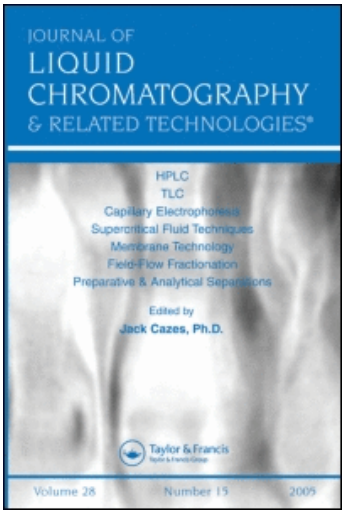

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Separation of Enantiomers in Ion-Pair Chromatographic Systems

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SEPARATION OF ENANTIOMERS IN ION-PAIR CHROMATOGRAPHIC SYSTEMS

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ABSTRACT

Enantiomers of amines and acids have been separated in ion-pair chromatographic systems with chiral agents present in mobile or stationary phase. Chiral counter ions such as quinine or camphorsulfonate have been used in liquid solid systems with organic mobile phases, while the chiral complexing agents albumin and (+)-n-dibutyltartrate have been used in reversed phase systems. Regulation of retention and stereoselectivity as well as improvement of detection sensitivity by the composition of the system is discussed.

INTRODUCTION

During the last decade much interest has been focused on the development of new techniques for separation of enantiomers (optical isomers). The fact that the two enantiomeric forms of a molecule (drug, herbicide, insecticide) might have different effects in biological systems (1) has given rise to a strong

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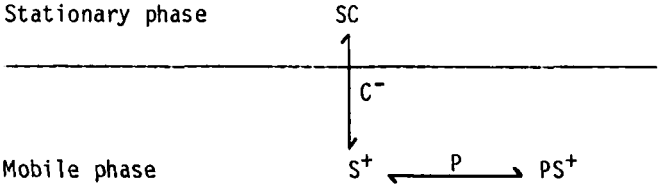
demand for analytical methods that enable resolution of racemic mixtures and quantitative determination of the individual antipodes in samples of different origin.

Chromatography has for a long time been used to resolve enantiomers and for determination of enantiomeric composition. The chromatographic technique is easy to operate and has furthermore the advantage that the determination of optical purity is not dependent on the degree of specific rotation as other types of detectors, not based on measuring the rotation of planpolarized light can be used.

Many different approaches have been used to separate enantiomers in liquid chromatography (cf. 2,3). Chiral solid phases have considerable practical advantages but the use of chiral agents in the mobile phase in combination with non-chiral adsorbents often gives more versatile systems. This paper presents approaches to separate enantiomeric ionic compounds using chiral complexing agents in ion-pair chromatographic systems.

Ion pair chromatography is a technique commonly used for separation of ionized compounds in liquid chromatography. A charged solute (S^+) is retained in the stationary phase as a neutral complex (ion pair, SC) with an ion of opposite charge (counter ion, C^-). The retention can be regulated by the nature and the concentration of the counter ion but also by other complexing agents in the mobile phase. The principle is outlined in the following scheme.



(1)

It is also possible to influence the retention by a complexing agent in the stationary phase.

Stereoselective separation can be achieved by a chiral counter ion or by a chiral complexing agent. Table 1 summarizes

TABLE 1

Separation of Enantiomeric Compounds in Ion-pair Chromatographic Systems

Substrate	Chiral agent	Mobile phase	References
Cations and anions with H-bonding groups	Counter ion in mobile phase	Organic	(4-8)
Anions of carboxylic acids	Albumin in mobile phase	Aqueous	(9)
1,2-Aminoalcohols as cations	(+)-di-n-Butyltartrate in stationary phase or mobile phase	Aqueous Organic	(10) (11)

the ion-pair chromatographic systems for separation of enantiomers that have been studied by our group.

SEPARATION OF ENANTIOMERS AS DIASTEREOMERIC ION PAIRS

The separation is based on the fact that a chiral counter ion can bind enantiomeric substrates as diastereomeric ion pairs, which will have different distribution properties if the counter ion has a suitable structure. The binding forces are usually a combination of electrostatic attraction and hydrogen bonding (cf. 12), and solvents of low polarity are normally used to obtain a high degree of ion pair formation. The ion pairs can have different distribution properties due to differences in solvation in the mobile phase or to different strength in the binding to the adsorbent.

