Pharmaceutical Research, Vol. 26, No. 6, June 2009 (© 2009)
DOI: 10.1007/s11095-009-9842-1

Research Paper

Accurate Potentiometric Determination of Lipid Membrane–Water Partition Coefficients and Apparent Dissociation Constants of Ionizable Drugs: Electrostatic Corrections

Mustafa M. A. Elsayed,[1] Ulrich Vierl,[1] and Gregor Cevc[1,2]

Received November 17, 2008; accepted January 26, 2009; published online March 13, 2009

Purpose. Potentiometric lipid membrane–water partition coefficient studies neglect electrostatic interactions to date; this leads to incorrect results. We herein show how to account properly for such interactions in potentiometric data analysis.
Materials and Methods. We conducted potentiometric titration experiments to determine lipid membrane–water partition coefficients of four illustrative drugs, bupivacaine, diclofenac, ketoprofen and terbinafine. We then analyzed the results conventionally and with an improved analytical approach that considers Coulombic electrostatic interactions.
Results. The new analytical approach delivers robust partition coefficient values. In contrast, the conventional data analysis yields apparent partition coefficients of the ionized drug forms that depend on experimental conditions (mainly the lipid-drug ratio and the bulk ionic strength). This is due to changing electrostatic effects originating either from bound drug and/or lipid charges. A membrane comprising 10 mol-% mono-charged molecules in a 150 mM (monovalent) electrolyte solution yields results that differ by a factor of 4 from uncharged membranes results.
Conclusion. Allowance for the Coulombic electrostatic interactions is a prerequisite for accurate and reliable determination of lipid membrane–water partition coefficients of ionizable drugs from potentiometric titration data. The same conclusion applies to all analytical methods involving drug binding to a surface.

KEY WORDS: bupivacaine; diclofenac; drug-binding; ketoprofen; potentiometric titration; terbinafine.

INTRODUCTION

Lipophilicity and ionization are key physicochemical characteristics that control bioavailability and pharmacokinetics of drugs. They often play an important role in the pharmacodynamics of drugs, as well. They are influential in analytical studies, especially in all kinds of reverse-phase chromatography, where they influence drugs distribution in and retention by the matrix.

Traditionally, drug lipophilicity was characterized by studying the partitioning between octanol and water. Expressed in terms of partition coefficient, $P_{o/w}$, such lipophilicity parameter was then correlated with the drugs pharmacokinetics and pharmacodynamics. The octanol-water partition coefficients may be misleading, however. Octanol is a medium with a low dielectric constant, which can only model the hydrophobic molecular interactions. Biological membranes have more complex structure and interact with drugs through more diverse mechanisms, including hydrogen bonding and electrostatic interactions. Octanol thus favors partitioning of the neutral form of ionizable drugs and underestimates partitioning of the ionized drug forms. Phospholipid bilayer membranes, preferably in the form of bio-mimetic phospholipid bilayer vesicles (liposomes), were therefore introduced as a better model than octanol to study biologically relevant drug partitioning (1–4).

Several methods are available to study lipid membrane–water partition coefficients, $P_{mem/w}$. Potentiometric titration (5–7), ultrafiltration (2,4), equilibrium dialysis (8,9), spectroscopy (10–12), isothermal titration calorimetry (13), and immobilized-liposome chromatography (6,14,15) are particularly popular, but other methods can be used as well (16–18).

Due to its experimental simplicity, the potentiometric titration method has gained special interest. The method employs a two-phase titration approach (Fig. 1). First, the test substance is titrated in an aqueous solution against a standard acid or base, to deduce the drug aqueous ionization/dissociation constant, expressed as pK_a. Second, the titration is repeated in presence of a second compartment (e.g. lipid membranes). A minimum of two titrations with different organic-aqueous compartment volumes ratios are needed. They yield apparent dissociation constants, pK_a^{app}, that differ from the aqueous pK_a as a function of the employed organic-aqueous compartment volumes ratio (Figs. 1–2). From the difference $\Delta pK_a = pK_a^{app} - pK_a$ one can calculate the partition coefficient of the neutral, P^N, as well as the ionized, P^I, test substance forms (5,7). Simultaneously, one can also calculate the test substance dissociation constant in the membrane, pK_a^{mem}.

[1] IDEA AG, Frankfurter Ring 193a, 80807 Munich, Germany.
[2] To whom correspondence should be addressed. (e-mail: cevc@idea-ag.de)

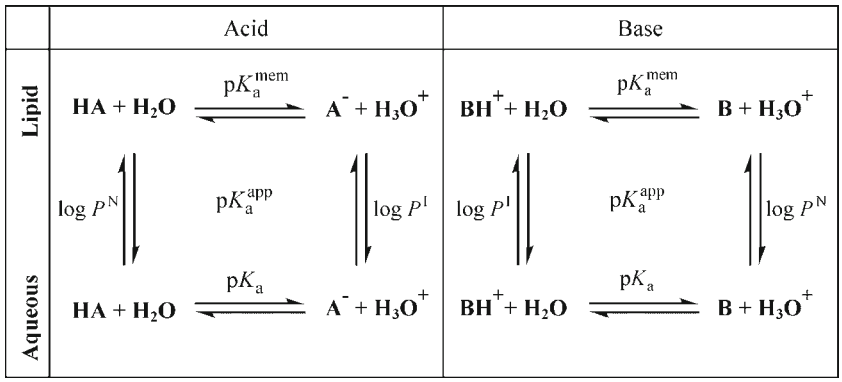


Fig. 1. Schematic illustration of dissociation and partitioning equilibria for acids and bases in a lipid membrane–water system.

The potentiometric titration method has many advantages. It requires no phase separation, a time consuming process that can disturb the equilibrium state of samples. It can be used over a wide range of partition coefficients. It affords complete lipophilicity profiles of ionizable drugs in the partition system, since being a continuous method (as opposed to the point-by-point methods, such as dialysis). Gaining information on the apparent dissociation constant of the drug in the membrane, in addition to the partition coefficients, is attractive as well. The method gained popularity with the introduction of Sirius pH-metric log *P* titration equipment, which includes analytical software (Sirius Analytical Instruments Ltd., UK). It is equally possible, however, to use any common titrator or a simple pH-meter to get the necessary experimental data, if one can write an own analytical program.

Interpretation of potentiometric titration results must be done with utmost prudence. Incorporation of an ionized drug into a neutral phospholipid membrane charges-up the membrane (or modifies the membrane's surface charge density, if the membrane is intrinsically charged). This hinders further drug incorporation and affects the observed drug distribution (4,6,19) and pK_a^{app} . Potentiometrically determined P^I values of different organic acids and bases were thus somewhat smaller than the corresponding values determined with alternative methods (6). The assumption that P^I is constant over the employed range of lipid-aqueous compartment volumes ratios (Eq. 3) is therefore also inevitably inaccurate. As a consequence, the published uncorrected P^I values, derived potentiometrically, are often doubtful. The corresponding P^N values are usually more reliable as they are not directly affected by electrostatic interactions[1], which cause the P^I variability.

The aim of this work was to improve the understanding of potentiometric titrations in complex systems and to progress the potentiometric partition coefficient determination by developing an improved analytical approach. We specifically intended to improve the method reliability by taking Coulombic electrostatic interactions into account. This should deliver intrinsic P_0^N and P_0^I values rather than experiment conditions dependent apparent P^N and P^I values. To the effect, we potentiometrically studied the partitioning of four ionizable drugs, the cationic bupivacaine and terbinafine and the anionic diclofenac and ketoprofen, into neutral, phosphatidylcholine, and/or negatively charged, mixed phosphatidylcholine-phosphatidylglycerol, bilayer membranes.

