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Comparison of the retention behavior of β-blockers using immobilized artificial membrane chromatography and lysophospholipid micellar electrokinetic chromatography

John A. Masucci[a,b,*], Gary W. Caldwell[a], Joe P. Foley[b]

[a]*The R.W. Johnson Pharmaceutical Research Institute, Spring House, PA 19477, USA*

[b]*Department of Chemistry, Villanova University, Villanova, PA 19085, USA*

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Abstract

We have demonstrated that significant differences exist between the retention of eight β-blockers analyzed with immobilized artificial membrane (IAM) and lysophospholipid micellar electrokinetic capillary (LMEKC) chromatographic methods. The general retention trends are maintained with highly hydrophilic compounds such as atenolol eluting first and more hydrophobic compounds such as propranolol eluting last. The retention order, however, is different and would result in major ranking differences. LMEKC demonstrates a better correlation with liposomal partitioning (R^2=0.95) than does IAM chromatography (R^2=0.60). LMEKC, with its higher efficiency, can allow a more specific evaluation of lipophilicity than IAM chromatography and is useful in the analysis of pharmaceutical candidates, particularly for ranking purposes.

Keywords: Immobilized artificial membrane; Lysophospholipid micellar electrokinetic capillary; Pharmaceutical analysis; β-Blockers; Lysophospholipids; Phospholipids

1. Introduction

The ability to predict oral adsorption of therapeutic agents is important to the drug discovery process. The main barrier to oral adsorption is the intestinal cell membrane which consists of oriented phospholipid bilayers. To mimic the phospholipid bilayer barrier, several approaches have been used including octanol–water partition coefficients (log P_{OW}) [1], liposomal partitioning [2], and retention in reversed-phase chromatography [3], microemulsion electrokinetic chromatography [4], and immobilized liposome chromatography [5].

Recently, chromatographic packings have become available which consist of phospholipids bonded to silica particles [6]. These packings are thought to simulate the solution–cell membrane interface and are useful in predicting transport of molecules across the intestinal cell membrane [7,8]. Unfortunately, these packings are difficult to prepare and are relatively expensive. In addition, it has been observed, in our laboratory, that some of these columns have a limited working life of 3000–5000 column volumes, sufficient for only 200–300 injections.

The use of liposomes (phospholipid vesicles) in electrokinetic chromatography (EKC) has been reported [9]. However, it was demonstrated that the slower kinetics of the transport process through the

*Corresponding author.

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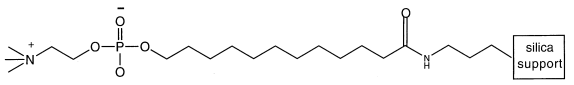
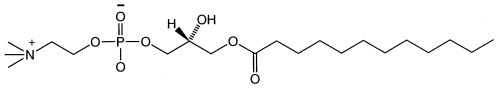
vesicle wall results in broad peaks. In addition, the preparation of homogeneous unilamellar vesicles is a tedious process, but is necessary for consistent results.

In an effort to reproduce the dynamics of partitioning in immobilized artificial membrane (IAM) chromatography, but avoid the problems associated with vesicle preparation, we have investigated the use of lysophospholipid micelles in electrokinetic chromatography. Lysophospholipids consist of a phosphate head group and a single aliphatic tail (see Fig. 1) [10]. These single tail lipids form micelles in solution above the critical micelle concentration (CMC) [11], in contrast to double tail phospholipids which form heterogeneous spherical vesicles. The micelles form spontaneously in solution at relatively low concentrations [11] and these lysophospholipids are readily available from commercial sources. The use of these lysophospholipid surfactants in micellar electrokinetic chromatography (MEKC) has not been previously reported.

β-Blockers comprise a class of compounds having antihypertensive, antianginal and antiarrhythmic properties. This study compares the retention behavior of eight β-blockers using IAM and LMEKC chromatographies. In addition, correlations of LMEKC and IAM data with liposomal partitioning, which closely simulates the cell membrane [2,7], is presented. A comparison of these methods may lead to a better understanding of the overall biological membrane partitioning mechanism.



