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pH effects on drug interactions with lipid bilayers by liposome electrokinetic chromatography

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Abstract

Liposome electrokinetic chromatography (LEKC) provides convenient and rapid methods for studying drug interactions with lipid bilayers using liposomes as a pseudostationary phase. LEKC was used to determine the effects of pH on the partitioning of basic drugs into liposomes composed of zwitterionic phosphatidylcholine (PC), anionic phosphatidylglycerol (PG), and cholesterol, which mimic the composition of natural cell membranes. An increase in pH results in a smaller degree of ionization of the basic drugs and consequently leads to a lower degree of interaction with the negatively charged membranes. From the LEKC retention data, the fractions of drugs distributed in the bulk aqueous and the liposome phase were determined at various pH values. Finally, lipid mediated shifts in the ionization constants of drugs were examined.

Keywords: Liposome electrokinetic chromatography; Drug; Lipid bilayers; Partition coefficient; Lipophilicity; Passive transmembrane permeability; Caco-2 cell permeability; Oral bioavailability

1. Introduction

The interaction of drugs with membranes is an important field of study due to the significant and useful applications in quantitative structure–activity relationship (QSAR) studies [1–7]. Liposomes are suitable models for biomembranes. Partition coefficients of drugs between a bulk aqueous and liposome phase (K_{LW}) can be used as a measure of the extent of drugs affinities toward lipid bilayers of cell membranes, or drugs *lipophilicity* [8]. Partitioning of uncharged solutes into vesicles and liposomes is controlled by a combination of hydrophobic, dipolarity/polarizability, and hydrogen bonding interactions [9,10]. A great majority of drug molecules have ionizable functional groups, thus electrostatic interactions also play a major role in their partitioning behavior, as measured by the liposome–water distribution coefficient (D_{LW}), which is dependent on the extent of solute ionization as determined by the pH.

Partitioning into a cell membrane is generally considered the first step in the passage of drugs across biological membranes [11,12]. Transport of drugs through biological membranes (membrane permeability) is primarily by passive diffusion for large numbers of drugs. Passive transport through a cell membrane involves an initial partitioning into the lipid bilayer of the membrane, followed by diffusion through the bilayer, and finally a partitioning out of the membrane. This initial partitioning into liposomes is measured by K_{LW} or D_{LW}.

The liposome–water distribution coefficient depends on a number of variables, such as solute lipophilicity, composition of the membrane, temperature, and among others, pH. At a physiological pH (5.5–7.5), many drugs are partially or fully charged and are electrostatically attracted or repelled by many biological membranes that are composed of acidic lipids, and carry a net negative charge. Basic functional groups are ubiquitous among drugs that impart a net positive charge on the molecule and interact favorably with the charged membrane.

For ionizable compounds, partitioning into liposomes is influenced by acid–base equilibria as illustrated for a basic drug in Fig. 1. The extent of drug ionization is determined by their pK_a and solution pH, which in turn influence a drug's

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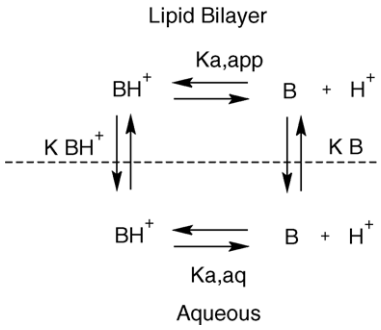


Fig. 1. Ionization equilibrium and partitioning of a basic drug into a lipid bilayer. K_B and K_{BH^+} are the partition coefficients for the neutral and charged forms, respectively. $K_{a,aq}$ and $K_{a,app}$ are the ionization constants in the aqueous and lipid bilayer phases, respectively.

ability to interact with (or partition into) cell membranes, and is of great importance to the behavior, activity, and usefulness of the drug. The observed distribution coefficient of a charged drug takes into account the partitioning of both the charged and neutral forms of the drug. Each of these forms will partition into the liposomes as illustrated in Fig. 1; K_B is the partition coefficient of the neutral form and K_{BH^+} is the distribution coefficient of the charged form of a basic drug.

Much work has been done studying the pH-dependent drug–membrane binding and lipid-mediated pK_a shifts of drugs, especially anesthetics, including tetracaine [13–15], which is used in this work. pH-dependent partitioning of charged solutes into liposomes has been determined using pH-metric, ultrafiltration, and equilibrium dialysis methods [16–19]. Additionally, authors have examined the differences between liposome–water and octanol–water partitioning as a function of pH, where the octanol–water partition coefficient is the standard model for drug lipophilicity [17]. The use of LEKC to study electrostatic interactions influencing charged drug partitioning into liposomes has been discussed in a previous paper [20]. This work included studies on the effect of membrane and buffer compositions on the retention of basic drugs. Recently, LEKC has been used in QSAR correlations with membrane permeability (Caco-2, MDCK, human jejunal) and intestinal absorption of a series of charged and neutral drugs including the influence of pH on these QSARs [21].

LEKC is a capillary electrophoresis (CE) technique that uses phospholipid vesicles as a pseudostationary phase in CE. In LEKC, solutes interact with certain sites on the pseudostationary phase by hydrophobic and/or electrostatic interactions and are separated based on their differential partitioning into the liposome phase [9,10,22–25]. The retention factor, *k*, represents the fraction of the solute in the liposome versus the aqueous phase, which is used to describe the degree of interaction with the liposome.

Determining distribution coefficients for the binding of charged solutes to liposomes can be accomplished via LEKC much in the same manner as it is done for micelles via micellar electrokinetic chromatography (MEKC) [26,27]. The exception is that liposomes constitute the pseudostationary phase instead of micelles. LEKC is a simple approach to determining liposome–water partition coefficients for small organic molecules [9] and drugs compounds [28].

In this work the pH-dependent affinity for net negatively charged liposomes was determined for a series of basic drugs using LEKC methods. Quantitative models were applied to examine the mobility and retention as a function of drug ionization.

2. Materials and methods

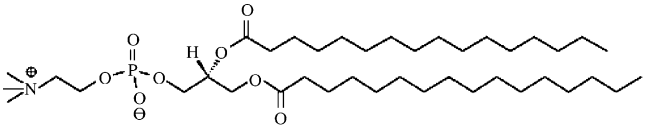
2.1. Reagents

2-(*N*-Morpholino)ethanesulfonic acid (MES), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), and tris(hydroxymethyl)aminopropane (Tris) were purchased from Sigma (St. Louis, MO, USA). Tetracaine, nefopam, and lidocaine were purchased from Aldrich. 1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (sodium salt) (DPPG), were obtained from Avanti Polar Lipids (Alabastar, AL).