The method is mainly used for separation of enantiomeric amines and acids, but the principle has also been applied to resolution of enantiomeric metal complexes (13) and the amino acid tryptophane (14).

Counter Ion Structure and Stereoselectivity

The selection of chiral complexing agents is often made according to the "three point rule" which states that a three point interaction between the chiral agent and at least one of the enantiomers is necessary to obtain stereoselective retention (15). However, the principle is not readily applicable to diastereomeric ion pairs and counter ions are usually selected as to give a two point binding with the substrates (cf. ref. 16). Some examples are given below.

(+)-10-Camphorsulfonic acid has been used as the chiral counter ion for separation of enantiomeric aminoalcohols (4,5). Szepesi **et al.** (17) used the acid to resolve enantiomers of some alkaloids. It is a strong acid with rigid structure due to the bridge in the ring system. The two point binding to a cationic substrate can be achieved by the charged group and by the hydrogen accepting oxo group that can give a strong interaction with a hydrogen donating function in the substrate (see Fig. 1). The camphorsulfonic acid and bromo analogues thereof give a fairly low stereoselectivity with separation factors (α) of about 1.1 (4) for enantiomers of 1,2-aminoalcohols.

A much higher stereoselectivity with α=1.4 for these aminoalcohols can be obtained with N-carbobenzoxycarbonyl-glycine-L-proline (ZGP) as chiral counter ion (Fig. 2) (8).

Racemic sulfonic and carboxylic acid have been separated with optically active aminoalcohols as chiral counter ions in the mobile phase (Fig. 3) (6,7). The chiral selectivity is highly dependent on the structure of the chiral counter ion as demonstrated in a study with 10-camphorsulfonic acid as substrate (Table 2). Alprenolol with the binding groups (amine and alcohol) in an alkyl chain gave fairly low stereoselectivity. Quinine, quinidine and cinchonidine, which also have a two carbon chain between the hydroxyl and the amine, gave considerably higher separation factors probably due to the fact that the binding groups are situated in a rigid ring system with bulky groups in

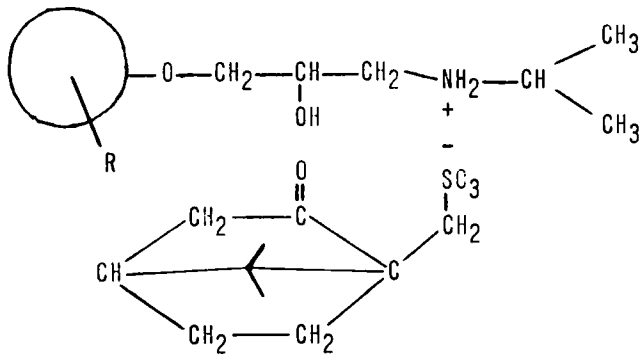


FIGURE 1. Model for Interaction between the (+)-10-Camphorsulfonic acid and Aminoalcohols.

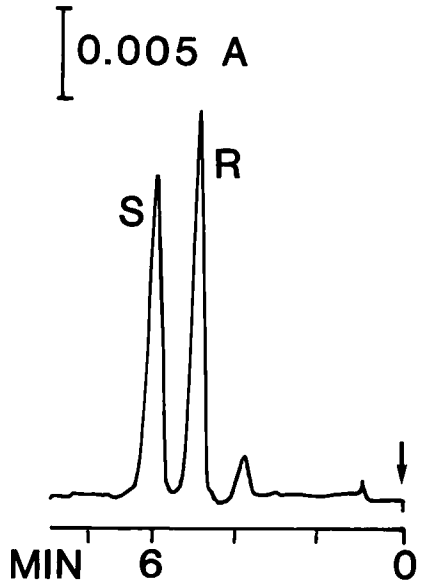
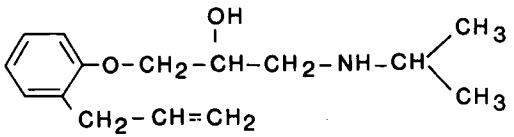


FIGURE 2. Separation of (R,S)-alprenolol.
Solid phase: LiChrosorb DIOL. Mobile phase: ZGP 2,5 mM and triethylamine 1 mM in dichloromethane (80 ppm H_2O)

1.



