Interaction of Antiinflammatory Drugs with EPC Liposomes: Calorimetric Study in a Broad Concentration Range

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ABSTRACT Isothermal titration calorimetry was used to characterize and quantify the partition of indomethacin and acemetacin between the bulk aqueous phase and the membrane of egg phosphatidylcholine vesicles. Significant electrostatic effects were observed due to binding of the charged drugs to the membrane, which implied the use of the Gouy-Chapman theory to calculate the interfacial concentrations. The binding/partition phenomenon was quantified in terms of the partition coefficient (K_p), and/or the equilibrium constant (K_b). Mathematical expressions were developed, either to encompass the electrostatic effects in the partition model, or to numerically relate partition coefficients and binding constants. Calorimetric titrations conducted under a lipid/drug ratio >100:1 lead to a constant heat release and were used to directly calculate the enthalpy of the process, ΔH, and indirectly, ΔG and ΔS. As the lipid/drug ratio decreased, the constancy of reaction enthalpy was tested in the fitting process. Under low lipid/drug ratio conditions simple partition was no longer valid and the interaction phenomenon was interpreted in terms of binding isotherms. A mathematical expression was deduced for quantification of the binding constants and the number of lipid molecules associated with one drug molecule. The broad range of concentrations used stressed the biphasic nature of the interaction under study. As the lipid/drug ratio was varied, the results showed that the interaction of both drugs does not present a unique behavior in all studied regimes: the extent of the interaction, as well as the binding stoichiometry, is affected by the lipid/drug ratio. The change in these parameters reflects the biphasic behavior of the interaction—possibly the consequence of a modification of the membrane's physical properties as it becomes saturated with the drug.

INTRODUCTION

Efficient interaction with phospholipidic biological bilayers and the ability to permeate cell membranes are parameters of indubitable importance in the design, development, and study of pharmacological active molecules. Historically, these molecular characteristics were evaluated by the octanol/water partition coefficient ($K_{O/W}$), which was used to predict the degree of a drug's affinity to the lipid membrane (Leo et al., 1971). Nevertheless, an isotropic bulk organic solvent such as *n*-octanol is indeed unable to mimic the structural and functional features of biological membranes, and as a result the lack of $K_{O/W}$/activity correlation is very common (De Young and Dill, 1988).

The aim to get better membrane models led to the use of several organized structures: cellular lines (involving very time-consuming procedures), micelles (whose structure is a poor bilayer membrane model), and mixed micelles, monolayers, and liposomes (Betageri and Rogers, 1988). Liposomes are phospholipidic vesicles, formed by one or more concentric bilayers, offering a simple, but structurally complete membrane model; these specific membrane features can be isolated from other factors and studied separately (Lasic, 1993).

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The antiinflammatory, analgesic, and antipyretic drugs indomethacin and acemetacin are widely used in the treatment of inflammatory and degenerative diseases. The mechanism of action of these drugs is associated with the prostaglandin synthesis inhibition, by interaction of the drugs with the enzyme cyclooxygenase, which is involved in phospholipidic metabolism (Vane, 1971). However, the nonsteroidal antiinflammatory drugs (NSAIDs) have been associated with serious adverse effects, especially those concerning the gastrointestinal tract. Because the etiology of these gastrointestinal effects has recently been related with the interaction of the drugs with the membrane phospholipids (Lichtenberger et al., 1995; Giraud et al., 1999; Srinath et al., 2000), drug encapsulation in liposomes provides some degree of protection against these adverse effects. There are already in vivo studies of preparations of indomethacin and acemetacin associated with liposomes as pharmaceutical formulation (Katare et al., 1995; Lichtenberger et al., 1996), which show advantages in parameters like bioavailability and therapeutic index, when compared with the free drug.

In this context, the evaluation and characterization of indomethacin and acemetacin liposome association is a useful step in the process of fully understanding the mechanism of the drug's interaction with the bilayer. Calorimetric techniques have significantly contributed to the understanding of biological processes at molecular level. In this respect, isothermal titration calorimetry (ITC) has proven a useful technique, inasmuch as it allows the determination of thermodynamic parameters for biological reactions with

high sensibility and accuracy, at constant temperature (Freire et al., 1990). The heat associated with the reaction is directly measured, and related thermodynamic parameters, such as enthalpy (ΔH), entropy (ΔS), and Gibbs free energy (ΔG), can be calculated and used to quantify the extension and energetics of the reaction under study. The data can be treated to calculate the binding constant, K_b, and the binding stoichiometry, n (Bäuerle and Seelig, 1991; Thomas and Seelig, 1993; Milhaud et al., 1996; Rowe et al., 1998).

In this article, a study of the interaction of two NSAIDs (indomethacin and acemetacin) with the membrane of neutral liposomes, at pH 7.4 and 298.15 K, is presented. The purpose of this study was to quantify the extent of the lipid/drug association in a broad concentration range, and to provide a thermodynamic analysis of the interaction.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (EPC), indomethacin, and acemetacin were from Sigma (St. Louis, MO), and used as received. All other chemicals were from Merck (Darmstadt, Germany). Solutions were prepared with HEPES buffer (10 mM, $I = 0.1$ M, pH 7.4). The ionic strength was adjusted with NaCl.