Lysophosphatidylcholine IAM surface



1-Lauroyl-2-Hydroxy-s*n*-Glycero-3-Phosphocholine

Fig. 1. Structure of immobilized artificial membrane packing surface and the lysophospholipid (CMC = 0.7 m*M*) used in this study.

2. Experimental

2.1. Instrumentation

IAM chromatography experiments were performed using a Rainin (Woburn, MA, USA) dual pump HPLC system with Dynamax autosampler and detector. The column used was a Regis (Morton Grove, IL, USA) IAM.PC.DD, 100×4.6 mm, packed with 5 μm×300 Å phosphatidylcholine derivatized aminopropyl silica particles. The mobile phase consisted of 0.017 *M* Dulbecco's phosphate buffered saline (DPBS) solution (pH 7.0) at 1.0 ml/min. Detection was performed at 214 and 254 nm coupled to a Dynamax data system (Macintosh). All analyte concentrations were 1 mg/ml with an injection volume of 20 μl for all experiments. Dimethyl sulfoxide (DMSO) was used as the void (t_0) marker and *m*-nitroaniline was used as an internal reference analyte to correct for run to run variation.

LMEKC experiments were performed using a Beckman (Fullerton, CA, USA) P/ACE Model 2200 with Windows controller software. The capillary was 58 cm (effective length 50 cm)×75 μm I.D. uncoated fused-silica thermostated at 25°C. The running buffer consisted of 20 m*M* phosphate (pH 7.0) with and without 10 m*M* 1-lauroyl-2-hydroxy-s*n*-glycero-3-phosphocholine (Fig. 1). Separation conditions were 15 kV positive inlet, which produced approximately 50 μA of current. Detection was performed at 200 nm. All analyte concentrations were 0.34 m*M* with hydrodynamic injections (0.034 bar) of 1 s each followed by a 5-s injection of 5% methanol for determination of electroosmotic flow (EOF). The micellar elution time (t_{MC}) was estimated using a saturated solution of Sudan III in methanol–buffer (50:50, v/v) and by analysis of a homologous alkylphenone series.

2.2. Reagents

1-Lauroyl-2-hydroxy-s*n*-glycero-3-phosphocholine was purchased from Avanti Polar Lipids (Alabaster, AL, USA) and was stored at −80°C prior to use. All analytes and DPBS buffer were obtained from Sigma (St. Louis, MO, USA). Other reagents were pur-

chased from Fisher Scientific (Fair Lawn, NJ, USA). All reagents were used without further purification.

2.3. Methods

The IAM chromatographic buffer was prepared by dissolving the preweighed DPBS salt mixture into 1 l of deionized water and adjusting to pH 7.0 with 1 *M* hydrochloric acid. This solution was used without further purification.

A 20 m*M* phosphate LMEKC buffer solution was prepared by titration of 20 m*M* sodium phosphate dibasic (2.84 mg/ml deionized water) with 20 m*M* sodium phosphate monobasic (2.76 mg/ml deionized water) to pH 7.0. All analyte and lysophospholipid solutions were prepared by dissolution of pure compound into the 20 m*M* phosphate buffer and diluting appropriately. All solutions were filtered through a Whatman (Clifton, NJ, USA) 0.45-μm cellulose acetate syringe filter prior to analysis.

The IAM column was conditioned by passing 50 ml of buffer prior to analyses and making repetitive injections of *p*-toluidine to ensure identical (<5% deviation) retention times.

The fused-silica capillary was conditioned prior to use by rinsing at 1.36 bar with 0.1 *M* sodium hydroxide for 5 min followed by a 10-min deionized water rinse. All experiments were preceded by a 2-min rinse with the specific running buffer.

3. Results and discussion

In Fig. 1 the structures for the IAM surface phospholipid coating used in this study and the corresponding lysophospholipid are shown. The similarities include a zwitterionic head group and comparable aliphatic chain lengths.

