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CHIRAL SEPARATIONS OF CATIONIC AND ANIONIC DRUGS ON AN α_1-ACID GLYCOPROTEIN-BONDED STATIONARY PHASE (ENANTIOPAC®)

II. INFLUENCE OF MOBILE PHASE ADDITIVES AND pH ON CHIRAL RESOLUTION AND RETENTION

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SUMMARY

The influence of mobile phase additives and pH on chiral resolution and retention on a high performance liquid chromatographic chiral stationary phase composed of α_1-acid glycoprotein bonded to diethylaminoethyl silica (EnantioPac®) has been investigated. Cationic and anionic compounds of widely differing structures were chromatographed and drastic effects on stereoselectivity were observed with hydrophobic charged modifiers. For cationic solutes, a decrease in the pH of the mobile phase from 7.0 to 6.0 gave reduced retention and, in some cases, improved selectivity when tetrabutylammonium or tetrapropylammonium bromide was used as modifier. For anionic solutes, a pH decrease from 6.6 to 6.1 gave enhanced retention but without a significant change in stereoselectivity. The steric bulk and hydrophobic moieties of the solute seem to have a strong influence on chiral selectivity. Widely different separating efficiencies were obtained with molecules of different structures.

INTRODUCTION

Analytical methods for separation and determination of enantiomeric compounds are often of vital importance in the development of new drugs and new therapeutic principles. During latter years, such studies have been significantly promoted by the development of a series of new separation methods based on the formation of diastereomeric complexes in the mobile or stationary phase of a chromatographic system.

Chromatographic systems with the enantiomeric complexing agent (the chiral selector) bound to a solid phase that can be used with aqueous mobile phases are of particular interest in the pharmacological studies of enantiomeric compounds. The

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mobile phases are compatible with biological fluids, and work-up procedures such as extraction and derivatization of ionized molecules are unnecessary.

One of the most promising of these phases has been developed by Hermansson[1,2] and is now commercially available as EnantioPac® (LKB, Bromma, Sweden). The chiral selector is α_1-acid glycoprotein (AGP) ionically bound to diethylaminoethyl silica and cross-linked by a procedure that involves oxidation, Schiff base formation and reduction of the enamines to secondary amines[3]. AGP is the main cationic binding protein in the human organism and it has an isoelectric point of 2.7 in phosphate buffer. It is composed of a peptide chain containing 181 amino acid units and five carbohydrate units which include fourteen residues of sialic acid. The carbohydrate units comprise 45% of the molecular weight, which is 41 000[4].

The stereoselective binding of cationic molecules to AGP in aqueous media has been studied by a dialysis technique using propranolol as the substrate[5,6]. Hermansson[7] has also determined the binding constants of the enantiomers of some cationic drugs by a chromatographic technique with a non-chiral solid phase and AGP present in the mobile phase. However, very little is known about how AGP binds ionized molecules. It seems that sialic acid is involved for some compounds, since Pike *et al.*[8] found that enzymatic desialylation of AGP reduces the binding affinity for cationic molecules while the binding of neutral and anionic molecules is unaffected.

EnantioPac appears to have a wide applicability to the resolution of molecules of pharmacological interest[2,9,10]. In our last paper[10], we demonstrated that the AGP-bonded column can be applied to chiral separations of cationic compounds of widely different structures, from simple aminoalcohols like ephedrine and pseudoephedrine to polycylic compounds such as methorphan and atropine. The retention and chiral selectivity is highly dependent on temperature and pH. Charged and uncharged modifiers will affect the retention, and in many cases significant improvements of the chiral separations could be obtained by the addition of hydrophobic, ionized mobile phase additives such as tetrabutylammonium bromide and octanoic acid. The conditions for the separation and chiral resolution of over 40 cationic molecules of pharmacological interest were presented.

This paper presents studies of the influence of mobile phase additives and pH on the chiral resolution and retention of cationic and anionic compounds of widely different structures. The often dramatic effect of the hydrophobic charged modifiers is demonstrated by studies on diastereomeric compounds and groups of closely related substances. The influence on the chiral selectivity of the steric bulk and hydrophobic moities of the solute is also illustrated, as is the widely different separating efficiency obtained with molecules of different structures. On the basis of these observations, the background to the large variations of the binding properties of the chiral phase with changes in the mobile phase composition and solute structure is discussed.

EXPERIMENTAL

Apparatus

The chromatographic experiments were performed with Spectra-Physics (Santa Clara, CA, U.S.A.) Model 8700 liquid chromatographs equipped with Spectra-

Physics Model 770 and Model 8773XR variable-wavelength detectors. The temperature of the columns and the mobile phase reservoirs was regulated by using thermostatically controlled circulating water. The injectors were Rheodyne Model 7125 with 10-μl loops.