The fluorescent probe, *n*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoetanolamine, triethylammonium salt (DHPE), palmitoyl-oleoyl-phosphatidylcholine (POPC), and dimyristoyl-phosphatidylglycerol (DMPG) were from Avanti Polar Lipids (Alabaster, AL).

Vesicle preparation

Multilamellar vesicles (MLVs) were prepared by the thin film hydration method (Lasic, 1993), in which a lipid solution in chloroform/methanol was evaporated to dryness with a stream of nitrogen. The resultant lipid film was hydrated with HEPES buffer and the mixture was vortexed at room temperature (inasmuch as the transition temperature for EPC ranges from −15°C to −7°C; Tyrrell et al., 1976; New, 1990) to yield MLVs. Large unilamellar vesicles (LUVs) were obtained from the MLV, by extrusion in a 10-ml stainless-steel extruder (Lipex Biomembranes, Vancouver, BC, Canada), maintained at constant temperature by a circulating water bath. The liposome suspensions were passed 10 times through polycarbonate filters of 100-nm pore size (Nucleopore, Pleasanton, CA) under inert (N_2) atmosphere. Size distribution of the extruded vesicles was determined by quasi-elastic light scattering analysis (Malvern ZetaSizer 5000, Malvern Instruments, Malvern, Worcestershire, UK), using a helium-neon laser (633 nm) as a source of incident light, operating at a scattering angle of 90° and a temperature of 25°C. The mean particle size of the LUV was 141.5 ± 5.8 nm (average and standard deviation of six independent measurements). EPC concentration in the vesicle suspensions was determined by the phosphomolibdate method (McClare, 1971).

Calorimetry

The calorimetric technique used was stepwise isothermal titration microcalorimetry. The water bath and peripheral units were built at Lund University (Lund, Sweden), and a twin heat conduction calorimeter (ThermoMetric AB, Järfälla, Sweden) was used with a 1-cm^3 titration cell equipped with a gold stirrer. The instrument was calibrated electrically, using an insertion heater (Briggner and Wadsö, 1991). The detailed calorimetric procedure has been described previously (Bastos et al., 1997). In each titration, 0.9848 ± 0.0008 ml of sample (either liposome suspension or drug solution) were placed in the titration cell and sequences of 20 successive 9.978 μl injections were made, at 298.15 K, at 4-min intervals (injections with 20-min intervals did not reveal the presence of slow reactions). Experiments were performed in the fast titration mode, the resulting curves deconvoluted (Bastos et al., 1991), and the integrals calculated with the program PSIGCALC (Sven Hägg, Lund, Sweden). The enthalpies were corrected for the dilution effects determined in separate experiments.

Different types of experiments were performed, regarding the relative positions of the drugs and the vesicles:

Type A1: drug solution in the syringe (1.2–4.3 mM), lipid suspension in the cell (32–45 mM).
Type A2: drug solution in the syringe (1.2–4.3 mM), lipid suspension in the cell (4–13 mM).
Type B: lipid in the syringe (40–46 mM), drug solution in the cell (0.75–1.92 mM).

ζ-potential measurements

The vesicles ζ-potential was measured by quasielastic light scattering analysis in the same instrument used for the diameter analysis (Malvern ZetaSizer 5000). The measurements were performed in a ZET 5104 cell, at a temperature of 298 K (25.0°C), and a scattering angle of 90°. The solvent viscosity and refractive index values were 0.890 cP and 1.330, respectively (apparatus default).

Fluorescence measurements

Fluorescence spectra were obtained using a SPEX Fluorolog 212 system (Spex CertiPrep, Metuchen, NJ) at a right-angle geometry. The excitation wavelength was set to 488 nm (DHPE absorption maximum) and the emission covered the spectra from 400 to 600 nm (emission maximum ~510 nm) The aqueous pH was measured with a combined Orion Ross glass electrode in a Orion 720A pH-meter (Thermo Electron, Beverly, MA).

MODELS AND DATA ANALYSIS

When a solute A is in the presence of two nonmiscible phases (in this case, aqueous and lipid phases), it distributes between the two according to its affinity to each medium. This affinity can be quantified in terms of a partition coefficient,

$$K_p = \frac{[A_m]/V_m}{[A_w]/V_w} \approx \frac{[A_m]}{[A_w][L]V_\Phi}, \quad (1)$$

where the subscripts w and m stand for aqueous- and membrane-bound species, V_m and V_w for lipid and aqueous solution volumes, $[L]$ for lipid concentration, and V_ϕ for the lipid molar volume. All concentrations are expressed as a function of total suspension volume. As the lipid molar volume is not always known, the partition coefficient, K_p, can also be expressed in M^{-1}, being K_p (in M^{-1}) $= V_\phi K_p$ (dimensionless). In the present text we will be using K_p expressed in M^{-1}.

Besides this simple-partition model, the distribution of a solute between the two immiscible phases can also be regarded as a drug-lipid association. The association constant, K_b, for the binding equilibrium

$$A_w + L \rightleftarrows A_m \quad (2)$$

is given by (Berezin et al., 1973)

$$K_b = \frac{[A_m]}{[A_w]([L] - [A_m])}, \quad (3)$$

where the symbols have the same meaning as above. If the drug concentration is much lower than the lipid concentration, the equilibrium free lipid concentration is practically equal to the initial lipid concentration and in this case, $K_b \approx K_p$. Therefore, although conceptually different, the mathematical expressions for partition and association are equivalent, under these conditions.

