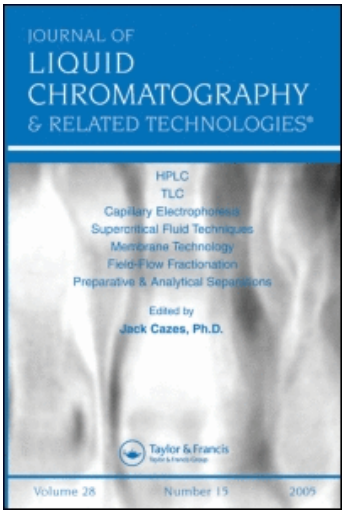

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Influence of Amphiphilic Mobile Phase Additives Upon the Direct Liquid Chromatographic Optical Resolution by Means of BSA-Based Chiral Sorbents

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INFLUENCE OF AMPHIPHILIC MOBILE PHASE ADDITIVES UPON THE DIRECT LIQUID CHROMATOGRAPHIC OPTICAL RESOLUTION BY MEANS OF BSA-BASED CHIRAL SORBENTS

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ABSTRACT

The effect of amphiphilic retention modifiers in liquid chromatography on BSA as a chiral stationary phase has been investigated. In all cases studied, capacity ratios are decreased or essentially unchanged. Decreasing capacity ratios affected the separation factors quite differently, depending on the nature of the analyte. In some cases increased α-values were found. Anionic modifiers (C_6 - C_{10} alkanoic acids) cause the largest reduction of the capacity ratios in a series of carboxylic acid analytes and are also most tightly bound to the stationary phase. The effects of hexylamine, hexanol and hexanoic acid upon the retention and resolution of an uncharged analyte (oxazepam) were very similar and not very large, indicating very little influence on the chiral binding site. The large effects on anionic analytes were not in good agreement with an ion-pair distribution model.

INTRODUCTION

Interaction of serum albumin with various ions has been a subject of considerable research. The affinity of the protein for ionic ligands bearing alkyl side chains has been shown to depend on the hydrophobic character of the side chain as well as the sign of the charged group. Anions with long chains such as fatty acids are bound very tightly, while molecules containing shorter side chains, or positively charged ionic groups, are less strongly bound (1).

The binding of fatty acids to bovine serum albumin (BSA) and their effect on the binding of drugs to BSA has been studied mainly by the technique of ultrafiltration, equilibrium partition and column affinity chromatography (2-5).

This paper describes the effect of amphiphilic and uncharged mobile phase additives on the retention and enantioselectivity of small chiral molecules using immobilized bovine serum albumin as a stationary phase (6) in a liquid chromatographic system.

EXPERIMENTAL

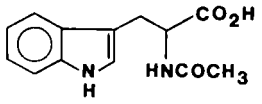
Chemicals

N-Acetyl-DL-tryptophan (I) was obtained from the Sigma Chem. Co. (St. Louis, Mo.). N-Benzoyl-DL-phenylglycine (II) was prepared using standard methods (7). The racemic barbiturates Benzonal (IIIa) and its analog (IIIb) were kindly supplied by Dr. J. Bojarski, Krakow, Poland. Synthesis of dansyl derivatives of racemic aspartic- (IVa) and glutamic acid (IVb) and of N-(2,4-dinitrophenyl)-(DNP-) derivatives of these acids (Va, Vb) was performed as described previously (8). Phtalimido-DL-alanine (VIa) and -DL-threonine (VIb) were prepared as described elsewhere (9). Oxazepam (VII), polythiazide (VIII), ibuprofen (IXa) and ketoprofen (IXb) were obtained from the Department of Drug Control, Biomedical Centre, Uppsala, Sweden. The dihydropyridine derivative H167/72 (X) was a gift from AB Hässle, Mölndal, Sweden.

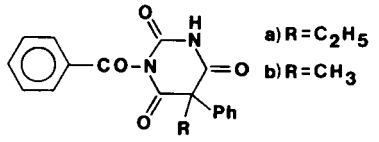
The mobile phase additives used in the study, hexylamine, octylamine, hexanoic acid, octanoic acid and decanoic acid were obtained from Aldrich,

SCHEME 1

Structures of the different compounds investigated.