The separations were performed on commercially available EnantioPac columns (100 mm × 4.0 mm I.D.) containing cross-linked α_1-acid glycoprotein bound to diethylaminoethyl silica (180 mg/g). The columns were generously supplied by LKB.

Materials

The terbutaline and bambuterol analogues were supplied by Draco (Lund, Sweden), UH 106 and 104 by Astra Pharmaceuticals (Sodertalje, Sweden) and nadolol A and B by Squibb (Princeton, NJ, U.S.A.). All other solutes were obtained from the stores of the U.S. Food and Drug Administration (Washington, DC, U.S.A.). The structures of some compounds of particular interest are given in Fig. 1.

The tetrapropylammonium and tetrabutylammonium bromides, aspartic, butyric and octanoic acids, ethylene and propylene glycols, 1,2-butanediol, N,N-dimethylethylamine, L-2,4-diaminobutyric and octylsulfate were purchased from Aldrich (Milwaukee, WI, U.S.A.). Decanoic and 6-aminohexanoic acids were purchased from Fluka (Hauppage, NY, U.S.A.). The 2-propanol was high-performance liquid chromatographic (HPLC) grade from Burdic & Jackson (Muskegon, MI, U.S.A.). All other chemicals were reagent grade and used without further purifications.

Chromatographic conditions

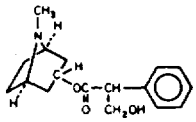
The standard conditions used during the chromatography were a flow-rate of 0.30 ml/min and a mobile phase temperature of 20.0°C. The mobile phases were 0.02 *M* phosphate buffers to which modifiers were added. The pH was adjusted to the desired level by the addition of sodium hydroxide or phosphoric acid. In most cases, a UV absorption maximum of the solute was chosen as the wavelength of detection. During most of the chromatographic studies, an EnantioPac column was placed in series before the injector to prevent changes in the properties of the separation column.

RESULTS AND DISCUSSION

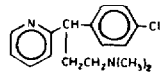
Influence of solute structure

In our previous paper on chiral separations of organic cations on the EnantioPac column[10], we used a working hypothesis implying that the stereoselectivity (α) is dependent upon the presence of hydrogen bonding groups (HB), large substituents or cyclic structures at the ammonium ion, N^+, and/or the HB group and the distance between the HB and N^+. This was illustrated using two series of compounds, one related to metoprolol and the other related to tocainide. Separation factors and separation conditions were also given for about 40 other compounds, all containing more or less strongly hydrogen bonding groups such as alcohol, amide, ester, ether, indene, oxo, phenol, pyridine and phenothiazine moieties.

The applicability of the chiral phase is, however, so large that these require-



Atropine



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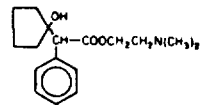
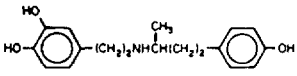
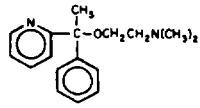


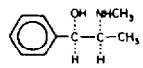
Cyclopentolate



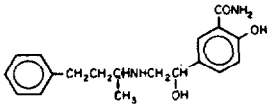
Dobutamine



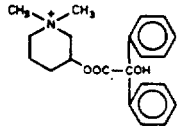
Doxylamine



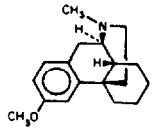
Ephedrine



Labetalol



Mepensolate



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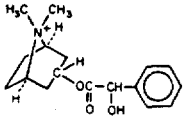
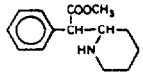
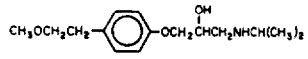


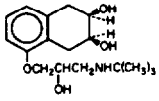
Methylhomatropine



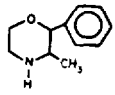
Methylphendiate



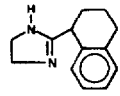
Metoprolol



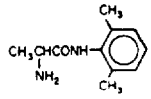
Nadolol



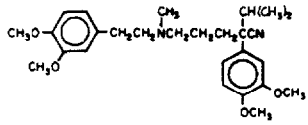
Phenmetrazine



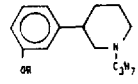
Tetrahydrozoline



Tocainide



Verapamil



UH 106 R = H

UH 104 R = CH_3

Fig. 1. Structures of compounds used in the studies.

ments can be considered only as basic observations on important structural features. More specific rules for the relationship between structure and chiral selectivity can be given only for groups of closely related compounds.

Further studies on cations containing several rings or ring systems have indi-

TABLE I

SEPARATION OF ENANTIOMERIC CATIONS

Mobile phase: modifier in 0.02 *M* phosphate buffer. k'_1 = capacity ratio of first eluted enantiomer; α = separation factor (k'_2/k'_1).