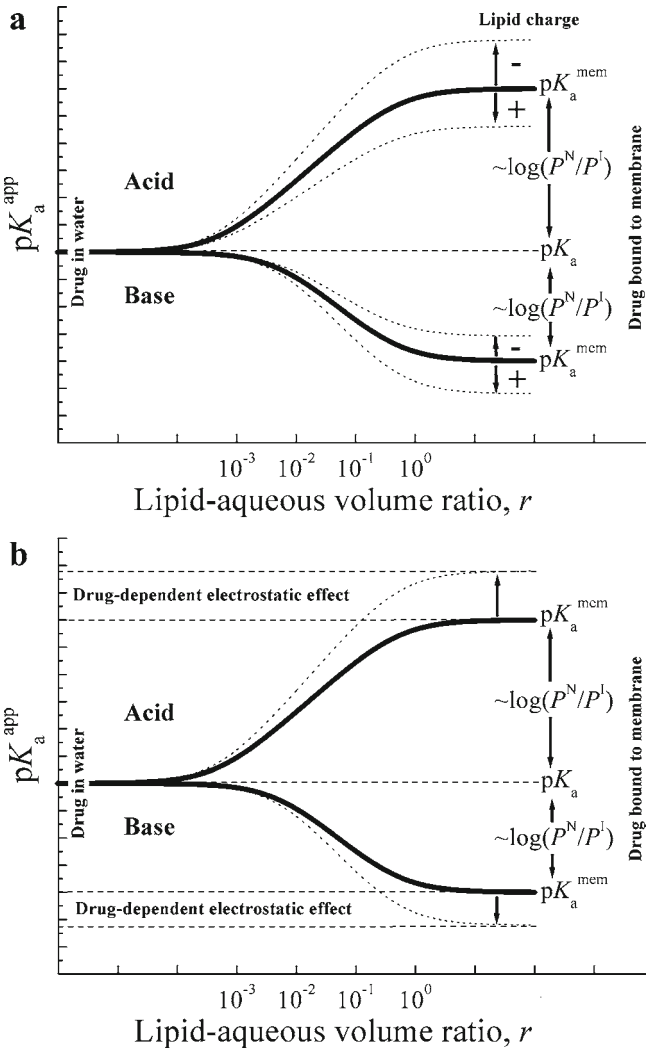


Fig. 2. The apparent dissociation constant, pK_a^{app} , of acidic and basic drugs as a function of the lipid-aqueous compartment volumes ratio, *r*. The practically usable range is $10^{-3}<r<10^{-1}$. **a** Effect of intrinsic membrane charges. **b** Effect of drug-dependent membrane charging.

[1] When P^N is close to P^I, the wrong assumption of a constant P^I can also affect the calculated P^N value, as the two parameters are typically derived simultaneously from the same data set.

MATERIALS AND METHODS

MATERIALS

We obtained soybean phosphatidylcholine (SPC, Lipoid S 100, purity >95%, the assumed average molecular weight ~800 g/mol) from Lipoid GmbH (Ludwigshafen, Germany). The sodium salt of 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG, Na, purity >99.5%, molecular weight= 688.86 g/mol) was from Genzyme Pharmaceuticals (Liestal, Switzerland). Bupivacaine hydrochloride monohydrate was purchased from Heumann PCS GmbH (Feucht, Germany), diclofenac sodium from Fagron GmbH & Co. KG (Barsbüttel, Germany), ketoprofen from Bidachem (Fornovo S. Giovanni, Italy), and terbinafine hydrochloride from Amino Chemicals Ltd. (Marsa, Malta). All were of pharmaceutical quality. All other chemicals and reagents were of analytical grade.

Preparation of the Liposomes

We prepared large unilamellar vesicles (LUV) by extrusion. In brief, we dissolved the selected lipids (SPC for the neutral membranes or SPC/DMPG$^-$ (93.36:6.74 mol/mol) for the negatively charged membranes) in chloroform (SPC) or 3/1 v/v chloroform/methanol mixture (SPC/DMPG$^-$) in a 500 mL round-bottom flask. Vacuum evaporation of solvents at 50°C in a rotary evaporator yielded a thin lipid film, which we hydrated at the same temperature with 150 mM aqueous sodium chloride solution. This produced multilamellar vesicles (MLV) suspension with a total phospholipid concentration of 120 mg/mL. We then prepared LUV suspension by extruding such MLV suspension eight times through 0.1 μm polycarbonate membranes (GE Water & Process Technologies, Trevose, PA, USA) under 1.2 MPa nitrogen gas pressure. The mean diameter of the neutral LUVs was 145 nm and of the charged LUVs 130 nm, as determined by dynamic light scattering (ALV-NIBS/HPPS particle sizer, ALV-Laser Vertriebsgesellschaft mbH, Langen, Germany). The polydispersity index was in either case smaller than 0.1.

Potentiometric Titration Measurements

For samples preparation, we dissolved the drug in aqueous NaCl solution adjusted to pH ~3.5 with HCl (for bupivacaine and terbinafine) or to pH ~9.0 with NaOH (for diclofenac and ketoprofen). We then mixed the drug solution with the LUV suspension and thoroughly stirred the mixture for 15 min. Additional equilibration for up to 3 hours did not affect the results. For diclofenac and ketoprofen, we finally adjusted the mixture pH to ~3.5 with HCl. For each set of experiments, we used samples with at least four different lipid-water volumes ratios (between 0.006 and 0.11, corresponding to SPC concentrations between 7 and 126 mM) and maintained total drug concentration at 5 mM and ionic strength at either 150 mM or 600 mM.

For titrations, we used Mettler DL 67 and DL50 graphix automatic titrators (Mettler-Toledo, Switzerland), equipped with Lab X pro version 2.a instrument controlling software. We conducted all titrations alkalimetrically at 37°C. We adjusted the titrant (0.1 M NaOH) with NaCl to maintain constant ionic strength during titrations. The titrant volume per addition was automatically adjusted within the range of 0.005–0.020 mL to limit the pH change to about 0.04 pH units. The sample was allowed to equilibrate under stirring for 1–10 min after each titration step.

We determined bupivacaine's and ketoprofen's aqueous pK_a by fitting the measured potentiometric titration curves, over the pH range in which the tested drug is soluble, with the conventional titration equations. For diclofenac and terbinafine we used the published aqueous pK_a values of 4.01 and 7.05, respectively (20).

Potentiometric Data Analysis

We programmed a Mathcad calculation sheet (Mathcad version 11.0b, Mathsoft Engineering & Education, Inc., Cambridge, MA, USA) with the set of equations given in "RESULTS AND DISCUSSION" and in APPENDIX. This included expressions for the intrinsic (lipid-dependent) membrane surface charge density, σ_{mem}, the surface charge density due to membrane associated drug (drug-dependent), σ_D, and the resulting membrane electrostatic potential, ψ. We then used the program to extract the test drugs partition coefficients from the measured pH titration data with non-linear regression analysis using Mathcad's equations solving routine (Levenberg–Marquardt algorithm). We checked analytical sensitivity by determining the lower and upper limit values for each parameter that yielded residual sum of squares 10% higher than the optimum parameters values. We confirmed that starting parameters variation over a reasonable range had no effect on the final analytical result. We used the same program to model the theoretical cases illustrated in some of the figures.

RESULTS AND DISCUSSION

Theoretical Considerations

We used the partition coefficient most conventional expression, as the ratio of the molar drug concentration in the membrane, $C_{mem,V}$, and in the aqueous compartment, $C_{aq,V}$:

$$P = \frac{C_{mem,V}}{C_{aq,V}}. \quad (1)$$

The subscript V denotes concentrations based on the lipid, V_{mem}, and the aqueous, V_{aq}, compartments volumes. The often used alternative definition:

$$P = \frac{C_{mem}V_{aq}}{C_{aq}V_{mem}} = \frac{C_{mem}}{C_{aq}r} \quad (2)$$

relies on C_{mem} and C_{aq}, defined analogously but based on the total suspension volumes, with r is the lipid-aqueous compartment volumes ratio. Both definitions presume a large excess of lipid over the lipid-associated drug fraction (limiting case). As is evident from Table I, the numeric partition coefficient values depend on the concentration units used (21).

Introduction of a second compartment (e.g. octanol) influences the dissociation behavior of the tested compound (Fig. 1) and shifts its dissociation constant from pK_a to p$K_a^{app\#}$.

Table I. Partition Coefficients, Expressed in Various Concentration Units and the Corresponding Lipid-Aqueous Compartment Ratios

Partition coefficient, P	Lipid–aqueous compartment ratios, r	
	Volume ratio, r_V	Molar ratio, r_M
Molar concentration, P_M	$= \frac{C_{mem,V}}{C_{aq,V}} = \frac{C_{mem} V_{aq}}{C_{aq} V_{mem}}$	$= \frac{V_{mem}}{V_{aq}} = \frac{LM_{mem}}{(1000\rho_{mem} - LM_{mem})}$
Mole fraction, P_x	$= \frac{C_{mem} W}{C_{aq} L} = P_M \frac{\rho_w M_{mem}}{\rho_{mem} M_w} = P_M \frac{V^0_{mem}}{V^0_w}$ $\approx P_M \cdot 44$ (for $M_{mem} = 800$ g/mol)	$= \frac{L}{W} = r_V \cdot \frac{V^0_w}{V^0_{mem}}$ $\approx r_V/44$ (for $M_{mem} = 800$ g/mol)
Lipid membrane related, P_L	$= \frac{C_{mem}}{C_{aq} L} = P_M \cdot \frac{V^0_{mem}}{V^0_w} \cdot \frac{1}{W}$ $\approx P_M \cdot 0.79$ (for $M_{mem} = 800$ g/mol)	Molar concentration, L $= L = r_V \cdot \frac{V^0_w}{V^0_{mem}} \cdot W$ $\approx r_V \cdot 1.26$ (for $M_{mem} = 800$ g/mol)