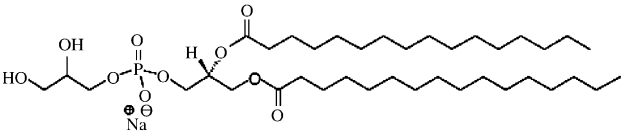
2.2. Liposome preparation

A mixed buffer was used for the CE studies in order to maintain a high buffer capacity and constant ionic strength across the pH range 6–11.5. The importance of using a constant ionic strength buffer in LEKC studies involving charged drugs is discussed in reference [20]. Buffers containing multiple components and constant ionic strengths were prepared according to the software program developed by Okamoto, which is used to determine the quantity of various buffer components required to achieve a certain pH at given ionic strength and temperature conditions [29]. A description and application of the buffer program is found in reference [29] by Okamoto who has graciously donated a copy of the software. The desired buffer conditions (buffer type, concentration, total solution ionic strength, pH, and temperature) were entered into the program, and the software program determined the quantities of buffer components (buffer amounts and NaCl) as well as acid–base (HCl or NaOH) required to prepare the buffer at the specified conditions. All buffers consisted of 10 mM MES, 10 mM Tris, and 10 mM CAPS. The total ionic strength of all buffers was held constant at 29 mM by the addition of NaCl; the quantity of NaCl added was determined by the buffer program. All buffers were prepared and the pH was measured at 36 °C, the temperature for CE studies. Buffers were prepared to cover the pH range of 6–11.5.

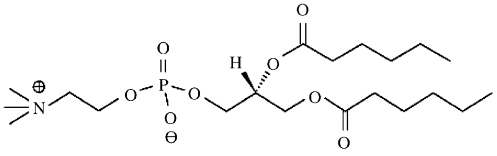
Liposomes for use in CE experiments were prepared using a mixture of the short and long chain phosphatidylcholines, DHPC and DPPC, respectively, (both zwitterionic lipids) and the long chain anionic phospholipid, DPPG. DPPG was added to provide a net negative charge to the liposomes.



1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)



1,2-Dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (DPPG)



1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC)

Fig. 2. Phospholipid structures.

Phospholipid structures are shown in Fig. 2. The composition consisted of $DPPG_{20}DPPC_{30}DHPC_{50}$, where the subscripts represent molar percentages. The total lipid concentration was 10 mM. Similarly, mixtures of the short chain DHPC and long chain dimyristoylphosphatidylcholine (DMPC) have been used as a pseudostationary phase in electrokinetic chromatography [30]. Mixtures of short and long chain lipids are known to form bicelles, or biomimetric vesicles [31,32]. The lipid solution consisting of DPPG, DPPC, and DHPC in this work is referred to as "liposomes" even though it has not been verified whether it is actually mixed micelles, bicelles, or liposomes in solution. Bicelles form under very specific of conditions including concentration, molar ratio of lipids, buffer, temperature, etc. [33].

The appropriate amounts of phospholipids were dissolved in a 9:1 (v/v) mixture of chloroform and methanol (respectively). The organic solvent was removed under reduced pressure using a rotary evaporator in a water bath maintained at 70 °C. The thin lipid film was hydrated with the buffer solution. The solution was then vortex mixed for one minute and sonicated in a bath sonicator for approximately 5 min before use.

2.3. CE methods

CZE and LEKC experiments were carried out on a laboratory-built CE instrument. A Spellman SL30 high-voltage power supply was used to apply a positive voltage over the length of the fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA), with an inner diameter of 50 μm and an outer diameter of 375 μm. The temperature of the system was maintained at 36 °C using a circulating oil bath. The absorbance was measured at 214 nm using a SSI 500 variable-wavelength UV detector.

The retention factor, k, for a neutral drug was calculated from the LEKC data using Eq. (1), where t_R is the retention time of the drug of interest, t_{eo} is the retention time of the electroosmotic flow marker, methanol, and t_{lip} is the retention time of decanophenone, the marker of the liposomes.

$$k = \frac{(t_R - t_{eo})}{t_{eo}(1 - (t_R/t_{lip}))} \quad (1)$$

Charged solutes will possess their own electrophoretic mobility in the aqueous phase in addition to partitioning into the liposomes and migrating at the liposome mobility. As a result, the migration of the solutes in the bulk aqueous (t_o, measured in CZE), needs to be included in the calculation of retention factor. Eq. (2) is used to calculate the retention factors of charged solutes using CZE and LEKC measured retention times.

$$k = \frac{(t_R - t_o)}{t_o(1 - (t_R/t_{lip}))} \quad (2)$$

The capillary was conditioned in the following manner: 10 min with Milli-Q water; 20 min with 1.0 M NaOH; 10 min with Milli-Q water; 10 min with methanol; 10 min with Milli-Q water. For LEKC experiments, the capillary was rinsed for 30 min with the liposome solution following the capillary conditioning. Following this rinse procedure, a voltage was applied for approximately 30 min to further equilibrate the column with the liposomes before sample injections were performed. At the end of the day, the capillary was rinsed for 10 min with Milli-Q water.

Since the rinse procedure has not yet been optimized, the conditions used in these experiments were longer than necessary to insure complete equilibration. The rinse procedure as described above could be shortened upon optimization. Additionally, liposomes do not need to be prepared immediately prior to use. In order to avoid the daily preparation time before running experiments, the liposomes can be prepared ahead of time and stored in the refrigerator for later use. Optimizing the rinse procedure and preparing liposomes ahead of time will significantly reduce the time required to prepare for data analysis.

As mentioned above and described by Eq. (2), in order to determine retention factor, k, for charged solutes in LEKC, the migration times, t_R, in the presence of liposome (LEKC condition) and in the absence of liposome, t_o (CZE condition) have to be determined. All CZE and LEKC solute mobility and retention factor values are the average of four measurements. The CZE data for each sample was collected immediately following the LEKC data after rinsing the capillary for 2 min with the buffer solution (i.e. in the absence of liposomes).

All buffers, liposomes, and solutions used for rinsing were filtered through a 0.45 μm filter disk (Scientific Resources)

prior to use. The capillary was rinsed with the liposome solution for 1–2 min between LEKC injections. Approximately 0.02–0.06 g of each solute was dissolved in 3 mL of methanol to prepare stock solutions. Drugs for LEKC experiments are tetracaine, nefopam, and lidocaine. Drug structures can be found in Figs. 7–9. To prepare a sample for injection, approximately 50–200 μL of the stock sample was used, decanophenone dissolved in methanol was added where appropriate, and enough methanol to make 1–1.5 mL total sample volume. Generally mixtures of samples were injected for 1–2 s by hydrodynamic injection.

