Interaction of Basic Drugs with Lipid Bilayers Using Liposome Electrokinetic Chromatography

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Purpose. This study explores factors influencing the interactions of positively charged drugs with liposomes using liposome electrokinetic chromatography (LEKC) for the development of LEKC as a rapid screening method for drug-membrane interactions.

Methods. Liposomes were prepared and the retention factors were measured for a series of basic drugs under a variety of buffer conditions, including various buffer types, concentrations, and ionic strengths as well as using different phospholipids and liposome compositions. LEKC retention is compared with octanol-water partitioning.

Results. The interaction of ionizable solutes with liposomes decreased with increasing ionic strength of the aqueous buffer. The type of buffer also influences positively charged drug partitioning into liposomes. Varying the surface charge on the liposomes by the selection of phospholipids influences the electrostatic interactions, causing an increase in retention with increasing percentages of anionic lipids in the membrane. Poor correlations are observed between LEKC retention and octanol-water partitioning.

Conclusions. These studies demonstrate the overall buffer ionic strength at a given pH is more important than buffer type and concentration. The interaction of positively charged drugs with charged lipid bilayer membranes is selectively influenced by the pK_a of the drug. Liposomes are more biologically relevant *in vitro* models for cell membranes than octanol, and LEKC provides a unique combination of advantages for rapid screening of drug-membrane interactions.

KEY WORDS: basic drugs; liposomes; octanol; partitioning.

INTRODUCTION

Partitioning of solutes (drugs, metabolites, toxins, etc.) into lipid bilayers of biological membranes plays a significant role in their uptake, transport, bioavailability, and distribution (1–23). As most drugs are administered orally, their ability to transport across the intestinal epithelium, a monolayer of cells that line the interior of the intestine, is an important issue. The primary mechanism of gastrointestinal absorption of the vast majority of drugs is believed to involve initial partitioning into cell membranes followed by passive transmembrane diffusion (4,5). Other factors such as dissolution of drugs in the gastrointestinal (GI) fluid, metabolism, and active transporters (such as P-glycoprotein) could also influence the intestinal absorption of certain drugs.

Assessment of absorption and distribution of numerous drug candidates in combinatorial libraries is a crucial step in the early stages of drug discovery (6). Recent advances of combinatorial chemistry in drug discovery have provided the opportunity of rapidly synthesizing staggering numbers of drug candidates. This, however, has created enormous challenges and the need for development of high-throughput screening (HTS) of physicochemical properties of potential drug candidates in compound libraries for characterization of pharmacokinetic properties that involve absorption-distribution-metabolism-excretion (ADME) (7,8). The most important solute property in such studies is lipophilicity, which is measured by a partition coefficient between an aqueous and organic phase. There exists a great deal of interest from both a scientific and practical standpoint in pharmaceutical research for a better understanding and quantification of solute partitioning between water and lipid bilayers of cell membranes.

Octanol-water partition coefficient ($\log P_{ow}$) has become the most widely used scale for solute lipophilicity and a model for drug interactions with cell membranes (9). However, there exists a plethora of evidence that solute distribution between isotropic bulk solvents is quite inadequate in modeling solute partitioning into anisotropic, heterogeneous, and organized environments of lipid bilayers in cell membranes (10–12). The lack of electrostatic interactions in octanol-water partitioning is a serious misrepresentation of the actual affinity of drugs for cell membranes. Additionally, direct measurement of octanol-water partition coefficients of numerous new compounds in combinatorial libraries is a major obstacle. Nevertheless, in the absence of a more chemically relevant model, octanol-water partition coefficient ($\log P_{ow}$) is presently the most widely used scale for estimating solute-membrane interactions.

Liposome electrokinetic chromatography (LEKC) provides distinct advantages for determination of lipophilicity in pharmacokinetic and quantitative structure activity relationship (QSAR) studies. LEKC is a capillary electrophoresis (CE) method where liposomes are incorporated in the buffer solutions and serve as pseudo-stationary phases for separations of uncharged and charged molecules. There are several significant advantages in using LEKC for assessment of drug-membrane interactions over the existing models such as octanol-water partitioning, retention in HPLC systems such as immobilized artificial membrane (IAM-HPLC) (13,14), or immobilized liposome chromatography (ILC) (15) as summarized below:

(a) Liposomes are spherical lipid bilayer microstructures that are made of phospholipids and closely resemble biologic cell membranes (16). This makes them more suitable models for the dynamic and fluid lipid bilayer environment of cell membranes than octanol or HPLC bonded stationary phases (IAM columns) where a single phospholipid is chemically attached to silica gel. Successful applications of liposome-water partition coefficients in QSARs ranging from correlations with intestinal absorption to pharmacokinetic parameters like binding to plasma proteins have been demonstrated (17–20).

(b) LEKC offers advantages such as speed, convenience, small sample size, and lack of sample purity requirement compared to the existing techniques such as potentiometry, solid-phase extraction, dialysis, spectrophotometry, etc., where such measurements are simply too cumbersome and/or pro-

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hibitively time consuming for widespread use in drug discovery applications (21–25). In fact, LEKC is the only technique for measurement of partition coefficients with the capability of high throughput screening of large compound libraries. Multiplexed 96-capillary CE systems with both absorbance and fluorescence detection are now commercially available.