In experimental conditions where the lipid/drug concentration ratio is lower, additional effects can invalidate the partition model due to interactive phenomena, such as electrostatic effects (if the drug is charged) (Matos et al., 2004) or saturation of the membrane (Milhaud et al., 1996; Dimitrova et al., 2000).

Determination of the enthalpy of reaction

When the concentration of added drug is kept very small (lipid/drug ratio $>\approx$100:1), the heat effect is practically constant in each addition of drug. The area under each peak gives the amount of heat released after injection i, q_i, and it can be easily related to the reaction enthalpy (ΔH), by (Breslauer et al., 1992; Freire et al., 1990)

$$\Delta H = q_i / n_{m,i}, \quad (4)$$

where $n_{m,i}$ is the number of moles of drug bound per injection.

Partition model

From Eqs. 1 and 4, an expression can be deduced, relating the binding polynomial to the heat released (for the case where the drug is being titrated with lipid),

$$Q_i = n_T \Delta H \frac{K_p [L]_i}{1 + K_p [L]_i}, \quad (5)$$

where n_T represents the total number of moles of drug present in the system, equal to the sum of bound (n_m) and free drug (n_w); and $[L]_i$, which is the lipid concentration in the cell after each injection.

Consideration of electrostatic effects

When dealing with charged drugs and when the lipid/drug ratio in the system is significantly low, as in the case of titration of drug solution with lipid suspension, the electrostatic contribution cannot be neglected. If the phospholipidic membrane is initially neutral, the partition of a negatively charged species will lead to a charging up of the membrane. This implies that the concentration of the charged drug at the interface ($[A_i^-]$) will be lower than its bulk concentration ($[A_w^-]$). The interface concentration can be related to the bulk concentration by the Boltzmann equation (McLaughlin and Harary, 1976),

$$[A_i^-] = [A_w^-] \exp(-z_i F \Psi_0 / RT), \quad (6)$$

where Ψ_0 is the surface potential, z_i is the valence of the ion ($^{-1}$), and F, R, and T have the usual meanings.

Correction of Eq. 5 with Eq. 6 allowed us to establish a relationship between the heat released and lipid concentration, but where the interfacial concentration of the drug is taken into account,

$$Q_i = n_T \Delta H \frac{K_p \exp(-z_i F \Psi_0 / RT)[L]}{1 + K_p \exp(-z_i F \Psi_0 / RT)[L]}. \quad (7)$$

Determination of the surface potential (Ψ_0)

The surface potential (Ψ_0) can be obtained in a number of different methodologies. In the present work we used three methods to obtain Ψ_0, as follows.

Method a

Direct experimental determination of ζ-potential values (Ψ_x) of the liposomes, as described in the experimental part, for different drug and lipid concentrations, and calculation of Ψ_0 according to

$$\Psi_0 = \frac{2kT}{e} \ln \frac{1 + \alpha}{1 - \alpha} \quad (8)$$

and

$$\alpha = \frac{\exp(e\Psi_x / 2kT) - 1}{\exp(-\kappa x)[\exp(e\Psi_x / 2kT) + 1]}, \quad (9)$$

where k is the Boltzmann constant, e is the electron charge, κ is the reciprocal of the Debye screening length, and α is a parameter dependent on the value of Ψ_0 (the mathematical formalism is described further in Matos et al., 2004).

Method b

Theoretical calculation of the surface charge density, σ, as described by Seelig and his co-workers (Bäuerle and Seelig, 1991; Thomas and Seelig, 1993),

$$\sigma = n_m e_0 / (n_m a_D + n_L a_L), \quad (10)$$

where a_L is the surface area of the lipid molecule, a_D is the surface area of the drug molecule, n_m is the number of moles of bound drug, and n_L the total number of moles of lipid. The lipid surface area, $a_L = 68$ Å^2, was taken from the literature (Bäuerle and Seelig, 1991; Beschiaschvili and Seelig, 1992), and the surface areas for the two drugs were calculated in QUANTA (QUANTA version 00.1110, 2000, Molecular Simulations, Burlington, MA), using a probe of 0.5 Å^2. The

values obtained were 59.6 $Å^2$ for acemetacin and 30.6 $Å^2$ for indomethacin. From the n_m values obtained from the calorimetric titration, the experimental surface charge density was calculated according to Eq. 10. The surface potential can then be calculated from the Gouy-Chapman theory by (McLaughlin et al., 1971)

$$\sigma^2 = 2000\varepsilon_r\varepsilon_0 RT\,\Sigma\, c_i[\exp(-z_i F\Psi_0/RT) - 1]. \qquad (11)$$

The values for the surface potential Ψ_0 were then obtained by computer fitting, matching the experimentally derived surface charge density with the corresponding values predicted from the Gouy-Chapman theory.