The β-blockers used for this study are shown in Fig. 2. These compounds have very similar pK_a values and molecular masses as listed in Table 1. However, these compounds were chosen primarily because their hydrophobicities (distribution coefficients by 'shake flask' method, K'_{SF}) span four orders of magnitude (Table 1) [3]. By exploiting this property in LMEKC we should observe significant differentiation of these analytes.

3.1. Calculation of capacity factors and mobilities

The IAM chromatography capacity factors (k') were calculated using the conventional chromatographic relation [12]:

$$k' = \frac{t_R - t_0}{t_0} \quad (1)$$

where t_R is the observed retention time for each analyte and t_0 is the elution time for an unretained analyte.

The free solution capillary electrophoretic (CE) mobilities were calculated using the following equation [13]:

$$\mu_A = \frac{L_d/t_m}{V/L_t} \quad (2)$$

where μ_A is the apparent electrophoretic mobility, L_d is capillary length to the detector, L_t is the total capillary length, V is the applied electric voltage, and t_m is the measured migration time. Eq. (2) was used to calculate μ_A and μ_{eo} (coefficient of EOF at t_0) for each analyte. To calculate the true electrophoretic mobility (μ_{ep}), the equation $\mu_{ep} = \mu_A - \mu_{eo}$ [13] was used.

The following equation was used to calculate capacity factors (k') for LMEKC experiments [14]. This equation incorporates a correction for the electrophoretic mobility of charged species as predetermined by free solution CE analysis of each analyte.

$$k' = \frac{t_R(1 + \mu_r) - t_0}{t_0(1 - t_R/t_{MC})} \quad (3)$$

In this equation μ_r is the defined as the relative electrophoretic mobility ($\mu_{ep,LMEKC}/\mu_{eo,LMEKC}$) determined independently for each analyte. The value of $\mu_{ep,LMEKC}$ was calculated by correcting the value of $\mu_{ep,CE}$ for differences in viscosity between solution with and without lysophospholipid. The difference was calculated by injecting a small plug of analyte and monitoring its elution time upon application of a low pressure (0.034 bar) rinse. The difference in elution times (more viscous micellar solution elutes later by 4%) was used to calculate $\mu_{ep,LMEKC}$ from $\mu_{ep,CE}$. Also it was determined that $t_{MC} = t_0$

