Journal of Pharmaceutical & Biomedical Analysis
Vol. 4, No. 2, pp. 221–235, 1986
Printed in Great Britain

0731-7085/86 $3.00 + 0.00
Pergamon Journals Ltd.

Chromatographic resolution of enantiomers using albumin as complexing agent in the mobile phase*†

C. PETTERSSON‡, T. ARVIDSSON, A.-L. KARLSSON and I. MARLE

Department of Analytical Pharmaceutical Chemistry, Biomedical Center, University of Uppsala, Box 574, S-751 23 Uppsala, Sweden

Abstract: Enantiomers of carboxylic acids have been separated with albumin as a chiral complexing agent in the mobile phase. Stereoselective separation has been obtained for different types of acids, in some cases with very high separation factor, as shown for di-*p*-toluoyltartaric acid ($\alpha_s = 5.8$). The stereoselectivity and retention properties depend on pH and on the concentration of albumin in the mobile phase. Retention can also be regulated by modifying the nature of the solid phase as well as by the use of additives in the mobile phase. Acids with low molar absorptivity, or with absorbance in the same wavelength range as albumin, can be detected by an indirect technique based on the use of a cationic mobile phase additive, such as 1-ethylquinolinium, which has high UV-absorptivity at a wavelength remote from that of albumin.

Keywords: *Resolution of enantiomers; albumin as complexing agent; reversed-phase liquid chromatography; carboxylic acids; indirect UV detection.*

Introduction

Albumin is one of the main transport proteins for drugs in blood plasma and it can give stereoselective binding of substances of widely different structures [1]. The stereoselective binding of enantiomers (optical isomers) to albumin has been studied by various techniques, such as equilibrium dialysis, ultrafiltration and gel filtration [1].

The stereoselective interaction of albumin with solutes has also been used in chromatographic systems for the resolution of enantiomers. A number of chromatographic techniques have been applied, including systems where albumin is present in the mobile phase [2] as well as systems where albumin is bound to a solid phase such as agarose or silica [3–5].

The present investigation reports a detailed study of the stereoselective properties of albumin as a complexing agent in an aqueous mobile phase of a chromatographic system,

* Presented at the Symposium on Liquid Chromatography in the Biomedical Sciences, June 1984, Ronneby, Sweden.

† Taken in part from Dr Pharm. Sc. Thesis, C. Pettersson, Uppsala, 1984.

‡ To whom correspondence should be addressed.

where a surface-modified silica is employed as an adsorbing stationary phase. The influence of albumin and other mobile phase components on the retention and on the stereoselectivity for carboxylic acids has been studied. An indirect UV-detection technique (cf. [6]) was employed for solutes with low detectability, or in cases where high concentrations of albumin were present in the mobile phase.

Experimental

Apparatus

An Altex Model 110 A solvent metering pump (Beckman Instruments, Berkeley, CA, USA) was used, equipped with a pulse damper (Touzard et Matignon, Vitry, France). The UV detectors used were obtained from LDC, Riviera Beach, FL, USA and were Model 1203, UV III Monitor, wavelength 280 nm, and a variable-wavelength Spectromonitor III Model 1204. The injector employed was a Rheodyne Model 7120 (Rheodyne, Berkeley, CA, USA) with a 20-μl loop.

The columns were of stainless steel with polished inner surface, equipped with modified Swagelok connectors and Altex stainless-steel frits (2 μm). The separation column was 100 × 4.6 mm i.d. A pre-column, 50 × 4.6 mm i.d., packed with LiChroprep RP-18, was inserted before the injector in order to protect the analytical column.

A HETO waterbath (Type 02 PT 923, Birkerød, Denmark) was used when the indirect detection principle was employed.

Chemicals

LiChrosorb DIOL (5 μm), LiChrosorb RP-2 (5 μm), LiChrosorb RP-18 (5 μm) and LiChroprep RP-18 (25–40 μm) were obtained from E. Merck (Darmstadt, FRG); μ-Bondapak Phenyl (10 μm) was from Waters Associates (Milford, MA, USA) and Phenyl Hypersil (5 μm) was from Shandon (UK).

Human albumin fraction V, human albumin fraction V (essentially fatty-acid free), bovine albumin fraction V (essentially fatty-acid free) and horse albumin (essentially fatty-acid free), as well as D- and L-tryptophan and D- and L-*N*-acetyltryptophan, were all obtained from Sigma Chemical Company (St Louis, MO, USA).