I



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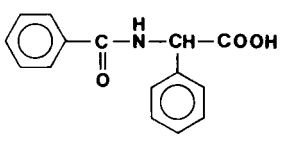

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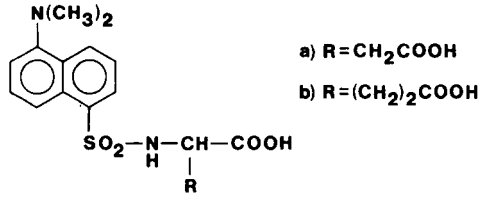

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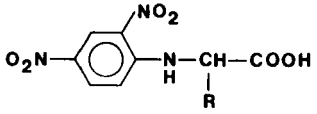
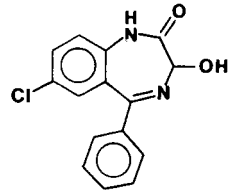
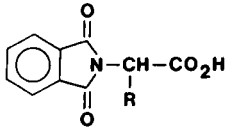


a) R=CH_2COOH
b) R=$(CH_2)_2COOH$

V

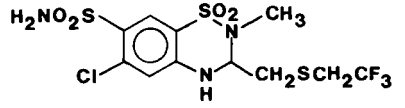


VII

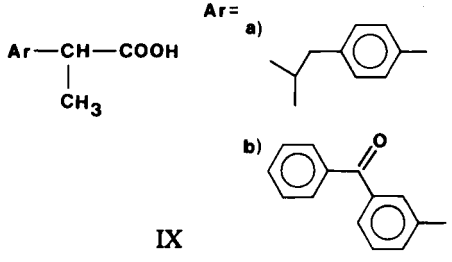


a) R=CH_3
b) R=$CHOHCH_3$

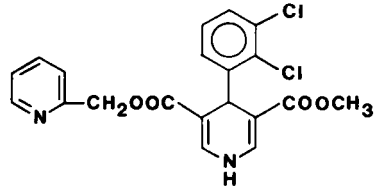
VI



VIII



IX



X

Milwaukee WI, USA. All other chemicals used were of analytical grade. Structures of all the compounds studied are given in Scheme 1.

Chromatography

The chromatographic system consisted of an LKB mod. 2150 solvent pump, a Rheodyne mod. 7125 sample injector equipped with a 20 µl loop, a 150 x 4.6 mm crosslinked BSA-silica (7µ) column (10), an LKB model 2151 variable wavelength UV detector, an LKB model 2210 potentiometric recorder and a Hewlett-Packard model 3390A electronic integrator. All chromatography was carried out isocratically using 50 mM phosphate buffers containing 0-5 mM of retention modifiers at a flow rate of 1.0 ml/min. The void volume was calculated from the position of the solvent front. Mobile phase systems: Phosphate buffer(50 mM) with 4% 1-propanol of pH 6.0 or 7.9 (system A); phosphate buffer (50 mM) with 3% of 1-propanol of pH 6.5 or 8.0 (system B); phosphate buffer (50 mM) with 10% of 1-propanol of pH 8.0 (system C).

RESULTS AND DISCUSSION

Column performance

The column performance and general chromatographic appearance is shown in Fig. 1, which demonstrates the resolution of polythiazide. The separation corresponds to $\alpha = 2.1$ and a resolution factor $R_s = 4.5$.

The effect of anionic mobile phase additives upon retention and resolution

Generally, BSA has a greater affinity for long chain fatty acids than for most other compounds (3). Addition of medium chained (C_6 - C_{10}) fatty acids to the mobile phase therefore promotes a competitive effect, leading to reduced k'-values, as illustrated by Table I.

The retention times of all the compounds studied decreased significantly with the increase in chain length of the fatty acid additive.

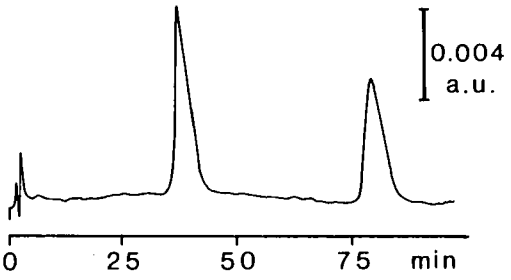


FIGURE 1. Separation of polythiazide enantiomers ($\alpha = 2.1$). Conditions Used: Phosphate buffer (50 mM, pH 8.0, 3% 1-propanol); UV 269 nm; flow rate 1.0 ml/min.