$W \approx 55.6$ M is water molar concentration, L lipid molar concentration, ρ_w the density (g mL^{-1}) of water and ρ_{mem} of lipid, M_w the molecular weight (g mol^{-1}) of water and M_{mem} of lipid, $V_w{}^0$ the molar volume (mL mol^{-1}) of water and V^0_{mem} of lipid. In the current study, the partition coefficient expressed using molar concentration units, P_M, and the corresponding lipid–aqueous compartment volumes ratio, r_V, are used

The shift mainly reflects the different local dielectric constants around drug molecules in different compartments. The shift depends also on the organic–aqueous compartment volumes ratio, *r*. One can accordingly deduce the partition coefficient of the protonated drug form, P^{XH}, and of the deprotonated drug form, P^X, from (7,22,23):

$$pK_a^{app\#} = pK_a + \log\left(1 + P^{XH} r^{\#}\right) - \log\left(1 + P^X r^{\#}\right), \quad (3)$$

which can be rewritten simply as:

$$\Delta pK_a^{\#}\left(r^{\#}, P^X, P^{XH}\right) = \log\left(1 + P^{XH} r^{\#}\right) - \log\left(1 + P^X r^{\#}\right). \quad (4)$$

pK_a represents the aqueous dissociation constant and $pK_a^{app\#}$ the apparent dissociation constant determined in the two-compartment mixture with an organic–aqueous compartment volumes ratio $r^{\#}$. Equations 3 and 4 are commonly used for potentiometric determination of partition coefficients. At least two $pK_a^{app\#} - r^{\#}$ data pairs are needed to deduce P^{XH} and P^X.

The problem with Eq. 4 lies on the fact that the dissociation constant shift, ΔpK_a, of a drug molecule bound to a surface (e.g. a lipid membrane or a micelle) is affected by electrostatic and other non-hydrophobic interactions at the drug binding sites, as well (Fig. 2). Equation 4 neglects such interactions and fails to capture the potentially influential Coulombic electrostatic effects. This raises at least two problems. First, protons attraction into vicinity of a negatively charged surface and repulsion from a positively charged surface shifts the interfacial pH down and up, respectively. The interfacial pH experienced by the bound drug molecules consequently differs from the experimentally measured bulk pH ("local pH shift"). Second, the surface electrostatic potential directly influences the ionized drugs distribution, analogous to its effect on the protons distribution.

The local pH shift can be accounted for simply by introducing an electrostatic correction term, based on the Gouy–Chapman approximation, $\Delta pK_a^{el} = \log[\exp(-\Phi)] = -\Phi \log e = -\Phi/2.3$ into Eq. 4, to get:

$$\Delta pK_a^{\#}\left(r^{\#}, P^X, P^{XH}, \Phi\right) = \log\left(1 + P^{XH} r^{\#}\right) - \log\left(1 + P^X r^{\#}\right) - \frac{\Phi}{2.3}. \quad (5)$$

Φ is the normalized dimensionless electrostatic potential (cf. APPENDIX).

The membrane electrostatic potential originates from two sources. The first is the charged lipid membrane components, creating an intrinsic (lipid-dependent) membrane surface charge density, σ_{mem}. One can easily calculate such contribution from the known membrane composition (cf. APPENDIX). The second source of the membrane electrostatic potential is the ionized drug molecules partitioned into the membrane, which yields the drug-dependent surface charge density, σ_D. This density is, in the first approximation, proportional to the number of membrane associated ionized drug molecules per unit area (cf. APPENDIX), as has been also demonstrated directly with zeta potential measurements (24,25).

Despite awareness of the drug-dependent electrostatic potential existence, and recognition of its possible influence on titration curves (3,6,26), most researchers in the field

continue to work with uncorrected potentiometric data. In contrast, it is now customary to include corrections for the drug-dependent electrostatic effects in the analyses of drug–membrane association measurements conducted at a constant pH, where it is admittedly easier to achieve the task. Table II provides a summary of some drug distribution studies (4,19,25,27–30), in which analysts have applied these corrections. The drug-dependent electrostatic contribution results in distribution coefficient values that are sensitive to experimental conditions and can be up to more than 90% below the correct value (cf. Table II).

In the above-mentioned studies, the correction was done simply as an iterative, post hoc correction. More specifically, analysts first determined the apparent drug distribution coefficient. Subsequently, they calculated the drug-dependent electrostatic potential, based on the bound drug concentration, and used the result to correct the originally measured apparent distribution coefficient. Point-by-point methods, during which each experiment is carried out at a constant pH value and a constant lipid–drug ratio, allow such iterative post-correction. Potentiometric studies need more complex correction procedure, however. The reason is that the drug-dependent electrostatic potential changes during each single titration as a function of the pH. Lipid–water volumes ratio varies within each set of experiments, which affects the drug-dependent electrostatic potential as well. One must therefore apply electrostatic correction consistently to all data points.

To cope with the task, we developed an analytical approach that explicitly considers Coulombic interactions and thus provides accurate intrinsic partition coefficient values for the ionized as well as the neutral drug forms independent of experimental conditions. The underlying principle is that the ionized drug binding charges-up the membrane and consequently affects the apparent partition coefficient of the ionized drug. The relationship between the observed apparent partition coefficient, P^{I}, and the real intrinsic partition coefficient, P_0^{I}, is given by the Boltzmann factor:

$$P^{\mathrm{I}} = P_0^{\mathrm{I}} \exp[-\Phi]. \tag{6}$$

The membrane normalized dimensionless electrostatic potential, Φ, or the corresponding electrostatic potential at the drug binding site, $\psi = \Phi k_{\mathrm{B}}T/ze_0$, is a function of the net surface charge density, σ, given by the sum of the lipid-dependent membrane surface charge density, σ_{mem}, and the drug-dependent surface charge density, σ_{D}. Equation 4 therefore has to be replaced with Eq. 7 for the acidic or Eq. 8 for the basic drugs:

$$\Delta \mathrm{p}K_{\mathrm{a}}^{\#} = \log\left(1 + P_0^{\mathrm{XH}} r^{\#}\right) - \log\left\{1 + P_0^{\mathrm{X}} r^{\#} \exp\left[-\Phi\left(\sigma_{\mathrm{mem}} + \sigma_{\mathrm{D}}^{\#}\right)\right]\right\} \quad \text{for an acid} \tag{7}$$

Table II. Effect of the Drug-Dependent Electrostatic Potential on Measured Apparent Lipid Membrane–Water Distribution Coefficient, D, of Selected Drugs

Compound	Analytical technique	Distribution coefficient, D		D/D_0
		Neglecting electrostatics	Considering electrostatics	
Dibucaine (30)	Ultracentrifugation Using α-deuterated POPC vesicles: 0.1 M NaCl, 50 mM buffer, at pH=5.5, 25°C	52.6–259.5 M^{-1} [a,b,f]	660±80 M^{-1} [a,f]	0.080–0.393
Etidocaine (30)	Ultracentrifugation Using α-deuterated POPC vesicles: 0.1 M NaCl, 50 mM buffer, at pH=5.5, 25°C	3.2–7.9 M^{-1} [a,b,f]	11±2 M^{-1} [a,f]	0.290–0.716
Melittin (27)	Ultracentrifugation Using POPC vesicles: 40 mM buffer, at pH 6.8–6.9, 25°C	$(0.4–1.6)\times10^3$ M^{-1} [b,f]	$(2.1\pm0.2)\times10^3$ M^{-1} [f]	0.177–0.779
Flunarizine (25)	Ultracentrifugation Using POPC vesicles: 0.1 M NaCl, 30 mM buffer, at pH=5.0, 20°C	6,885–19,430 M^{-1} [a,b,f]	28,700±3,350 M^{-1} [a,f]	0.240–0.677
Amlodipine (28)	Ultracentrifugation Using POPC vesicles: 0.1 M NaCl, 10 mM buffer, at pH=7.25, 25°C	3,984–10,476 M^{-1} [a,b,f]	15,500±1,000 M^{-1} [a, f]	0.257–0.672
Proxicromil (4)	Ultrafiltration Using DOPC vesicles at pH=7.4, 25°C	1,650–7,172 [a,c,e]	8,154 [a,e]	0.202–0.880
Salmeterol (4)	Ultrafiltration Using DOPC vesicles at pH=7.4, 25°C	3,640–11,674 [c, e]	9,552 [e]	0.381–1.222
Sodium cholate (29)	Isothermal titration calorimetry Using DPPC vesicles: water at pH=7.4, 60°C	7,750 [a,d,g]	6.4×10^6 [a, g]	0.001
Sodium cholate (29)	Isothermal titration calorimetry Using DPPC vesicles: 0.1 M NaCl at pH=7.4, 60°C	2.0×10^5 [a,d,g]	9.8×10^5 [a,g]	0.204

[a] Experiments were carried out at pH values where the compounds are assumed by the authors to be more than 99% ionized (i.e. $D \approx P^I$)
[b] Range of D values measured through different drug concentrations
[c] Range of D values measured through different ionic strengths and drug concentrations
[d] Average partition coefficient through the whole concentration range used
[e] D calculated using molar concentration units
[f] D calculated is lipid membrane related (c.f. Table I)
[g] D calculated using mole fraction concentration units (c.f. Table I)

$$\Delta pK_a^{\#} = \log\{1 + P_0^{XH} r^{\#} \exp[-\Phi(\sigma_{mem} + \sigma_D^{\#})]\} - \log(1 + P_0^{X} r^{\#}) \quad \text{for a base} \tag{8}$$

These equations contain interdependent parameters and should be solved in a self-consistent fashion. The procedure for calculating Φ as a function of σ_{mem} and σ_D, and for solving such problem, is described in the APPENDIX. The dissociation constant shift caused by Coulombic interactions, ΔpK_a^{el}, is positive or negative depending on the net membrane charge sign and the drug charge sign (cf. Fig. 2).