Racemic tropic acid, 3-phenyllactic acid, 2-methoxymandelic acid, 3-methoxymandelic acid, as well as (+)- and (−)-α-methoxyphenylacetic acid, (+)- and (−)-α-methoxy-α-trifluoromethylphenylacetic acid, (+)- and (−)-mandelic acid, (+)- and (−)-di-benzoyltartaric acid and (+)- and (−)-di-*p*-toluoyltartaric acid were obtained from Fluka AG (Buchs SG, Switzerland).

Racemic 2-phenylpropionic acid, 2-phenoxypropionic acid, atrolactic acid hemihydrate and (−)-3-phenyllactic acid were from Janssen Chimica (Beerse, Belgium). Samples of (+)- and (−)-*N*-(1-phenylethyl)phthalamic acid were from E. Merck. (+)-2-Phenoxypropionic acid was kindly donated by A. Collet (Collège de France, Paris).

1-Ethylquinolinium iodide was from Eastman–Kodak (Rochester, NY, USA) and was converted into its hydroxide form using an anion exchanger, AG 1-X8 20–50 mesh (Bio Rad Richmond, CA, USA), before addition to the mobile phase. All other substances were of *pro analysi* or reagent grade and were used without further purification.

Column preparation

The LiChrosorb DIOL column was packed by a slurry technique using chloroform as suspending medium. LiChrosorb RP-18 and LiChrosorb RP-2 were packed with

tetrachloromethane–dioxane (4:1, v/v) and (1:1, v/v), respectively, as suspending media. μ-Bondapak Phenyl was packed with ethanol–dichloromethane (1:1, v/v) and Phenyl Hypersil with isopropanol as suspending media. The DIOL column was tested with hexane–*n*-butanol (199:1, v/v) as mobile phase using 2,4-dinitrotoluene and 2-phenylethanol as retained solutes, giving k' values of 1 and 8, respectively. The other columns were tested with mixtures of water and methanol as mobile phases giving capacity factors between 1 and 10 for the test solutes 2-phenylethanol, 2,6-xylenol and 2,3,5-trimethylphenol. Only columns that gave reduced plate heights ($h = H/d_p$) of less than 10 were used.

Chromatographic technique

A pre-column was inserted in front of the injector to protect the analytical column from impurities in the mobile phase. In systems intended for indirect detection with a UV-absorbing cation in the mobile phase, columns, injector and mobile phase reservoir were thermostatted at 25.0°C in a water-bath.

The elution volume of albumin was used as the void volume (V_a) when calculating the capacity factor, k'. All solutes were dissolved in the mobile phase prior to injection.

The asymmetry factor (asf) was calculated by dropping a perpendicular from the vertex (formed by the two peak tangents) to cut the baseline into two segments. The asymmetry factor is defined as the ratio between the second segment and the first segment of the base-line under the peak.

Unless otherwise stated, the albumin used was essentially fatty acid free. Stock solutions of albumin were stored in the refrigerator at 6°C.

Results and Discussion

Retention model

The stereoselectivity of the chromatographic system is attributable to the ability of albumin to bind enantiomers with differing strength.

Albumin is a complex molecule and a solute can be bound at several different positions (or binding-sites). The binding is normally a reversible equilibrium and the various binding-sites have limited capacities. The binding of small molecules to several independent binding-sites on the protein is usually described by the Scatchard equation [7]:

$$\bar{r} = \sum_{i=1}^{m} \frac{n_i K_{XP(i)}[X]}{1 + K_{XP(i)}[X]} \tag{1}$$

where $\bar{r}$ represents the number of moles of solute bound per mole of protein, $K_{XP(i)}$ is the equilibrium constant for the binding of the solute X to binding-site P_i on the protein, [X] is the concentration of unbound solute and n_i is the number of binding-sites P_i on the protein molecule. One type of strong binding-site on the protein often dominates, so that when the solute concentration is low, binding to other sites can usually be neglected.

Assuming the presence of one dominating binding-site, the total binding capacity (n_1C_p) at low solute concentration can be expressed by the equation:

$$n_1C_p = [P_1] + [XP_1] \tag{2}$$

where n_1 is the number of strong binding-sites, C_p is the total concentration of albumin, and $[P_1]$ and $[XP_1]$ are the concentrations of free and occupied binding-sites on the protein, respectively.

