pH-Metric logP 10. Determination of Liposomal Membrane-Water Partition Coefficients of Ionizable Drugs

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Purpose. To investigate a novel approach for the determination of liposomal membrane-water partition coefficients and lipophilicity profiles of ionizable drugs.

Methods. The measurements were performed by using a pH-metric technique in a system consisting of dioleylphosphatidylcholine (DOPC) unilamellar vesicles in 0.15 M KCl at 25°C. The DOPC unilamellar vesicle suspension was prepared via an extrusion process.

Results. The liposomal membrane-water partition coefficients of eight ionizable drugs: ibuprofen, diclofenac, 5-phenylvaleric acid, warfarin, propranolol, lidocaine, tetracaine and procaine were determined and the values for neutral and ionized species were found to be in the ranges of approximately 4.5 to 2.4 and 2.6 to 0.8 logarithmic units, respectively.

Conclusions. It has been shown that the liposomal membrane-water partition coefficients as derived from the pH-metric technique are consistent with those obtained from alternative methods such as ultrafiltration and dialysis. It was found that in liposome system, partitioning of the ionized species is significant and is influenced by electrostatic interaction with the membranes. We have demonstrated that the pH-metric technique is an efficient and accurate way to determine the liposomal membrane-water partition coefficients of ionizable substances.

KEY WORDS: partition coefficient; liposome; ionizable drug; drug-lipid membrane interaction.

INTRODUCTION

The bio-availability of a drug to the therapeutic target is an important consideration in rational drug design (1). Before the drug elicits an effect, it has to pass through various cellular barriers either by passive diffusion and/or carrier-mediated uptake. Depending on the location of the target site, the pH of the environment may vary considerably. In this context, the affinity of the drug molecule to the lipophilic environment at different pH values has to be quantified for a proper prediction of its ability to interact with the biological target. Traditionally, the octanol-water partition coefficient was used to study the lipophilic character of drug compounds and the correlation of lipophilicity to pharmacodynamics and pharmacokinetics (2–4). In the octanol-water system, the partition of drug molecules in neutral form is relatively favored to the ionized form. In the membrane-water system, it has been found that partitioning of the ionized species is significantly enhanced over that shown in the octanol-water system (5–7). Clearly, the octanol-water system is insufficient to account for certain critical characteristics of biological membranes, which comprise of lipid bilayers consisting of amphipathic groups with strong electrostatic interactions (8).

Liposomes, bilayers in the form of vesicles, have been proposed as a delivery system for water insoluble drugs (9,10), and also have been used as a model membrane system for drug partition studies (5). It has been pointed out that the partition parameters derived from liposome-water systems outperform the octanol-water system for the prediction of biological activities of certain classes of drugs (11). Recently, chromatographic methods using immobilized artificial membrane (IAM) surfaces (12,13), dialysis methods (14–16) and ultrafiltration methods (7,15) have been employed to measure the liposomal membrane-water partition coefficients for compounds of pharmaceutical interest. However, these methods have their own restrictions; for instance, some methods require radiolabeled compounds; the chromatographic approach is laborious to apply over a wide pH range.

In the earlier work of this series, we have developed the pH-metric titration method as a universal technique to determine the partition coefficients of ionizable compounds (17–25). The method is based on a two-phase potentiometric titration approach. Specifically, the substance in aqueous solution is titrated against standard acid or base to deduce the ionization constant (pK_a). Then, the experiment is repeated in the presence of water-saturated octanol (a water-immiscible phase) where partitioning of the substance occurs. The apparent ionization constant obtained from octanol titrations (p_oK_a) may deviate from the aqueous pK_a and this shift in constants is utilized to calculate $\log P_{oct}^{XH}$ (partition coefficient for protonated species) and $\log P_{oct}^{X}$ (partition coefficient for non-protonated species) as below (17,18)