Method c

Experimental determination of the surface potential values (Ψ_0) by the use of a fluorescent probe. The apparent pK_a of a lipophilic probe in the vesicle suspension, pK_a^{obs}, can be related with Ψ_0 by the following equation (Mukerjee and Banerjee, 1964; Fernandéz, 1981; Fromherz, 1989),

$$pK_a^{obs} = pK_a^0 - F\Psi_0/2.3RT, \qquad (12)$$

where pK_a^0 is the pK_a when $\sigma = 0$, and, consequently, $\Psi_0 = 0$. The pK_a^0 is an intrinsic pK_a of the molecule in the interface region, and must be ascertained in the same local conditions that exist in the charged interface.

The calibration of the pK_a^{obs} versus Ψ_0 was done using POPC as the neutral phospholipid system (pK_a^0) and mixtures of POPC/DMPG with increasing amounts of charge (Lukac, 1983). From previous experiments we knew that in these mixtures the charged component, DMPG, is randomly distributed in the vesicles; thus we can assume a homogeneous distribution of charges within the bilayers.

The values of pK_a^{obs} for the fluorescent probe DHPE were determined by fluorescence, fitting the intensity as a function of the aqueous pH, as stated by the equation $pK_a^{obs} = pH - \log(\alpha/1-\alpha)$ with $\alpha = (F-F_{AH+})/(F_A-F_{AH+})$, where F is the fluorescence intensity at the band maximum of the conjugate acid-base forms at the particulate pH being examined. F_{AH+} and F_A are the fluorescence intensities at the same wavelength at pH values such that only AH^+ or A exist (García-Soto and Fernandéz, 1983).

The values of $pK_a{}^{obs}$ were determined in the lipid dispersions in different drug concentrations and used to calculate the Ψ_0 values using Eq. 12.

Multiple independent binding sites model

The multiple independent binding sites model (Freire et al., 1990; Breslauer et al., 1992) assumes that the drug molecule possesses n independent binding sites, all with the same affinity for the lipid molecule (Turner et al., 1995). The binding constant, K_b^n, quantifies the association of one lipid molecule to one binding site in the drug molecule. Although a real binding in the usual strict sense is not applicable in lipid/drug association, the use of this conceptual model is useful as it provides us with the lipid/drug association number, n. Being that n_L is the total number of moles of lipid present in the system, Eq. 3 will now be expressed as

$$K_b^n = \frac{n \times n_m}{n[A_w](n_L - n \times n_m)} \qquad (13)$$

Considering Eq. 4 we have deduced Eq. 14,

$$Q_i = n_L \Delta H \frac{K_b^n[A_w]}{1 + nK_b^n[A_w]}. \qquad (14)$$

To relate the obtained binding constant, K_b^n, with the partition coefficient, K_p, we have deduced Eq. 15,

$$K_p = \frac{K_b^n}{1 + nK_b^n[A_w]}. \qquad (15)$$

As the concentration of drug in the cell is always $<10^{-3}$ M, in practical terms the free drug concentration, $[A_w]$, is relatively small, being $n \times K_b \times [A_w] \ll 1$, so $K_b^n \approx K_p$.

RESULTS AND DISCUSSION

Calorimetric titration of liposome suspensions with drug solutions

In titrations of type A1 (see Materials and Methods), lipid/drug concentration ratio in the titration cell was kept $>\approx$100:1. In this experimental condition, the heat release was constant, and allowed the calculation of the enthalpy change, per mole of drug added, by Eq. 4 (Bains and Freire, 1991; Bäuerle and Seelig, 1991; Seelig and Ganz, 1991; Beschiaschvili and Seelig, 1992; Thomas and Seelig, 1993; Terzi et al., 1994; Seelig et al., 1996; Schote and Seelig, 1998; Wenk and Seelig, 1998).

A typical calorimetric tracing of the injection of aliquots of drug solution into liposome suspensions can be seen in Fig. 1.

The number of moles of bound drug, n_m, was calculated assuming the drug's partition coefficients (previously determined) as 791 M^{-1} and 2465 M^{-1} in MLV (Castro et al., 2001a) and 992 M^{-1} and 1527 M^{-1} in LUV (Castro et al., 2001b), for indomethacin and acemetacin, respectively. These values of partition coefficients quantify an almost complete binding of both drugs to the membrane (96–99%), considering the lipid concentration and the pH used. The results were nevertheless corrected according to the obtained percentage of bound drug in each case. From the determined values of ΔH, the values ΔS and ΔG were calculated and results are presented in Table 1.

To evaluate the possibility of concomitant phenomena

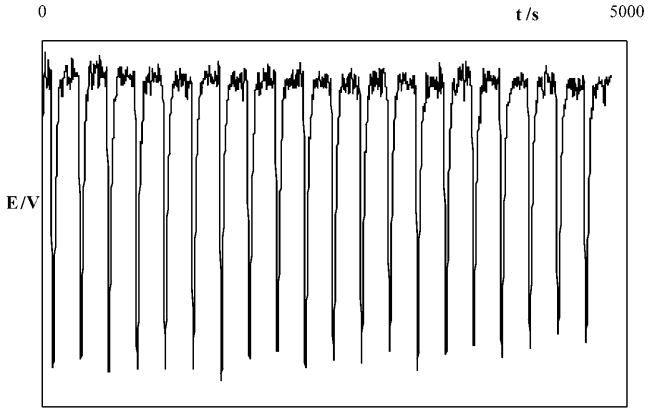

FIGURE 1 Titration of a suspension of LUV (45 mM) with an acemetacin solution (2.7 mM) in HEPES buffer at 298.15 K. Each peak corresponds to a 9.978 μl injection. Lipid/drug ratio ranged from 1500:1 to 80:1 at the end of the titration. The reaction is exothermic and the reaction enthalpy is constant because the lipid is in large excess over the added drug.