Retention factor in LEKC is directly related to the liposome–water partition coefficient, K_{LW} as in Eq. (3). Similarly, the liposome–water distribution coefficient (D_{LW}) can be substituted for K_{LW} in Eq. (3).

$$K_{LW} = \frac{k}{\phi^{LEKC}} \tag{3}$$

The LEKC phase ratio, Φ^{LEKC}, is defined as the ratio of the volume of the liposome pseudo-phase (V_{lip}) over that of the aqueous phase (V_{aq}) and can be determined from the intrinsic properties of the phospholipids such as molar volume (v), critical aggregation concentration (CAC), and phospholipid concentration (C_{PL}) using the following relationship (Eq. (4)):

$$\phi^{LEKC} = \frac{V_{lip}}{V_{aq}} = \frac{v(C_{PL} - CAC)}{1 - v(C_{PL} - CAC)} \tag{4}$$

0.554 L mol^{-1} was used as an estimate of the partial specific molar volume of the lipid solution (v), which was obtained as the weighted sum of the partial specific volumes of the individual lipids. This is similar to the estimation of partial specific molar volume in reference 25. Values of v_{DPPG}, v_{DPPC}, and v_{DHPC} were taken as 1.01, 0.954, and 0.851 mL g^{-1}, for DPPG, DPPC, and DHPC, respectively [34]. A value of zero was used for the CAC.

3. Results and discussion

3.1. LEKC experiments

LEKC is a method that allows the rapid determination of liposome–water partition and distribution coefficients of neutral or charged drugs. This approach can be applied to determine the pH-dependent partitioning behavior of ionizable drugs where the migration parameters (retention factor or mobility) are measured as a function of varying aqueous buffer pH values. Applying quantitative models to the LEKC data allows very specific assessment of drug–membrane interactions as a function of drug ionization and pH. Models have previously been developed in this laboratory to quantitatively describe the migration (retention factor and mobility) of ionizable compounds in MEKC [26,27]. These universal methods can be applied to the description of retention in LEKC.

The retention factor (k) of a basic drug is the weighted average of the retention factors of the charged and neutral forms, described by Eq. (5). α^{aq}_{BH+} and α^{aq}_{B} are the fractions of the charged and neutral drug forms in the aqueous phase, respectively. k_{BH^+} and k_B are the limiting retention factors of the fully charged and neutral forms, respectively.

$$k = \alpha^{aq}_{BH^+} k_{BH^+} + \alpha^{aq}_{B} k_B \tag{5}$$

Using Eq. (5), one can derive the following equation (Eq. (6)), which is used to model the retention factor of a basic drug as a function of pH.

$$k = \frac{k_{BH^+} + k_B(K_{a,aq}/[H^+])}{1 + (K_{a,aq}/[H^+])} \tag{6}$$

$K_{a,aq}$ is the aqueous ionization constant of the drug in the absence of the liposomes, as noted in Fig. 1. The sigmoidal retention profile (versus pH) reaches a plateau at low pH where the limiting retention factor is determined (i.e. this value of k_{BH^+} is the retention factor of the fully ionized drug). Likewise, there is a plateau region at high pH where k_B is the limiting retention factor for the fully ionized form of the drug.

Similar to Eq. (6), the sigmoidal relationship between distribution coefficient and pH is given by Eq. (7), where D_{LW} is the observed liposome–water distribution coefficient; K_{BH^+} and K_B are the limiting distribution coefficients of the fully protonated and neutral drug forms, respectively.

$$D_{LW} = \frac{K_{BH^+} + K_B(K_{a,aq}/[H^+])}{1 + (K_{a,aq}/[H^+])} \tag{7}$$

Fig. 3 illustrates the effect of the buffer pH on the distribution coefficients of the three basic drugs tetracaine (▲), nefopam (●), and lidocaine (♦). The symbols are the measured D_{LW} values and the lines are the calculated values according to Eq. (7). The regression results for the fit of the data in Fig. 3 (using Eq. (7)) are given in Table 1.

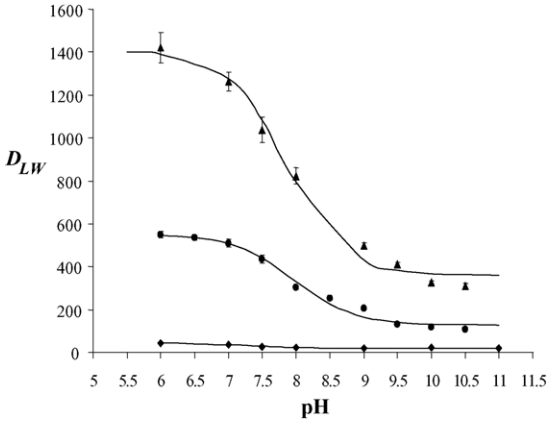


Fig. 3. Distribution coefficient of tetracaine (▲), nefopam (●), and lidocaine (♦) as a function of pH. Symbols represent measured K_{LW} values and lines are the fit of the data using Eq. (7). Distribution coefficients were measured using 10 mM $DPPG_{20}DPPC_{30}DHPC_{50}$ at 36 °C in a buffer consisting of 10 mM each of MES, Tris, and CAPS with a total ionic strength of 29 mM.

Table 1
Estimates of partition coefficients (K_{BH^+}, K_B) and p$K_{a,aq}$ (±standard deviation) for tetracaine, nefopam, and lidocaine and the R^2 value from the nonlinear regression determined using Eq. (7)

Drug name	K_{BH^+}	K_B	p$K_{a,aq}$	R^2
Tetracaine	1406 (±47)	360 (±29)	7.9 (±0.1)	0.990
Nefopam	549 (±16)	129 (±13)	8.0 (±0.1)	0.986
Lidocaine	46 (±2)	20 (±1)	7.2 (±0.2)	0.982

Partition coefficients were measured using 10 mM $DPPG_{20}DPPC_{30}DHPC_{50}$ at 36 °C in a buffer consisting of 10 mM each of MES, Tris, and CAPS with a total ionic strength of 29 mM.

The differences in partitioning of the three drugs can be explained by examining a combination of drug mobility in the presence and absence of the liposomes, along with the fractions of B and BH^+ associated with the lipid and aqueous phases. These fractions of association (f) were calculated using Eqs. (8)–(12), derived much like those in ref. [26] for acidic solutes in MEKC. The derivation is described briefly below.