TABLE 1

Effect of Alkanoic Acids as Mobile Phase Additives.
Mobile phase system A.

Compound no	pH	Mobile phase A		Mobile phase additives (0.75mM) Hexanoic acid		Octanoic acid		Decanoic acid	
		k_1'	α	k_1'	α	k_1'	α	k_1'	α
I	6.0	18.3	1.5	3.40	1.3	0.36	1.0	0.36	1.0
	7.9	13.4	5.4	2.00	1.6	0.38	1.7	0.11	1.0
II	6.0	15.3	1.6	4.70	1.8	1.90	1.4	0.87	1.0
	7.9	13.6	1.5	3.10	1.8	1.30	1.1	0.33	1.0
IIIa	6.0	43.2	1.2	29.7	1.2	24.2	1.2	17.5	1.1
	7.9	25.6	1.4	21.3	1.4	15.8	1.4	5.20	1.5
IVa	6.0	73.6	1.4	23.7	1.4	9.90	1.6	2.90	1.3
	7.9	16.3	1.6	8.50	1.6	2.50	1.7	0.39	1.8
IVb	6.0	33.8	1.1	13.3	1.2	5.80	1.3	1.90	1.3
	7.9	8.80	1.5	4.20	1.5	1.30	1.6	0.31	1.6
Va	6.0	63.2	3.0	16.0	1.7	11.8	1.9	2.00	1.4
	7.9	10.0	3.0	4.20	1.9	2.20	2.2	0.33	1.7
Vb	6.0	34.9	1.3	14.3	1.4	6.70	1.3	2.20	1.0
	7.9	9.40	1.2	4.50	1.2	2.10	1.1	0.36	1.0
VIa	6.0	34.6	1.9	7.60	2.2	0.55	1.6	0.24	1.0
	7.9	13.7	2.1	4.60	2.3	0.22	2.0	0.11	1.0

N-Acetyl-DL-tryptophan (I) is readily resolved on the BSA-silica column and the enantioselectivity is greatly enhanced when the pH of the mobile phase is increased. This effect is largely due to the decrease in the retention of the first eluted enantiomer. The presence of fatty acids (C_6 - C_{10}) as retention modifiers results in a significant decrease or complete loss of the optical resolution of the enantiomers. Both enantiomers are eluted faster from the column, but the effect is greater for the last eluted enantiomer (L-). These results are consistent with the ultrafiltration studies by Cunningham et al. who concluded that medium chained fatty acids inhibit the binding of L-tryptophan (5).

Similarly, phtalimido-DL-alanine (VIa) shows a larger separation factor when the pH of the mobile phase is increased, while the opposite is true for N-bensoyl-DL-phenylglycine (II). The somewhat faster elution of the least retained enantiomer in the presence of hexanoic acid (0.75mM) results in larger α-values for both the compounds. This is illustrated by Fig. 2 which shows the optical resolution of phtalimido-DL-alanine. Addition of octanoic acid to the

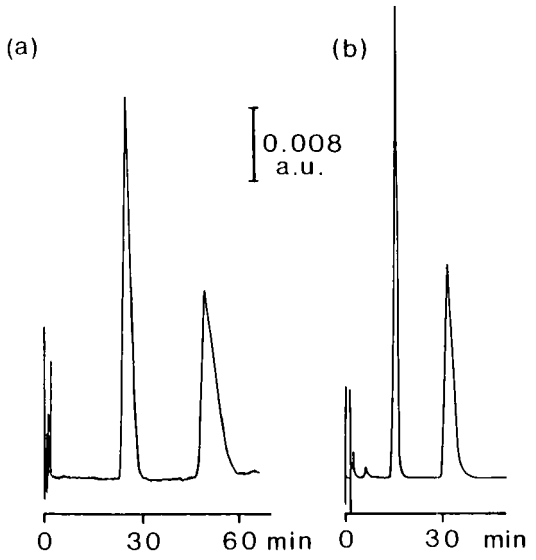


FIGURE 2. Chromatograms showing the large decrease in the retention of phtalimido-DL-alanine due to addition of hexanoic acid to the mobile phase. Mobile phase: a)Phosphate buffer (50 mM, pH 7.9, 4% 1-propanol); b) As in a) and containing 0,75mM hexanoic acid. UV 225 nm; flow rate 1.0 ml/min.

mobile phase results in a significant decrease of the α-values, whereas in the presence of decanoic acid a complete loss of resolution occurs. These data indicate that the binding of compounds II and VIa to the stationary phase involve both hydrophobic and electrostatic interactions. It is reasonable to assume that the inbinding of a fatty acid affects both these interactions, partly due to its hydrophobic side chain, but also because of the increase in the negative net charge of the stationary phase.