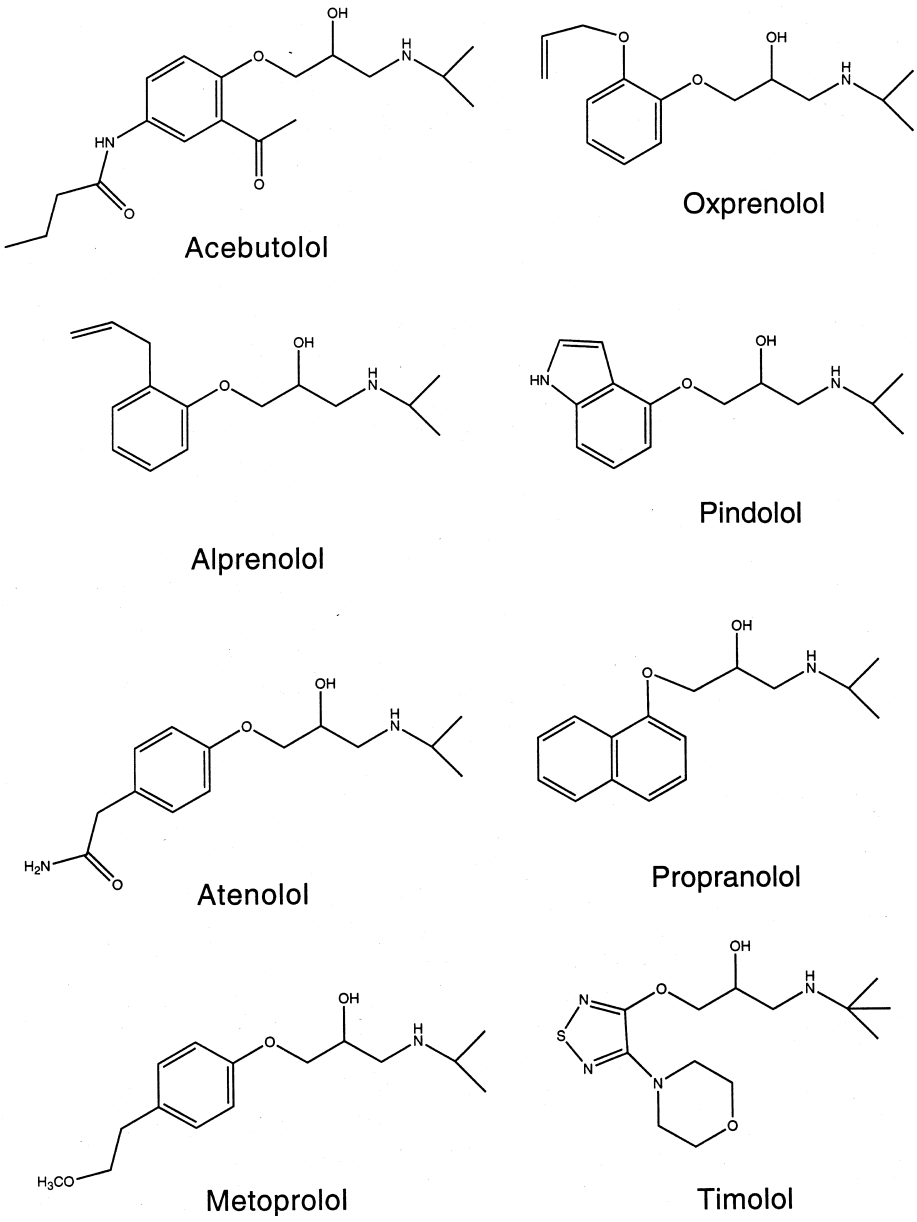


Fig. 2. Structures of β-blockers.

since the lysophospholipid micelles are zwitterionic and therefore neutral. This was verified by monitoring the retention of Sudan III, which coeluted with the t_0 marker, methanol, and by analysis of five alkylphenones in series from acetophenone to hexanophenone all of which eluted at t_0. This simplifies Eq. (3) to:

$$k' = \frac{t_R(1 + \mu_r) - t_0}{t_0 - t_R} \quad (4)$$

Table 1
Molecular mass, pK_a, K'_{SF}, LMEKC and IAM chromatography of β-blockers

Analyte	Molecular mass	pK_a [3]	K'^{a}_{SF} [3]	k' (LMEKC)	k' (IAM)
(1) Acebutolol	336	9.67	0.25	0.12±0.06	5.35±0.57
(2) Alprenolol	249	9.65	3.79	1.24±0.24	8.78±0.58
(3) Atenolol	266	9.44	0.01	0.0	0.53±0.08
(4) Metoprelol	267	9.70	0.20	0.17±0.03	2.01±0.30
(5) Oxprenolol	265	9.50	0.64	0.33±0.04	3.49±0.35
(6) Pindolol	248	8.80	0.17	0.23±0.02	7.08±0.15
(7) Propranolol	259	9.45	5.40	1.37±0.28	28.28±1.18
(8) Timolol	316	8.80	0.24	0.11±0.05	1.53±0.15

LMEKC: 10 m*M* lysophospholipid, 20 m*M* phosphate (pH 7.0); k' (LMEKC)$=[t_R(1+\mu_r)-t_0]/(t_0-t_R)$.
IAM: 0.01 *M* DPBS buffer, pH 7.0; $k'(\text{IAM})=(t_R-t_0)/t_0$.
Other conditions as specified in text.
All k' values are averages of three measurements±1 S.D.
[a] Octanol–buffer distribution coefficient by shake-flask method corrected to pH 7.0 [3].

Eq. (4) was used to calculate k' for our experiments.

3.2. IAM chromatography of β-blockers

A typical IAM chromatogram is shown in Fig. 3. This analysis of pindolol was obtained as described using DMSO as a t_0 marker and *m*-nitroaniline as a reference analyte. Significant deterioration of this column is evident by peak broadening and decreased retention after only 4200 column volumes. The peaks eventually split into two (data not shown), indicative of a possible void in the column. This deterioration of performance was observed for six different columns from four separate lots.