The fraction of the neutral drug (B) in the lipid phase is given by the concentration of the neutral drug in the lipid phase ($[B]_{lip}$) over the total concentration of drug. The total concentration of the drug includes the concentration of the charged and neutral drug in the lipid phase ($[BH^+]_{lip}$ and $[B]_{lip}$, respectively), and the concentration of the charged and neutral drug in the aqueous phase ($[BH^+]_{aq}$ and $[B]_{aq}$, respectively). This relationship is given in Eq. (8).

$$f_B^{lip} = \frac{[B]_{lip}}{[B]_{lip} + [B]_{aq} + [BH^+]_{lip} + [BH^+]_{aq}} \quad (8)$$

Substituting $K_{b,B}{}^{lip}[B]_{aq}C_{PL}$ for $[B]_{lip}$, $K_{b,BH^+}[BH^+]_{aq}C_{PL}$ for $[BH^+]_{lip}$, and $[B]_{aq}[H^+]/K_{a,aq}$ for $[BH^+]_{aq}$, results in Eq. (9), which is used to calculated the fraction of B in the lipid phase (f_B^{lip}) as a function of aqueous pH, where values of $K_{b,B}$ and K_{b,BH^+} are the limiting binding constant values (Table 2), described below; $K_{a,aq}$ is from Table 1; C_{PL} is 10 mM.

$$f_B^{lip} = \frac{K_{b,B}C_{PL}}{(1 + K_{b,B}C_{PL}) + ([H^+]/K_{a,aq})(1 + K_{b,BH^+}C_{PL})} \quad (9)$$

Likewise, the fraction of the charged drug (BH^+) in the lipid phase is given by the concentration of the charged drug in the lipid phase over the total concentration of drug. After the same substitutions, Eq. (10) is derived which is used to calculate the fraction of BH^+ in the lipid phase ($f_{BH^+}^{lip}$) as a function of pH.

$$f_{BH^+}^{lip} = \frac{(K_{b,BH^+}C_{PL})([H^+]/K_{a,aq})}{(1 + K_{b,B}C_{PL}) + ([H^+]/K_{a,aq})(1 + K_{b,BH^+}C_{PL})} \quad (10)$$

Similarly, the fraction of charged drug (BH^+) in the aqueous phase is given by the concentration of the charged drug in the aqueous phase over the total drug concentration. Using Eq. (11), the fraction of the charged drug, BH^+, in the aqueous phase ($f_{BH^+}^{aq}$) as a function of pH can be determined.

$$f_{BH^+}^{aq} = \frac{([H^+]/K_{a,aq})}{(1 + K_{b,B}C_{PL}) + ([H^+]/K_{a,aq})(1 + K_{b,BH^+}C_{PL})} \quad (11)$$

The fraction of the neutral drug, B in the aqueous phase (f_B^{aq}) is given by Eq. (12).

$$f_B^{aq} = 1 - f_B^{lip} - f_{BH^+}^{lip} - f_{BH^+}^{aq} \quad (12)$$

According to ref. [35], the binding constant of a solute to liposomes (K_b) is directly related to the liposome–water partition coefficient (K_{LW}) as in Eq. (13) [35]; v is the molar volume of the lipids. Converting K_{LW,BH^+} and $K_{LW,B}$ (Table 1), to their respective binding constants (according to Eq. (13)) results in the values of K_{b,BH^+} (binding constant of the charged form) and $K_{b,B}$ (binding constant of the neutral form) listed in Table 2. Values of K_{b,BH^+} and $K_{b,B}$ were used in the above equations (Eqs. (9)–(12)) to calculate the fraction of association.

$$K_b = K_{LW}v \quad (13)$$

Figs. 4–6 show the various fractions of the neutral and charged form of tetracaine, nefopam, and lidocaine, respectively, in the lipid and aqueous phases. Solid lines represent the fraction of the drug in the lipid phase, and dashed lines represent the fraction in the aqueous phase. The neutral form (B) is represented by circles (●), and the ionized form (BH^+) is represented by triangles (▲).

Equations can also be used to describe the mobility behavior of charged drugs in the presence and absence of the lipids [26,27]. In a CZE system (in the absence of the liposomes), the mobility of a charged solute in the aqueous phase is given by Eq. (14), where μ_0 is the observed mobility of the solute, μ_{BH^+} is the mobility of the fully protonated cation, while $[H^+]$ is from the aqueous buffer pH, and $K_{a,aq}$ is the dissociation constant of the solute in the aqueous phase. All mobilities are calculated from the measured retention times, capillary length and voltage used in the CE experiments. Regression results of the CZE mobility data using Eq. (14) are given in Table 3.

$$\mu_0 = \frac{\mu_{BH^+}([H^+]/K_{a,aq})}{1 + ([H^+]/K_{a,aq})} \quad (14)$$

Table 2
Values of binding constants (K_{b,BH^+} and $K_{b,B}$) for tetracaine, nefopam, and lidocaine determined using Eq. (13), using the limiting distribution coefficient values in Table 1

Drug name	K_{b,BH^+}	$K_{b,B}$	ΔpK_a
Tetracaine	778 (±26)	199 (±16)	0.47
Nefopam	304 (±9)	71 (±7)	0.37
Lidocaine	26 (±1)	11 (±1)	0.05

ΔpK_a values are calculated according to Eq. (17). Distribution coefficients were measured using 10 mM $DPPG_{20}DPPC_{30}DHPC_{50}$ at 36 °C in a buffer consisting of 10 mM each of MES, Tris, and CAPS with a total ionic strength of 29 mM.

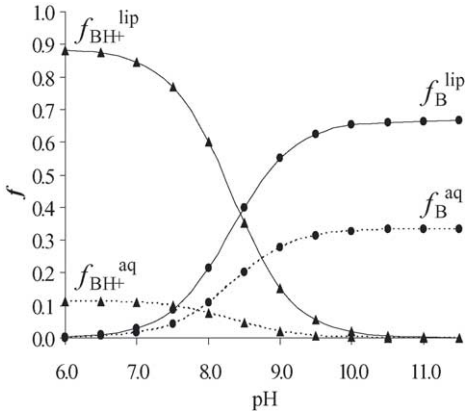


Fig. 4. Fractions of the ionized (▲) and neutral (●) forms of tetracaine in the liposome and aqueous phases. Solid lines represent the fraction of the drug in the liposome phase (Eqs. (9) and (10)), and dashed lines represent the fraction in the aqueous phase (Eqs. (11) and (12)). Distribution coefficients were measured with 10 mM $DPPG_{20}DPPC_{30}DHPC_{50}$ at 36 °C in a buffer consisting of 10 mM each of MES, Tris and CAPS with a total ionic strength of 29 mM. Values of $K_{b,B}$ and K_{b,BH^+} used to calculate the curves are from Table 2; $K_{a,aq}$ is from Table 1; C_{PL} is 10 mM.