$$P_{oct}^{XH} = \frac{r_2\, 10^{p_oK_a(2)-pK_a} - r_1\, 10^{p_oK_a(1)-pK_a}}{-(r_2 - r_1)\, 10^{p_oK_a(1)+p_oK_a(2)-2pK_a}} \Big/ r_1\, r_2 \left(10^{p_oK_a(1)-pK_a} - 10^{p_oK_a(2)-pK_a}\right)$$

$$P_{oct}^{X} = \frac{r_1\, 10^{p_oK_a(2)-pK_a} - r_2\, 10^{p_oK_a(1)-pK_a} + r_2 - r_1}{r_1\, r_2 \left(10^{p_oK_a(1)-pK_a} - 10^{p_oK_a(2)-pK_a}\right)}$$

where r_1 and r_2 represent the volume ratios of octanol-to-water phase obtained from two independent experiments. It should be noted that for ionizable substances, $\log P_{oct}^{XH}$ and $\log P_{oct}^{X}$ are constants and refer, respectively, to a single protonated and non-protonated species partitioning between two phases. On the other hand, the distribution coefficient, D_{oct}, indicates the apparent partition coefficient of all the protolytic forms of the substance, and is given by the following equation.

$$logD_{oct} = \log\left(P_{oct}^{X} + P_{oct}^{XH}\, 10^{-pH+pK_a}\right) - \log\left(1 + 10^{-pH+pK_a}\right)$$

Obviously, $\log D_{oct}$ can vary as a function of pH. Plots of $\log D_{oct}$ vs. pH are denoted as lipophilicity profiles in this paper.

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To the best of our knowledge, no extensive study has been reported for the use of the pH-metric technique to scrutinize the partition of drug substances into liposomal membranes, though some preliminary results have already been described (24). In the present work, the pH-metric technique is systematically applied to measure the liposomal membrane-water partition coefficients and lipophilicity profiles of eight ionizable drugs, namely, ibuprofen (anti-arthritic), diclofenac (anti-inflammatory agent), 5-phenylvaleric acid, warfarin (anticoagulant), propranolol (beta-blocker), lidocaine (anaesthetic), tetracaine (anaesthetic) and procaine (anaesthetic) in a DOPC liposome/0.15 M KCl system at 25 ± 0.5°C.

As in the octanol-water system, the shifts in ionization constants can be rationalized as the partitioning of drug molecules, either in neutral or ionized forms, to the liposomal layer. In contrast to the octanol-water system, the membrane acts as a polar interface which may function differently from an isotropic fluid (octanol). Here, we use subscripts mem (liposomal membrane) or oct (octanol) to denote the nature of the partitioning medium and superscripts I (ionized) or N (neutral) to indicate the electrical state of the solute. It is envisaged that eqns. 1, 2 and 3 are applicable in the liposomal membrane-water system to deduce the partition coefficient of neutral species ($\log P^N_{mem}$), the partition coefficient of ionized species ($\log P^I_{mem}$) and the distribution coefficient (D_{mem}) since the liposomal phase is electrically neutral in the medium utilized in our experiments (0.15 M KCl, see experimental section). In subsequent discussion, we will demonstrate that the results obtained from these treatments are justified by considering the electrostatic interaction between the charged membrane surface and the solute. Finally, we define

$$\delta_{mem} = \log \frac{P^N_{mem}}{P^I_{mem}}$$

δ_{mem} to symbolize the logarithm of the ratio of P^N_{mem} to P^I_{mem} for the liposome-water system. Note that the larger the δ_{mem} value, the smaller the affinity of ionized species for the liposome phase, relative to that of the neutral-species partitioning.

EXPERIMENTAL

Dioleylphosphatidylcholine (DOPC) of 5 to 400 mg and a weighed amount of drug sample, usually in its most water-soluble form, were used in experiments. Typical DOPC and drug concentrations were at millimolar level. In case of drug samples with high $\log P^N_{mem}$ values, the relative concentration of DOPC must be increased accordingly to ensure the sites for drug partitioning are well in excess. To this end, we have determined, on a trial-and-error basis, the optimal [DOPC]/[drug] ratios for different ranges of $\log P^N_{mem}$ values by varying the concentrations of DOPC and various drugs until the resulting partition parameters remain constant. For the three ranges of $\log P^N_{mem} > 5$, within 3–4, and <3, we found that the [DOPC]/[drug] ratio has to be ≥5, ≥2 and ≥1, respectively.