At pH 6.0, the enantiomers of the Dns- and 2,4-DNP- derivatives of aspartic acid (IVa, Va) are highly retained and show rather large separation factors. An increase in the pH of the mobile phase drastically decreases the k'-values with little or no change in the α-values. In the presence of alkanoic acid modifiers, no uniform trend in the separation factors of the aspartic acid derivatives is found, since a slight increase in the enantioselectivity is obtained when octanoic acid acts as the modifier (Table 1).

The separation factor α of Dns-DL-glu (IVb) increases with higher pH and also on addition of fatty acids (0.75 mM). The 2,4-DNP-derivative shows no great variation in its α-value except when decanoic acid is used as a modifier, in which case no enantioselectivity is found.

The racemic barbiturate Benzonal (IIIa), follows to a lesser extent the trend of rapidly decreasing k'- values. It would seem that the fatty acids do not bind to the same site as the analyte in this case.

Comparison between the effects of anionic, cationic and uncharged mobile phase additives on retention and selectivity

The effect of hexyl amine, hexanol and hexanoic acid on the k'- and α-values is illustrated in Table 2.

Compared to the retention obtained with phosphate buffer (3% 1-propanol) as the mobile phase, the presence of a second modifier resulted in a reduction of k'-values in all cases.

The acidic compounds N-acetyl-DL-tryptophan (I) and phtalimido-DL-threonine (VIb) are least retained on the column in the presence of hexanoic acid which also leads to a drastic decrease in α, especially for the threonine derivative. A more positive effect on the separation of both these compounds is shown at pH 8.0 on addition of hexylamine to the mobile phase.

TABLE 2

A Comparison of the Effects Caused by Different Types (Cationic, Neutral and Anionic) of Retention Modifiers. Mobile phase system B.

Compound no	pH	Mobile phase B		Mobile phase additives (0.75mM) Hexyl amine		Hexanol		Hexanoic acid	
		k_1'	α	k_1'	α	k_1'	α	k_1'	α
I	6.5	15.2	1.7	12.0	1.7	8.50	1.7	2.80	1.6
	8.0	10.1	2.0	8.00	2.1	4.80	2.0	1.80	1.8
IIIb	6.5	39.0	1.3	27.8	1.2	35.6	1.3	23.8	1.2
	8.0	18.4	1.6	16.4	1.6	15.7	1.7	13.3	1.6
VIb	6.5	3.60	6.1	2.70	5.4	1.90	5.8	2.50	2.7
	8.0	2.30	4.9	1.50	5.9	1.40	4.8	1.40	2.6
VII	6.5	15.2	3.5	14.7	3.1	14.0	3.1	14.1	3.2
	8.0	16.5	3.1	15.9	2.6	15.2	2.6	15.9	2.6
VIII	6.5	23.1	2.0	22.6	1.8	21.1	1.9	21.9	1.6
	8.0	20.8	2.1	20.9	1.9	20.6	1.9	20.9	1.8

Optical resolution of compound IIIb shows a significant pH dependence and the largest α-value is obtained with acidic buffers. Increased separation of the enantiomers is observed in the presence of hexanol, mainly due to faster elution of the least retained antipode.

Retention of polythiazide (VIII) is not greatly influenced by the mobile phase composition. The reduction in the k'-values is more pronounced for the last eluted enantiomer resulting in slightly lower α-values.

Hexanol as a second modifier exerts the largest influence on the retention and separation of the enantiomers of oxazepam (VII) resulting in lower k'- and α-values. These data and the fact that oxazepam does not respond significantly to a change in the pH of the mobile phase indicates that an essential part of its overall binding to the stationary phase is caused by hydrophobic interaction.

To better understand the large effects of the mobile phase additives, a study of the relationship between selectivity and concentration of the charged modifiers was carried out. The results are shown in Tables 3 and 4.