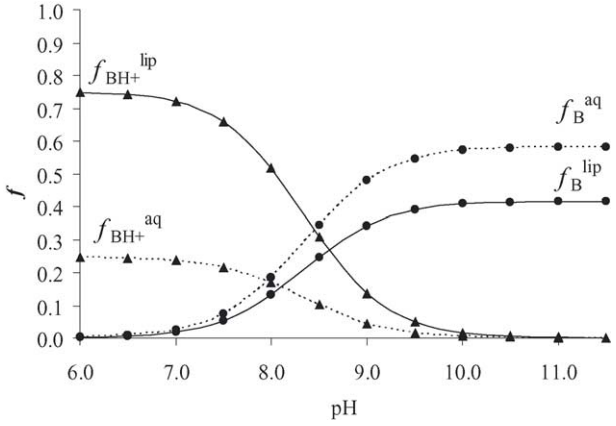


Fig. 5. Fractions of the ionized (▲) and neutral (●) forms of nefopam in the liposome and aqueous phases. Solid lines represent the fraction of the drug in the liposome phase (Eqs. (9) and (10)), and dashed lines represent the fraction in the aqueous phase (Eqs. (11) and (12)). Distribution coefficients were measured with 10 mM $DPPG_{20}DPPC_{30}DHPC_{50}$ at 36 °C in a buffer consisting of 10 mM each of MES, Tris, and CAPS with a total ionic strength of 29 mM. Values of $K_{b,B}$ and K_{b,BH^+} used to calculate the curves are from Table 2; $K_{a,aq}$ is from Table 1; C_{PL} is 10 mM.

Table 3
Estimates of μ_{BH^+} (±standard deviation) and $pK_{a,aq}$ for tetracaine, nefopam, and lidocaine, and the R^2 value from the nonlinear regression of the CZE mobility data using Eq. (15)

Drug name	μ_{BH^+} ($cm^2\ kV^{-1}\ min^{-1}$)	$pK_{a,aq}$	R^2
Tetracaine	12.7 (±0.2)	8.48 (±0.04)	0.995
Nefopam	14.2 (±0.3)	8.17 (±0.05)	0.993
Lidocaine	13.3 (±0.3)	7.80 (±0.05)	0.994

Drug mobility values were measured at 36 °C in a buffer consisting of 10 mM each of MES, Tris, and CAPS with a total ionic strength of 29 mM.

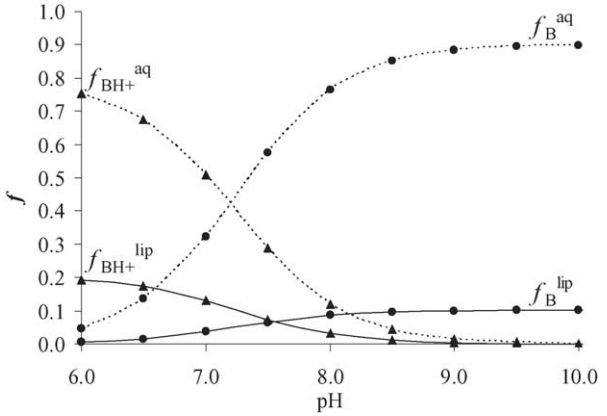


Fig. 6. Fractions of the ionized (▲) and neutral (●) forms of lidocaine in the liposome and aqueous phases. Solid lines represent the fraction of the drug in the liposome phase (Eqs. (9) and (10)), and dashed lines represent the fraction in the aqueous phase (Eqs. (11) and (12)). Distribution coefficients were measured with 10 mM $DPPG_{20}DPPC_{30}DHPC_{50}$ at 36 °C in a buffer consisting of 10 mM each of MES, Tris, and CAPS with a total ionic strength of 29 mM. Values of $K_{b,B}$ and K_{b,BH^+} used to calculate the curves are from Table 2; $K_{a,aq}$ is from Table 1; C_{PL} is 10 mM.

The net mobility of the drug in LEKC is a weighted average of the mobility of the charged (BH^+) and neutral (B) drug in the aqueous (aq) and the lipid (lip) phase and is described by Eq. (15).

$$\mu = f_{BH^+}^{aq}\mu_{BH^+}^{aq} + f_{BH^+}^{lip}\mu_{lip} + f_B^{lip}\mu_{lip} \quad (15)$$

μ is the observed mobility, $f_{BH^+}^{aq}$ and $f_{BH^+}^{lip}$ are the fractions of the charged drug (BH^+) associated with the aqueous (aq) and lipid (lip) phases, respectively. f_B^{lip} is the fraction of the neutral drug form in the lipid phase. $\mu_{BH^+}^{aq}$ is the aqueous mobility of the drug and μ_{lip} is the mobility of the liposomes. In the neutral form, the drug mobility is a result of its interaction with the lipids. The neutral form has zero mobility in the aqueous phase. The liposomes used in this work ($DPPG_{20}DPPC_{30}DHPC_{50}$) have a net negative charge, resulting in a negative mobility in LEKC. In the charged form, the drug mobility is a function of its interaction with the liposomes as well as its own aqueous mobility.

Using Eq. (15), one could derive the following equation (Eq. (16)) to model the LEKC mobility of a basic drug as a function of pH.

$$\mu = \frac{\mu_B + \mu_{BH^+}([H^+]/K_{a,app})}{1 + ([H^+]/K_{a,app})} \quad (16)$$

In this equation, μ is the observed LEKC mobility of a basic drug at a given $[H^+]$. μ_B and μ_{BH^+} are the limiting mobilities of the neutral and protonated forms of the drug, respectively. $K_{a,app}$ is the apparent ionization constant of the drug in the presence of the lipids. Regression results for the LEKC mobility as a function of pH (using Eq. (16)) are given in Table 4.