The eight analytes are well differentiated by IAM chromatography with capacity factors ranging from 0.525 for atenolol to 28.28 for propranolol as shown in Table 1.

3.3. Free solution capillary electrophoresis of β-blockers

Fig. 4 illustrates the electrophoretic analysis of pindolol. Methanol is used as the t_0 marker. Since

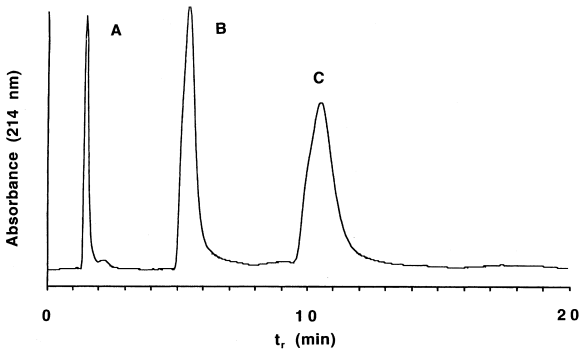


Fig. 3. Typical IAM chromatogram. Peaks correspond to the t_0 marker DMSO (A), the internal reference *m*-nitroaniline (B) and pindolol (C).

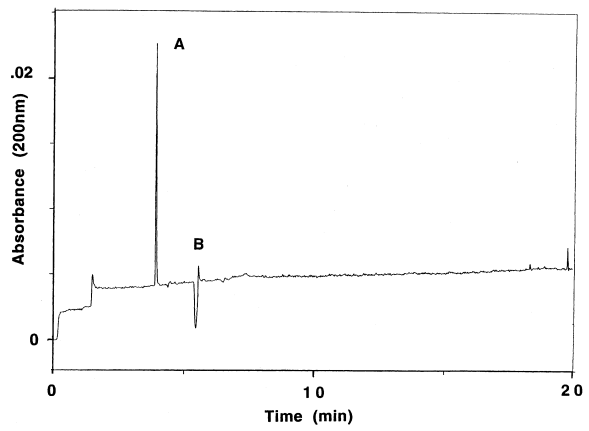


Fig. 4. Typical free solution electropherogram. Analysis of pindolol (A); methanol (B) is used as t_0 marker.

Table 2
Free solution CE analysis of β-blockers

Analyte	t_m (min)	t_0 (min)	μ_A ($cm^2/V\,s$)	μ_{eo} ($cm^2/V\,s$)	μ_{ep} ($cm^2/V\,s$)
(1) Acebutolol	4.39	5.72	$7.33\cdot10^{-4}$	$5.63\cdot10^{-4}$	$1.70\cdot10^{-4}$
(2) Alprenolol	3.98	5.59	$8.09\cdot10^{-4}$	$5.76\cdot10^{-4}$	$2.33\cdot10^{-4}$
(3) Atenolol	4.03	5.53	$7.98\cdot10^{-4}$	$5.82\cdot10^{-4}$	$2.16\cdot10^{-4}$
(4) Metoprelol	4.03	5.54	$7.99\cdot10^{-4}$	$5.81\cdot10^{-4}$	$2.18\cdot10^{-4}$
(5) Oxprenolol	3.97	5.56	$8.11\cdot10^{-4}$	$5.78\cdot10^{-4}$	$2.33\cdot10^{-4}$
(6) Pindolol	3.95	5.60	$8.14\cdot10^{-4}$	$5.74\cdot10^{-4}$	$2.40\cdot10^{-4}$
(7) Propranolol	4.03	5.65	$7.47\cdot10^{-4}$	$5.69\cdot10^{-4}$	$1.78\cdot10^{-4}$
(8) Timolol	4.14	5.69	$7.77\cdot10^{-4}$	$5.66\cdot10^{-4}$	$2.11\cdot10^{-4}$

Conditions: 20 m*M* phosphate (pH 7.0); $L=58$ cm, $l=50$ cm, $V=15\,000$ V; t_0 marker = methanol; $\mu=(Ll)/(Vt)$; $\mu_{ep}=\mu_A-\mu_{eo}$.

we are operating in the normal polarity mode (positive inlet), and since all the analytes are positively charged at pH 7.0, we observe all analytes migrating faster than the bulk EOF. Since free solution CE separations occur due to differences in charge to frictional drag (size) ratio, it is not surprising that these analytes are poorly differentiated as noted by similarities in their electrophoretic mobilities in Table 2.