(c) Using LEKC, it is possible to establish universal and consistent partition coefficient scales for drug-membrane interaction studies. A major shortcoming of HPLC methods (IAM or RPLC) is the lack of a universal and consistent partition coefficient scale (for interlaboratory use) for quantifying drug-membrane interaction. Retention factor, k', in HPLC depends on the partition coefficient into the stationary phase (K) and the phase ratio (Φ) as $k' = K\,\Phi$. However, the uncertainties in measuring the phase ratio and more importantly, the variability of phase ratio among different HPLC columns have hindered the development of a coherent, consistent, and reproducible partition coefficient scale for interlaboratory use in drug-membrane interaction studies.

On the contrary, phase ratio in LEKC can be determined for a given pseudo-phase, and more importantly, phase ratio does not vary between instruments, capillaries, or with use (which is the case for HPLC). The LEKC phase ratio, Φ^{LEKC}, is defined as the ratio of the volume of the liposome phase (V_{lip}) over the aqueous phase (V_{aq}) and is related to the intrinsic properties of the phospholipids such as molar volume, v, critical aggregation concentration (CAC) as well as concentration (C_{PL}, the concentration of phospholipids), and can be reproducibly determined with relatively high accuracy according to Eq. 1.

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

The intrinsic characteristics of the liposomal pseudo-stationary phase remain constant for a given temperature and ionic strength, and do not depend on the CE system or the capillary. This would make it possible to accurately determine K_{lw} (the liposome-water partition coefficient) from retention factor in LEKC (using Eq. 2) for a variety of liposomes systems that could be readily used in different laboratories.

$$K_{lw} = \frac{k}{\phi^{LEKC}} \quad (2)$$

The retention factor, k, for neutral solutes is calculated from the LEKC data using the retention times and Eq. 3, where t_R is the retention time of the solute, 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.

$$k = \frac{(t_R - t_{eo})}{t_{eo}[1 - (t_R/t_{lip})]} \quad (3)$$

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 solutes in the bulk aqueous (t_0) needs to be included in the calculation of retention factor. Equation 4 is used to calculate the retention factors of charged solutes.

$$k = \frac{(t_R - t_o)}{t_o[1 - (t_R/t_{lip})]} \quad (4)$$

The liposome-water distribution coefficient (D_{lw}) of charged solutes can be substituted for the partition coefficient and calculated according to Eq. 2.

(d) In LEKC, the pseudo-stationary phase is a part of the buffer solution, which renders the flexibility of controlling the composition of the lipid bilayer pseudo-phase. The composition of these "artificial membranes" can be carefully controlled to nearly mimic the properties of the natural membranes through adjustment of the type and mole fractions of phospholipids as well as incorporating "additives" such as cholesterol and even proteins. For example, in an attempt to emulate the *in vivo* situation for studying drug partitioning using equilibrium dialysis, Kramer *et al.* used liposomes composed of a complex lipid mixture extracted from MDCK cells (26) (termed "MDCKsomes") which mimic the composition of the intestinal epithelium cells.

A large number of pharmaceutical compounds are basic drugs and consequently possess a positive charge at physiologic pH. Therefore investigating several factors influencing the retention of basic drugs in LEKC is important for the development of LEKC as a method for screening of drug-liposome interactions. Liposomes were prepared and the retention factors were measured for a series of basic drugs under a variety of buffer conditions, including various buffer types, concentrations, and ionic strengths as well as using different phospholipids and liposome compositions.

MATERIALS AND METHODS

Reagents

N-[2-Hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid], *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid], sodium salt (HEPES buffer), 2-[*N*-morpholino]ethanesulfonic acid (MES buffer), sodium phosphate monobasic (phosphate buffer), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS buffer), Tris(hydroxymethyl)-aminopropane (TRIS buffer), and 2-(cyclohexylamino)ethanesulfonic acid (CHES buffer) were purchased from Sigma (St. Louis, MO, USA). Cholesterol (Chol), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC), 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-L-serine) (sodium salt) (PS), 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt), (PG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (PE), L-α-phosphatidylinositol (soy, sodium salt) (PI), and sphingomyelin, (egg, chicken) (SPH) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All samples used in the LEKC studies were purchased from Sigma.

Vesicle Preparation

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 to achieve a certain pH at given ionic strength and temperature conditions (27). A description and application of the buffer program is found in reference 27 by Okamoto. Dr Okamoto has graciously donated a copy of the software.

The appropriate amounts of phospholipids and cholesterol were dissolved in a 9:1 volume 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 appropriate buffer. Liposomes were prepared according to the extrusion method where the multilamellar vesicles (MLVs) were processed to small unilamellar vesicles (SUV's) to achieve a uniform size distribution and smaller size liposomes. Extrusion was performed through polycarbonate membranes using a Northern Lipids Lipex extruder (Vancouver, BC, Canada) maintained at a temperature of 60°C by a circulating water bath. Extrusion was performed a total of 5 times through 200 nm pore size filters, 5 times through filters with a pore size of 100 nm, and finally 10 times through the smallest pore size filter, 50 nm.

CE System

The 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. Methanol was used as the electroosmotic flow marker, t_{eo}, and decanophenone were used as the liposome marker, t_{lip}.

LEKC Methods

At the beginning of each day, the capillary was rinsed 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 then rinsed for 30 min with the liposome solution. 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. Typically liposome solutions were prepared simultaneously while the initial capillary rinse procedure was carried out. At the end of the day, the capillary was rinsed for 10 min with Milli-Q water.

As mentioned above and described by Eq. 4, in order to determine retention factors, k, for charged solutes in LEKC, the migration times, t_R, in the presence of liposomes (LEKC condition) and in the absence of liposomes, t_o, (CZE condition) have to be determined. All CZE and LEKC solute mobilities are the average of 4 repeated 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). This was found to be the optimum method for creating the same capillary wall conditions (i.e., keeping the wall coated with the liposomes) between LEKC and CZE runs, as liposomes have been found to coat the walls of the capillary (28).