TABLE 3

The Effect of Increasing Concentration of Octanoic Acid Upon Retention and Resolution of Ibuprofen. Mobile phase system B, pH 8.0.

Octanoic acid Conc. (mM)	k_1'	k_2'	α
1.0	19.95	47.40	2.38
2.0	16.45	38.75	2.36
3.0	11.47	24.42	2.13
4.0	10.42	22.00	2.11
5.0	9.00	18.68	2.08

TABLE 4

The Effect of Increasing Concentration of Octylamine Upon Retention and Resolution of Ketoprofen and a Dihydropyridine Derivative. Mobile phase system C.

Octylamine Conc. (mM)	Compound IXb		Compound X	
	k_1'	α	k_1'	α
0	40.0	1.25	--	--
1.0	36.2	1.20	65.3	1.21
2.0	32.7	1.21	62.9	1.17
3.0	29.0	1.21	58.2	1.11
4.0	22.1	1.20	49.4	1.07

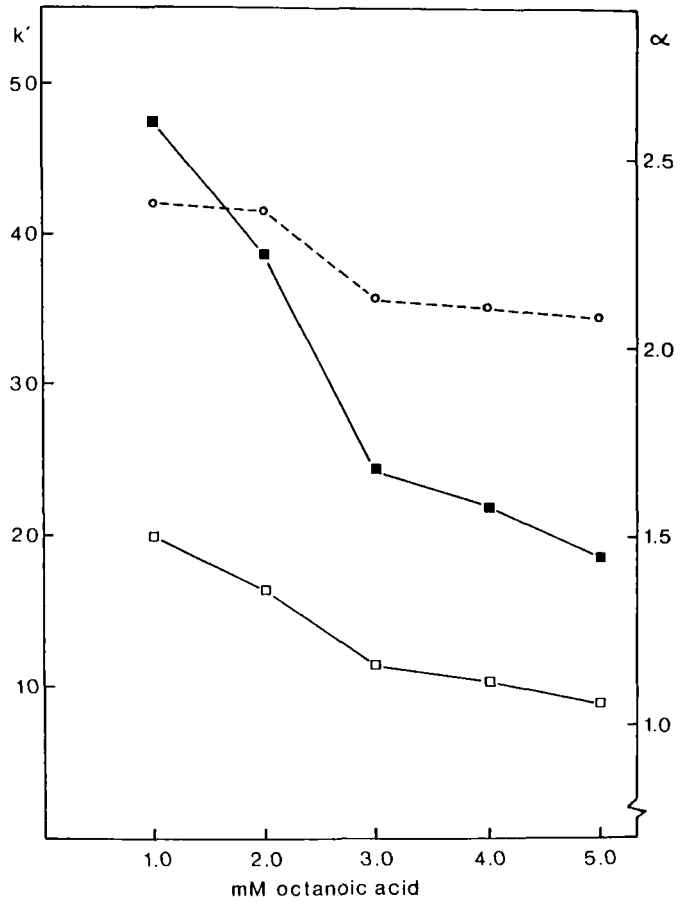


FIGURE 3. Influence of octanoic acid on the capacity ratios (k') and the separation factor (α) of ibuprofen. Mobile phase: Phosphate buffer (50 mM, pH 8.0, 3% 1-propanol) containing different amounts of octanoic acid. Symbols used: □ = k_1', ■ = k_2', o = α.

The effect of increasing concentrations of octanoic acid in the mobile phase upon k' and α of ibuprofen (IXa) is shown in Fig. 3. The greatest change occurs at a modifier concentration between 2.0 and 3.0 mM.

A similar effect is shown in Fig. 4 where octylamine is used as a modifier. Despite the different nature of the two analytes there is a decreasing retention of both enantiomers in each case, leading to decreased α in the case of compound X.