The mobility of tetracaine as a function of pH in the presence (●) and absence (♦) of $DPPG_{20}DPPC_{30}DHPC_{50}$ (subscripts represent molar percentage) is shown in Fig. 7. The

Table 4
Estimates of μ_{BH^+}, μ_B, (±standard deviation) and p$K_{a,app}$ of tetracaine, nefopam, and lidocaine and the R^2 value from the nonlinear regression of the LEKC mobility data using Eq. (17)

Drug name	μ_{BH^+} (cm^2 kV^{-1} min^{-1})	μ_B (cm^2 kV^{-1} min^{-1})	p$K_{a,app}$	R^2
Tetracaine	−15.8 (±0.3)	−12.2 (±0.2)	8.6 (±0.2)	0.961
Nefopam	−12.3 (±0.2)	−7.9 (±0.2)	8.5 (±0.2)	0.978
Lidocaine	6.1 (±0.1)	−2.2 (±0.1)	8.02 (±0.03)	0.999

LEKC drug mobility values were measured using 10 mM $DPPG_{20}DPPC_{30}DHPC_{50}$ at 36 °C in a buffer consisting of 10 mM each of MES, Tris, and CAPS with a total ionic strength of 29 mM.

structure of tetracaine is included in the figure as well. The corresponding plots for nefopam and lidocaine are given in Figs. 8 and 9, respectively. The symbols are the measured data points and the lines are the calculated mobility values in the presence and absence of $DPPG_{20}DPPC_{30}DHPC_{50}$ lipids, according to Eqs. (16) and (14), respectively. Error bars are included for all data points, however, due to the scaling, the bars are generally not seen since they are smaller than size of the symbols.

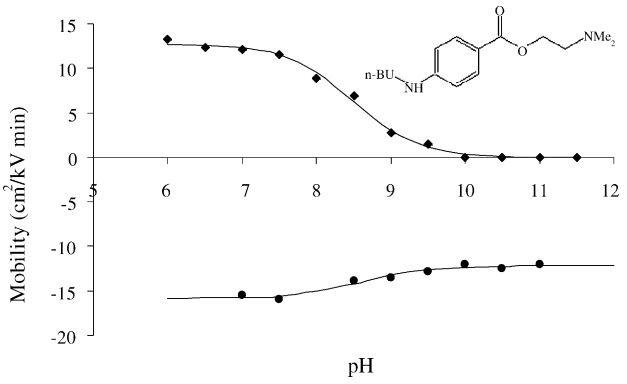


Fig. 7. Mobility of tetracaine as a function of pH in the presence (●) and absence (♦) of $DPPG_{20}DPPC_{30}DHPC_{50}$. Symbols are the measured mobilities and solid lines are the fit of the data according to Eqs. (14) and (16). Drug mobility values were measured in the presence and absence of 10 mM $DPPG_{20}DPPC_{30}DHPC_{50}$ at 36 °C in a buffer consisting of 10 mM each of MES, Tris, and CAPS with a total ionic strength of 29 mM.

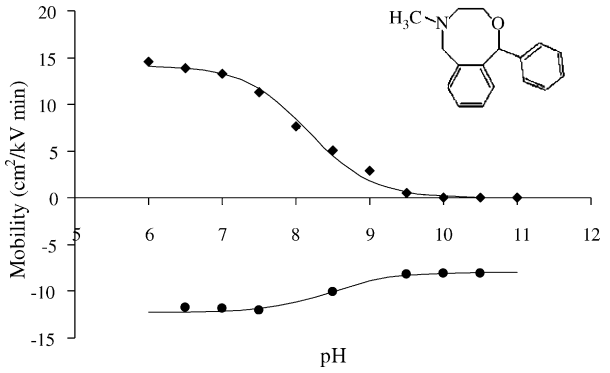


Fig. 8. Mobility of nefopam as a function of pH in the presence (●) and absence (♦) of $DPPG_{20}DPPC_{30}DHPC_{50}$. Symbols are the measured mobilities and solid lines are the fit of the data according to Eqs. (14) and (16). Drug mobility values were measured in the presence and absence of 10 mM $DPPG_{20}DPPC_{30}DHPC_{50}$ at 36 °C in a buffer consisting of 10 mM each of MES, Tris, and CAPS with a total ionic strength of 29 mM.

Simultaneously examining the three types of plots discussed above (retention versus pH, fraction of association versus pH, mobility versus pH) provides a better understanding of the partitioning behavior of the three drugs. For the lipid composition used in these studies, $DPPG_{20}DPPC_{30}DPHC_{50}$ (i.e. possessing a net negative charge), the basic drugs exhibit a sigmoidal relationship between K_{LW} and pH (Fig. 3), such that they have a larger distribution coefficient at low pH, which decreases as the pH is increased (i.e. $K_{BH^+} > K_B$). At low pH when the drugs are protonated, they have a greater interaction with the lipids compared to the interaction of the drug in the neutral form. The enhanced retention at low pH values is due to the electrostatic attraction of the positively charged drug to the net negatively charged lipid bilayer membrane. Electrostatics plays a significant role in the interactions of charged drugs with membranes which is discussed further in ref. [20].

Out of the three drugs illustrated in Fig. 3, the distribution coefficients (at all pH values) decrease in the order tetracaine > nefopam > lidocaine. The largest difference in distribution coefficient between the three drugs is at the low pH values, when the drugs are in the completely protonated form. According to Eq. (7), the values for the limiting distribution coefficients (listed in Table 1) of the charged form (K_{BH^+}), are 1406 (±47), 549 (±16), and 46 (±2) for tetracaine, nefopam, and lidocaine, respectively. As seen in the fraction plots for tetracaine and nefopam (Figs. 4 and 5), at the lower

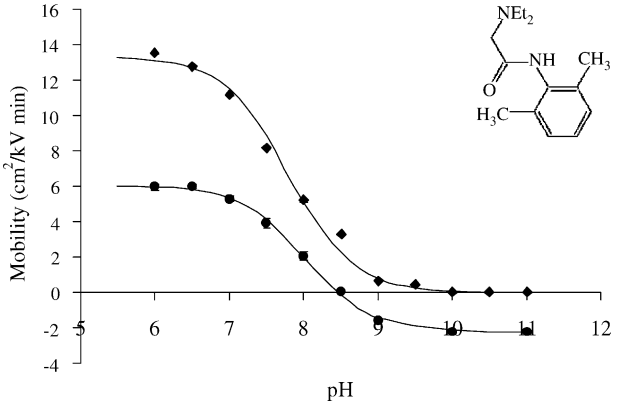


Fig. 9. Mobility of lidocaine as a function of pH in the presence (●) and absence (♦) of $DPPG_{20}DPPC_{30}DHPC_{50}$. Symbols are the measured mobilities and solid lines are the fit of the data according to Eqs. (14) and (16). Drug mobility values were measured in the presence and absence of 10 mM $DPPG_{20}DPPC_{30}DHPC_{50}$ at 36 °C in a buffer consisting of 10 mM each of MES, Tris, and CAPS with a total ionic strength of 29 mM.

pH values there is a significant fraction of the charged drug associated with the lipids. There is only a very small fraction of the drug associated with the aqueous phase at low pH (pH 6). Lidocaine is quite different due to its very small interaction with the liposomes. The lower retention of lidocaine (seen in Fig. 3) can be explained by the larger fraction of charged lidocaine residing in the aqueous as opposed to the lipid phase (Fig. 6). There is only a small fraction of BH^+ for lidocaine associated with the lipids at pH 6.