Large unilamellar vesicle (LUV) suspensions were prepared by using the freeze-and-thaw extrusion technique which has been detailed elsewhere (26). Mixtures of DOPC and drug sample were reconstituted as liposomes in the form of multilamellar vesicles (MLV), by vortexing in 5 ml of 0.15 M KCl solution. The MLV suspension was then subjected to five freeze-and-thaw cycles to ensure the internal trapped volume of the vesicles reached an equilibrated distribution of drug and salt within the suspension. Finally, LUVs were made from the MLV suspension by using an extruder (Lipex Biomembranes, Vancouver, Canada; distributed in Europe by Sirius, East Sussex, England). Mayer et al. showed that freeze-thawing before extrusion gives better results regarding the unilammelarity of the liposomes than extrusion without freeze-thawing (27). In addition, it has been reported that LUVs with trapped volume of about 2.6 μL solute/μmol DOPC and diameter of about 100 nm can be obtained by using this method (26).

pH-Metric measurements were carried out using the PCA101 instrument (Sirius Analytical Instruments, East Sussex, England) (17–25). All titrations were performed in 0.15 M KCl solution under argon atmosphere at 25 ± 0.5°C using standard 0.5 M HCl or 0.5 M KOH solutions. The pH electrode (Orion, Ross™ type, Beverly, MA, US) was calibrated titrimetrically in the pH range of 1.8–12.2 (28). Ionization constants of the drug samples were determined by pH-metric titration. As pointed out by Pauletti and Wunderli-Allenspach (16), a 1 to 5-minute time period is sufficient for the pH-equilibration between the inside and outside of the liposome vesicle. The rate of titrant addition was optimized to make sure this condition was satisfied. In all experiments, the pH change per titrant addition was limited to about 0.2 pH units. Typically, more than 30 pH readings were collected from each titration. The partition parameters were derived by fitting the experimental data to eqns. 1 and 2 using a least-squares procedure as reported previously (17–18). All calculations were performed using *pKaLOGP™ for Windows* program (v4.05c, Sirius Analytical Instruments, East Sussex, England).

DOPC (Avanti, Alabama, US), ibuprofen (Aldrich), diclofenac (Sigma, sodium salt), 5-phenylvaleric acid (Aldrich), warfarin (Sigma), propranolol (Aldrich), lidocaine (Sigma) and tetracaine (Sigma, HCl salt) were used as received. Procaine was a gift from K. Takács-Novák (Semmelweis University of Medicine, Budapest). Solutions were made up of deionized water of resistivity > 10^{14} Ω-cm.

RESULTS AND DISCUSSION

We deliberately selected four acids (ibuprofen, diclofenac, 5-phenylvaleric acid, warfarin) and four bases (propranolol, lidocaine, tetracaine, procaine) to exemplify several classes of commonly used drug compounds. Their structures are depicted in Fig. 1. The criteria used for selection of molecules were firstly that some independently measured $\log P^N_{mem}$ and $\log P^I_{mem}$ values were available for comparison. Secondly, because of their low molecular weight, moderately high solubility and pK_a values between 2 and 10, the molecules selected were likely to produce good-quality titration curves in pH-metric experiments, which was thought to be important for the initial development of the analytical method. The molecules chosen were all more-or-less "cigar-shaped", with the ionization centres close to one end, which probably influences the interpretation of results using "pH-piston hypothesis" as discussed below.