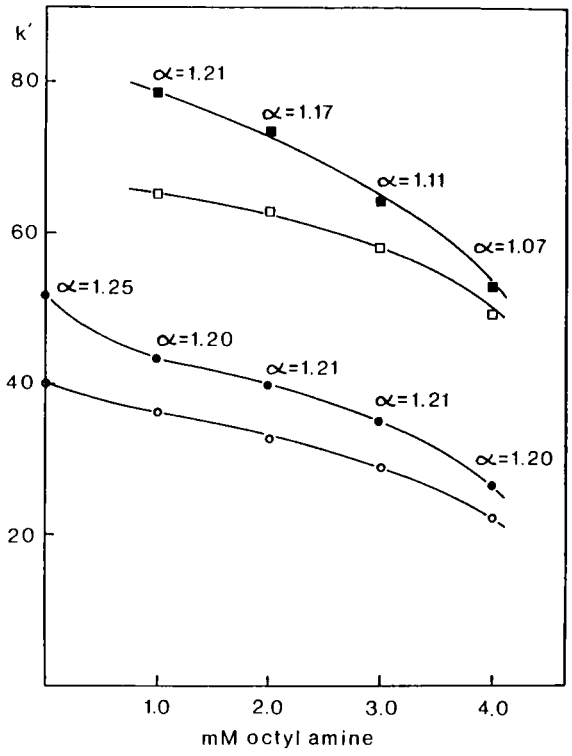


FIGURE 4. The effect of octylamine concentration on the k'- and α-values of ketoprofen (IXb) and H 167/72 (X), respectively. Mobile phase: Phosphate buffer(50mM, pH 8.0, 10% 1-propanol) with 0-4 mM octyl amine. Symbols used: o = k_1', ● =k_2' (IXb); □ = k_1',■ = k_2' (X).

Equations for the reversed-phase liquid chromatography of protolytes as ion-pairs have been developed by Schill and coworkers (11). Provided certain assumptions are made, an expression of the form given in eqn. 1, which relates the capacity ratio of an anionic analyte to the concentration of a retention modifier Q^+Z^-, can be derived.

$$k'_x = A \cdot K_{QX} \cdot [Q^+]_m / (1 + K_{QZ} \cdot [Q^+]_m \cdot [Z^-]_m) \quad (1)$$

In eqn. 1 K_{QX} and K_{QZ} are the equilibrium constants for the analyte and the modifier anion, respectively, as Q^+ ion pairs between the stationary and

mobile phase; A is a constant. Eqn. 1 does not take into account any retention of X^- in the absence of Q^+. To compensate for this, a second term, independent of QZ, will be required (11).

A linearization of eqn. 1 is obtained by inversion, yielding the relation:

$$1/k'_x = B / [Q^+]_m + C \cdot [Z^-]_m \qquad (2)$$

Linear relationships between 1/k' and $1/[Q^+]$ have been obtained experimentally for some aromatic carboxylic acids on C_{18}-columns in phosphate buffer/acetonitrile with tetrabutylammonium ion as modifier (11).

These equations have later been applied to the retention of amines (12) as well as carboxylic acids (13) on EnantioPac columns (immobilized α_1-acid glycoprotein as stationary phase). The significance of such correlations may, however, be questioned. In the authors' opinion, protein phases are extremely complex, giving rise to a multitude of different types of interactions (thereby causing enantiomer discrimination), making the situation far more complicated from the point of view of chemical equilibria and ion-pair distribution. In none of these cases has the correlation been applied to any increased retention by ion-pairing effects. Hermansson and Eriksson (13) make use of a second term, assuming a modifier-independent binding site, thereby introducing a variable for optimization of linearity. Further, their examples involved only uncharged analytes. We feel that the assumptions of one or two different types of binding sites are quite arbitrary and not justified. The reported cases represent competitive interactions by modifier and analyte with the protein phase resulting in decreased retention.

Our results show that a main function of the lipophilic retention modifiers is to compete with the analyte for hydrophobic binding sites and thereby often cause a significant decrease in retention of both enantiomers. Charge effects contribute, however, as found from the different influence on retention caused by alkanoic acids, alkanols and alkylamines. Another interesting example is the reported large effect of N,N-dimethyloctylamine (DMOA) upon the α-values of some carboxylic acids, notably naproxen (13). The large increase in α with increasing DMOA-concentration was mainly the result of a pronounced increase of k'_2 in this case. This means that several kinds of binding sites are engaged, since otherwise no change in α would result from a change in modifier concentration (12).

The failure of our as well as other data to fit within the framework of the ion-pair distribution model is indicative of a high complexity in the retention of analytes on stationary phases based on biopolymers. Further studies in this area are clearly needed.

ACKNOWLEDGEMENTS

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