Other authors have accounted for differences in CZE and EKC buffers by the addition of high concentrations of NaCl to the CZE buffer to make up for the differences in ionic strength between the two conditions (29). This was necessary in these studies due to the high ionic strength of the sodium dodecyl sulfate (SDS), which was used as the pseudostationary phase (29). However, the situation is different for the LEKC system. The addition of the liposomes has a much smaller contribution to the total ionic strength of the buffer solution, as phospholipid concentrations were small (10 mM) and they consist largely of the zwitterionic phospholipid, PC. Therefore this effect was considered negligible.

All solutions used for rinsing were filtered through a 0.45 μm filter disk (Scientific Resources Inc.) 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 around 3 ml of methanol to prepare stock solutions. 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. Samples were injected for 1–2 s by hydrodynamic injection.

Fig. 1 is a sample electropherogram for 10 mM $PG_{20}PC_{80}$ with 3 mM cholesterol. There is a defined elution window in LEKC, which is marked by the retention times of the t_{eo} (MeOH) and t_{lip} (decanophenone) markers, indicated in Fig. 1 as peaks 2 and 5, respectively. Atenolol, peak 1, has very little interaction with the liposomes and its mobility in LEKC is mostly due to its own mobility in the aqueous phase. Imipramine and amitriptyline, peaks 3 and 4, respectively, have a greater interaction with the liposomes.

Typically (for normal buffer conditions) the elution of the final peak (the liposome marker) was between 4 and 7 min for liposomes with different charge densities. CZE runs were on the order of 2.5 min. Therefore the retention factor (and thus, partition coefficient) of one drug can be determined in approximately 30–35 min (for an average of three measurements). Typically sample mixtures were injected consisting of 2 to 5 drugs. This, of course, greatly increases the number of compounds that could be analyzed simultaneously.

Because the rinse procedure has not been optimized, the conditions used in these experiments were longer than necessary to ensure complete equilibration. The rinse procedure

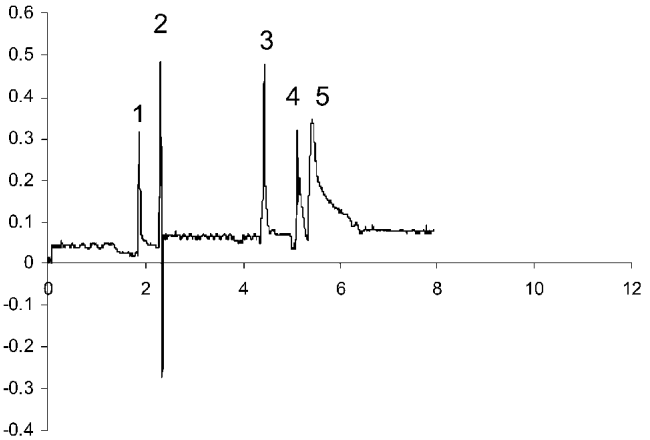

Fig. 1. Electropherogram conditions: 10 mM $PG_{20}PC_{80}$ with 3 mM Chol. Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C. Peak identification: 1, atenolol (sample ID 4); 2, MeOH; 3, imipramine (sample ID 9); 4, amitriptyline (sample ID 3); 5, decanophenone.

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 use at a later date. Optimizing the rinse procedure and preparing liposomes in advance will significantly reduce the time required to prepare for data acquisition.

Determination of Liposome Size

The average liposome size was determined using Photon Correlation Spectroscopy (PCS) using a Zetasizer $1000HS_A$ (Malvern Instruments Ltd, Malvern, UK) with a 5 mW helium neon laser at 633 nm. The scattered light was collected at an angle of 90°. All measurements were made at 36°C. The Malvern PCS software algorithm chosen to analyze the data was Contin and the size distribution profiles were analyzed using the method of volume. Average liposome sizes were obtained from at least three repeat measurements of the mean diameter of the liposome. The average size of the liposomes used in this work is 52.3 (± 0.6) nm.

RESULTS AND DISCUSSION

Effect of Buffer Concentration

The buffer type, concentration, and ionic strength were varied to investigate the effect of the aqueous buffer on the partitioning into liposomes as determined by the LEKC retention factor. The liposome composition chosen was 10 mM PG_{24} PC_{46} $Chol_{30}$, where the subscripts represent the molar percentages of the components used. The liposome composition was held constant while the buffer composition was varied. Three different buffers were studied at pH 7.0: HEPES, MES, and phosphate with pK_a values of 7.66, 6.21, and 7.20, respectively (30). Retention data was collected using 10, 25, and 40 mM of each of the three buffers. 40 mM was selected as the highest buffer concentration due to stability issues of the liposomes in LEKC at higher concentrations. At this pH, a set of positively charged drugs including tetracaine and acebutolol as well as the neutral solute phenol were selected as test solutes. For this work, the measurement of the average liposome diameter was used to compare liposome properties with different buffer conditions. The liposome size remained constant over the range of buffers studied.

For the neutral solutes, there is essentially no difference in log k for a given buffer type at different concentrations. For example, phenol has log k values of –1.22 (± 0.01) and –1.20 (± 0.01) for 10 and 40 mM phosphate buffer concentrations. In addition, the type of buffer does not affect the measured log k value. The log k values of phenol in 10 mM HEPES, MES, and phosphate are –1.21 (± 0.01), –1.20 (± 0.01), and –1.22 (± 0.01), respectively. This is in agreement with previous determinations from this lab, where in a linear solvation energy relationship (LSER) study using LEKC, the type and concentration of buffer had no influence on the LSER coefficients for a series of neutral solutes (31).