Experimental Results

The aqueous ionization/dissociation constants of bupivacaine and ketoprofen, measured at 37°C, are given in Table III. They agree with the published data (31–33).

For the neutral membranes, simple use of the conventional model (Eq. 4), which neglects electrostatic interactions, leads to apparent partition coefficient values of the ionized drug forms that are typically lower than the correct intrinsic values (Eqs. 7–8). This is due to the drug-dependent electrostatic potential. We found the error to be around 12% (0.05 difference on the log scale) for bupivacaine and ketoprofen and around 19% (0.09 difference on the log scale) for cholate[2], under the experimental conditions used in this work (Table IV).

Partition coefficient of the neutral drug forms should be unaffected by Coulombic interactions. For ketoprofen, with widely different P^N and P^I values, this is true. In contrast, the directly calculated P^N value of bupivacaine, with relatively close P^N and P^I values, is approximately 11% wrong (0.04 difference on the log scale), owing to the error-spillover from the uncorrected, and thus erroneous, P^I.

The problem is even more evident with diclofenac and terbinafine, for which potentiometric measurements deliver no sensible P^N and P^I values without proper correction for the Coulombic interactions. The unusual $pK_a^{app} - r$ behavior illustrated in Fig. 3, which the conventional model completely fails to describe, highlights the quandary. Instead of the common, continuous, and unidirectional pK_a^{app} shift with the lipid-aqueous compartment volumes ratio, r (cf. Fig. 2), the directly measured terbinafine pK_a^{app} initially decreases and then increases with r (cf. Fig. 3). Diclofenac behaves similarly but, being an acid, in the opposite direction: the pK_a^{app} of diclofenac thus initially increases and then decreases with r. We can explain this behavior by separating the non-Coulombic (due to the relatively low local dielectric constant around bound drug molecules) and Coulombic contributions to the net ΔpK_a^{app} (Fig. 3 insets). The progressive partitioning into lipid bilayers, driven by the drug lipophilicity, imparts high surface charge density to the originally neutral lipid membranes. At low lipid concentration, the strong charge-charge repulsion, expressed as the initial high ΔpK_a^{el}, causes the initial pK_a^{app} extra-decrease for terbinafine and extra-increase for diclofenac. Raising relative lipid concentration lowers the drug-dependent surface charge density and thus ΔpK_a^{el}, causing net pK_a^{app} increase for terbinafine and decrease for diclofenac. Only the analytical approach proposed herein (Eqs. 7 and 8), which considers the Coulombic electrostatic interactions, successfully describes this behavior (cf. Fig. 3). We calculated the pK_a^{app} as a function of r and the intrinsic P_0^I from Eqs. 7 and 8, considering Coulombic interactions. We conclude that any drug with a log P_0^I >2, will exhibit such non-ideal $pK_a^{app} - r$ dependency, even if drug concentration is as low as 2 mM (for $0.006<r<0.11$). We also expect similar behavior for drugs with lower P_0^I, if higher drug concentrations are used.

Table III. The Aqueous Dissociation Constants (pK_a) of Tested Drugs at 37°C

	Ionic strength (mM)	pK_a
Bupivacaine	150	8.02±0.01
	600	8.10±0.00
Ketoprofen	150	4.01±0.02
	600	3.88±0.03
Terbinafine (20)	150	7.05
Diclofenac (20)	150	4.01
Cholic acid[a]	150	4.80±0.00

[a] Data from another study (not published yet) conducted in our laboratory

Ionic strength influences the apparent P^I values; comparison of data measured for bupivacaine and ketoprofen in 150 and 600 mM electrolyte solutions shows that. We generally find that the apparent P^I values are higher in more concentrated electrolyte solutions[3] (cf. Table IV), arguably due to decreasing the surface electrostatic potential with increasing ionic strength (electrostatic repulsion shielding caused by the electrolyte counter-ions, see APPENDIX). Ionic strength therefore affects relatively little the potentiometrically derived partition coefficient values corrected for Coulombic interactions. It is noteworthy that simple adjustment of the test medium ionic strength to biological salt concentrations does not ensure getting a biologically relevant partition coefficient. One must in any case correct for the drug-dependent membrane charging effects. The ionic strength effect, which is small in the current study for bupivacaine and ketoprofen, depends on the drug pK_a^{app} and the lipid-drug molar ratio (up to 27 in the current work). The effect is greater for drugs with higher P_0^I, for experiments conducted with lower lipid-drug molar ratios, or for experiments conducted in more dilute electrolyte solutions.

For terbinafine titrated in 600 mM electrolyte solution inclusion of the correction for Coulombic interactions was insufficient for a sensitive derivation of P_0^N and P_0^I values; we could only determine the lower limit of each parameter under such experimental conditions (Table IV). To get more meaningful data, experiments would have to be done with very low r values, i.e. in the steep part of the $pK_a^{app} - r$ curve (cf. Fig. 3), which is practically precluded by possible

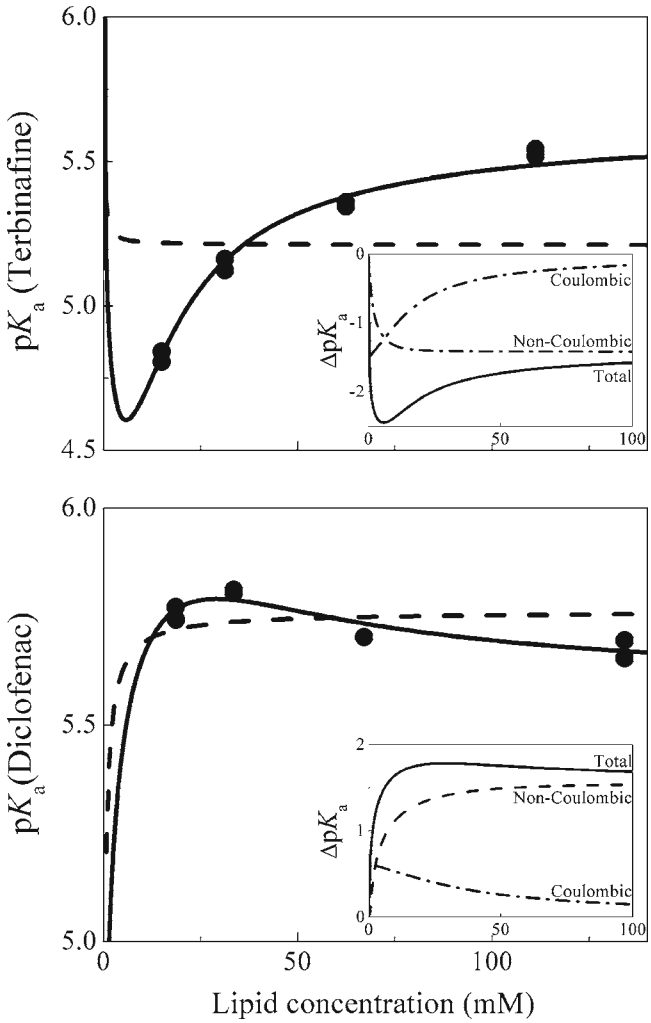
[2] Data from another study (not published yet) conducted in our laboratory.

[3] This is also evident for terbinafine, although the conventional model, neglecting electrostatic interactions, could not be used to obtain P^I values. The pK_a^{app} values at the same lipid concentrations were higher at higher bulk ionic strength, which indicates higher apparent P^I (data not shown).

