indeed, the behavior in Figure 6 can be explained by the average *diff*(log P^{N-I}) parameter for the two series (23,24). The *diff*(log P^{N-I}) term for the (p-methylbenzyl)alkylamines is on average about 0.5 units

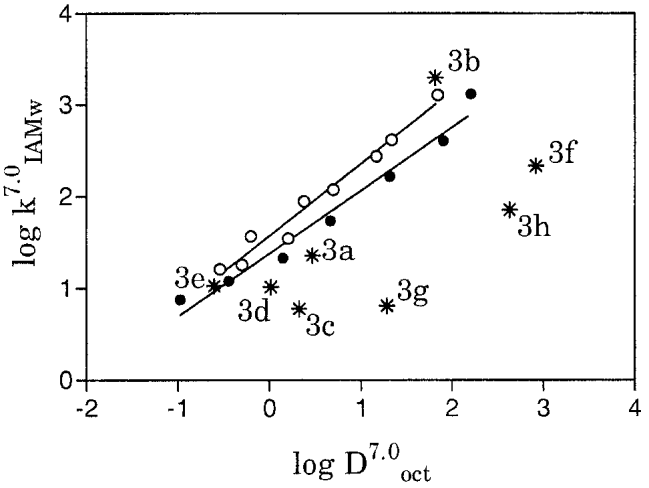

Fig. 5. Comparison between retention on the IAM.PC.DD2 stationary phase and partitioning in the *n*-octanol/water system at pH 7.0. Log $k^{7.0}_{IAMw}$ vs. $D^{7.0}_{oct}$ for the three investigated series. (●): (p-methylbenzyl)alkylamines, (○): β-blockers and (✳): heterogeneous series of ionizable acidic and basic compounds.

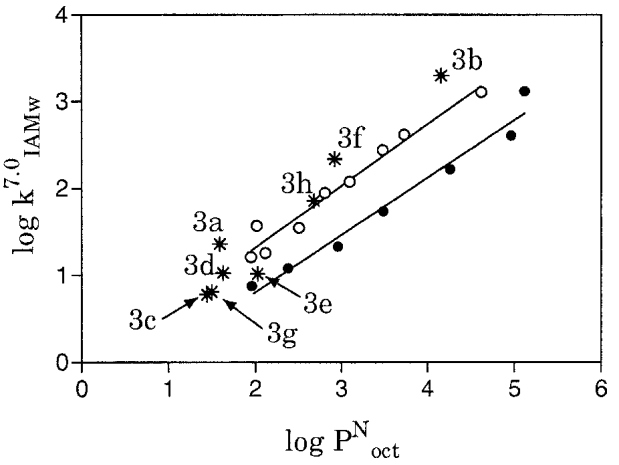

Fig. 6. Comparison between retention on the IAM.PC.DD2 stationary phase at pH 7.0 and partitioning of the neutral forms in the *n*-octanol/water system. Log $k^{7.0}_{IAMw}$ vs. P^{n}_{oct} for the three investigated series. (●): (p-methylbenzyl)alkylamines, (○): β-blockers and (✳): heterogeneous series of ionizable acidic and basic compounds.

higher than for the β-blockers, a difference which leads to a better separation of regression lines in Figure 6 than in Figure 5.

CONCLUSIONS

To assess the influence of molecular size and charge on IAM.PC.DD2 retention compared to partitioning in PhC-liposomes and in *n*-octanol, three series of ionizable compounds were investigated, namely a homologous set of (p-methylbenzyl)alkylamines, a congeneric series of β-blockers, and a heterogeneous series of basic and acidic (N-acidic) drugs.

The results indicate that for series of rather large molecules such as the β-blockers or the long-chain (p-methylbenzyl)alkylamines **1e–1g**, IAM.PC.DD2 retention correlates well with PhC-liposomes partitioning. Hydrophobic recognition forces seem to be mainly responsible for their partitioning in phospholipids. Moreover, for these solutes partition coefficients measured in liposomes/water and IAM capacity factors correlate well with *n*-octanol/water partitioning. However, further analysis shows that the liposomes/water partitioning of β-blockers is increased compared to that of the long-chain (p-methylbenzyl)alkylamines. The formation of a H-bond between the hydroxy group of β-blockers and the ester bond of phospholipids seems likely.

For the more hydrophilic short-chain (p-methylbenzyl)alkylamines **1a–1c**, the balance between electrostatic and hydrophobic interactions is not the same in the two systems. Even if only one type of electrostatic interaction occurs, namely between the protonated amino group and the polar head-groups of phospholipids, their affinity is higher for liposomes than for the IAM phases. On the other hand, IAM.PC.DD2 retention for the entire series **1a–1g** correlates well with *n*-octanol partitioning. Thus, it appears that electrostatic interactions play only a minor role for the IAM retention of these model solutes, presumably due to the smaller density of phospholipids in IAMs compared to liposomes, the lack of lateral and axial mobility in IAM columns and the monolayer nature of IAMs.

Summarizing the above observations, partitioning in immobilized artificial membranes depends on size, hydrophobicity and charge. In contrast, the solute's capacity to form H-bonds, which is important for partitioning in liposomes, plays only a minor role in IAMs. Thus, for a set of structurally unrelated basic and acidic compounds, partitioning in *n*-octanol/water, IAM retention and liposomes/water partitioning are governed by a different balance of intermolecular interactions. The results presented here demonstrate that these three indices of lipophilicity are not exchangeable, especially when hydrophilic compounds are considered. Their relative usefulness to derive adequate structure-pharmacokinetics relationships remains to be established. However, it should be underlined that for hydrophobic congeneric series, IAM retention is a fast promising technique requiring a minimal amount of compound.

ACKNOWLEDGMENTS

P.-A. C. and B. T. are grateful for financial support by the Swiss National Science Foundation. The authors gratefully acknowledge Drs M. Neuwels and A. Ducarme (UCB, Braine l'Alleud, Belgium) for stimulating discussions and exchange of results on the heterogeneous series under study.

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