2.

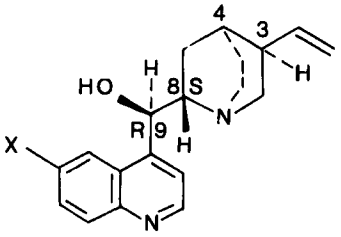


FIGURE 3. Counter ion structures.
1. alprenolol
2. quinine, X = OCH_3, [3(R), 4(S), 8(S), 9(R)]
   quinidine, X = OCH_3, [3(R), 4(S), 8(R), 9(S)]
   cinchonidine, X = H, [3(R), 4(S), 8(S), 9(R)]

TABLE 2

Influence of Counter Ion Structure on the Stereoselective Retention of 10-camforsulphonic Acid

Solid phase: LiChrosorb DIOL. Mobile phase: salt of chiral cation in methylene chloride + 1-pentanol.

Chiral salt	Content mol/L x 10^4	1-Pentanol %	$\alpha_{+/-}$
(+)-Alprenolol chloride	10.0	0	0.95
(-)-Quinine chloride	3.0	1	1.33
(-)-Quinine acetate	3.5	1	1.47
(+)-Quinidine acetate	3.5	1	0.77
(-)-Cinchonidine acetate	3.5	1	1.24

$$\alpha_{+/-} = \frac{k' \text{ of (+)-form}}{k' \text{ of (+)-form}}$$

the vicinity of the chiral centers. Similar results have also been obtained with enantiomeric carboxylic acids as substrates.

Sample Structure and Stereoselectivity

A study of the structural requirements for chiral resolution of aminoalcohols with (+)-10-camphorsulfonic acid as chiral counter ion is presented in Table 3 (5). No separation of enantiomers was obtained for compounds with more than two alkyl carbons between the amine and the hydroxyl group, which emphazises the importance of the two point binding for the stereoselective retention of the diastereomeric ion pairs.

Introducing substituents in the aromatic ring or having the aromatic ring directly bound to the asymmetric carbon atom had no significant effect on the stereoselectivity in most cases.

The size of the substituent at the nitrogen seems to be of less importance than its binding properties. No stereoselectivity was obtained when an alkylaryl group was attached to the amine function, while separation of enantiomers was obtained when the group contained a polar function e.g. amide.

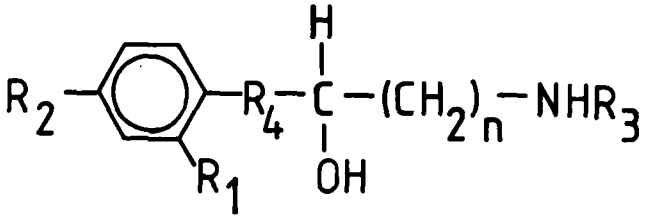
A two carbon distance between the hydroxyl and the amine group also seems to be vital for the separation of enantiomeric aminoalcohols with ZPG (N-carbobenzoxycarbonyl-glycine-L-proline) as the chiral counter ion (8).

The stereoselective retention of enantiomers of acids when using quinine or one of the related aminoalcohols as chiral counter ion also seems to require a two point binding between the ion pair components. The substrate must contain a polar function, beside the carboxylic group, that can give further interactions, e.g. a hydrogen bonding in addition to the electrostatic attraction. Some examples of enantiomer separations are given in Table 4 (6).