The positively charged solutes have a different behavior such that the buffer type and concentration significantly influence log k values for all drugs studied. The results of the effect of buffer type on LEKC retention for tetracaine and acebutolol is illustrated in Fig. 2. Error bars are included however they are smaller than the size of the symbols. For the solutes shown here, in all the three buffer types, log k values decrease with increasing buffer concentrations. HEPES results in the largest log k values followed by MES and then phosphate buffer results in the lowest log k values. This follows along with the ranking of the ionic strength of the three buffers. For the same concentration, the organic buffers HEPES and MES have the lowest ionic strengths, and the inorganic phosphate buffer has the highest ionic strength of the three buffers.

To study the effect of ionic strength on the interaction of charged drugs with liposomes, the buffer concentration was held constant at pH 7.0 using 10 mM HEPES. Sodium chloride was added with concentrations ranging from 0 to 40 mM NaCl. The effect of ionic strength can be seen for several positively charged and neutral solutes in Fig. 3. Neutral solutes (nonfilled symbols) show no change in log k values with increasing ionic strength of the buffer solution. Positively charged solutes (filled symbols) show a significant decrease in log k with increasing ionic strength. Since log k is directly proportional to $\log D_{lw}$, the distribution coefficient would also decrease with increasing ionic strengths. This is true for all basic solutes studied. Lidocaine has a pK_a value around 7.9 (32), and is about 89% ionized and still follows the same trend as acebutolol and alprenolol, both of which are fully charged with a pK_a values of 9.2 or greater (32). The trend of a decrease in retention is the same for all positively charged solutes examined, which indicates that ionic strength does not have a selectivity effect on the drug - liposome interactions with the positively charged solutes studied here.

The decrease in partitioning with increase in ionic strength or increase in buffer concentration is consistent with previous determinations (33). This reduced interaction is due to the shielding of the charge on the liposome surface in addition to the shielding of the charge on the drug molecule by the buffer counter ions, thus decreasing the electrostatic interactions. This would also explain the lack of difference in partitioning with the neutral solutes as the buffer is varied since they have no electrostatic interactions. A stronger effect

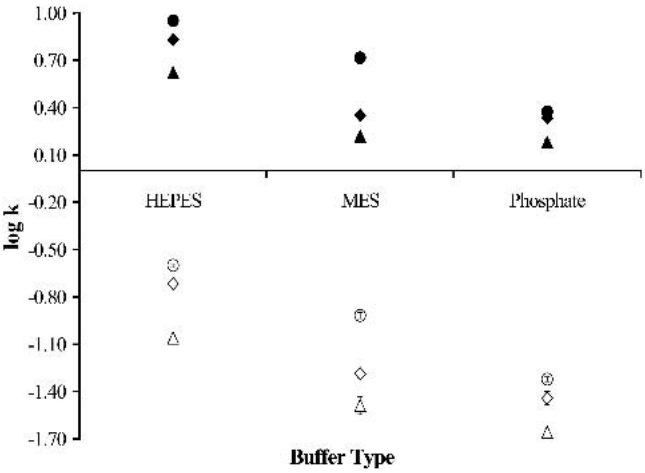

Fig. 2. Log k of tetracaine (filled symbols) and acebutolol (open symbols) as a function of HEPES, MES, and phosphate buffers at various concentrations: 10 mM (●, ○), 25 mM (♦, ◇), and 40 mM (▲, Δ).

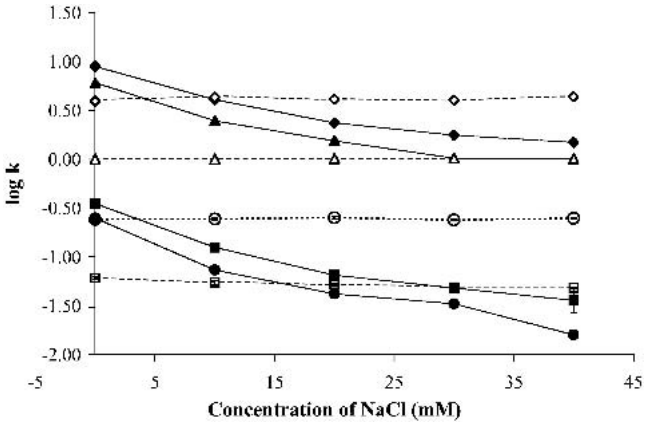

Fig. 3. Plot of log k vs. the concentration of NaCl added to the aqueous buffer (10mM Hepes, pH 7.0): tetracaine (♦); lidocaine (■); acebutolol (●); alprenolol (▲); phenol (□); 4-ethylphenol (○); 2-chlorophenol (△); 3,4-dichlorophenol (◇).

of the ionic strength has been observed when more anionic vesicles are used (33).

To study the effect of buffer concentration at a constant ionic strength, the ionic strength was held constant at 29 mM by the addition of NaCl while different concentrations and types of buffers were investigated. This data is shown in Table I for 10, 20, 28, and 40 mM HEPES as well as 10 mM MES and two mixed buffers. Mixed buffers consisted of three buffer components: 10 mM each of HEPES, CHES, and CAPS buffers compose the first mixed buffer (*Mixbuf*), and 10mM each of MES, TRIS, and CAPS compose the second mixed buffer (*Mixbuf-a*). It is noted that the zeta potential (calculated according to Eq. 5, below) was constant for this series of buffers (average zeta potential for the series = –49.2 ± 0.4 mV), indicating a constant liposome charge density.