The difference in distribution coefficients (among the three drugs) is much smaller at high pH values (pH 11) when the drug is completely in the non-protonated (neutral) form. Values of K_B are 360 (±29), 129 (±13), and 20 (±1) for tetracaine, nefopam, and lidocaine, respectively (Table 1). For nefopam, the fractions of the neutral form of the drug in the aqueous and lipid phase are fairly close at high pH. For tetracaine, there is a significant difference, with a much greater fraction of the neutral drug (B) associated with the lipids. Tetracaine also has a larger distribution coefficient than nefopam at high pH (Fig. 3). For the high pH values, the neutral lidocaine is almost exclusively in the aqueous. There is only a very small fraction of neutral lidocaine associated with the liposomes. As a result, this drug has the least amount of retention in the liposomes.

The mobility of tetracaine in the absence of the liposomes (CZE) is positive at low pH values and sigmoidally decreases to zero as the pH increases and the equilibrium shifts to the neutral drug form, which has zero mobility in CZE (Fig. 7). This is the typical migration profile for a basic drug in CE. The mobility of tetracaine in the presence of the liposomes (LEKC) is negative both at low and high pH due to a large interaction with the lipids (see Fig. 4). The liposomes have a large negative mobility ($-19.51\,cm^2\,kV^{-1}\,min^{-1}$ at pH 7.0), and therefore, once the drug partitions into the liposomes, it will travel at this negative liposome mobility. The mobility sigmoidally decreases (becomes less negative) as the pH increases. The limiting mobilities of tetracaine in LEKC, μ_B and μ_{BH^+} are −12.2 (±0.2) and −15.8 (±0.3) $cm^2\,kV^{-1}\,min^{-1}$, respectively, calculated according to Eq. (16). In this case, there is only a small difference in mobility between the charged and neutral forms of the drug because both forms interact with the lipids significantly, as seen in the corresponding fraction plot, Fig. 4. In the low pH range, the first two terms in Eq. (15) are the dominant ones, while the third term dominates at high pH values. For tetracaine, $f^{aq}_{BH^+}$ is small (see Fig. 4), therefore, $f^{lip}_{BH^+}$ is the dominant term, and the mobility at low pH is primarily due to the interaction with the liposomes (and hence, the mobility of the liposome).

The mobility of nefopam as a function of pH in the presence (●) and absence (♦) of the liposomes, and the structure of nefopam is seen in Fig. 8. Nefopam has a similar profile to tetracaine (Fig. 7). Nefopam has a slightly reduced negative mobility in the presence of the liposomes compared with tetracaine. The limiting mobilities of nefopam in LEKC, μ_B and μ_{BH^+} are −7.9 (±0.2) and −12.3 (±0.2) $cm^2\,kV^{-1}\,min^{-1}$, respectively (Eq. (16), Table 4). Again, $f^{lip}_{BH^+}$ is very important at low pH.

The mobility profile of lidocaine as a function of pH in the presence (●) and absence (♦) of liposomes is different than tetracaine and nefopam and is displayed in Fig. 9. Lidocaine has a very small interaction with the lipids (see Fig. 3). The overall mobility observed in Fig. 9 is mostly due to the drug mobility in the aqueous phase (as seen in Fig. 6). In this case (contrary to tetracaine and nefopam), $f^{aq}_{BH^+}$ is the dominant term in Eq. (15) at low pH. The limiting mobility of lidocaine in LEKC, is −2.2 (±0.1) and 6.1 (±0.1) $cm^2\,kV^{-1}\,min^{-1}$, for μ_B and μ_{BH^+}, respectively (Eq. (16)). The difference in mobility (between CZE and LEKC) is greater at the lower pH values when the drug is in the protonated form. At high pH, there is only a small fraction of B associated with the lipids (f^{lip}_B is small), therefore, the time the drug is traveling at the mobility of the liposome (μ_{lip}) is very small (see Eq. (15)). Thus the mobility of lidocaine in LEKC at high pH is close to zero, because the neutral form has zero mobility in CE.

Austin et al. studied the distribution coefficients of charged drugs into the zwitterionic DMPC as a function of pH using ultrafiltration methods [17]. They found a sigmoidal relationship between distribution coefficient and pH, however, they found larger partitioning of the neutral form of the drug into the net neutral phospholipid liposomes. Similarly, Avdeef et al. determined membrane–water distribution coefficients for basic drugs (including lidocaine and tetracaine) into zwitterionic liposomes of dioleyphosphatidylcholine (DOPC) using the pH-metric technique [18]. A sigmoidal partitioning-pH profile is observed, with the charged drug partitioning to a lesser extent into the zwitterionic membranes than the neutral drug form. The authors rationalized these findings reporting that an uncharged amphiphilic species will be favorably bound to the membrane if the hydrophobic portion is embedded in the interior of the lipid bilayer, while the polar headgroup is oriented towards the membrane surface. An electrostatic pairing of the positively charged drug and the negatively charged phosphate group in the headgroup region of the lipid bilayer would require a movement away from the bilayer interior and cause the drug to reposition in the bilayer, moving to a less bound position. This is not surprising since the protonated form (BH^+) has an overall larger polarity than the unprotonated form (B); thus has smaller affinity towards the neutrally charged medium of DOPC bilayers. In fact, a similar behavior is observed for partitioning into octanol; that is drugs ionization leads to smaller partitioning into octanol. The situation, however, is different in natural membranes that possess a net negative charge due to the presence of anionic lipids.

The partitioning profiles obtained for PC membranes discussed above are quite different than those presented in this work for the net negatively charged $DPPG_{20}DPPC_{30}DHPC_{50}$ membranes (membranes containing PG). In this work, the electrostatic pairing of the charged drug with the net negatively charged membrane is stronger than the partitioning of

the neutral drug form. The result is a greater retention factor at low pH (Fig. 3). Kramer et al. reported on the pH dependent interactions of the basic drug propranolol in membranes composed of phosphatidylcholine (PC) and mixtures of PC and the anionic lipid, phosphatidylinositol (PI) using equilibrium dialysis [36]. The authors reported the neutral propranolol is more strongly attracted to the PC membranes (net zero charge) than the protonated drug. In contrast, the protonated propranolol has a larger affinity for the PI containing membranes than the neutral form of the drug [36]. The results of Kramer for PC/PI mixed membranes is in agreement with the results presented here for mixed DPPC/DPPG membranes.