2-Phenylpropionic acid, without additional polar function, shows no stereoselective separation in contrast to

TABLE 3

Separation of Enantiomeric Carboxylic Acids



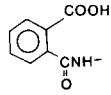
n	R_1	R_2	R_3	R_4	α
1	H	$CH_2.CH_2.OCH_3$	$CH(CH_3)_2$	$-O-CH_2-$	1.09
2	H	-"-	-"-	-"-	1.00
3	H	-"-	-"-	-"-	1.00
1	H	-"-	-"-		1.09
1	H	$O-CH_2.CH=CH_2$	$CH.(CH_3)_2$	$-O-CH_2-$	1.08
1	$CH_2.CH=CH_2$	H	-"-	-"-	1.08
1	$OCH_2.CH=CH_2$	H	-"-	-"-	1.00
1	H	OCH_3	-"-	-"-	1.08
1	H	$CH_2.CH_3$	-"-	-"-	1.09
1	H	$OCH_2.CH_2.OCH_3$	-"-	-"-	1.09
1	$CH_2.CH=CH_2$	H	$CH_2.CH_2O.C_6H_4.CONH_2$	-"-	1.11
1	Cl	-"-	$-CH_2O.C_6H_4.CONH_2$	-"-	1.11
1	Br	$CH_2.CH_2.OCH_3$	-"-	-"-	1.11
1	CH_3	H	$CH_2.CH_2.CH_2.C_6H_5$	-"-	1.00
1	H	-"-	$CH_2.CH_2.C_6H_4.CH_3$	-"-	1.00

Solid phase: LiChrosorb DIOL. Mobile phase: (+)-10-camphorsulfonate $2.2 \cdot 10^{-3}$ M in methylene chloride + 1-pentanol (199+1).

TABLE 4

Separation of Enantiomeric Carboxylic Acids

Solid phase: LiChrosorb DIOL. Mobile phase: quinine and acetic acid 0.30 mM in CH_2Cl_2 + 1-pentanol (99+1)

Acid	R_1	R_2	R_3	R_4	α
O-Methylmandelic	-COOH	-Ph	$-OCH_3$	-H	1.18
α-Methoxy-α-trifluoro-methylphenylacetic	-COOH	-Ph	$-OCH_3$	$-CF_3$	1.11
Tropic	-COOH	-Ph	$-CH_2OH$	-H	1.15
Atrolactic	-COOH	-Ph	-OH	$-CH_3$	1.00
2-Phenoxypropionic	-COOH	-OPh	-H	$-CH_3$	1.30
2-Phenylpropionic	-COOH	-Ph	-H	$-CH_3$	1.00
Naproxen	-COOH	$-NaphOCH_3$	-H	$-CH_3$	1.00
N-(1-Phenylethyl)-phthalamic		-Ph	-H	$-CH_3$	1.15

R_1 - R_4 are the groups bound to the chiral carbon.

2-phenoxypropionic acid. Even rather weakly hydrogen bonding functions seem to be sufficient for enantioselectivity as illustrated by the fairly high separation factors obtained for methoxyderivatives of mandelic acid. The absence of chiral resolution for atrolactic acid is so far not explained. The resolution of (+/-)-N-tert-butoxycarbonyl-phenylalanine, containing a carboxylic and an amide group using quinidine as chiral counter ion is shown in Fig. 4 (6).

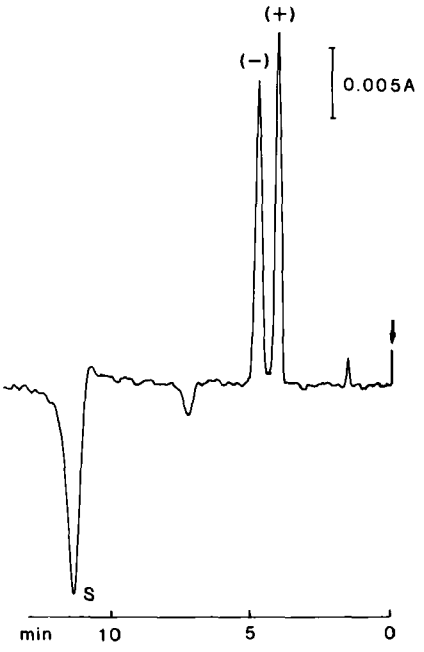


FIGURE 4. Resolution of (+/-)-N-t-BOC-phenylalanine.
Solid phase: LiChrosorb DIOL. Mobile phase: quinidine 0.35 mM and acetic acid 0.35 mM in methylene chloride + 1-pentanol [99+1]

Regulation of Retention and Stereoselectivity

The capacity factors for the enantiomers usually decrease as the concentration of the counter ion increases, probably due to a competitive distribution of the counter ion and the diastereomeric ion pairs to the same adsorption sites on the stationary phase.