There are no differences in retention between all buffer compositions for the one neutral test solute, benzocaine. For the positively charged solutes, there is very little difference in retention as the HEPES concentration is increased with a constant ionic strength. This is different than the previously discussed data where the buffer concentration was increased with no set ionic strength. There are only slight variations in log k for the remaining buffer types as well. These studies emphasize the overall buffer ionic strength at a given pH is more important than the buffer type and concentration. This is significant for studies on the pH effect on the interactions of charged drugs with liposomes (34). Mixed buffers are commonly used in studies of the effect of pH on liposome partitioning in order to maintain a constant environment for the liposomes. A mixed buffer of constant ionic strength must be used.

Effect of Liposome Composition

Electrostatic interactions between ionized drugs and charged membranes are an important factor influencing drug partitioning. The extent of interaction increases with the charge density of the membrane at a given pH. Cell membranes are often negatively charged due to the presence of lipids with anionic headgroups such as PS and PI. Membrane compositions vary widely in terms of the type and mole fraction of phospholipids, including the anionic lipids that determine the charge density of the membranes. For these reasons the effect of liposome composition was studied in terms of the type and percentage of anionic phospholipids. To compare the effect of the selection of anionic lipid, $PS_{20}PC_{80}$ liposomes were prepared to compare with the $PG_{20}PC_{80}$ liposomes. In addition, a liposome composition denoted as *cell-mimic* was prepared to simulate the phospholipids present in Caco-2 cells (35). This composition consisted of $PC_{52.6}$ $PE_{19.3}$ $PS_{16.6}$ $PI_{8.4}$ $SPH_{3.1}$, where PS and PI are the negative lipids in this mixture, making the total anionic lipid content 25%, leaving 75% composed of zwitterionic lipids including PC, PE, and SPH. All liposomes in this study had a total lipid concentration of 10 mM and a constant concentration of cholesterol of 3 mM (30% of the total lipid concentration).

Listed in Table II is a series of drugs having a net positive charge at pH 7.4 which were selected to study the effect of liposome composition. Also included in Table II are the drug pK_a values (32,36). A constant buffer composition was used and the percentage of anionic phospholipid was varied from 5% to 50% while the percent PC (zwitterionic phospholipid) ranged from 95 to 50%, respectively. Previous studies have shown that the effect of liposome composition has little effect on the retention of neutral drugs in LEKC. (M.G. Khaledi, unpublished results). The retention factor increases with increasing percentage of anionic lipids for all basic drugs studied. This can be seen in Figs. 4a and 4b for imipramine, labetalol, nadolol, terbutaline, and metoprolol. The lines in Figs. 4a and 4b are second-order polynomial fits of the data. For many drugs studied, there is little interaction with liposomes consisting of 5% PG; therefore this data is not included

Table I. Log k for Various Buffers with a Constant pH (7.0) and Ionic Strength (29 mM) for One Neutral Drug (Benzocaine) and Six Positively Charged Drugs

	10 mM HEPES	20 mM HEPES	28 mM HEPES	40 mM HEPES	10 mM MES	*Mixbuf*[a]	*Mixbuf-a*[b]
Benzocaine	–0.77 (0.01)	–0.72 (0.00)	–0.74 (0.01)	–0.72 (0.00)	–0.75 (0.01)	–0.76 (0.01)	–0.72 (0.00)
Lidocaine	–1.20 (0.03)	–1.14 (0.03)	–1.14 (0.02)	–1.06 (0.03)	–1.15 (0.04)	–1.12 (0.03)	–1.32 (0.04)
Tetracaine	0.29 (0.01)	0.30 (0.02)	0.19 (0.02)	0.31 (0.01)	0.16 (0.01)	0.23 (0.02)	0.32 (0.02)
Amitriptyline	0.92 (0.04)	0.94 (0.03)	0.94 (0.04)	0.89 (0.01)	0.90 (0.03)	0.90 (0.06)	0.91 (0.03)
Terbutaline	–1.00 (0.02)	–1.04 (0.02)	–1.03 (0.03)	–1.01 (0.02)	–0.97 (0.01)	–1.00 (0.01)	–1.01 (0.01)
Alprenolol	0.18 (0.01)	0.16 (0.01)	0.15 (0.02)	0.18 (0.01)	0.13 (0.01)	0.15 (0.01)	0.20 (0.01)
Doxepin	0.67 (0.02)	0.69 (0.01)	0.64 (0.01)	0.63 (0.02)	0.66 (0.01)	0.64 (0.01)	0.69 (0.02)

[a] *Mixbuf* consisted of 10 mM each of HEPES, CHES, and CAPS buffer.
[b] *Mixbuf-a* consisted of 10 mM each of MES, TRIS, and CAPS buffers.

Table II. List of Basic Drugs Studied and pK_a Values (pKa Values Shown are Basic pKa Values (BH+) Unless Otherwise Noted)[a]

Sample ID	Solute name	pK_a
1	Acebutolol	9.20
2	Alprenolol	9.65
3	Amitriptyline	9.42
4	Atenolol	9.6
5	Chlorpheniramine	9.16
6	Desipramine	10.44
7	Doxepin	9.0
8	Doxylamine	4.4; 9.2
9	Imipramine	9.5
10	Labetalol	7.4; 8.7[a]
11	Lidocaine	7.9
12	Maprotiline	10.2
13	Metoprolol	9.7
14	Mianserin	7.1
15	Nadolol	9.39
16	Nefopam	9.2
17	Nortriptyline	9.7
18	Orphenadrine	8.4
19	Oxprenolol	9.5
20	Pheniramine	4.2, 9.3
21	Pindolol	8.8
22	Propranolol	9.45
23	Terbutaline	8.8; 10.1[a]; 11.2[a]
24	Tetracaine	8.39
25	Trimeprazine	9.0
26	Trimipramine	8.0

[a] pKa value denotes acidic pKa (HA).