Table I lists the aqueous ionization constants and the partition coefficients of these drugs in octanol-water and liposomal

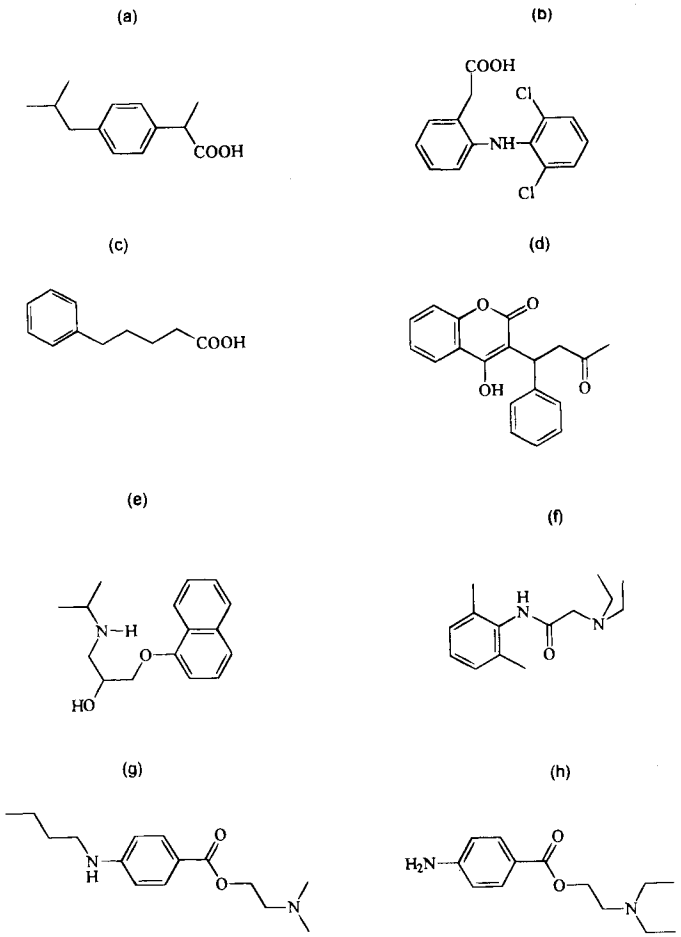

Fig. 1. Structures of the eight ionizable drugs. (a) ibuprofen, (b) diclofenac, (c) 5-phenylvaleric acid, (d) warfarin, (e) propranolol, (f) lidocaine, (g) tetracaine, (h) procaine.

membrane-water systems determined by the pH-metric method. As shown in Table I, the relationships between the $logP^N_{mem}$ and $logP^I_{mem}$ values are consistent with those reported by others (7,16,29). This validates the pH-metric approach. Note that for each compound in this study, $logP^N_{mem}$ and $logP^N_{oct}$ values for unionized species are similar, with $|logP^N_{mem} - logP^N_{oct}|$ varying between 0.03 (propranolol) up to 0.28 (tetracaine). On the other hand, $logP^I_{mem}$ values are in every case considerably higher than $logP^I_{oct}$, with $|logP^I_{mem} - logP^I_{oct}|$ varying between 1.57 (procaine) and 2.61 (5-phenylvaleric acid). Figs. 2a and 2b show, respectively, the lipophilicity profiles of the acidic and basic drugs which are generated using eqn. 3 for octanol-water and liposomal membrane-water systems. As the drugs ionize, the partitioning into lipid membranes is significantly higher than into octanol. This is expected because the ionized species are strongly associated with the membrane by means of electrostatic interactions with the zwitterionic phospholipid (9).

Next, we turn our attention to the influence of the acid/base character on the partitioning of the ionized drug to lipid membrane. This can be visualized by considering δ_{mem}, listed in Table I. It can be seen that for basic drugs, the δ_{mem} values vary from 0.84 to 1.62 while for acidic drugs, the δ_{mem} values change from 1.51 to 2.08. Miyazaki et al. observed similar trends with anaesthetics (analogues of tertiary amine) partitioning into dimyristoylphosphatidylcholine liposomes (6). Note that the average value of δ_{mem} for acidic drugs in this work is 55% higher than that of the basic drugs. This is in marked contrast with the analogous parameter, δ_{oct} ($log(P^N_{oct}/P^I_{oct})$, see Table I), of the octanol-water system, which shows only 30% enhancement for acidic drugs. The fact that δ_{mem} values tend to be higher than δ_{oct} values may be related to the anisotropic nature of the liposomal phase for acidic and basic drugs partitioning. It should be pointed out that the compounds utilized in this work only cover certain classes of drug. More experimental results are required to fully generalize this trend. However, it is clear from our observations that charged forms of amphiphilic acids (anionic) have lesser affinity for phosphatidycholine-based membranes than charged forms of amphiphilic bases (cationic), compared to the uncharged species, respectively. That is, δ_{mem} of bases is typically lesser than that of acids. We invoke the *principle of charge and hydrogen-bond complementarity* to rationalize this.