Solute	*Modifier*	*pH*	k'_1	α
Dobutamine	Tetrapropylammonium bromide (0.001 *M*)	6.0	14	1.56
Methorphan	Tetrabutylammonium bromide (0.003 *M*)	6.0	5.2	2.69
Tetrahydrozoline	Dimethylethylammonium (0.1 *M*)	7.0	5.8	1.66
Verapamil	Decanoic acid (0.005 *M*) + Tetraethylammonium bromide (0.05 *M*)	7.0	13	1.75
UH 104	Decanoic acid (0.01 *M*)	7.0	6.4	1.42
UH 106	Decanoic acid (0.01 *M*)	7.0	9.3	1.30

cated that the presence and position of the hydrogen bonding groups is of minor importance for the chiral selectivity of such molecules. Some examples are given in Table I. The bulky and very weakly hydrogen bonding compounds, tetrahydrozoline and methorphan, show high separation factors in systems with a cationic modifier in the mobile phase. The closely related compounds UH 106 and 104 give a further illustration: a change from a methoxy substituent in UH 104 to a phenolic group in UH 106 gives rise to a decrease in α.

In the previous paper[10], we also demonstrated that under certain conditions both anionic and cationic enantiomeric compounds can be resolved on the Enantio-Pac column. Further examples of the chiral resolution of anionic compounds are given in Table II. Resolution can be obtained with quaternary ammonium modifiers as well as with uncharged modifiers, but the former give higher separation factors.

It is important to notice that 2-phenylbutyric, 3-phenylbutyric and 2-phenylpropionic acids contain no hydrogen bonding moieties and that the separation factor increases with increasing length of the alkyl chain coupled to the chiral carbon. The results in Table II also indicate that the separation factor increases in the presence of a hydrogen bonding group and with increasing bulk of the aromatic group (*cf.* 2-phenylpropionic acid, 2-phenoxypropionic acid and ibuprofen).

It must be emphasized that the relationship between solute structure and chiral selectivity can be drastically changed by the properties of a modifier added to the mobile phase. Some examples of this are given in Table III, which contains the results for aminoalcohols related to terbutaline and bambuterol when decanoic acid and 6-aminohexanoic acid were used as modifiers. The chiral selectivity changes dramatically with the substitution in the aromatic ring, but in quite different directions with the two modifiers. For the diphenols, the highest separation factors are achieved with 6-aminohexanoic acid as modifier, while the monophenols have significantly higher separation factors in the presence of decanoic acid. It should be noted that no chiral resolution is obtained when there is a methyl substituent at the chiral carbon.

The bulkiness of the molecule seems to be of vital importance for the chiral resolution. Terbutaline with a tertiary butyl substituent at N^+ is easily resolved. However, it has not, as of yet, been possible to obtain a chiral resolution for metaproterenol (with an isopropyl substituent at N^+) or any other phenylethanolamine

TABLE II

SEPARATION OF ENANTIOMERIC CARBOXYLIC ACIDS

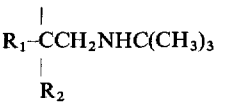
Mobile phase: modifier in 0.02 *M* phosphate buffer, pH 7.0.

Solute	*Modifier*			
	Dimethylethylammonium (0.1 M)		*Propylene glycol (0.25 M) + sodium chloride (0.1 M)*	
	k'_1	α	k'_1	α
2-Phenylbutyric acid	3.4	1.97	1.8	1.46
3-Phenylbutyric acid	2.5	1.20	–	–
2-Phenoxypropionic acid	1.3	1.99	–	–
2-Phenylpropionic acid (PhPA)	1.7	1.15	–	–
Ibuprofen (4-isobutyl-PhPA)	8.3	1.90	4.3	1.25
Naproxen (6-methoxy-2-naphthyl-PA)	–	–	10.8	1.20
Fenoprofen (3-phenoxy-PhPA)	–	–	18.2	1.31

TABLE III

SEPARATION OF ENANTIOMERIC COMPOUNDS RELATED TO TERBUTALINE

Mobile phase: modifier in 0.02 *M* phosphate buffer, pH 7.0.



Solute		*Modifier*			
R_1	R_2	*6-Aminohexanoic acid (0.10 M)*		*Decanoic acid (0.005 M)*	
		k'_1	α	k'_1	α
3,5-Dihydroxyphenyl*	H	0.9	1.33	1.1	1.21
2,5-Dihydroxyphenyl	H	1.2	1.47	0.8	1.0
4-Hydroxyphenyl	H	0.8	1.0	0.9	2.00
2-Hydroxyphenyl	H	6.7	1.0	1.6	1.78
Phenyl	H	–	–	1.7	1.13
3,5-Dihydroxyphenyl	CH_3	0.8	1.0	0.8	1.0
3,5-bis(Dimethylcarbamyl)phenyl**	H	2.2	1.50	1.2	1.0
3-Hydroxy-5-dimethylcarbamylphenyl	H	37	1.16	2.3	1.32

* Terbutaline.
** Bambuterol.

derivative that does not contain a bulky substituent in the phenyl ring and where there is a propyl or smaller group at N^+.