Addition of competing protolytes of the same kind as the solute are further means to affect the retention (5-7). The protolyte can compete in uncharged form or as ion pair for the binding capacity of the stationary phase. Studies on carboxylic acids with quinine as counter ion have indicated a complex retention mechanism (6,7).

The retention can also be decreased by addition of polar components such as 1-pentanol to the mobile phase. The decrease in retention is usually accompanied by a decrease in stereoselectivity, probably due to a competition by 1-pentanol for the hydrogen bonding groups in the ion pair components (4-6). The effect is particularly strong for substrates having strongly hydrogen bonding substituents.

The water content of the mobile phase also has a pronounced influence on retention and stereoselectivity, and the effect seems to be dependent on the nature of the solute. Systems with a very low water content give practical problems such as long equilibration time and unstable retentions. A water content of 80-90 ppm can be recommended. It gives fast equilibration and constant retention without siginificant loss of stereoselectivity (7).

The retention and the chromatographic behaviour of the diastereomeric ion pairs is highly dependent on the properties of the adsorbing stationary phase. LiChrosorb DIOL, a surface modified silica with hydrophilic properties, is usually preferred as adsorbent since it gives good stereoselectivity and better chromatographic performance for both cationic and anionic enantiomers than more hydrophobic adsorbents (5,7). The chiral resolution often changes significantly with the properties of the adsorbent and a change of stationary phase present means to optimize the stereoselectivity.

Indirect Photometric Detection in Systems with UV Absorbing Counter Ions

For compounds with low UV absorptivity the detection sensitivity is drastically increased in systems with a UV absorbing counter ion such as quinine. It is due to the fact that the injection of a solute gives rise to a displacement of the equilibria in the injection zone and as a consequence the solute

will migrate surrounded by a mobile phase with another composition than the normal (cf. ref. 6). The detector will register the change in the concentration of the UV absorbing mobile phase component, which is proportional to the concentration of the migrating solute.

An example is given in Fig. 5 which shows the separation of the enantiomeric forms of 10-camphorsulfonate in a system with quinine as chiral counter ion (6). The detection is made at 337 nm where 10-camphorsulfonate is without absorbance and the enantiomers are detected solely by changes in counter ion concentration. The negative peak, S, is characteristic for the system (system peak). It will appear with the same retention on any injection in the system, independent of the nature of the injected solute.

The detector response is proportional to the amount of solute injected. A quantitative expression for the detection sensitivity is given by the conditional molar absorptivity. ε^*, which is the peak area in absorbance units divided by the amount of solute injected (in moles) (6).

The conditional molar absorptivity has a maximum when the solute peak and the system peak coincide and it decreases at lower and higher retention. However, the conditional molar absorptivity is dependent not only on the retention and the molar absorptivity of the UV absorbing mobile phase component but to some extent also on the nature of the solute (6).

SEPARATION OF ENANTIOMERS WITH ALBUMIN AS CHIRAL COMPLEXING AGENT

Albumin is one of the main transport proteins in blood plasma and it is known to give stereospecific interactions with organic molecules (18). These stereospecific binding properties of albumin have been utilized in liquid chromatography for separation of optical isomers. Initially the protein was bound to a solid matrix (19-21), but lately it has also been used as chiral additive in

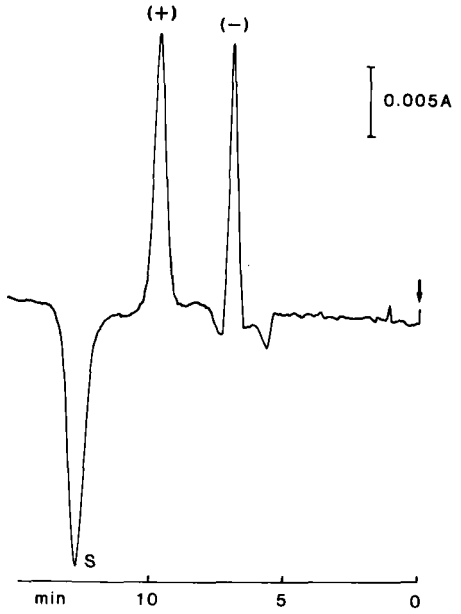


FIGURE 5. Resolution of (+/-)-10-camphorsulfonic acid. Solid phase: LiChrosorb DIOL. Mobile phase: quinine 0.35 mM and acetic acid 0.35 mM in methylene chloride + 1-pentanol [99+1].

the mobile phase (9,22). The binding mechanism is not known in detail but it is believed to involve electrostatic attraction, hydrogen bonding and hydrophobic interactions.