3.4. Determination of critical micelle concentration

In Fig. 5, we have used the method of Jacquier and Desbène [15] to measure the CMC for 1-lauroyl-2-hydroxy-*sn*-glycero-3-phosphocholine. By monitoring the change in retention with increasing lysophospholipid concentration, the CMC is determined. Calculation of the intersection of the two linear portions of the curve, resulted in a value of

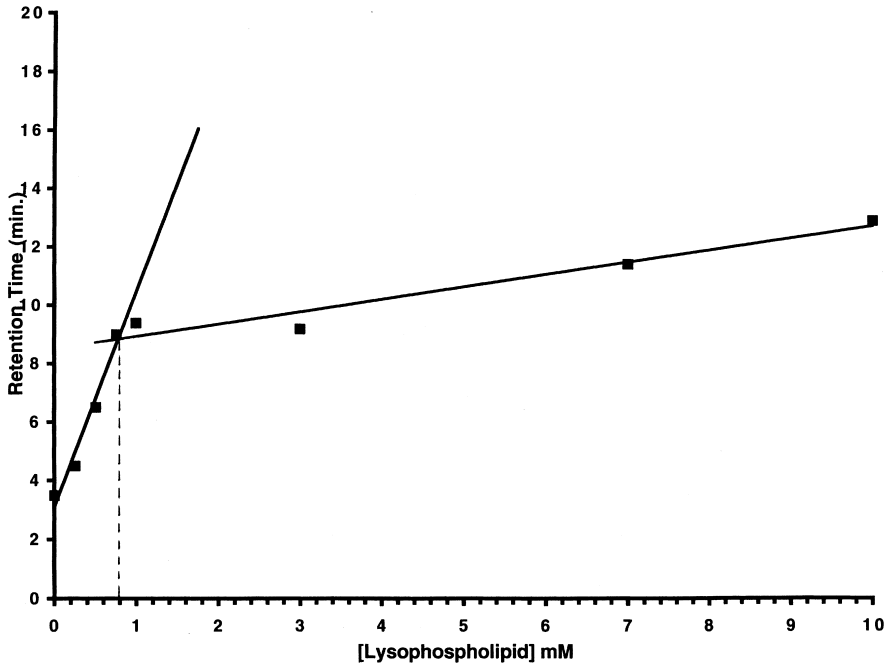


Fig. 5. Retention of propranolol vs. lysophospholipid concentration. Equations of linear portions are $y=7.4x+3.1$ and $y=0.42x+8.6$.

0.79 m*M* which agrees well with the literature value of 0.70 [10]. The lysophospholipid concentration of 10 m*M* chosen for the LMEKC experiments is about 14 times the CMC.

3.5. LMEKC of β-blockers

In Fig. 6 we demonstrate the analysis of pindolol with 10 m*M* lysophospholipid in the running buffer. Methanol is used as the EOF marker. A large decrease in EOF is evident, as t_0 increased from about 5.6 min to 15.5 min on the addition of lysophospholipid (Figs. 4 and 6). This decrease is not unusual for zwitterionic surfactants and has been previously reported [16], however, the magnitude of the decrease in EOF should be noted. This can be explained as a decrease in the net capillary wall charge, as the cationic quaternary ammonium head group of the lysophospholipid pairs with the anionic silanol groups on the capillary wall. This directs the aliphatic lipid tails away from the capillary wall, thus shielding the wall charge.

In Table 1, we see that these analytes are well differentiated, with LMEKC capacity factors ranging from 0.0 for atenolol to 1.4 for propranolol.