Table IV. The Lipid Membrane–Water Partition Coefficients of Tested Drugs at 37°C

	Ionic strength (mM)	Lipid	Neglecting electrostatics		Considering electrostatics	
			log P^N	log P^I	log P_0^N	log P_0^I
Bupivacaine	150	SPC	2.715 (2.693–2.738)	1.483 (1.443–1.522)	2.671 (2.657–2.684)	1.534 (1.505–1.562)
	600	SPC	2.846 (2.826–2.866)	1.688 (1.656–1.721)	2.787 (2.770–2.803)	1.680 (1.649–1.711)
	150	SPC/PG	2.715 [a]	1.710 (1.697–1.723)	2.671 [a]	1.534[b] [1.377 (1.355–1.398)] [d]
Ketoprofen	150	SPC	3.284 (3.268–3.300)	0.940 (0.890–0.987)	3.280 (3.266–3.294)	0.997 (0.948–1.043)
	600	SPC	3.410 (3.390–3.431)	0.994 (0.932–1.052)	3.407 (3.388–3.427)	1.023 (0.963–1.081)
	150	SPC/PG	3.317 (3.304–3.330)	0.788 (0.737–0.836)	3.316 (3.304–3.328)	1.010[c] [1.247 (1.196–1.295)] [d]
Terbinafine	150	SPC	–[e]	–[e]	5.194 (5.122–5.398)	3.775 (3.697–3.989)
	600	SPC	–[e]	–[e]	5.298 (4.996–∞)	3.917 (3.610–∞)
Diclofenac	150	SPC	–[e]	–[e]	3.924 (3.902–3.946)	2.356 (2.326–2.387)
Cholic acid [f]	150	SPC	3.902 (3.781–4.050)	1.479 (1.260–1.708)	3.876 (3.784–3.973)	1.573 (1.385–1.747)

Values between parentheses are the lower and upper limits of each parameter
[a] Fixed to the value observed for neutral membrane (SPC) at 150 mM ionic strength
[b] Using intercharge distance=0.228 nm
[c] Using intercharge distance=0.461 nm
[d] Without allowing for separation between intrinsic membrane charges and drug-dependent charges
[e] The conventional analytical approach, neglecting electrostatic interactions, fails to describe terbinafine and diclofenac titration data (cf. Fig. 3) and delivers no sensible partition coefficient values
[f] Calculating using data from another study (not published yet, r=0.006-0.11, total cholate concentration=5 mM) conducted in our laboratory, presuming that the herein proposed model is applicable to cholate-membrane interaction



membrane saturation with the drug. The problem is less evident in 150 mM electrolyte, where stronger Coulombic effects shift the steep part of the $pK_a^{app} - r$ curve towards the higher, and thus experimentally accessible, r values (cf. Fig. 3).

To further explore the drug-dependent electrostatic effects, we calculated the error resulting from neglect of Coulombic interactions (Figs. 4 and 5). Avdeef *et al.* (5) suggested that drugs with high P^N require use of high lipid-drug molar ratios to ensure an excess of the available drug partitioning sites. They suggested the optimum lipid-drug molar ratios to be ≥5, ≥2, and ≥1 for drugs with log P^N>5, within 3–4, and <3, respectively (5). Such recommendation considers the possible membrane saturation with the drug neutral form but neglects the equally possible "electrostatic saturation" due to the drug-dependent membrane charging. We addressed this latter problem by calculating the error (expressed as the ratio of the ionized drug form apparent and intrinsic partition coefficients, P^I/P_0^I), resulting from neglect of Coulombic interactions, as a function of the intrinsic ionized drug form partition coefficient and the selected lipid–drug molar ratio, at 150 mM ionic strength and 37°C (Fig. 4). The difference between the correct intrinsic P_0^I and the apparent P^I can be quite large. Fig. 4 implies that the selected lipid–drug molar ratio should be >31 for drugs with log P_0^I =1 and >63 for drugs with log P_0^I =2 to keep the error in the derived P^I below 10%. Using such a high lipid-drug molar ratio is impractical, however, as it precludes accurate potentiometric measurements. Moreover, even if a high lipid–drug molar ratio is used (by using

◀**Fig. 3.** Terbinafine and diclofenac apparent dissociation constant, pK_a^{app}, as a function of the lipid–aqueous compartment volumes ratio, r, at 37°C and 150 mM bulk ionic strength. The *symbols* show results of individual potentiometric titration experiments. The *dashed curve* describes the best fitting results using conventional data analysis that neglects electrostatic interactions (Eq. 4). The *thick curve* describes the best fitting results using the proposed analytical approach (Eqs. 7 and 8). Inset: The Coulombic and non-Coulombic contributions to the overall dissociation constant shift.

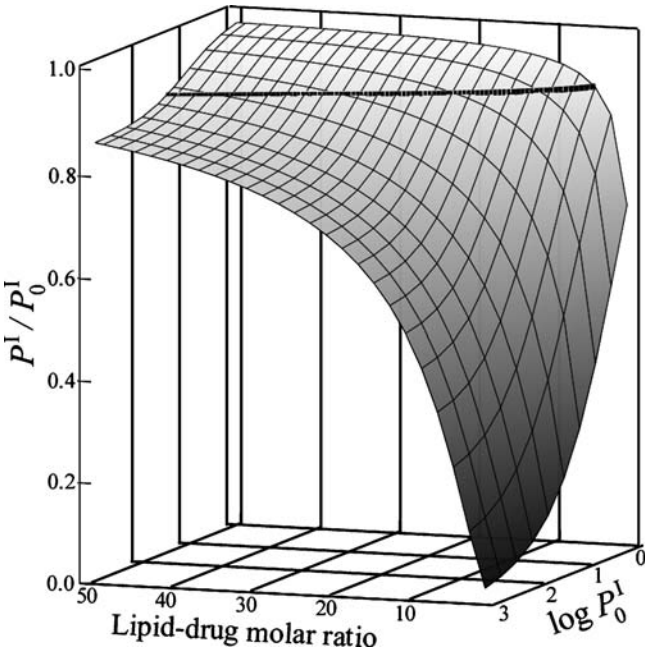


Fig. 4. The effect of the drug-dependent Coulombic electrostatic interactions on the ionized drug form partitioning as a function of the lipid-drug molar ratio and the intrinsic partition coefficient of the ionized drug form, expressed as log P_0^I. The *figure* gives the calculated error, expressed as the ratio of the ionized drug form apparent and intrinsic partition coefficients, P^I/P_0^I, at 150 mM bulk ionic strength and 37°C. The *thick curve* shows the 0.9 limit, i.e. the 10% error boundary.

a very large sample volume containing a considerable drug amount while maintaining a high lipid–drug molar ratio), the electrostatic correction is a prerequisite for analytical accuracy. Increasing the bulk ionic strength diminishes this need and reduces the error (cf. Fig. 5), but the error still exists even in a 1 M solution at lipid–drug molar ratio of 5. Electrostatic interactions must therefore be properly allowed for to ensure accurate potentiometric data analysis.

We collected complementary evidence by studying test drugs partitioning into anionic liposomes prepared from a SPC/DMPG$^-$ 93.36:6.74 mol/mol mixture. We expected more binding of the cationic bupivacaine and less binding of the anionic ketoprofen to such negatively charged membranes surfaces; the directly measured ΔpK_a^{app} values should be accordingly lower for bupivacaine and higher for ketoprofen relative to the neutral membranes (cf. Fig. 2). Fig. 6 shows that this was indeed the case. Similar results were reported for tetracaine, where increasing the percentage of anionic lipids in bilayer membranes from 0% to 30% lowered ΔpK_a^{app} from 0.43 to 0.24 pH units (34). This confirms the membrane electrostatics effect on drug partitioning.

Simple allowance for Coulombic interactions expressed through bilayer "surface potential" is reasonably accurate for the intrinsically neutral membranes, where $\Phi=\Phi(\sigma_D)$. The surface potential $\Phi=\Phi(\sigma_D+\sigma_{mem})$, calculated from the known negatively charged lipids concentration in the membrane, is often too high, however. It thus gives too low intrinsic P_0^I values for bupivacaine and too high such values for ketoprofen in the negatively charged membranes relative to the corresponding neutral membranes values (Table IV). Two most likely explanations are: (1) the charged drug molecules bind at some distance from lipid charges, which lowers the effective electrostatic potential experienced by the former and diminishes discrepancy between P^I and P_0^I (cf. Eq. 19); (2) the assumed interfacial dielectric constant is too low. Assuming the highest possible interfacial dielectric constant (ε_r=78 for water) was not enough to correct the results, suggesting that the first explanation is more likely.

To test the hypothesis, we reanalyzed the data allowing for a finite distance between the charges on DMPG$^-$ and on the bound drug molecules. We derived the effective electrostatic potential experienced by the drug molecules at their binding sites from the P_0^I values pertaining to the neutral membranes. This gave −16.6 mV for bupivacaine and −10.9 mV for ketoprofen. For comparison, the nominal surface potential calculated based on the known membrane composition is −25.8 mV. To experience such potential values, the distance between DMPG$^-$ charges and bound drug charges should be 0.228 nm for bupivacaine and 0.461 nm for ketoprofen. Other factors, such as membrane hydration, could influence the calculated distance, but would not change the fundamental conclusion that intrinsic membrane and drug charges are dislocated. The calculated distances thus give an estimate of the relative drug positions in the membrane.