If an ionizable substance binds nearly equally well in both its uncharged and charged forms, then δ_{mem} will be small. An uncharged amphiphilic species (acid or base) is favorably bound to the membrane if the hydrophobic portion of the substance is buried in the acyl-chain interior of the bilayer while the polar head group stays near the surface of the membrane. Added stabilization is achieved if the drug species is an effec-

Table I. Partition Coefficients and Ionization Constants of the Eight Ionizable Drugs (see Fig. 1) Measured Using the pH-metric Technique (literature values are printed in italics)

		Otanol-water system			Liposomal membrane-water system		
Drug	pK_a	$logP^N_{oct}$	$logP^I_{oct}$	δ^a_{oct}	$logP^N_{mem}$	$logP^I_{mem}$	δ_{mem}
Ibuprofen	4.45 ± 0.04	3.97 ± 0.01	–0.05 ± 0.01	4.02	3.80 ± 0.03	1.81 ± 0.05	1.99
Diclofenac	3.99 ± 0.01	4.51 ± 0.01	0.68 ± 0.02	3.83	4.45 ± 0.02	2.64 ± 0.04	1.81
5-Phenyl valeric acid	4.59 ± 0.02	2.92 ± 0.02	–0.95 ± 0.02	3.87	3.17 ± 0.02 *2.94*[b]	1.66 ± 0.03 *0.50*[b]	1.51
Warfarin	4.90 ± 0.01	3.25 ± 0.01	–0.46 ± 0.02	3.71	3.46 ± 0.01 *3.4*[c]	1.38 ± 0.03 *1.4*[c]	2.08
Propranolol	9.53 ± 0.01	3.48 ± 0.01	0.78 ± 0.02	2.70	3.45 ± 0.01 *3.51*[d] *3.28*[e]	2.61 ± 0.02 *2.56*[d] *2.76*[e]	0.84
Lidocaine	7.96 ± 0.02	2.45 ± 0.02	–0.53 ± 0.36	2.98	2.39 ± 0.02 *2.1*[c] *2.39*[d]	1.22 ± 0.04 *0.9*[c] *1.49*[d]	1.17
Tetracaine	2.39 ± 0.02 8.49 ± 0.01	3.51 ± 0.01	0.22 ± 0.02	3.29	3.23 ± 0.02	2.11 ± 0.03	1.12
Procaine	2.29 ± 0.01 9.04 ± 0.01	2.14 ± 0.01	–0.81 ± 0.03	2.95	2.38 ± 0.02	0.76 ± 0.09	1.62

[a] $\delta_{oct} = \log(P^N_{oct}/P^I_{oct})$
[b] Dimyristoylphosphatidylcholine liposome using ultrafiltration method (7).
[c] Egg phosphatidylcholine liposome using dialysis method at 37°C (30).
[d] Egg phosphatidylcholine liposome using pH-metric method (31).
[e] Egg phosphatidylcholine liposome using dialysis method at 37°C (16).