Our studies have been concentrated on separations of anionic enantiomers in ion-pair chromatographic systems with a hydrophobic adsorbent and albumin as chiral complexing agent in the aqueous mobile phase. Different kinds of racemic solutes have been separated, e.g., mono- and divalent carboxylic acids (9) and tryptophane (9,22). The chromatographic technique has also been used for determination of the binding affinity to the protein (23). A complete resolution of tryptophane enantiomers with human serum albumin in the mobile phase is shown in Fig. 6 (9).

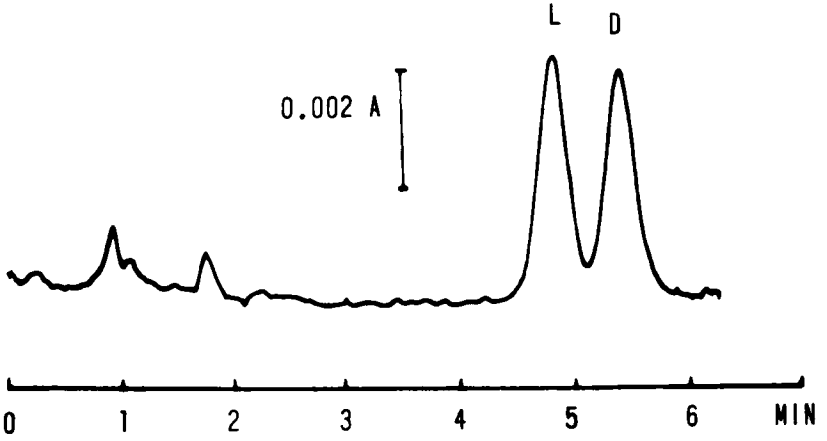


FIGURE 6. Resolution of D,L-tryptophan.
Solid phase: LiChrosorb RP-2. Mobile phase: 20 μM HSA in phosphate buffer pH 6.5 (μ=0.1). Solute concentration: 3.0 10^{-5} M. Detection: 280 nm.

Regulation of Retention and Stereoselactivity

The principles for regulation of the retention in an ion-pair chromatographic system with a complexing agent such as albumin in the mobile phase have already been outlined above (eq. 1). The retention of an anion is regulated by the concentration of albumin and by the nature and the concentration of the counter ion in the mobile phase. Increase of the concentration of the protein will increase the binding of the anion to the mobile phase and decrease the retention. Increase of the concentration and hydrophobicity of the counter ion will increase the binding to the adsorbent and increase retention. When the counter ion concentration is constant, albumin (P) is used in sufficient excess and the adsorbent has such pore size that albumin is excluded, the retention of the anion, X^-, will follow the relationship (9):

$$\frac{1}{k'_X} = B + B.n_i.K_{XP(i)}.(P) \qquad (2)$$

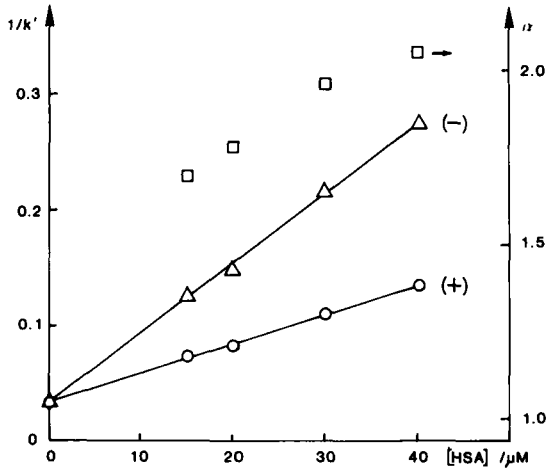


FIGURE 7. Influence of Albumin on Retention and Stereoselectivity for Enantiomers of 2-phenoxypropionic acid.
○ and △ = $\frac{1}{k'}$ for (+)- and (-)-enantiomer, respectively.
□ = separation factor.
Solid phase: LiChrosorb RP-18. Mobile phase: HSA in phosphate buffer pH 6.5 (μ=0.1). Solute concentration: 9.8 10^{-5}M

($K_{XP_{(i)}}$ = binding constant to the binding site P_i on albumin, n_i = number of binding sites P_i and B is a constant).