Insufficient bulkiness might also be the reason for the inability of the AGP chiral stationary phase to resolve nicotine.

Modifier effects

Charged and uncharged mobile phase additives were primarily used to reduce the retention of highly retained compounds. However, our previously published studies showed that under certain conditions charged compounds are retained as ion pairs, and thus charged modifiers will produce different effects than uncharged modifiers. Our studies also gave rise to the assumption that the retaining phase contains chiral and non-chiral binding sites, both with limited capacities. The modifiers, therefore, can compete for both kinds of binding sites, resulting not only in a decrease in retention but also in a change in chiral selectivity.

Our initial studies also indicated a rather complex retention mechanism. It was observed that a decrease of the retention of cations was obtained not only by cationic mobile phase additives but also with anionic modifiers, *i.e.* counter ions, such as butyrate and octanoate. Closer studies (ref. 10, Fig. 4) gave results indicating an ion-exchange mechanism, but on different levels depending on the nature of the counter ion.

In order to improve the understanding of the effect of the mobile phase ad-

ditives, the studies were continued with modifiers of different kinds such as diols (ethylene and propylene glycol and 1,2-butanediol); uncharged amino acids (6-aminohexanoic acid, beta-alanine, leucine); cations (dimethylethylamine, 2,4-diaminobutyric acid); and anions (decanoic and aspartic acid).

Diols

The retention of compounds that are strongly bound to the chiral phase can be considerably reduced by monovalent alcohols such as 2-propanol, but these additives will often give a significant reduction of the chiral selectivity. The diols present an interesting alternative, as they are known to give low-energy interactions with proteins (cf. refs. 11 and 12) and might be used in relatively high concentrations without risk of denaturation of the protein.

A comparison of the effects of some diols, ethanol and 2-propanol is presented in Table IV. The results show that the addition of a diol to the mobile phase gives a decrease in retention, as does the addition of ethanol and 2-propanol. The stereoselectivity is reduced when the diols are used as modifiers but, for some compounds, to a lesser extent than the reductions observed with ethanol and 2-propanol. The diols, however, increase the viscosity of the mobile phase, which might affect the separating efficiency as discussed below.

Amino acids

Uncharged amino acids have a rather limited effect on the retention and chiral selectivity within the concentration ranges studied. For example, an increase of the concentration of aminohexanoic acid from 0.01 to 0.10 *M* at pH 7.0 decreases the

TABLE IV

INFLUENCE OF ALCOHOLS AS MODIFIERS

Mobile phase: modifier + 0.1 *M* sodium chloride in 0.02 *M* phosphate buffer, pH 7.0.

Modifier	*Solute*							
	Cyclopentolate		*Labetalol A*		*Labetalol B*		*Methylhomatropine*	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α
Ethylene glycol (0.32 *M*)	23	1.73	27	1.69	25	1.37	1.8	2.65
Ethylene glycol (1.29 *M*)	12	1.46	12	1.64	14	1.30	1.3	2.75
Propylene glycol (0.25 *M*)	–	–	19	1.71	23	1.28	2.0	3.27
1,2-Butanediol (0.25 *M*)	7.5	1.86	8.4	1.27	9.6	1.17	1.5	3.81
Ethanol (0.44 *M*)	21	1.95	15	1.45	19	1.28	1.6	3.10
Ethanol (1.74 M)	3.2	1.81	5.2	1.12	–	–	0.7	2.00
2-Propanol (0.33 *M*)	8.9	1.89	9.7	1.19	9.6	1.10	1.0	2.42

retention of most cationic solutes by about 30%. The stereoselectivity often remains the same as in the absence of a modifier, but an increase has also been observed in some cases.

Charged modifiers

The drastic effects of some cationic and anionic modifiers on chiral selectivity was discussed in our previous paper. The magnitude of these effects is particularly well demonstrated by some studies using diastereomeric solutes. Fig. 2 shows the effect of modifiers on the chiral resolution of the diastereomeric aminoalcohols ephedrine and pseudoephedrine. For both compounds, the chiral separation factor