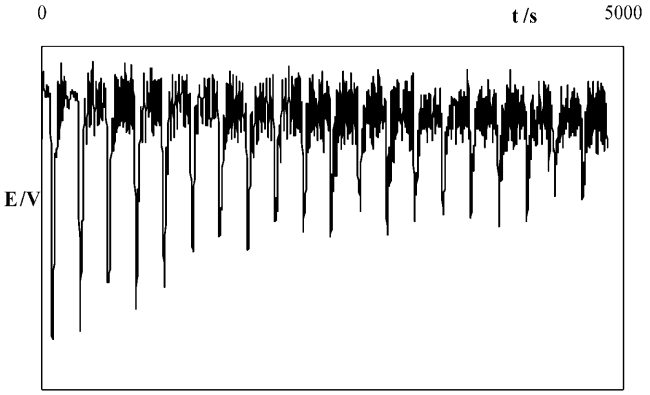

FIGURE 2 Titration of a suspension of LUV (4 mM) with an acemetacin solution (3.2 mM) in HEPES buffer at 298.15 K. Each peak corresponds to a 9.978 μl injection. Lipid/drug ratio ranged from 120:1 to 6:1 at the end of the titration.

such as drug aggregation or micellization, solutions of each drug were injected into buffer, and no measurable heat change was detected—an indication that no feature other than drug partition is being measured.

In titrations of type A2 (see Materials and Methods; the lipid/drug ratio is lower (between $\approx$150:1 and $\approx$6:1), and constant partition of the drug between water and lipid media can no longer be guaranteed, because concomitant saturation or electrostatic effects interfere in the process. The injection of aliquots of drug solution in a LUV suspension originates a series of decreasing heat releases, as can be observed in Fig. 2.

The amount of drug that has interacted with the lipid membrane after each injection can be calculated from the heat released, knowing ΔH and using Eq. 4. If a partition mechanism was to be considered, a linear relation would be expected between the bound fraction, X_b (given by the ratio of the bound drug moles, n_m, to the total number of lipid moles, n_L) and the amount of free drug in the aqueous media, $[A_w]$ (Bäuerle and Seelig, 1991; Thomas and Seelig, 1993; Seelig et al., 1996; Schote and Seelig, 1998; Wenk and Seelig, 1998). Results show, however, that less drug is taken up at higher concentrations than expected for simple partitioning. Plots of X_b versus $[A_a]$ were constructed for both drugs, as exemplified in Fig. 3, and do not show a linear relationship.

The decrease in drug uptake throughout the titration can be explained by several simultaneous phenomena, such as saturation of the lipid membrane (Ramsay et al., 1986; Myers et al., 1987), electrostatic effects due to the binding of a charged molecule to the membrane (Lehrmann and Seelig, 1994), or even decrease of ΔH throughout the titration as a result of the change in the nature of the interaction (Ramsay et al., 1986).

Actually, the partition coefficient, named under these conditions the apparent partition coefficient (Schote and Seelig, 1998), decreases throughout the titration. For indomethacin, the apparent partition coefficient decreases from 869 M^{-1} to 186 M^{-1} from the first to the last injection, whereas for acemetacin a decrease from 2224 M^{-1} to 374 M^{-1} is observed. Apparently, a saturation of the membrane

TABLE 1 Values of ΔH calculated from the experimental data obtained in the titration of a concentrated lipid suspension (32–45 mM) with a drug solution (1.2–4.3 mM)

Liposome type	Drug	ΔH (kJ.mol^{-1})	ΔG (kJ.mol^{-1})	$T\Delta S$ (kJ.mol^{-1})
LUV	Indomethacin	−17.3 (2.2)	−17.3 (0.2)	0 (2.0)
	Acemetacin	−19.7 (1.1)	−18.9 (0.5)	−0.8 (1.5)
MLV	Indomethacin	−14.2 (1.9)	−16.5 (0.2)	2.3 (2.0)
	Acemetacin	−17.8 (2.0)	−19.4 (0.2)	1.6 (2.0)

Values of ΔG and $T\Delta S$ were calculated by the trivial thermodynamic relationships: $\Delta G = -RT \ln K_b$ and $\Delta G = \Delta H - T\Delta S$. Values in parentheses are the standard deviations.