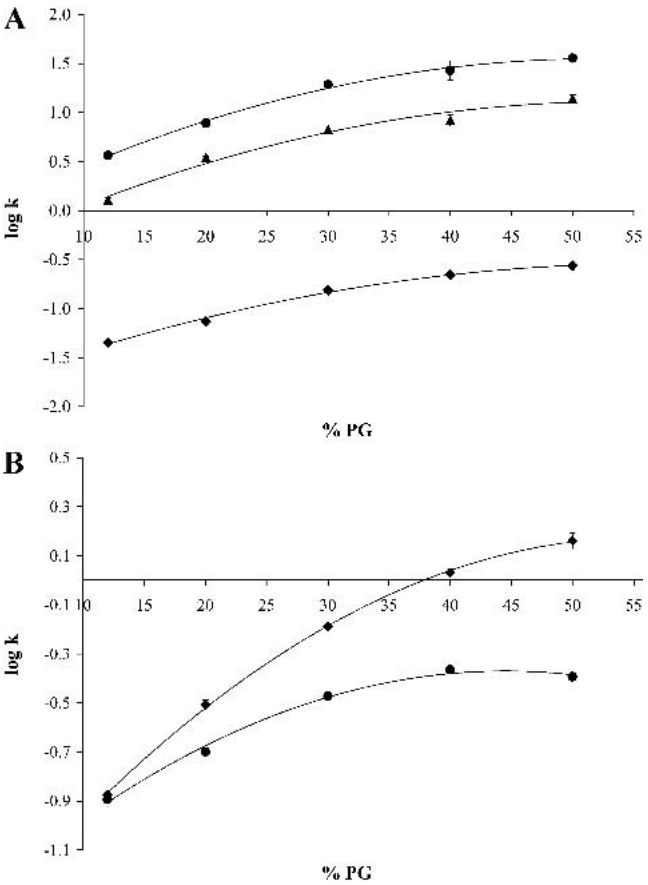

Fig. 4. (a) Plot of log k vs. the percentage of PG in the liposome composition for imipramine (●), labetalol (▲), and nadolol (♦). Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C. Symbols are the measured data points and lines are a second order polynomial fit of the data. (b) Plot of log k vs. the percentage of PG in the liposome composition for terbutaline (●) and metoprolol (♦). Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C. Symbols are the measured data points and lines are a second order polynomial fit of the data.

in Figs. 4a and 4b. This little interaction (with 5% PG) is likely due to the small total concentration of phospholipids used in this work (10 mM) that results in a low charge density of liposomes.

The increase in retention with an increase in PG content is attributed to an increase in electrostatic interactions between the positively charged drug and the increasing negative charge on the liposome surface. Positively charged drugs have been shown to interact stronger with liposomes containing larger PS content (37). This is consistent with other literature reports, for example, greater DMPG content in membranes also resulted in an increase in the degree of binding of Quinine to lipid membranes (33). Liposomes consisting of 30% PS had an affinity for the positively charged drug, flurazepam of about 4.8 times that of membranes consisting of PC only, determined using second derivative spectrophotometry (38).

In contrast to the buffer composition, the anionic phospholipid content of the vesicles has a selective effect on the drug-liposome interactions. When examining the electrostatic interactions of drugs, the charge of the drug in addition to the charge on the liposomes will influence the degree of electrostatic interaction. A fully protonated drug will have a greater degree of electrostatic attraction compared to a drug that may only be partially positively charged. Therefore, the increase in retention factor with increasing anionic lipid content may not be the same for all drugs. Figure 4a shows three solutes which are all >99% ionized. They show similar trends in the increase in log k with increasing PG content. Figure 4b shows the selective effect of changing the percent PG on the log k for 2 solutes which have very little interaction with the liposomes at the lower PG content (12% PG) while at higher percentages of PG (50% PG) the difference in interaction is significant. It is possible that the difference in retention with the increasingly negative liposomes is due to differences in the percent ionization (i.e. different charge) of the two drugs. Terbutaline has a basic pK_a of 8.8 while metoprolol has a pK_a of 9.7 (32), thus making metoprolol fully charged while terbutaline is only partially charged. The drug with the greater degree of ionization resulted in a greater increase in retention possibly because of enhanced electrostatic effects. There is only a slight difference in the degree of ionization of the two drugs in this case. Comparing drugs with lower pK_a values (i.e., lower percentage ionized at the given pH) with the current data set would be beneficial in investigating the pK_a selectivity effect.