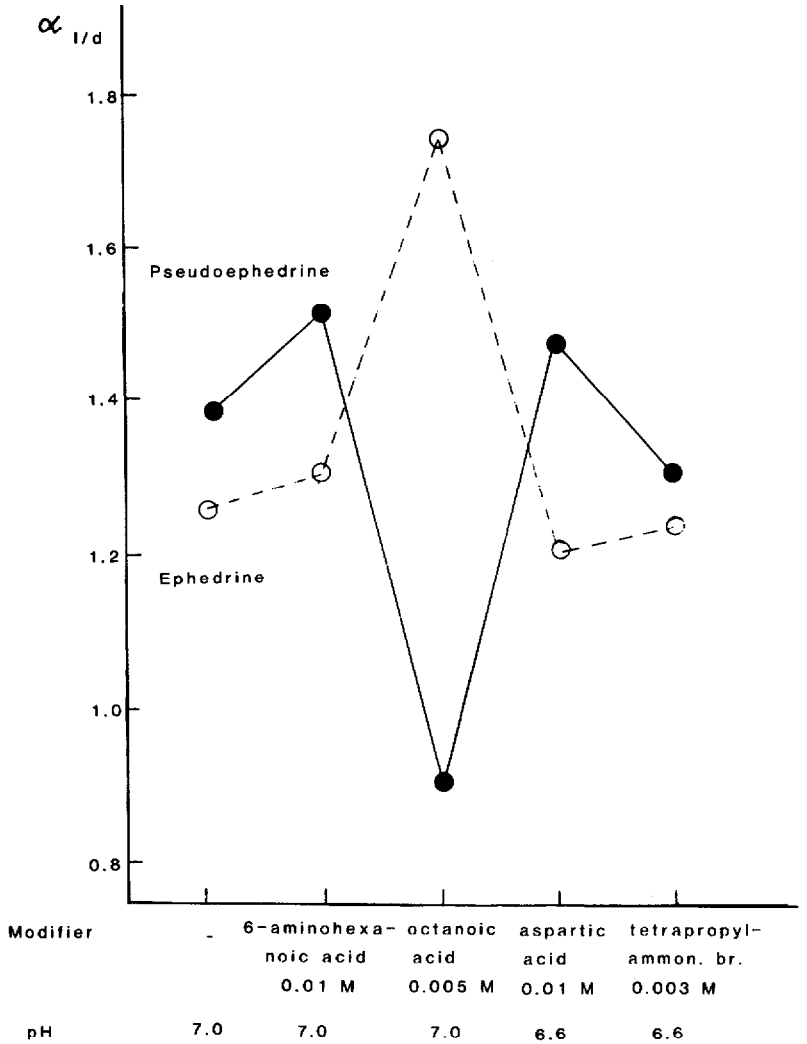


Fig. 2. Chiral separation of the diastereomeric ephedrine and pseudoephedrine. $\alpha_{L/D}$ = k' of L-form / k' of D-form.

is only slightly affected by the uncharged modifier 6-aminohexanoic acid and the cationic modifier tetrapropylammonium bromide and the D-enantiomers have shorter retentions than the corresponding L-enantiomers. A drastic change is obtained with the anionic modifier octanoic acid. The chiral resolution of ephedrine is significantly increased and that of pseudoephedrine is decreased. In addition, there is a reversal of the retention order between the enantiomers of pseudoephedrine, the L-form now having the shorter retention. It is interesting to note that the use of aspartic acid as a modifier does not give rise to the same effects.

The chiral resolution of the strongly retained diastereomers, labetalol A and

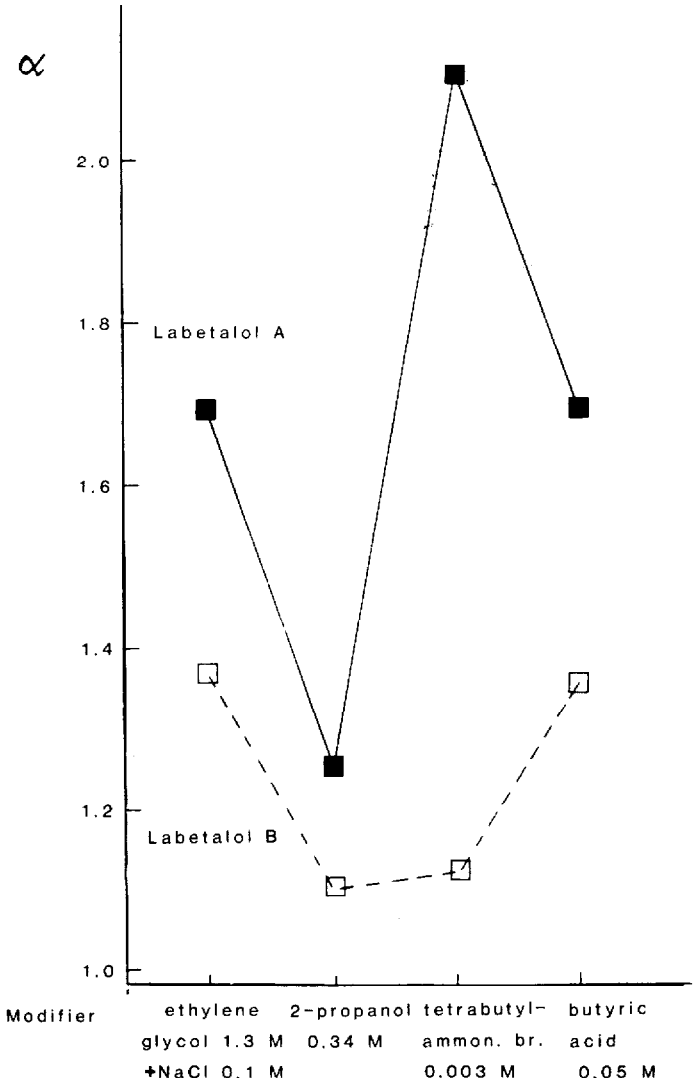


Fig. 3. Chiral separation of the diastereomeric labetalol A and B; pH 7.0. $\alpha = k'$ of the second peak/k' of the first peak.