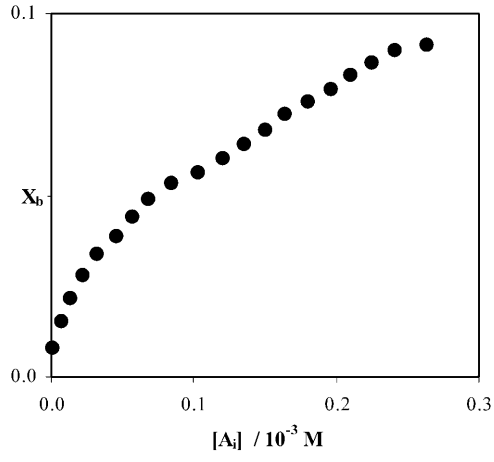

FIGURE 3 Plot of X_b versus $[A_a]$ for acemetacin. Results of the titration of a suspension of LUV (4 mM) with an acemetacin solution (3.2 mM) in HEPES buffer.

is taking place, as observed by Seelig and his co-workers (Bäuerle and Seelig, 1991; Thomas and Seelig, 1993; Seelig et al., 1993). These authors used the Gouy-Chapman theory to correct the data for electrostatic effects, and were able to determine the true partition constant (Thomas and Seelig, 1993). However, the use of the same mathematical formalism (see Determination of the Surface Potential, *Method b*) to our data did not lead to a complete linearization of the X_b vs. $[A_i]$ plots. The same is even more apparent in the calculations done with the drug's surface concentration calculated with the surface potential derived from the fluorescence method (see Determination of the Surface Potential, *Method c*; see also Fig. 4, *a* and *b*).

Fitting our results with fixed or varying ΔH shows that its value does not change significantly from the values in Table 1. Therefore, the observed decrease in heat release is possibly not related to a variation on ΔH value, but to parallel saturation phenomena.

To determine the stoichiometry of the reaction, n, the results were then evaluated by the multiple independent binding sites model (Eq. 14), but considering the interfacial concentrations, calculated according to Eq. 6. Nonlinear fitting of Eq. 14 was achieved only if two fitting zones were considered: the first encompassing the first 10 points of the titration and the second the latter 10. Fittings can be observed for acemetacin in Fig. 5.

The value of ΔH was fixed as -19.7 kJ/mol for acemetacin and -17.3 kJ/mol for indomethacin (note that fittings performed without fixing the value of ΔH yielded similar results). Results are depicted in Table 2. Three methodologies were used to calculate the surface potential, and a comparison between the results obtained (unpublished material). The three methodologies used to calculate the surface potential yielded similar fitting results. As an example, we include in Table 2 the values obtained by the three methodologies for the case of acemetacin. This kind of agreement was obtained throughout. In view of this agreement, the results presented hereafter will only be those obtained by use of the values of Ψ_0 obtained from ζ-potential measurements.

As presented in Table 2, the interaction of both drugs with the lipid membrane appears to be of a biphasic nature. For lower concentrations of drug, the binding constant is higher, whereas for lower lipid/drug ratio the process behavior apparently changes, with the drug having a lower affinity for the lipid as assessed by the decrease in K_b. The value of n also decreases, suggesting that the binding stoichiometry changes with the increase in bound drug. The turning-point happens at a lipid/drug ratio of 12:1 for acemetacin and 17:1 for indomethacin.

One can infer that once this ratio has been surpassed, the amount of drug in the membrane changes its composition and, consequently, its physical characteristics, which is reflected in a different association with the drug added afterwards. This interpretation is not new; an alteration of the interaction behavior during incorporation of a solute in organized systems has been reported previously, either with lipid membranes (Schuster et al., 1975) or micelles (Lissi and

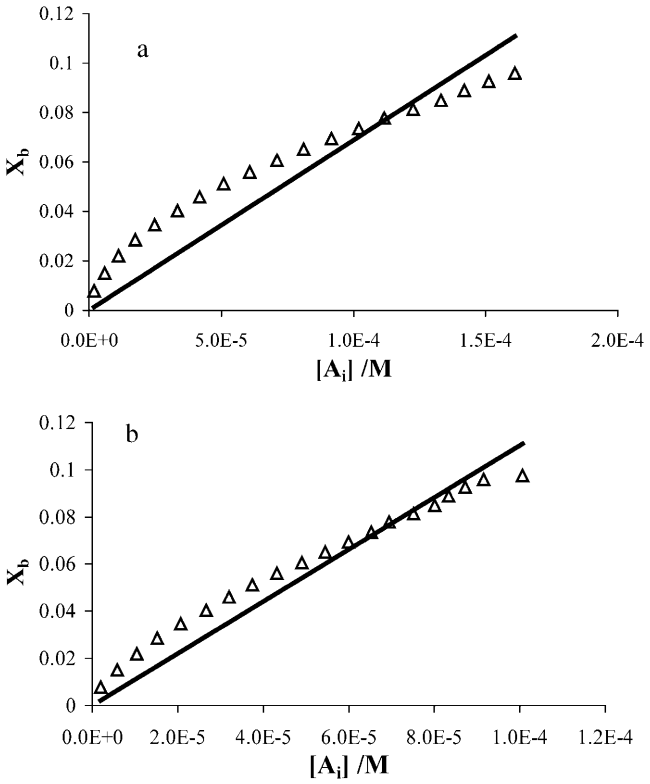

FIGURE 4 Plot of X_b versus $[A_i]$ for acemetacin. Results of the titration of a suspension of LUV (4 mM) with an acemetacin solution (3.2 mM) in HEPES buffer. In *a*, the surface potential is calculated from the fluorescence method, and in *b*, from the Gouy-Chapman theory (Eqs. 6, 8, and 9).