The eq. shows that there should be a linear relationship between $1/k'_X$ and the albumin concentration. Plots in accordance with this eq. for the enantiomers of 2-phenoxypropionic acid are shown in Fig. 7 (9). The binding affinity, $n_i \cdot K_{XP(i)}$, can be estimated from the quotient between the slope and the intercept.

The use of high concentrations of albumin in the mobile phase might impair a direct photometric detection of solutes at wavelengths below 280 nm. This difficulty can be avoided by an indirect detection technique, using a counter ion with absorbance at wavelengths higher than 280 nm. An example is given in Fig. 8. It shows the separation of the enantiomers of a carboxylic acid, α-methoxy-α-trifluoromethylphenylacetic acid, with albumin and

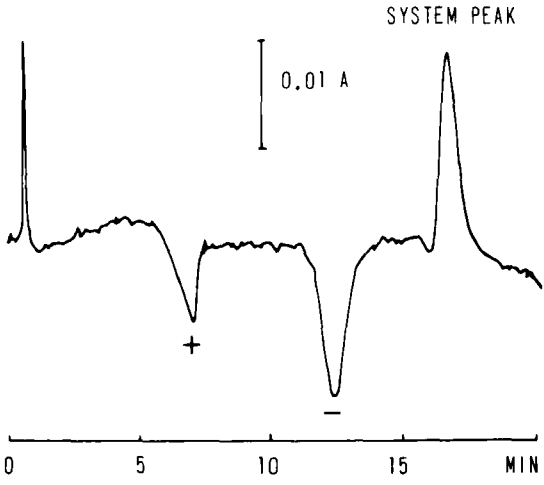


FIGURE 8. Resolution of (+/-)-α-methoxy-α-trifluoromethyl-phenylacetic acid.
Solid phase: Phenyl Hypersil. Mobile phase: HSA 24 μM + 1-ethylquinolinium 200 μM in phosphate buffer pH 6.5 (μ=0.1). Detection: 328 nm.

detected by the changes in the concentration of the UV absorbing counter ion, 1-ethylquinolinium, having an absorbance maximum at 328 nm (9).

The retention can also be varied by changing pH in the mobile phase. However, this might give rise to unpredictable variations in the stereoselectivity due to changes in the binding sites on the protein molecule (24).

Solute Structure and Stereoselectivity

The chiral binding sites on albumin and the mechanism for the stereoselective retention are not known in any detail. Studies on carboxylates have indicated that a polar group, in addition to the carboxylic function, is necessary to obtain stereoselective retention. However, small changes in the groups bound to the

chiral center and changes in the distances between the groups can have drastic effects on the separation factors. Some examples are given in Table 5.

Studies with albumin from different species have shown that they are unequal with respect to resolution of enantiomers. Differences in amino acid content and amino acid sequence might be the reason for the different binding properties (cf.20).

SEPARATION OF ENANTIOMERS WITH (+)-DI-n-BUTYLTARTRATE AS CHIRAL COMPLEXING AGENT

The stereoselective effects of tartaric acid esters were first shown by Prelog **et al.** (25) in liquid-liquid distribution studies on enantiomeric aminoalcohols. The tartaric acid esters were added to the organic phase and an unequal distribution of the enantiomers was observed when they were distributed as ion pairs with a non-chiral counter ion, e.g., hexafluorophosphate.

The principle of using esters of tartaric acid as chiral complexing agents was later transferred to HPLC (10). The dibutylester of tartaric acid (DBT) is applied as stationary phase on a hydrophobic adsorbent by pumping DBT equilibrated aqueous mobile phase through the column. Careful thermostatting is needed to obtain stable chromatographic systems.