B, can only be studied in the presence of alcohols or hydrophobic charged modifiers, which give a significant decrease in retention. The observed influence of tetrabutylammonium bromide is remarkable (Fig. 3). The chiral resolution of labetalol A is significantly increased, while there is no similar effect observed for labetalol B. Possible changes in retention order could not be studied since the pure enantiomers were not available.

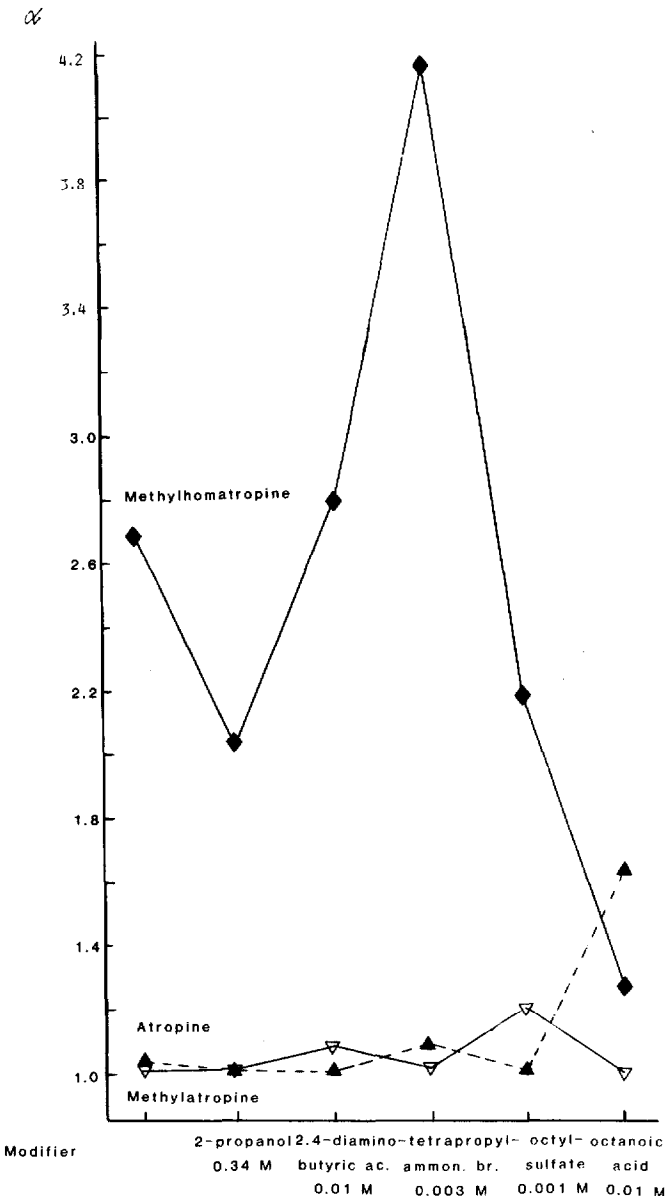


Fig. 4. Chiral separation of the structurally closely related atropine, methylatropine and methylhomatropine, pH 7.0. $\alpha = k'$ of the second peak / k' of the first peak.

Another drastic illustration of the highly complex nature of the relationship between stereoselectivity, solute structure and mobile phase additive is given in Fig. 4. This figure shows the effect of various mobile phase modifiers on the chiral separation factors for the enantiomers of three closely related compounds: the tertiary amine atropine, its N-methyl derivative (methylatropine) and another quaternary ammonium ion, N-methylhomatropine, which differs from methylatropine only by a methylene group in the side-chain coupled to the chiral carbon.

N-methylhomatropine is easily separated into enantiomeric forms, with and without a modifier in the mobile phase. In this study, the maximum α value was achieved after the addition of tetrapropylammonium bromide. The separation factor decreases in the presence of 0.01 *M* octanoic acid and no chiral separation was observed with an octanoic acid concentration of 0.03 *M* or higher. Decanoic acid gave a similar effect. However, a significantly smaller decrease in α was observed following the addition of butyric acid.

Unlike N-methylhomatropine, the closely related methylatropine is very difficult to resolve into its enantiomeric forms. A maximum α value of 1.2–1.3 is achieved using 0.001 *M* octylsulfate or 0.025 *M* cyclohexylsulfamate as modifier.