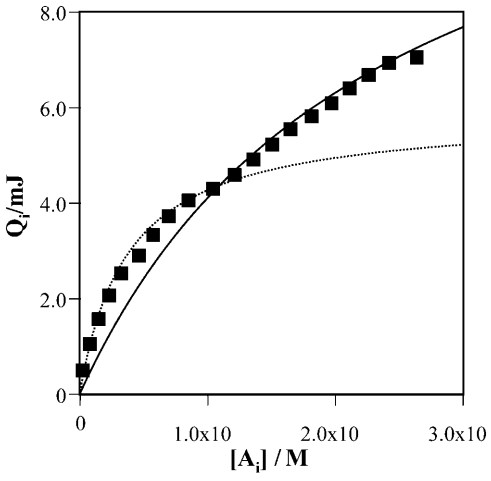

FIGURE 5 Fitting of the multiple independent binding sites model (Eq. 14) to the experimental data obtained from the titration of a suspension of LUV (4 mM) with an acemetacin solution (3.2 mM) in HEPES buffer. The value of ΔH was fixed at -19.7 kJ/mol.

TABLE 2 Values of binding constants, K_b, and number of lipid binding sites, *n*, obtained from fitting Eq. 14 to the experimental data collected from the titrations of LUV suspensions (4–13 mM) with drug solution

Drug	Fitting range	K_b^n (M^{-1})	*n*
Indomethacin	First 10 data	530 (43)	8.5 (2.9)
	Last 10 data	450 (24)	4.9 (1.3)
Acemetacin	First 10 data	2594 (198)	10.4 (0.8)
		2416 (196)*	11.2 (0.8)*
		2535 (165)†	8.0 (0.8)†
	Last 10 data	1110 (27)	1.6 (0.3)

Values in parenthesis are standard deviations obtained from the fitting. Values not marked were obtained with the interface drug concentration calculated by use of the surface potential calculated from the ζ-potential results.

*Values calculated with the surface potential determined by fluorescence (*Method c*).

†Values calculated with the theoretical surface potential (*Method b*).

Abuin, 1983). Recently, Coutinho and Prieto (2003) described a concentration-dependent cooperative partition of nystatin to liposomes. In particular, ITC technique has been used to characterize biphasic type binding of solutes to lipid membranes (Zachowski and Durand, 1988; Dimitrova et al., 2000).

Calorimetric titration of drug solutions with liposome suspensions

In titrations of type B (see Materials and Methods; lipid/drug ratio ranged between ≈0.3 and ≈6. An example of the results obtained from these titrations can be observed in Fig. 6.

With the progress of the titration, less and less free drug in solution is available for interacting, resulting in a decrease in the heat released (Terzi et al., 1994; Seelig et al., 1996; Schote and Seelig, 1998). The height of the individual titration peaks is correlated with the ligand concentration, whereas the steepness of the decrease depends primarily on the lipid concentration and the binding constant (or partition coefficient; see Wenk et al., 1997).

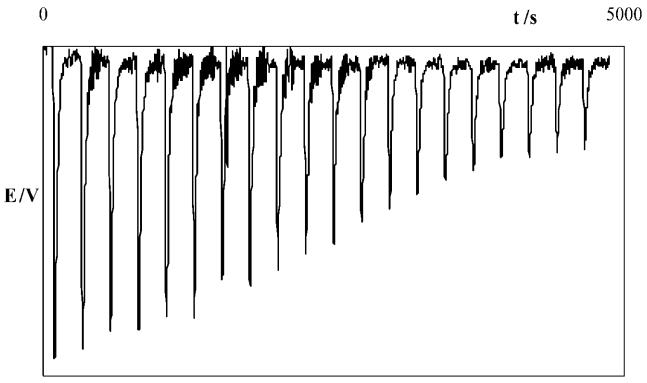

FIGURE 6 Titration of an acemetacin solution (1.6 mM) with a suspension of LUV (40 mM) in HEPES buffer at 298.15 K. Each peak corresponds to a 9.978 μl injection. Lipid/drug ratio in the titration cell ranged from 0.28:1 to 5.6:1 at the end of the titration.

Plots of X_b versus $[A_a]$ (i.e., free drug concentration in the bulk, with no correction for electrostatic effects) did not yield a linear correlation; besides, nonlinear fitting of the simple-partition model expressed by Eq. 5 (which is mathematically equivalent to the X_b vs. $[A_a]$ model) result in lack of fit (results not shown). The lack of success of such approaches is explained by the strong electrostatic interference, due to the high proportion of a charged ligand interacting with the lipid.

The experimental data were fit with Eq. 7, which accounts for electrostatic correction of the data; the fit is displayed in Fig. 7.