The electrostatic properties in the interface region of the lipid bilayer play a significant role in influencing the partitioning behavior of charged drugs. By changing the fraction of charged lipids in the liposomes, the surface charge density and zeta potential are altered. The zeta potential, ζ of liposomes of varying PG content was calculated from their mea-

sured mobility (μ_{lip}) according to the Smoluchowski equation (39):

$$\zeta = \frac{\mu_{lip}\eta}{\varepsilon_0 \varepsilon_r} \quad (5)$$

where η is the aqueous solution viscosity, ε_0 is the permittivity of free space, and ε_r is the relative permittivity of the medium, with values of 7.05×10^{-4} N s m^{-2}, 8.854×10^{-12} C^2 N^{-1} m^{-2}, and 75.8, respectively. The effect of the percentage of PG on the calculated zeta potential is seen in Fig. 5. Many detailed discussions on the effect of anionic lipid content on the zeta potential have been published (40–42). Zeta potentials have been found to have an exponential dependence on the PS content in liposomes (43). Estelrich *et al.* determined a zeta potential value of around –40 mV for SUVs composed of 20% PS and 80% soybean PC prepared by extrusion through 50 nm filters (43). This is on the order of the value of –49.6 (± 0.1) mV for $PS_{20}PC_{80}$ determined in this work. Differences in ionic strength between the buffers used in this work and by Estelrich may cause the difference in the two values. In addition, soybean PC contains phospholipids with different chain lengths and degrees of unsaturation, which influences the aggregation properties in the liposomes.

Wiedmer *et al.* calculated the total charge on liposomes consisting of various ratios of anionic lipids. With about 30% anionic lipid a flattening of the curve is seen such that the liposomes were almost saturated with negative charges (44). This same flattening after 30% PG can be seen with the zeta potential determinations here. The zeta potential only varies from –52.4 (± 0.2) mV to –54.1 (± 1.2) mV between 30 and 50% PG.

More negative zeta potentials were obtained with larger PG content in the liposomes. As a result, we see a greater retention of basic drugs with increasing PG content, or a more negative zeta potential. Consistent with the flattening of the charge on the liposome surface, the retention factor starts to plateau after 30% PG. McLaughlin *et al.* found a sigmoidal dependence on the percentage of bound peptide with the mol percent PG in the liposomes. Their data showed a similar trend of a plateau at concentrations of PG greater than 30% (45).

To further study the effect of liposome composition on the retention of basic drugs, two anionic phospholipids were chosen to investigate their effect on the retention of the drugs. For almost all drugs studied, there is a slightly greater log k with the PG containing liposomes compared to the PS containing liposomes. However, there is a very good correlation between the retention factors determined using these compositions. This correlation (R^2 is 0.992) is shown in Fig. 6 for the 26 drugs listed in Table II. Liposomes consisting of 20% PG or 20% PS also have a similar zeta potential value. Vesicles formed from 5:1 PC:PG or 5:1 PC:PS mixtures have been found to have similar zeta potentials (40,42). This correlation is slightly reduced at low percentages (5%) of anionic lipid, between liposomes composed of PG and PS, with a R^2 value of 0.977 for 17 drugs. This correlation is not as high as with the larger percentages of PG, perhaps due to a greater relative error associated with the measurements of smaller retention factors.

There is a good correlation for log k values determined using the *cell-mimic* liposomes and the $PG_{20}PC_{80}$ or $PG_{30}PC_{70}$ liposomes. The zeta potential of the *cell-mimic* liposomes fits in between the values for 20 and 30% negative lipid. Zeta potentials of PI have been found to be less negative than PS and PG (40). Although PE is a zwitterionic lipid, the incorporation of PE into PC vesicles has been found to slightly enhance the negative charge of the phosphate group resulting in a slightly negative zeta potential (43). Despite these differences, the simple composition of liposomes consisting of between 20 and 30% PG can be used to simulate interaction of drugs into the more complicated liposomes mimicking the intestinal epithelial cell membranes of Caco-2 cells.

Correlation with Octanol-Water Partitioning

The liposome—water partitioning data can be correlated with octanol—water partitioning ($LogD_{ow}$ or $ClogP_{ow}$) values obtained from the literature (32,46). The plot of log k *cell-mimic* liposomes vs. $LogD_{ow}$ is in Fig. 7. The correlation has

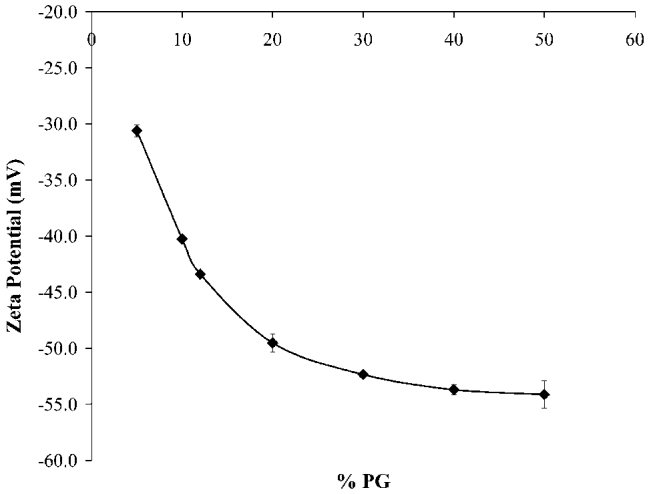

Fig. 5. Zeta potential as a function of percent PG in the liposomes. Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C.

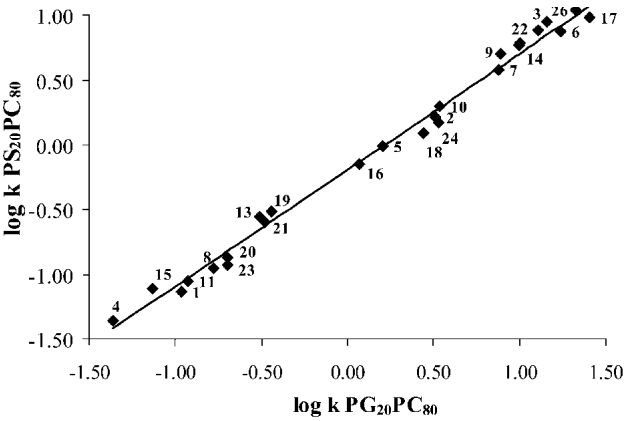

Fig. 6. Correlation between log k $PS_{20}PC_{80}$ and log k $PG_{20}PC_{80}$. The equation of the line is y = 0.900x – 0.194 with a correlation of 0.992 for 26 points as determined by linear regression. Points are labeled according to Table II. Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C.