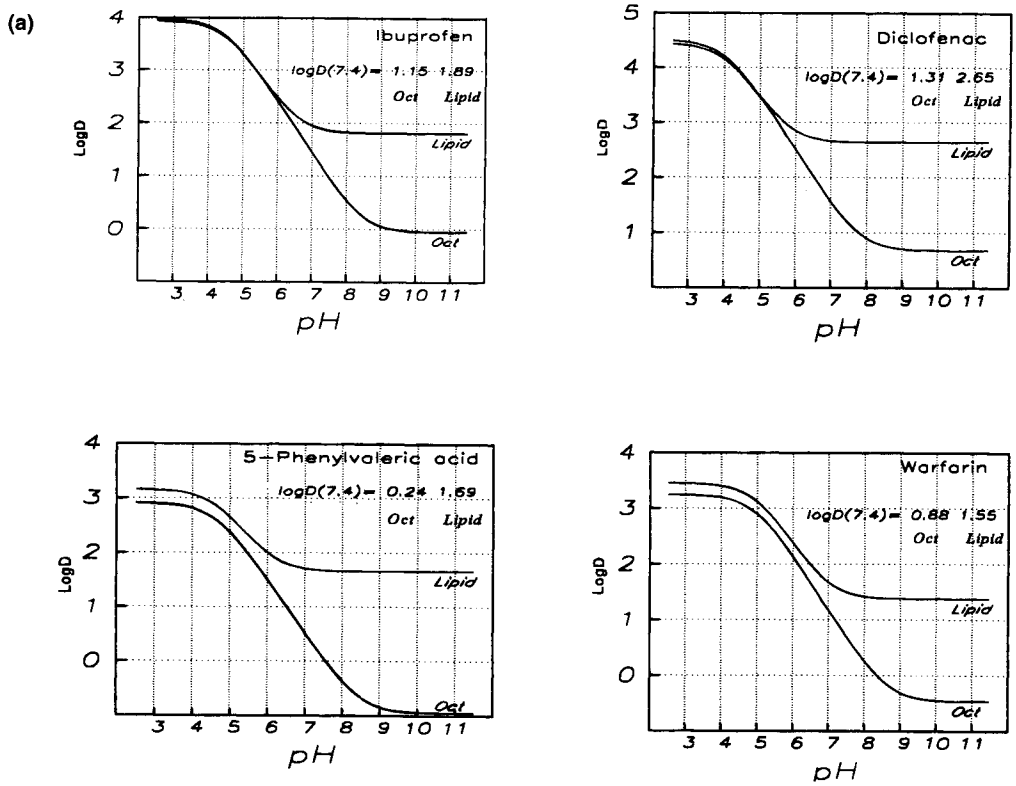

Fig. 2. Lipophilicity profiles of the (a) four acidic drugs and (b) the four basic drugs in octanol-water and liposomal membrane-water systems at 25°C and ionic strength of 0.15 M generated using eqn. 3 with Oct denotes the former and Lipid indicates the later, respectively.