The good fit obtained with this model implies that the electrostatic effects were satisfactorily corrected. The results of K_p (or K_b) in LUV obtained were 432 ± 19 M^{-1} for acemetacin and 350 ± 42 M^{-1} for indomethacin. If both ΔH and K_p are allowed to vary, the following set of results are obtained. For acemetacin, $\Delta H = -17.7 \pm 0.3$ kJ/mol and $K_p = 530 \pm 37\ M^{-1}$ and for indomethacin, $\Delta H = -15.3 \pm 0.7$ kJ/mol and $K_p = 414 \pm 12\ M^{-1}$. In this case, significantly smaller values are obtained for ΔH when it is allowed to vary. This is a further indication that we have in this case a highly perturbed membrane, due to the excess of drug present.

CONCLUSIONS

An important objective in the design of pharmacological active compounds is their ability to efficiently permeate and interact with biological lipid membranes, from which can depend either pharmacological or adverse effects of the drugs. The study and characterization of such drug/membrane interactions can lead to important assessments related to the drugs' action in biological media. As stated, the interaction of

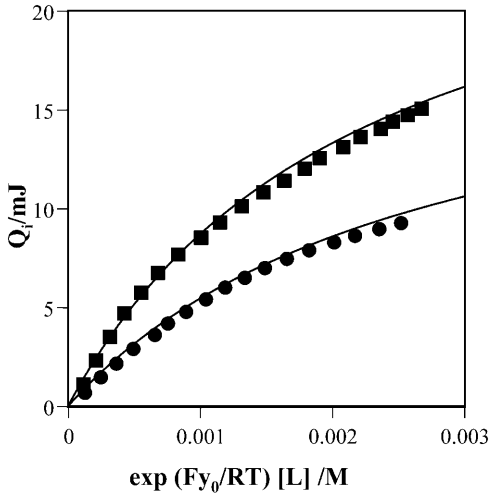

FIGURE 7 Fitting of the electrostatic corrected partition model (Eq. 7) to the experimental data obtained from the titration of acemetacin (*squares*) or indomethacin (*circles*) with a suspension of LUV in HEPES buffer. The value of ΔH was fixed at −19.7 kJ/mol for acemetacin and −17.3 kJ/mol for indomethacin.

both indomethacin and acemetacin with the lipid bilayer is exothermic. Further, the enthalpy is the driving force for the interaction, as the entropy variation is close to 0 for both drugs. The results point out that the interaction of these drugs can be classified, as suggested by Seelig et al. (Bäuerle and Seelig, 1991), as the nonclassical hydrophobic effect, usually observed in the interaction of solutes with lipid membranes.

In titrations performed under experimental conditions in which the lipid/drug ratio is low, saturation phenomena are observed, and the apparent partition coefficients are lower than the real partition coefficient. Application of the multiple independent binding sites model leads to the finding of a biphasic nature for these interactions. In fact, a modification of the membrane's physical properties apparently occurs as the membrane becomes saturated with the ligand, with a corresponding change in the values of the binding constant and the stoichiometry. Such modification in the membrane appears to take place at lipid/drug ratio of 12:1 for acemetacin and 17:1 for indomethacin. This assertion agrees with published results that describe a modification on the structure of the bilayer caused by indomethacin, assessed by differential scanning calorimetry studies (Bonina et al., 1994; Castelli et al., 1997).

Therefore, the use, in this study, of lipid/drug ratios that ranged between 1500:1 and 0.28:1 (taking all titrations effectuated) allowed us to verify the inconstancy of the extent and stoichiometry of the interaction under study, and infer that the binding of the drug to the molecule actually induces physical and structural changes in the lipid membrane.

At this point, some additional remarks should be made. Firstly, a word about the binding/partition approach developed in this work: the interaction of a solute with a dispersed lipid phase is conceptually related to a Nernstian distribution between two nonmiscible liquids. By this approach, only a partition of the solute is conceptually valid, and all the parameters contributing to the interaction extent—electrostatic, hydrophobic, steric, etc.—are enclosed in the notion of partition and globally quantified. Moreover, all these parameters are assumed to be nonvariable with changes in concentration. On the other hand, the consideration of a binding mechanism between the solute and the lipid describes the existence of binding sites in the membrane, receptor-like locals that accommodate the ligand. By its very nature the basic homogeneous distribution of the phospholipid molecules throughout the bilayer is not completely agreeable with this receptor nature of the membrane, and has been refuted by other authors.

Nevertheless, in this work, and to our knowledge for the first time, the two approaches of binding and partition have been complementarily used to fully describe a drug/lipid interaction. We have noticed that, for a wide range of lipid/drug ratios, the partition concept is adequate as long as we correct for electrostatic effects; but when different lipid/drug proportions are spanned, the saturation and electrostatic effects, or even membrane structural changes, are increasingly contributing to the overall interaction and this phenomenon can no longer be interpreted in terms of a simple partition. In this case, the process is better described by a binding isotherm, characterized by a number of lipid molecules associated with each drug molecule and by a binding constant. The introduction of the parameter n allowed flexibility into the binding curve, and showed that there actually was a change in association number along the binding isotherm, which is a parameter not possible to envisage in a simple partition model.

A second point to be stressed could be the need for enlarging the range of analytical concentrations under study, which allows concentration-dependent features to be put in evidence. In fact, one of the main advantages attributed to the ITC technique is the possibility of covering a wide range of concentrations. For other methods, such as spectroscopic, this constitutes a limitation; and the conclusions to be drawn from the data obtained are only a snapshot of a particular state of the interaction under study.

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