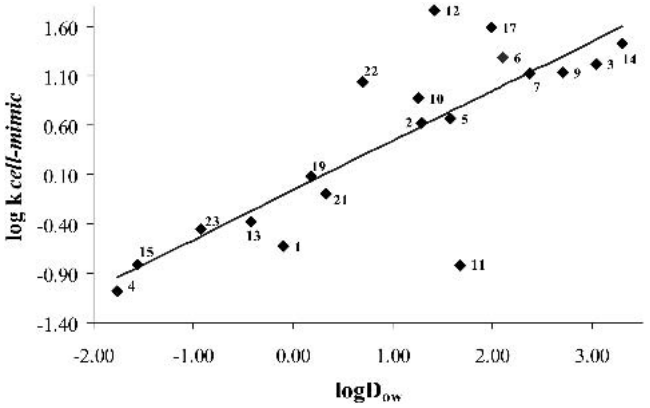

Fig. 7. Correlation between log k *cell-mimic* and log D_{ow}. The equation of the line is y = 0.505x – 0.056 with a correlation of 0.628 as determined by linear regression. Points are labeled according to Table II. Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C.

an R^2 equal to 0.628 for 19 solutes. A poor correlation is observed with $LogD_{ow}$ as reported in the literature due to the lack of electrostatic interactions in octanol. A better relationship is observed between log k *cell-mimic* and $ClogP_{ow}$ than between log k *cell-mimic* and $LogD_{ow}$. The plot of log k *cell-mimic* liposomes vs. $ClogP_{ow}$ results in a R^2 of 0.807 for the 26 solutes listed in Table II and is shown in Fig. 8.

Charged drugs are able to interact with liposomes by both hydrophobic and electrostatic interactions, while with octanol, only hydrophobic interactions are possible. A great majority of drug molecules have ionizable functional group(s); many of which are partially or fully charged under biologic conditions. As discussed above, electrostatic interactions have a significant impact on drug partitioning into liposomes. Clearly, the octanol-water system fails to mimic interfacial properties of biomembranes and the ionic interactions between charged drugs and the phospholipids that constitute cell membranes. Therefore a single scale such as P_{ow} cannot possibly represent drug interactions with various membranes with a wide range of compositions in QSAR studies. Ionized drugs typically have low octanol—water distribution coefficients, $logD_{ow}$ due to the absence of electrostatic interactions. On the other hand, basic solutes have an enhanced interaction with membranes due to electrostatic attraction (47). As a result, the correlation between the octanol—water distribution coefficient and the LEKC retention factor (directly related to D_{lw}) is poor.

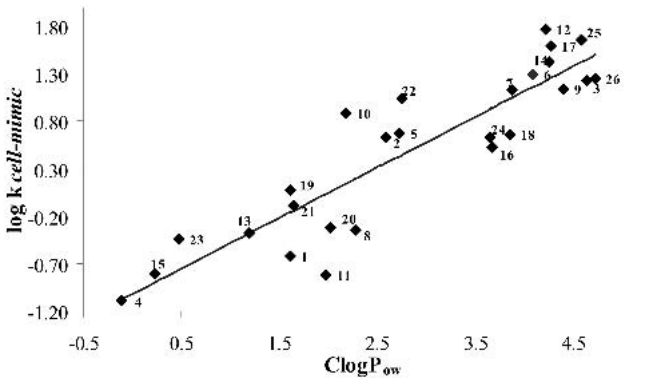

Fig. 8. Correlation between log k *cell-mimic* and $ClogP_{ow}$. The equation of the line is y = 0.533x – 1.022 with a correlation of 0.807 as determined by linear regression. Points are labeled according to Table II. Buffer composition consisted of 10 mM each of HEPES, CHES, and CAPS buffers, with a total ionic strength held constant at 11 mM at a pH of 7.4 at 36°C.

CONCLUSIONS

LEKC is a convenient and powerful method for rapid screening of drug-membrane interactions. Liposomes are more biologically relevant in - vitro models for cell membranes than octanol or HPLC stationary phases (such as IAM columns). LEKC provides a unique combination of advantages such as small sample size, no sample purity requirement, speed, convenience, automation, high throughput capability, and flexibility of adjusting the liposome pseudo—phase compositions to mimic natural membranes.

It is emphasized that a laboratory built CE system was used in this work, requiring the operator to manually inject every sample. The use of an automated instrument would significantly increase sample throughput Additionally, one could imagine the drastic increase in sample throughput with the use of 96-capillary CE systems, which are commercially available. The potential for high-throughput liposome-water distribution coefficient determination is realized by the comparison with other methods such as the pH-metric titration which can take up to 1 h per liposome titration (48). This method would also require pure samples and repeat titrations for an average value, while only analyzing one sample at a time.

In addition, the possibility of determining the phase ratio would make it possible to create universal scales for drug-membrane interactions, which is not possible using HPLC-based systems. Recent reports from this laboratory have also demonstrated the possibility of calculating liposome-water partition coefficients from solute structure (49). Such capability will be of great use in drug design and screening as drug candidates affinity for membranes could be predicted prior to their synthesis. The application of liposome-water partition coefficients for prediction of intestinal absorption and membrane permeability will be reported elsewhere (49).

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