3.6. Correlations

A comparison between the LMEKC and IAM chromatographic retention behavior of eight β-blockers is illustrated in Fig. 7. The correlation factor ($R^2 = 0.64$) is not significantly improved by omission of any single data point, indicating a generally poor correlation with points spaced about the trendline. However, the general retention trends are maintained with the hydrophilic atenolol eluting first (unretained in LMEKC) and more hydrophobic propranolol and alprenolol eluting last in both methods. It was observed, however, that the intermediate compounds (2 to 6), did change retention order.

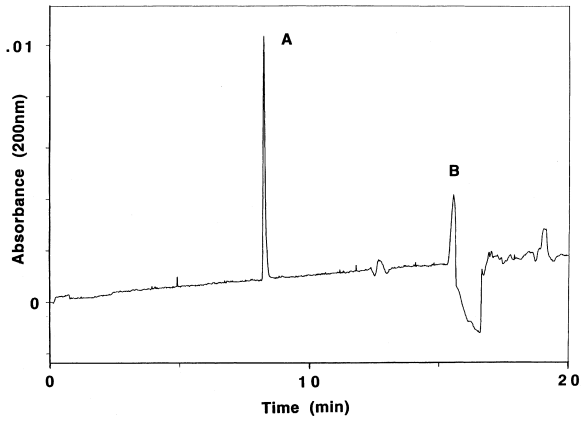


Fig. 6. Typical LMEKC chromatogram. Analysis of pindolol (A) using 10 m*M* lysophospholipid in running buffer; methanol (B) is used as t_0 marker.

The LMEKC buffer conditions (pH 7.0, (0.02 *M* total phosphate) were chosen to mimic the IAM chromatographic buffer conditions as was the purpose of this study. This resulted in k' values for LMEKC that were generally small (<1.5) (Table 1). If the objective of this work had been to develop a method to separate these analytes, conditions would have been altered to achieve larger k' values. This would have been possible by a combination of factors such as increasing pH, using less conductive buffers, and/or increasing lysophospholipid concentration.

In a study by Betageri and Rogers [2], dimyristoylphosphatidylcholine (DMPC) liposomes were used to measure partition coefficients (K') of all the β-blockers used in this study with the exception of timolol. LMEKC and IAM capacity factors (k') are compared with the liposome partition coefficients, corrected to pH 7.0, in Fig. 8. As these plots indicate, the LMEKC data correlates better ($R^2 = 0.95$ vs. 0.60) for this analyte set. This would indicate that the actual partitioning mechanism for the DMPC liposomes and the LMEKC micelles are similar, but significantly different for the IAM liquid coating. This difference could to due to the immobilized nature of the IAM surfactant vs. the free solution partitioning possible with both LMEKC and DMPC liposomes. Omission of acebutolol from the IAM data, improves the correlation to $R^2 = 0.8$ which is in agreement with values previously reported by Ong et al. [17]. This also demonstrates the inaccuracies of correlations on small data sets.