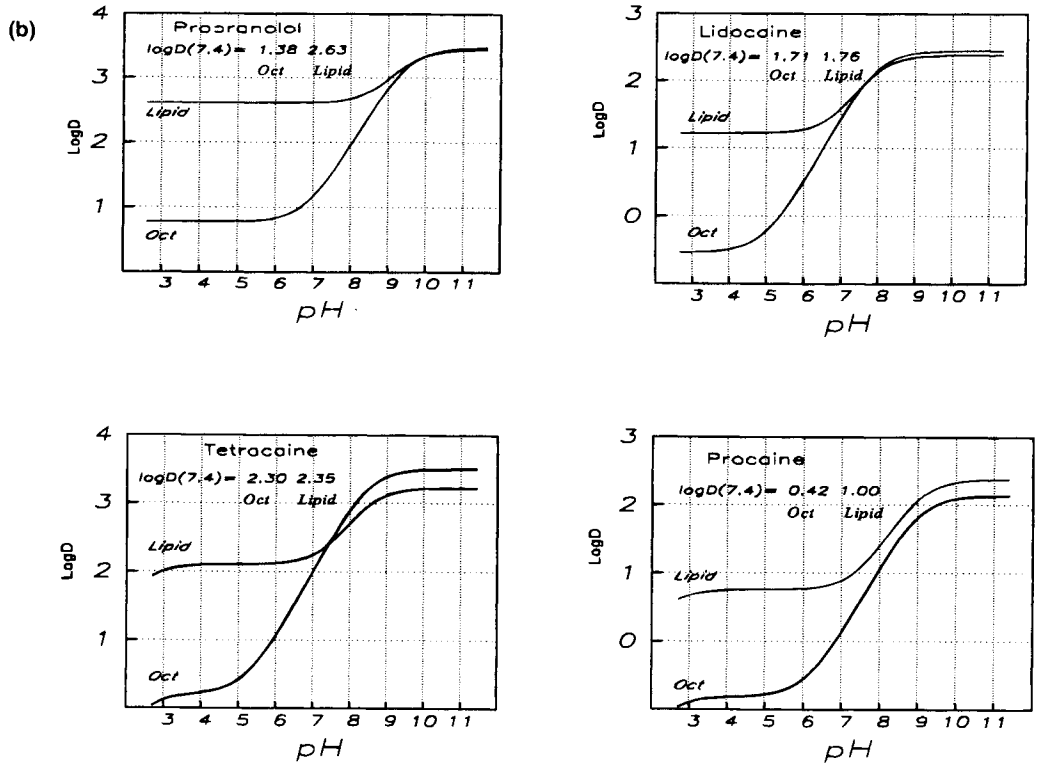

Fig. 2. Continued.

tive H-bond donor, due to interactions with the carbonyl groups, which are H-bond acceptors (Figs. 3a and 3b). If such a bound substance ionizes, then a re-positioning (in the direction of the aqueous exterior) may be required due to the added electrostatic interactions.

The charge distribution in a phosphatidylcholine membrane is anisotropic. As the ionized species moves in the direction of the aqueous exterior, the first charges it experiences in the membrane 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 greater movements for weak acids, compared to weak bases. In the case of ionized weak acids, the movement would expose more of the hydrophobic portion of the molecule to the aqueous exterior, an effect leading to weakened membrane binding. Furthermore, a sodium or potassium ion from the bulk solution would have to move deeper into the membrane to preserve charge neutrality, an effect not energetically favorable. Figs. 3c and 3d illustrate these "re-positionings" with changes in the bulk pH.

Thus ionized bases better complement the charge/H-bond structure of the phosphatidylcholine membranes than ionizable acids. By cycling the bulk pH between a unit above and a unit below the apparent pK_a, it is plausible that simple amphiphilic drugs can be induced to migrate between two types of binding environments, dominated by electrostatic effects in one case and by H-bond donor-receptor interactions in the other case, with energy differences between the two states proportional to δ_{mem}. Boulanger et al. reported similar observations in their deuterium nuclear magnetic resonance studies of local anaesthetics (bases) partitioning to membranes (30). They found that the uncharged forms at high pH were strongly bound to the membrane with the long molecular axes approximately parallel to the fatty acyl-chains (compare Fig. 3a). On the other hand, the charged forms at low pH were discerned to bind closer to the membrane-water interface (compare Fig. 3c). We have informally referred to this pH-positioning as the "pH-Piston Hypothesis". It is envisaged that the membrane acts as a "receptor" with which the drug can interact with different binding sites in a pH-dependent manner. In this simple view bases form better "pH pistons" than acids.

With multi-protic drug molecules or structurally more complicated molecules than those considered here, other models of drug-receptor complementarily could be invoked. Further work on this aspect is being carried out in our laboratory and results will be reported in due course.

CONCLUSIONS

Liposomal membrane-water partition coefficients of eight ionizable drugs were measured by the pH-metric method. It was found that for non-ionized solutes, the distribution coefficients in the octanol-water system are similar to those measured in the lipid membrane-water system, while for the ionized solutes, the latter are higher than the former suggesting ionized species partitioning into the membrane. A "pH piston hypothesis" based on electrostatic interactions is proposed to account for the difference between the partition behavior of ionized basic drugs and ionized acidic drugs. Liposomal membrane-water partition coefficients and lipophilicity profiles for the eight drug compounds were reported and are consistent with lit-