Much like the influence of pH on the retention and partitioning of charged drugs, pH will also significantly impact the membrane permeability of acidic and basic drugs. The relationship between permeability and pH is also sigmoidal in shape. Permeability coefficients of basic drugs through Caco-2 monolayers are small at low pH, and sigmoidally increase as the pH is increased, with the neutral form having a greater permeability coefficient. Palm et al. reported this for the cationic drugs cimetidine and alfentanil in the pH-dependent permeation through Caco-2 monolayers [37].

In general, the neutral form of drugs is thought to permeate through membranes to a greater extent than the charged form. Traditionally, the pH-partition theory has been applied to the transport of drugs across cell membranes. This idea relies on the assumption that only the unionized form of an ionizable drug is able to diffuse across the membrane. However, studies have shown that the contribution of the ionized form of the drug to membrane transport is significant when the drug has a fraction unionized less than 0.1 [37]. This is significant because many drugs will be fully ionized over the entire physiological pH range. Similar to the transport of the drug across membranes, the partitioning of the ionized form of the drug into charged membranes is significant.

3.2. Lipid-mediated pK_a shifts

The interactions of drugs with lipid bilayers alter their acid–base properties, thus shifting their ionization constants. The apparent ionization constants in lipid solutions are different from those in a purely aqueous phase. The magnitude of the pK_a shift ($\Delta pK_a = pK_{a,app} - pK_{a,aq}$) depends on the difference of binding (i.e. partitioning) of the charged and uncharged forms of the drugs with the membranes. The pK_a of the drug in the lipid (p$K_{a,app}$) and in the aqueous phase (p$K_{a,aq}$) are labeled on Fig. 1. The magnitude of the shift in pK_a (ΔpK_a) is a function of various properties of the solute and properties of the lipid bilayer microenvironment where the solute resides, including the dielectric constant and surface potential. Drugs that reside deeper in the lipid bilayer will experience a lower dielectric constant and as a result, a greater shift in pK_a value. Additionally, the charged surface of liposomes influences the shift in pK_a.

When a drug interacts with a lipid bilayer, it experiences a significantly different microenvironment from the bulk aqueous. For example, estimates for the dielectric constant in the region of the phospholipid head-groups is about 32, compared with 78 in the bulk aqueous [38]. On the other end, the dielectric constant deep in the hydrocarbon core of the lipid bilayer is reported to be around 2 [38]. Therefore, a drug residing in this headgroup region will be in a very different dipolarity region than a drug residing the aqueous or one embedded in the bilayer.

The pH-metric titration method uses these principles of a shift in ionization constant to determine the distribution coefficients of charged drugs [18,19]. This pH-metric method involves a two-phase potentiometric titration where the drug substance is titrated both in the presence and absence of liposomes. The apparent pK_a in the presence of liposomes may deviate from the pK_a in the absence of liposomes based on the differential partitioning of the charged and neutral form into liposomes. This shift in ionization constant is used to calculate the distribution coefficient of the charged drug.

From the LEKC retention data, it is possible to examine the lipid-induced shift in ionization constant. MEKC has previously been used to determine the micellar-mediated shifts in ionization constants upon binding of amino acids and peptides [39]. Eq. (17) is used to determine the ΔpK_a from the binding constants. K_{b,BH^+} and $K_{b,B}$ are from Table 2 and C_{PL} is 10 mM. ΔpK_a values calculated with Eq. (17) are included in Table 2.

$$\Delta pK_a = \log \frac{1 + K_{b,BH^+} C_{PL}}{1 + K_{b,B} C_{PL}} \quad (17)$$

The ΔpK_a values obtained (from Eq. (17)) are 0.47, 0.37, and 0.05 for tetracaine, nefopam, and lidocaine, respectively.

Tetracaine has the largest retention with the liposomes, and the greatest ΔpK_a (from Eq. (17)). On the other hand, lidocaine has a much smaller overall partitioning, and therefore, has a ΔpK_a close to zero. The positive shift in pK_a as observed here is obtained when the charged form of the drug has a stronger binding than the neutral form. This is seen in Fig. 3 comparing the retention factors at low and high pH, as well as by examining the binding constants in Table 2.

Shifts in pK_a values ($\Delta pK_a = pK_{a,app} - pK_{a,aq}$) can be examined using p$K_{a,app}$ as the apparent pK_a in the liposomes, from the regression data included in Table 4. p$K_{a,aq}$ is the aqueous ionization constant determined from the nonlinear regression fit of the CZE data (Table 3). ΔpK_a values determined this way for tetracaine, nefopam, and lidocaine are 0.12, 0.33, and 0.22, respectively. These values are also positive, indicating a stronger binding of the charged drug form as previously discussed. However, the ΔpK_a values are smaller than those determined by Eq. (17). In this case, nefopam has a larger ΔpK_a value, due to a larger differential partitioning of the charged and neutral drug forms. The difference in $\log K_{BH^+}$ and $\log K_B$ for nefopam is greater than for tetracaine. Also, the difference between the fraction of charged and neutral nefopam in the liposomes is greater than for tetracaine.

There are good fits of the CZE mobility data; however, the fits are not as good for the LEKC mobility data. This is likely due to the small difference in mobility of the associated and dissociated forms especially of the more hydrophobic drugs, similar to the work reported in ref. [26]. On the other hand, the fits of the retention data is better. In this case, the fit of k versus pH is better for the more hydrophobic drugs due to the greater difference in partitioning of the two forms, and lidocaine with the smallest interaction has a small differential partitioning and the worst fit out of the three. The ΔpK_a should be the same as calculated with Eq. (17), however, it is likely the error associated with fitting the plots which causes the differences.

When a drug partitions into a liposome, it primarily experiences the headgroup region. The outer headgroup area of a liposome is very complex with an interfacial region spanning the bulk aqueous to the hydrocarbon interior of the lipid bilayer. In this interfacial region there is a significant change of physical and chemical properties with location in the bilayer. Therefore, a charged drug that electrostatically binds to a charged lipid headgroup might reside in a different location than the neutral form of the same drug which might find a position in the bilayer interior. In this case, the charged and neutral drug forms will experience different microenvironments. Therefore, drugs that penetrate the lipid bilayer headgroup region to different depths will consequently experience varied dielectric constants thus influencing their shifts in ionization constants.

4. Concluding remarks

The effect of the aqueous pH on the partitioning of basic drugs can easily be determined by LEKC methods. Basic drugs partitioning into net negatively charged liposomes have a sigmoidal decrease in retention with increasing pH. Applying quantitative models allowed an investigation of the contributions of the charged and neutral forms of the drug to partitioning by examining the fractions of each of these forms of the drug associated with the liposomes.

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