The corresponding tertiary amine, atropine, shows a quite different response to the mobile phase additives than the other two related compounds. Very low chiral resolution is obtained with cationic modifiers, while a rather high separation factor of over 1.6 is obtained in the presence of octanoic acid.

It is important to note that particularly high chiral separation factors are observed for anionic solutes when cationic modifiers such as dimethylethylamine are used (see Table II), while no or very low α values are obtained in the presence of anionic modifiers.

A retention that is deemed too high can be decreased by the addition of a cationic or anionic modifier, as demonstrated in Tables V and VI. The method has, however, a limited application since high modifier concentrations, as a rule, decrease the chiral selectivity. It is also possible to reduce a high capacity ratio by using combinations of modifiers, but this approach has so far only been tested in a limited number of cases.

pH effects

Besides the addition of a modifier, a change in the pH of the mobile phase is

TABLE V

INFLUENCE OF MODIFIER CONCENTRATION: TETRABUTYLAMMONIUM

Mobile phase: tetrabutylammonium bromide in 0.02 *M* phosphate buffer, pH 7.0.

Concn. (M)	*Chlorpheniramine*		*Methorphan*		*Methylphenidate*	
	k'_1	α	k'_1	α	k'_1	α
0.001	15.5	2.24	13.1	2.73	4.0	2.16
0.003	11.0	2.26	8.1	2.72	2.3	2.17
0.010	4.9	1.51	4.3	1.94	1.0	1.53

TABLE VI

INFLUENCE OF MODIFIER CONCENTRATION: OCTANOIC ACID

Mobile phase: octanoic acid in 0.02 *M* phosphate buffer, pH 7.0.

Concn. (M)	*Atropine*		*Cyclopentolate*		*Labetalol A*		*Methorphan*	
	k'_1	α	k'_1	α	k'_1	α	k'_1	α
0.01	6.3	1.64	13.0	1.82	11.8	1.35	–	–
0.03	3.7	1.50	4.8	1.54	9.8	1.17	18.4	1.70
0.05	2.8	1.51	3.5	1.50	7.4	1.14	6.9	1.46

the main means of adjusting the retention. The pH of the mobile phase should be kept within the range 3.0–7.5, as the silica-based column is unstable outside this region.

For cationic solutes, a decrease in pH usually results in decreased retention.

TABLE VII

INFLUENCE OF pH: CATIONIC SOLUTES

Mobile phase: modifier in 0.02 *M* phosphate buffer.

Modifier	*pH*	*Solute*							
		Chlorpheniramine		*Cyclopentolate*		*Doxylamine*		*Methorphan*	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α
Tetrabutylammonium bromide (0.003 *M*)	7.0	11.0	2.26	18	1.70	9.5	1.23	8.1	2.72
	6.0	6.6	1.0	4.8	2.09	6.1	1.37	5.2	2.69
		Metoprolol		*Phenmetrazine*		*Tocainide*			
		k'_1	α	k'_1	α	k'_1	α		
Tetrapropylammonium bromide (0.001 *M*)	7.0	3.5	1.49	2.9	1.29	3.5	1.49		
	6.0	1.5	1.54	1.2	1.44	1.3	1.13		
		Atropine		*Methorphan*		*UH 104*			
		k'_1	α	k'_1	α	k'_1	α		
Octanoic acid (0.005 *M* 10°C)	7.0	8.9	1.66	9.8	2.26	5.3	2.15		
	6.0	1.8	1.32	8.6	1.23	3.5	2.13		
		Cyclopentolate		*Doxylamine*		*Mepensolate*			
		k'_1	α	k'_1	α	k'_1	α		
Sodium chloride (0.1 *M*) + 2-propanol (0.33 *M*)	7.5	14	1.96	9.8	1.15	10.0	1.32		
	6.5	4.8	1.79	6.2	1.16	6.8	1.31		

TABLE VIII

INFLUENCE OF pH: ANIONIC SOLUTES

Mobile phase: 0.003 *M* tetrapropylammonium in 0.02 *M* phosphate buffer (10°C).

pH	*Solute*					
	2-Phenylbutyric acid		*2-Phenylpropionic acid*		*2-Phenoxypropionic acid*	
	k'_1	α	k'_1	α	k'_1	α
6.6	5.3	1.70	2.6	1.15	2.4	1.26
6.1	10.8	1.65	4.5	1.16	4.3	1.22

However, the effect on retention and stereoselectivity is dependent on both the nature of the solute and the composition of the mobile phase, as demonstrated in Table VII. For example, when cyclopentolate is the solute, a decrease of pH from 7.0 to 6.0 results in increased chiral selectivity when the modifier is 0.003 *M* tetrabutylammonium bromide and decreased selectivity in a mobile phase of sodium chloride (0.1 *M*) and 2-propanol (0.33 *M*). For doxylamine, a decrease in pH also results in an increase in α when the modifier is 0.003 *M* tetrabutylammonium bromide, but has no effect in the sodium chloride–2-propanol system. The stereoselectivity of methorphan, on the other hand, is unchanged when the pH of the tetrabutylammonium bromide-containing mobile phase is decreased and drastically reduced when the pH of an octanoic acid (0.005 *M*)-containing mobile phase is changed from 7.0 to 6.0.