Biological membranes bear a net negative surface charge (3,35) that affects drug partitioning (36,37). For example, the negative surface charges on intestinal brush border membranes (35,37) reportedly affect partitioning of different anionic and cationic drugs (37). To deduce biologically relevant P^I values, one can take various approaches. The first is to use artificial membranes with a surface charge density similar to the biological membranes of interest. One can then use the analytical approach proposed in this publication to correct only for the drug-dependent electrostatic effects. The second possible approach is to use neutral membranes and then employ the analytical approach

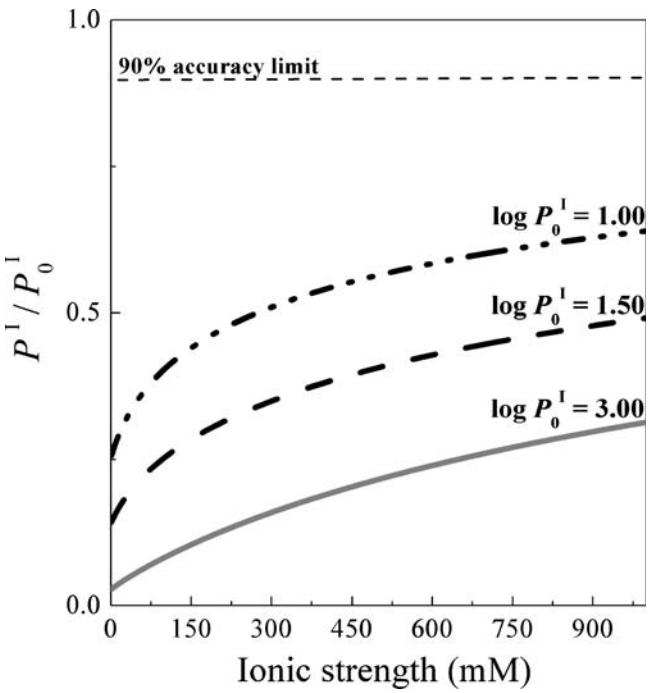


Fig. 5. The effect of the bulk ionic strength on the ionized drug form apparent partition coefficient, P^I, modeled for a lipid–drug molar ratio of 5 at 37°C.

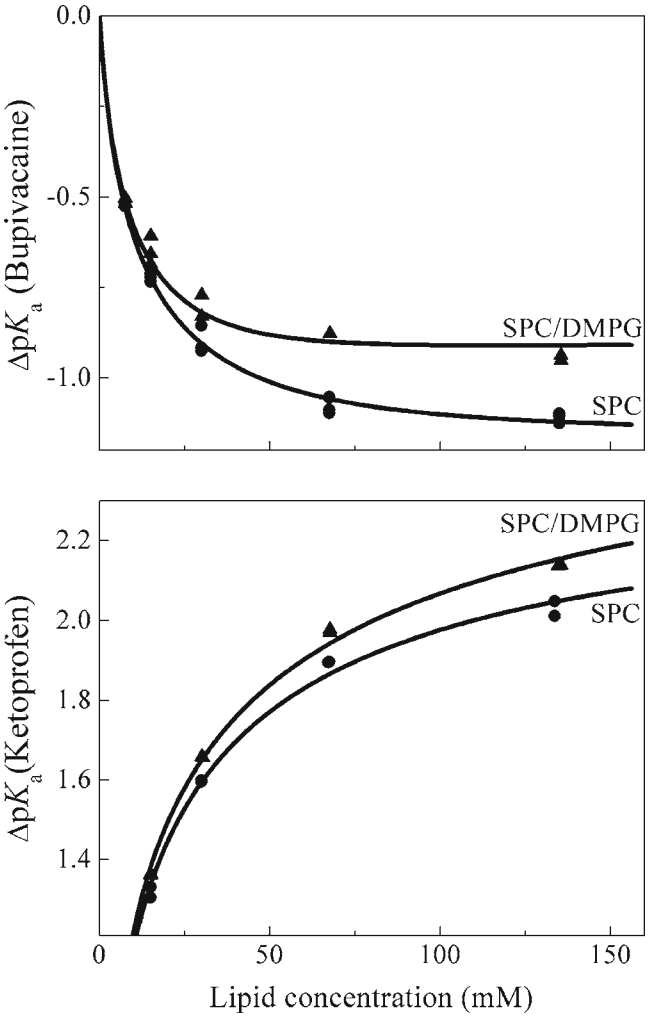


Fig. 6. The effect of membrane charges on the apparent dissociation constant, pK_a^{app}, of bupivacaine and ketoprofen at 37°C. The *symbols* show results of individual potentiometric titration experiments and the *curve* describe the best fitting results (Eqs. 7 and 8).

proposed in this article to correct for drug-dependent electrostatic effects. As a side benefit one then gets the correct intrinsic P_0^I value, from which one can finally calculate the apparent partition coefficient in the biological membrane of interest (cf. Eq. 6).

Experimental Recommendations

An earlier suggestion (7) is to cover experimentally a range of lipid-water compartment volumes ratios starting between the inverse partition coefficients values, i.e. $1/P^N < r < 1/P^I$, and ending at ratios $>1/P^I$, for optimum data fitting and partition coefficients values resolution; this covers the steep part of the pK_a^{app} –r curve shown in Fig. 2. We advocate testing the range of $1/P^N < r <$ approx. $1.5/P^I$, and to preselect the most useful specific r values, such that ultimately should deliver quasi-equidistant pK_a^{app} values; the proposed analytical model, used in predictive fashion, is helpful to select such r values. We furthermore recommend carrying out experiments with at least four different lipid-water compartment volumes ratios, and suggest repeating each measurement independently at least twice. For drugs with a relatively small $\log P^N - \log P^I$ difference, more r values may be needed to resolve P_0^N and P_0^I values. We counsel against using the method for drugs with too similar partition coefficients of the charged and uncharged drug forms (when $\log P^N - \log P^I < 0.5$).

We also remind that experimental range at and nearly above $r = 1/P^N$ is practically inaccessible for the lipophilic drugs. Especially the drugs with a high P^N as well as P^I values have often the optimum $1/P^I$ and $1/P^N$ values below the lowest practically achievable r unburdened with membrane saturation. The Coulombic repulsion may help testing such drugs, as it reduces the apparent P^I value and thus widens the $1/P^N - 1/P^I$ range. To enhance the effect, one could try to increase the drug concentration, but this can provoke membrane saturation with the tested substance and ultimately fails (increasing the drug concentration raises the lowest practically achievable r unburdened with membrane saturation). A better option is to decrease ionic strength of the suspending electrolyte, which is normally feasible. In the current study we were, indeed, able to sensitively determine lipid membrane–water partition coefficients of the highly lipophilic terbinafine from experimental data with 150 mM but not with 600 mM electrolyte (cf. Table IV).

The 'electrostatically enhanced' potentiometric method, which we are describing, is really useful—even at intermediate ionic strengths (~0.1 M)—just for the drugs with $\log P_0^I < 4$ and not higher; for the drugs with $\log P_0^I > 4$ the method can only afford the lower limit for each partition coefficient. We recommend using the bulk ionic strength of 150 mM, which is quasi-physiological and resolution enhancing, for the typical potentiometric partition coefficient measurements.

In general, the tested drug concentration should be high enough to ensure accurate potentiometric results. One should in any case adjust ionic strength of the titrant solution to that of the sample, to avoid ionic strength changes during the titration experiment, and keep temperature constant. The same ionic strength and temperature should be used with the simple aqueous (for aqueous pK_a determination) and the bi-compartment aqueous/lipid mixed systems.

We accept aqueous pK_a literature values only if they were published with sufficient experimental detail and reliable information on the used ionic strength and temperature. We prefer doing alkalimetric titrations, which are relatively more reliable, as they minimize the problem of CO_2 absorption (when starting an experiment at high pH); working under an inert gas atmosphere further reduces the problem.

CONCLUSIONS

We proposed and explained a new analytical approach suitable for analyzing results of potentiometric measurements. The model delivers accurate and reliable lipid membrane–water partition coefficients for the ionized and the neutral drug forms. In contrast to the conventional analytical approach, the new approach is essentially insensitive to experimental conditions, as it properly considers the Coulombic electrostatic interactions, which is responsible for ionic strength and surface charge density effects.

If one corrects experimental results for the drug-dependent electrostatic contributions, one can deal successfully with both neutral and charged lipid membranes, to which the

tested drugs are binding. The improved analytical procedure thus lends itself to applications beyond the potentiometric determination of partition coefficients, especially in bio-analytics, and in studies with bio-mimetic systems, having a composition similar to biologically interesting membranes.