4. Conclusions

In this study we have demonstrated significant

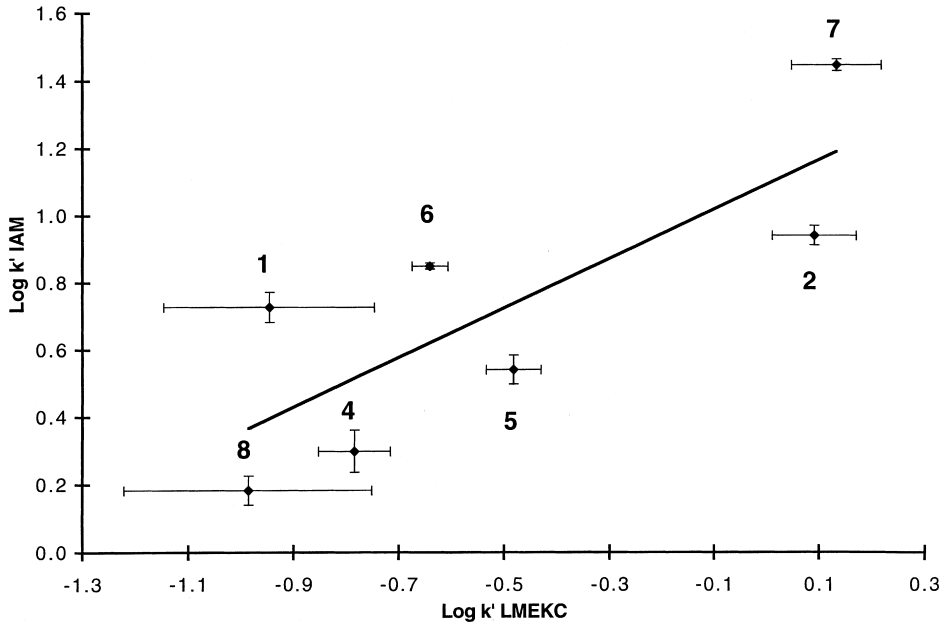


Fig. 7. Correlation of LMEKC and IAM chromatographic results. Analytes are labeled as in Table 1. Linear regression analysis yields $y=0.74x+1.10$, $R^2=0.64$. Log k' LMEKC and log k' IAM chromatography values are the average of three measurements. Error bars represent ± 1 S.D. Atenolol (compound 3) was unretained in LMEKC and not included.

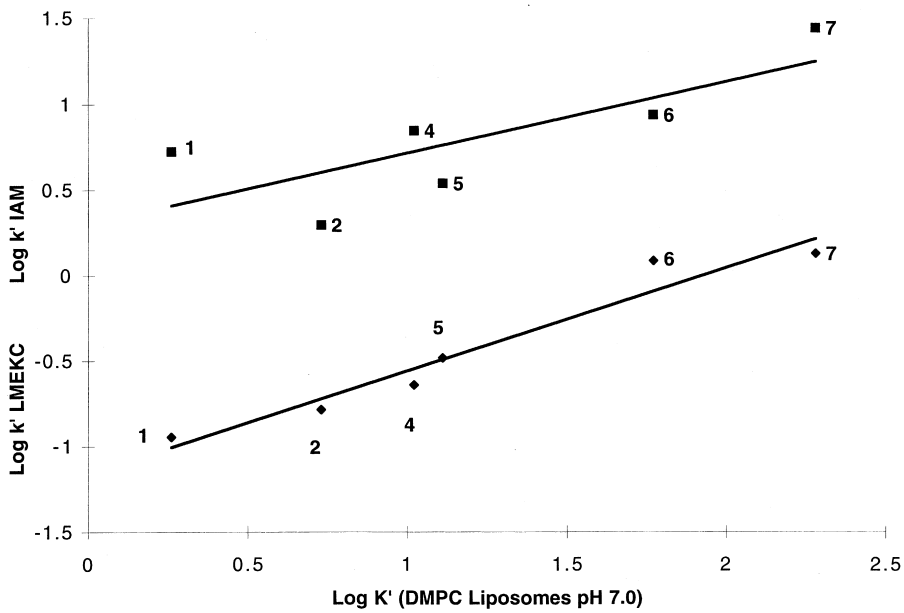


Fig. 8. Correlation of log K' of DMPC liposomes corrected to pH 7.0 with log k' LMEKC and log k' IAM. Linear regression analysis yields $y=0.61x-51.16$, $R^2=0.95$ for LMEKC and $y=0.42x+0.30$, $R^2=0.60$ for IAM. Analytes are labeled as in Table 1. Atenolol (compound 3) was unretained in LMEKC and not included. Timolol (compound 8) was not reported in the DMPC liposome study [2].

differences in retention for a set of eight β-blockers analyzed by IAM and LMEKC chromatographies. The data indicates that these techniques are useful for ranking purposes, with the most hydrophilic and hydrophobic eluting in similar manner with intermediate compounds eluting less predictably. Additionally, it was found that LMEKC has a much better correlation with liposome partitioning than does IAM chromatography for this small analyte set.

Finally, since LMEKC is much less expensive to use than IAM chromatography, which requires expensive columns with a limited working life, it can be considered a reasonable alternative.

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