It appears that a decrease in pH can give improved chiral selectivity for some compounds when tetrabutylammonium and tetrapropylammonium bromides are used as modifiers. Similar effects have not been observed in the presence of octanoic acid or sodium chloride–2-propanol.

For the anionic solutes studied, a decrease in pH increased retention without a significant change in chiral selectivity (Table VIII).

Separating efficiency

The theoretical plate height and peak symmetry obtained with the EnantioPac column was discussed briefly in our previous paper. It was shown that loadings higher than 3–4 nmol of a solute like methylhomatropine will give rise to peak asymmetry and a decrease in k'.

The present study of the separating efficiency was made using concentration ranges in which further reduction of the sample concentration did not affect k'. Only peaks with asymmetry factors in the range of 0.8 to 1.4 were evaluated. The results obtained with solutes of widely different structures and in the presence of different kinds of modifiers are given in Table IX. In cases of incomplete separation of enantiomers, evaluations were made from runs of pure enantiomers when they were available.

The results show that there is a significant difference in the separating efficiency for different classes of compounds. The carboxylic acids have *H* values below 0.10 mm. For atropine, methylhomatropine, phenmetrazine and tocainide the *H* values are in the range 0.10–0.13 mm, while ephedrine and metoprolol in most cases have

TABLE IX

SEPARATING EFFICIENCY (H) FOR ENANTIOMERIC SOLUTES

Mobile phase: 0.02 M phosphate buffer with and without modifier. H_1 and H_2 are plate heights in mm for first and second peak, respectively.

Solute	*Modifier*					
	Tetrapropylammonium bromide (0.003 M), pH 6.6		*Octanoic acid (0.005 M), pH 7.0*		*Without modifier, pH 6.6*	
	H_1	H_2	H_1	H_2	H_1	H_2
2-Phenylbutyric acid	0.08	0.08	–	–	0.08	0.07
2-Phenoxypropionic acid	0.10	0.09	–	–	0.09	0.07
Atropine	–	–	0.11	0.11	0.11	–
Methylhomatropine	0.13	0.12	0.10	0.09	0.13	0.12
Phenmetrazine	–	–	0.13	0.11	–	–
Tocainide	0.11	0.09	–	0.13	–	0.10
Nadolol A	0.15	0.12	0.16	–	0.12	–
Ephedrine	0.20	0.17	0.19	0.20	0.25	–
Metoprolol	0.26	0.20	–	–	0.28	0.21

a value for H of *ca.* 0.25 mm. It has not, as yet, been elucidated if the modifiers themselves give rise to significant differences in H values.

A change in pH from 7.0 to 6.0 did not result in any significant change in the separating efficiency. However, a decrease in temperature from 20.0 to 10.0°C increased H by *ca.* 20%.

In the presence of the diol modifiers, ethylene and propylene glycol, overloading effects such as peak asymmetry and concentration-dependent k' appeared even at loadings below 1 nmol, *i.e.* at significantly lower amounts than usual.

Properties of the chiral binding phase

The stereoselective binding ability of the AGP, which is the selector in the EnantioPac columns, has been demonstrated by different experimental techniques, both non-chromatographic[5,6] and chromatographic[7]. However, the structural background to the chiral binding ability has not been elucidated. The peptide chain as well as several components in the carbohydrate groups of the protein contain sites with chiral binding abilities.

The EnantioPac columns are prepared by binding AGP to diethylaminoethyl-silica by ionic forces. The protein is then immobilized by a cross-linking procedure. It is obvious that this procedure will denature the protein to a certain extent, but the product might still be rather loosely bound to the silica matrix.

The applicability of the EnantioPac column to chiral separations of structurally different molecules and the large differences in the separating efficiency for molecules of different structure and charge is very difficult to combine with the assumption of a single kind of chiral binding site. It is, furthermore, remarkable that depending on the structure of the solute a change in the properties of the mobile phase, such as type and concentration of a charged modifier or pH, can alter the chiral selectivity

and even reverse the retention order. Such drastic changes indicate that rather radical changes of the properties of the binding sites have occured.

It is well known that changes in the solvent can create conformational changes in protein molecules (*cf.* refs. 13 and 14) and it has been suggested that such processes might give rise to the drastic alterations of the chiral binding ability of the protein selector in the EnantioPac column. A decisive point is the question of whether the binding to the silica matrix and the cross-linking are of such a nature that the protein has sufficient flexibility for these changes. No definite conclusions can be drawn about conformational changes in the protein stationary phase from the present studies.

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