APPENDIX

The intrinsic (lipid-dependent) membrane surface charge density, σ_{mem}, is calculated from membrane composition:

$$\sigma_{mem} = z_L e_0 \cdot \frac{x_C}{(1-x_C)\cdot A_N + x_C \cdot A_C} \approx \frac{z_L e_0 x_C}{A_L}, \quad (9)$$

where z_L is the charged lipid valence, e_0 the elementary electric charge, x_C the molar fraction of the charged lipids with molecular area A_C, and A_N the average surface area of the neutral lipid molecules. In the current study, we used $A_N \approx A_C \approx 0.65\ nm^2$, which is a good approximation for typical fluid-phase phospholipids. During potentiometric titration, the relative proportion of the charged lipids may vary, if the employed pH range overlaps with the lipid titration range. If so, the resulting surface charge density variation must be considered.

The average drug-dependent membrane surface charge density, σ_D, is calculated analogously:

$$\sigma_D = z_D e_0 \frac{C^I_{mem}}{C_L A_L + (C^I_{mem} + C^N_{mem}) A_D} \approx z_D e_0 \frac{C^I_{mem}}{C_L A_L}. \quad (10)$$

z_D is the bound drug valence, C_L the membrane–forming lipid concentration, C^I_{mem} and C^N_{mem} the concentrations of the membrane associated ionized and neutral drug forms, respectively. The contribution of the membrane associated drug molecules to the lipid bilayer surface area, A_D, is normally relatively small. It can thus be neglected. We can now calculate σ_D once C^I_{mem} is known.

To calculate C^I_{mem}, we will start with the total drug concentration $C_{tot} = C_{mem} + C_{aq}$. Substitution of C_{aq} from Eq. 2 and rearrangement allow calculation of the total membrane bound drug concentration:

$$C_{mem} = C_{tot} \frac{Pr}{1+Pr}. \quad (11)$$

The ionized drug fraction, α, is:

$$\alpha = \begin{cases} \dfrac{10^{pH-pK_a^{app}}}{10^{pH-pK_a^{app}}+1} & \text{for an acid} \\ \dfrac{10^{pK_a^{app}-pH}}{10^{pK_a^{app}-pH}+1} & \text{for a base} \end{cases}. \quad (12)$$

Combining Eqs. 6, 11, and 12 yields:

$$C^N_{mem} = C_{tot} \cdot (1-\alpha) \cdot \frac{P^N_0 r}{1+P^N_0 r} \quad (13)$$

$$C^I_{mem} = C_{tot}\alpha \frac{P^I_0 r \exp[-\Phi(\sigma_{mem}+\sigma_D)]}{1+P^I_0 r \exp[-\Phi(\sigma_{mem}+\sigma_D)]}, \quad (14)$$

which takes into account the Coulombic electrostatic contributions from both σ_D and σ_{mem}. The superscript *I* denotes the deprotonated form, X, for an acidic drug and the protonated form, XH, for a basic drug (cf. Eqs. 7 and 8). All concentrations are defined relative to the total suspension volume. Combining Eqs. 10 and 14 finally yields the drug-dependent surface charge density:

$$\sigma_D \approx \frac{z_D e_0 C_{tot}\alpha}{C_L A_L} \cdot \frac{P^I_0 r \exp[-\Phi(\sigma_{mem}+\sigma_D)]}{1+P^I_0 r \exp[-\Phi(\sigma_{mem}+\sigma_D)]}. \quad (15)$$

The procedure is applicable at any pH value and ideally should involve the entire titration curve. σ_D is a function of Φ (or ψ) and vice versa, i.e. they are interdependent. The equation must thus be solved in a self-consistent fashion, and typically numerically. (We used Mathcad employing Secant and Mueller method for numerical solving.)

The Debye ion screening length, λ_D, is a property of the electrolyte solution and is given for 1:1 electrolytes by:

$$\lambda_D = \sqrt{\frac{\varepsilon_0 \varepsilon_r k_B T}{2000 e_0{}^2 N_A C_{el}}}. \quad (16)$$

ε_0 is the permittivity of free space (8.8542×10^{-12} As/Vm), ε_r the dielectric constant at the drug binding site (an average value of 40 for the lipid head group was used), k_B the Boltzmann constant (1.38×10^{-23} JK^{-1}), T the absolute temperature, e_0 the elementary electric charge (1.602×10^{-19} C), N_A Avogadro's number (6.02205×10^{23} mol^{-1}), C_{el} the bulk molar electrolyte concentration.

The electrostatic potential, ψ, of a uniformly charged surface in contact with a 1:1 electrolyte is given within the framework of Gouy–Chapman approximation (38), as a function of the total surface charge density, $\sigma = \sigma_D + \sigma_{mem}$, by:

$$\psi(\sigma) = \frac{2k_B T}{e_0} \operatorname{asinh}\left(\frac{\lambda_D e_0 \sigma}{2\varepsilon_0 \varepsilon_r k_B T}\right). \quad (17)$$

Table V. Numeric Approximations to Eqs. 16 and 18 used for Calculating of the Debye–Hückel Screening Length, λ_D, and the Normalized Electrostatic Potential, Φ, in a 1:1 Electrolyte

	For water (ε_r=78)		For ε_r=40	
	25°C	37°C	25°C	37°C
λ_D (nm)	$= 0.303 C_{el}^{-0.5}$	$= 0.309 C_{el}^{-0.5}$	$= 0.217 C_{el}^{-0.5}$	$= 0.221 C_{el}^{-0.5}$
Φ (σ)	$= 2\ z\ \operatorname{asinh}(8.547 \sigma C_{el}^{-0.5})$	$= 2\ z\ \operatorname{asinh}(8.380\ \sigma C_{el}^{-0.5})$	$= 2\ z\ \operatorname{asinh}(11.935\ \sigma\ C_{el}^{-0.5})$	$= 2\ z\ \operatorname{asinh}(11.702\ \sigma\ C_{el}^{-0.5})$
Φ (σ), for $\Phi \ll 1$[b]	$= 17.094\ z\sigma C_{el}^{-0.5}$	$= 16.760\ z\ \sigma C_{el}^{-0.5}$	$= 23.870\ z\ \sigma C_{el}^{-0.5}$	$= 23.404\ z\sigma C_{el}^{-0.5}$

[a] C_{el} is the electrolyte molar concentration, σ the total surface charge density in Cm^{-2}, z the charged component valence
[b] Calculated from the simplified asymptotic equation, $\psi = \lambda_D \sigma / \varepsilon_0 \varepsilon_r$, valid for $\psi \ll 25$ mV

σ is the surface charge density in Cm^{-2} and asinh the inverse hyperbolic sine (*areasinushyperbolicus*). The normalized dimensionless electrostatic potential, Φ, is defined as the ratio of electrostatic potential energy, $ze_0\psi$, and thermal energy, k_BT:

$$\Phi(\sigma) = \frac{ze_0\psi(\sigma)}{k_BT} \quad . \tag{18}$$

Numeric approximations to Eqs. 16 and 18 are given in Table V.

According to the Gouy–Chapman model, the relationship between the electrostatic potential $\psi(x)$ at distance x from a uniformly charged surface and the electrostatic surface potential $\psi(x=0)$, is:

$$x = \ln\left\{\frac{\tanh[e_0\psi(x=0,\sigma)/4k_BT]}{\tanh[e_0\psi(x,\sigma)/4k_BT]}\right\}\cdot\lambda_D \quad . \tag{19}$$

This provides means for estimating the effective distance between the lipid and the drug charges in a membrane.

The electrostatic correction described in this article allows only for the Coulombic, i.e. charge–charge interactions. Other contributions, such as hydration (polarity) effects, can be influential as well. If they are not small, such interactions should be considered, following the basic, self-consistent approach described in this work.