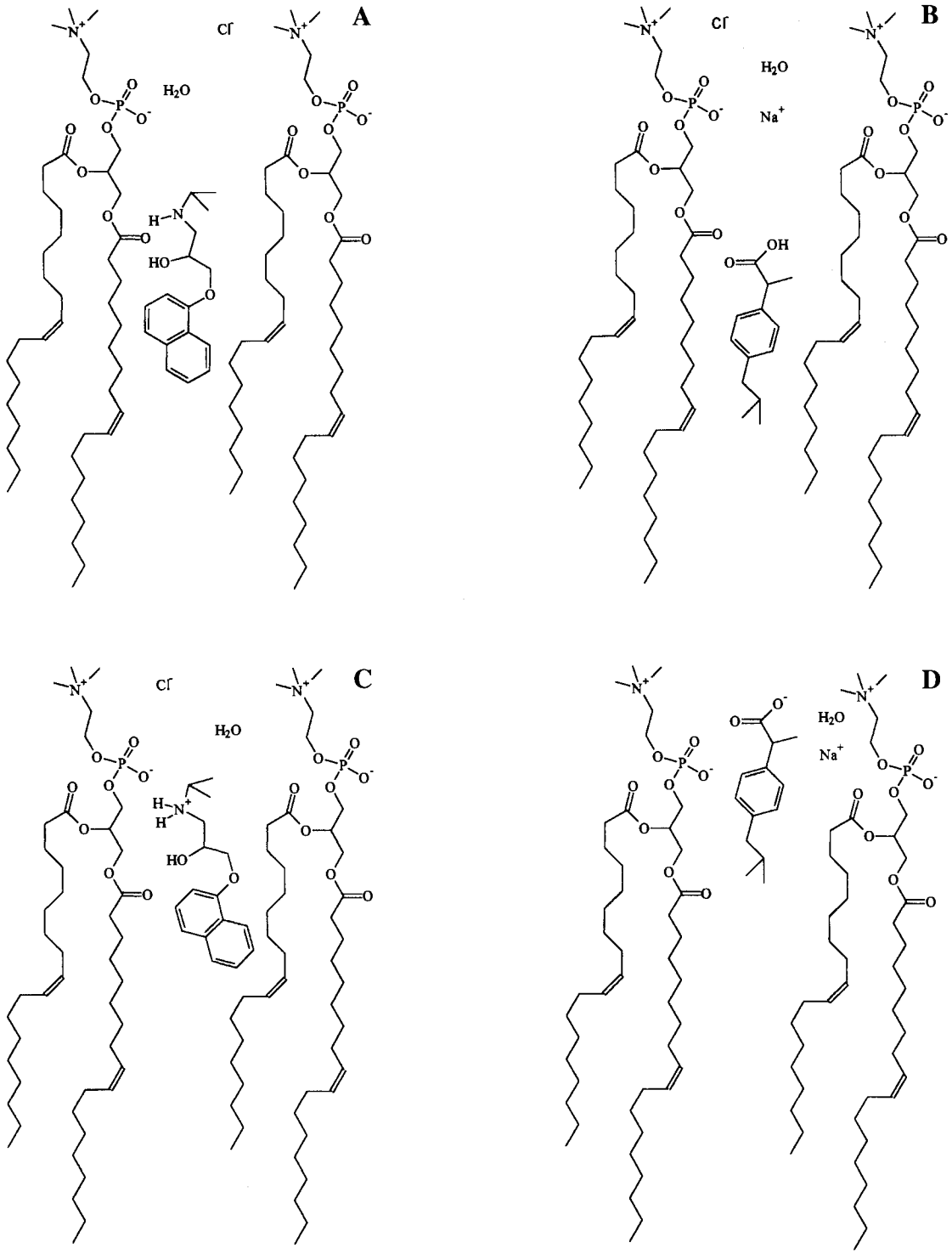

Fig. 3. Schematic of the pH piston hypothesis to show the partitioning of, (a) propranolol at high pH medium, (b) diclofenac at low pH medium (c) propranolol at low pH medium and (d) diclofenac at high pH medium, to DOPC liposomes.

erature when available. It may be concluded that the pH-metric technique is a versatile approach to determine the liposomal membrane-water partition coefficients of ionizable substances.

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