A separation of the enantiomers of the physiologically important aminoalcohol norephedrine is shown in Fig. 9 (10). Hexafluorophosphate is used as counter ion. Results from studies of other aminoalcolhols in the same chromatographic system are given in Table 6.

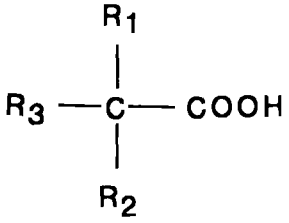
The introduction of methyl groups at the 2-carbon increase the stereoselectivity, whereas bulky substituents at the amine group seems to decrease the separation factor.

A resolution model has been proposed (25). It is based on differences in a three point hydrogen bonding between the

TABLE 5

Solute Structure and Stereoselectivity

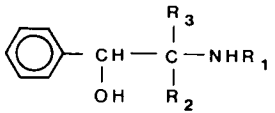
Solid phase: LiChrosorb RP-18. Mobile phase: 20 μM HSA in phosphate buffer ph 6.5 (μ = 0.1)

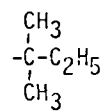


Solute	R_1	R_2	R_3	α
α-Methoxyphenylacetic acid	C_6H_5	OCH_3	H	1.30
Tropic acid	C_6H_5	CH_2OH	H	1.15
Mandelic acid	C_6H_5	OH	H	1.00
Atrolactic acid	C_6H_5	OH	CH_3	1.56
2-Phenylpropionic acid	C_6H_5	H	CH_3	1.00
2-Phenoxypropionic acid	C_6H_5O	H	CH_3	2.19
2-Methoxymandelic acid	CH_3O-C_6H_5	OH	H	1.00
3-Methoxymandelic acid	CH_3O-C_6H_5	OH	H	1.12
3-Phenyllactic acid	C_6H_5-CH_2	OH	H	1.16

TABLE 6

Retention and Stereoselectivity of Ephedrine and Analogues*



No.	Solute	R_1	R_2	R_3	k_1'	α
1	2-amino-1-phenylethanol	H	H	H	8.1	1.09
2	norephedrine	H	H	CH_3	8.0	1.15
3	1-(1-methyl-1-aminoethyl)benzylalcohol	H	CH_3	CH_3	8.2	1.18
4	ephedrine	CH_3	H	CH_3	8.5	1.15
5	pseudoephedrine	CH_3	H	CH_3	8.1	1.09
6	1-phenyl-N-(tert.pentyl)-2-aminopropanol		H	H	15.2	1.00
7	phenyramidol		H	H	50	1.05

* Conditions: see Figure 9.

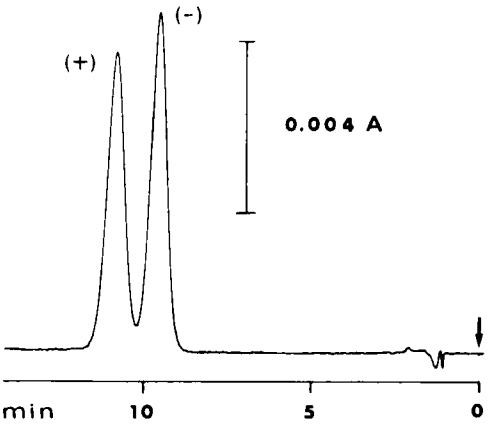


FIGURE 9. Separation of enantiomers of norephedrine. Solid Phase: Phenyl Hypersil. Stationary phase: Di-n-butyl-L-tartrate. Mobile phase: 90 mM hexafluorophasphate in phosphate buffer pH 6.4.

enantiomeric aminoalcohols and the tartaric acid ester. However, results from experiments with tertiary amines have indicated that the chiral complexing effect is not restricted to aminoalcohols.

Esters of tartaric acid can also be used as complexing agents in systems with an organic mobile phase and an aqueous stationary phase. The stereoselectivity is somewhat higher than that obtained with DBT in reversed phase systems (11).

CONCLUSION

The ion-pair chromatographic systems with chiral agents in mobile or stationary phase can be applied to separation of enantiomeric ions of widely different kinds. The systems are highly versatile and give good possibilities to a systematic regulation of retention and stereoselectivity.

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