REFERENCES

1. C. J. Alcorn, R. J. Simpson, D. E. Leahy, and T. J. Peters. Partition and distribution coefficients of solutes and drugs in brush border membrane vesicles. *Biochem. Pharmacol.* **45**:1775–1782 (1993). doi:10.1016/0006-2952(93)90433-W.
2. R. P. Austin, A. M. Davis, and C. N. Manners. Partitioning of ionizing molecules between aqueous buffers and phospholipid vesicles. *J. Pharm. Sci.* **84**:1180–1183 (1995). doi:10.1002/jps.2600841008.
3. A. Avdeef. *Absorption and drug development: solubility, permeability, and charge state*. Wiley, New Jersey, 2003.
4. R. P. Austin, P. Barton, A. M. Davis, C. N. Manners, and M. C. Stansfield. The effect of ionic strength on liposome-buffer and 1-octanol-buffer distribution coefficients. *J. Pharm. Sci.* **87**:599–607 (1998). doi:10.1021/js9703481.
5. A. Avdeef, K. J. Box, J. E. Comer, C. Hibbert, and K. Y. Tam. pH-metric logP 10. Determination of liposomal membrane–water partition coefficients of ionizable drugs. *Pharm. Res.* **15**:209–215 (1998). doi:10.1023/A:1011954332221.
6. B. I. Escher, R. Schwarzenbach, and J. C. Westall. Evaluation of liposome–water partitioning of organic acids and bases. 2. Comparison of experimental determination methods. *Environ. Sci. Technol.* **34**:3962–3968 (2000). doi:10.1021/es0010711.
7. S. D. Krämer, J. C. Gautier, and P. Saudemon. Considerations on the potentiometric log P determination. *Pharm. Res.* **15**:1310–1313 (1998). doi:10.1023/A:1011968630713.
8. C. Ottiger, and H. Wunderli-Allenspach. Partition behaviour of acids and bases in a phosphatidylcholine liposome–buffer equilibrium dialysis system. *Eur. J. Pharm. Sci.* **5**:223–231 (1997). doi:10.1016/S0928–0987(97)00278–9.
9. A. V. Thomae, T. Koch, C. Panse, H. Wunderli-Allenspach, and S. D. Kramer. Comparing the lipid membrane affinity and permeation of drug-like acids: The intriguing effects of cholesterol and charged lipids. *Pharm. Res.* **24**:1457–1472 (2007). doi:10.1007/s11095-007-9263-y.
10. B. de Castro, P. Gameiro, J. L. F. C. Lima, C. Matos, and S. Reis. A fast and reliable spectroscopic method for the determination of membrane–water partition coefficients of organic compounds. *Lipids*. **36**:89–96 (2001). doi:10.1007/s11745-01-673-0.
11. C. Rodrigues, P. Gameiro, S. Reis, J. L. F. C. Lima, and B. de castro. Derivative spectrophotometry as a tool for the determination of drug partition coefficients in water/dimyristoyl-α-phosphatidylglycerol (DMPG) liposomes. *Biophys. Chem.* **94**:97–106 (2001). doi:10.1016/S0301-4622(01)00227-7.
12. N. C. Santos, M. Prieto, and M. A. R. B. Castanho. Quantifying molecular partition into model systems of biomembranes: an emphasis on optical spectroscopic methods. *Biochim. Biophys. Acta*. **1612**:123–135 (2003). doi:10.1016/S0005-2736(03)00112-3.
13. R. P. Austin, P. Barton, A. M. Davis, R. E. Fessey, and M. C. Wenlock. The thermodynamics of the partitioning of ionizing molecules between aqueous buffers and phospholipid membranes. *Pharm. Res.* **22**:1649–1657 (2005). doi:10.1007/s11095-005-6336-7.
14. X. Y. Liu, Q. Yang, N. Kamo, and J. Miyake. Effect of liposome type and membrane fluidity on drug–membrane partitioning analyzed by immobilized liposome chromatography. *J. Chromatogr. A*. **913**:123–131 (2001). doi:10.1016/S0021-9673(00)01266-8.
15. Q. Yang, X. Y. Liu, S. Ajiki, M. Hara, P. Lundahl, and J. Miyake. Avidin–biotin immobilization of unilamellar liposomes in gel beads for chromatographic analysis of drug–membrane partitioning. *J. Chromatogr. B Biomed. Sci. Appl.* **707**:131–141 (1998). doi:10.1016/S0378-4347(97)00620-8.
16. C. Matos, B. de Castro, P. Gameiro, J. L. Lima, and S. Reis. Zeta-potential measurements as a tool to quantify the effect of charged drugs on the surface potential of egg phosphatidylcholine liposomes. *Langmuir*. **20**:369–377 (2004). doi:10.1021/la034780b.
17. M. Ikonen, L. Murtomäki, and K. Kontturia. An electrochemical method for the determination of liposome–water partition coefficients of drugs. *J. Electroanal. Chem.* **602**:189–194 (2007). doi:10.1016/j.jelechem.2006.12.014.
18. T. Hata, T. Sakamoto, H. Matsuki, and S. Kaneshina. Partition coefficients of charged and uncharged local anesthetics into dipalmitoylphosphatidylcholine bilayer membrane: estimation from pH dependence on the depression of phase transition temperatures. *Colloids Surf. B Biointerfaces*. **22**:77–84 (2001). doi:10.1016/S0927-7765(01)00160-6.
19. A. Hildebrand, K. Beyer, R. Neubert, P. Garidel, and A. Blume. Temperature dependence of the interaction of cholate and deoxycholate with fluid model membranes and their solubilization into mixed micelles. *Colloids Surf. B: Biointerfaces*. **32**:335–351 (2003). doi:10.1016/j.colsurfb.2003.08.001.
20. K. Balon, B. U. Riebesehl, and B. W. Muller. Drug liposome partitioning as a tool for the prediction of human passive intestinal absorption. *Pharm. Res.* **16**:882–888 (1999). doi:10.1023/A:1018882221008.
21. J. Lasch. Interaction of detergents with lipid vesicles. *Biochim. Biophys. Acta*. **1241**:269–292 (1995).
22. F. H. Clarke. Ionization constants by curve fitting: application to the determination of partition coefficients. *J. Pharm. Sci.* **73**:226–230 (1984). doi:10.1002/jps.2600730221.
23. F. H. Clarke, and N. M. Cahoon. Ionization constants by curve fitting: determination of partition and distribution coefficients of acids and bases and their ions. *J. Pharm. Sci.* **76**:611–620 (1987). doi:10.1002/jps.2600760806.
24. S. Ohki. Adsorption of local anesthetics on phospholipid membranes. *Biochim. Biophys. Acta*. **777**:56–66 (1984). doi:10.1016/0005-2736(84)90496-6.
25. P. G. Thomas, and J. Seelig. Binding of the calcium antagonist flunarizine to phosphatidylcholine bilayers: charge effects and thermodynamics. *Biochem. J.* **291**:397–402 (1993).
26. M. S. Fernandez, and P. Fromherz. Lipoid pH indicators as probes of electrical potential and polarity in micells. *J. Phys. Chem.* **81**:1755–1761 (1977). doi:10.1021/j100533a009.
27. E. Kuchinka, and J. Seelig. Interaction of melittin with phosphatidylcholine membranes. Binding isotherm and lipid head-group conformation. *Biochemistry*. **28**:4216–4221 (1989). doi:10.1021/bi00436a014.
28. H. D. Bäuerle, and J. Seelig. Interaction of charged and uncharged calcium channel antagonists with phospholipid membranes. Binding equilibrium, binding enthalpy, and membrane location. *Biochemistry*. **30**:7203–7211 (1991). doi:10.1021/bi00243a023.

29. A. Hildebrand, R. Neubert, P. Garidel, and A. Blume. Bile salt induced solubilization of synthetic phosphatidylcholine vesicles studied by isothermal titration calorimetry. *Langmuir*. **18**:2836–2847 (2002). doi:10.1021/la011421c.
30. A. Seelig, P. R. Allegrini, and J. Seelig. Partitioning of local anesthetics into membranes: surface charge effects monitored by the phospholipid head-group. *Biochim. Biophys. Acta*. **939**:267–276 (1988). doi:10.1016/0005-2736(88)90070-3.
31. P. Friberger, and G. Aberg. Some physiochemical properties of the racemates and the optically active isomers of two local anaesthetic compounds. *Acta Pharm. Suec*. **8**:361–364 (1971).
32. M. Meloun, S. Bordovská, and L. Galla. The thermodynamic dissociation constants of four non-steroidal anti-inflammatory drugs by the least-squares nonlinear regression of multiwavelength spectrophotometric pH-titration data. *J. Pharm. Biomed. Anal*. **45**:552–564 (2007). doi:10.1016/j.jpba.2007.07.029.
33. A. Avdeef, C. M. Berger, and C. Brownell. pH-metric solubility. 2: correlation between the acid–base titration and the saturation shake-flask solubility–pH methods. *Pharm. Res*. **17**:85–89 (2000). doi:10.1023/A:1007526826979.
34. J. M. Carrozzino. *Drug partitioning and solvation environments in lipid bilayers*. Department of Chemistry, North Carolina State University, Raleigh, 2004, p. 224.
35. K. Ohsawa, and H. Ohshima. Electrophoretic mobility and isoelectric point of purified brush border membrane vesicles. *Electrophoresis*. **5**:148–154 (1984). doi:10.1002/elps.1150050305.
36. M. Sugawara, H. Oikawa, M. Kobayashi, K. Iseki, and K. Miyazaki. Effect of membrane surface potential on the uptake and the inhibition of cationic compounds in rat intestinal brush-border membrane vesicles and liposomes. *Biochim. Biophys. Acta*. **1234**:22–28 (1995). doi:10.1016/0005-2736(94)00250-S.
37. C. Engvall, and P. Lundahl. Drug partition chromatography on immobilized porcine intestinal brush border membranes. *J. Chromatogr. A*. **1031**:107–112 (2004). doi:10.1016/j.chroma.2003.11.063.
38. Cevc. Electrostatic characterization of liposomes. *Chem. Phys. Lipids*. **64**:163–186 (1993). doi:10.1016/0009-3084(93)90064-A.