Under the experimental conditions used in the present work, i.e. pH 6 to 7 in the mobile phase, an acidic solute is ionized (X^-) and mainly retained as an ion pair with a counterion (Q^+). The distribution process can be illustrated by the formula:

$$X_m^- + Q_m^+ + A_s \overset{K_{QXA_s}}{\rightleftharpoons} QXA_s \quad (3)$$

where A_s is an adsorption site on the stationary phase and QXA_s represents an ion pair bound to such a site [8]. The term K_{QXA_s} is the equilibrium constant of the adsorption process and Q^+ represents any inorganic or organic cation in the mobile phase. The anion X^- can be distributed onto the solid phase with the aid of one or several of these counterions. For the sake of simplicity it is assumed in the following discussion that one of these counterions dominates. Assuming that the adsorption of the ion pairs follows the Langmuir adsorption model, the concentration of the ion pair QX in the stationary phase can be expressed by the equation (cf. [8]):

$$[QXA_s] = \frac{K^o K_{QXA_s} [Q]\,[X]}{1 + K_{QXA_s} [Q][X]} \quad (4)$$

where K^o is the monolayer capacity of the adsorbing stationary phase as defined by the equation:

$$K^o = [A_s] + [QXA_s]. \quad (5)$$

When albumin is introduced into the mobile phase it will complex with solute X as defined by the equilibrium constant:

$$K_{XP(1)} = \frac{[XP_1]}{[X][P_1]}\,. \quad (6)$$

It is assumed that albumin does not affect the adsorption properties of the solid phase, since the protein is excluded from the pores [9]. If that part of the aqueous phase in the column which is accessible to albumin is regarded as the mobile phase (the 'outer' phase: V_a ml), while the aqueous phase in the pores (the 'inner' phase: V_{is} ml) and the solid phase (W_s g) are considered to constitute the stationary phase, the capacity factor is given by:

$$k'_{X(O)} = \frac{\text{moles of X adsorbed + moles of X in the 'inner' phase}}{\text{moles of X in the 'outer' phase}}\,. \quad (7)$$

From equations (2)–(7) and the fact that the concentration of X^-, as well as of Q^+, is the same in both the 'outer' and the 'inner' phases (assuming no exclusion), the following expression can be derived for the capacity factor of the acidic solute X:

$$k'_{X(Q)} = \frac{W_s K^o K_{QXA_s} [Q]}{V_a (1 + K_{QXA_s} [X][Q])} \cdot \left\{1 + \frac{n_1 K_{XP(1)} C_p}{1 + K_{XP(1)} [X]}\right\}^{-1}$$

$$+ \frac{V_{is}}{V_a} \cdot \left\{1 + \frac{n_1 K_{XP(1)} C_p}{1 + K_{XP(1)} [X]}\right\}^{-1} . \tag{8}$$

When the concentration of the solute is low, so that $K_{QXA_s} [X][Q] \ll 1$ and $K_{XP(1)} [X] \ll 1$, the binding isotherms are linear and equation (8) reduces to:

$$k'_{X(Q)} = \frac{W_s K^o K_{QXA_s} [Q] + V_{is}}{V_a (1 + n_1 K_{XP(1)} C_p)} . \tag{9}$$

The retention of the anion can thus be regulated by: the nature of the solid phase (W_s, K^o, K_{QXA_s}); by the albumin concentration (C_p); and by the nature and concentration of the counterion (K_{QXA_s}, $[Q^+]$). Furthermore, an ion of the same charge as X^- present in the mobile phase may reduce the binding of X^- to albumin [9] in the mobile phase, and may also reduce the adsorption of X^- onto the stationary phase by a competitive effect [10]. Finally, the pH of the mobile phase may affect the retention by changing the protolysis of X^- and thereby its binding to the solid phase and to albumin. A pH change can also, however, affect the binding properties of albumin.

Albumin concentration

If the solute is bound to albumin, an increase of the concentration of albumin in the eluent will give rise to decreased retention (equation 9) due to an increased degree of complexation. Transformation of equation (9) to the form:

$$1/k'_{X(Q)} = B + B n_1 K_{XP(1)} C_p \tag{10}$$

where

$$B = \frac{V_a}{W_s K^o K_{QXA_s} [Q] + V_{is}}$$

indicates a linear relationship between $1/k'$ and the total concentration of albumin (C_p) at constant concentration of Q. Fairly good linearity was obtained for the enantiomers of 2-phenoxypropionic acid when plotting according to equation (10) (Fig. 1). The binding affinity $n_1 K_{XP(1)}$ may be estimated from the quotient between the slope and the intercept.

An increased albumin concentration will lead to increased stereoselectivity of the chromatographic system, $\alpha = k'_2/k'_1$, since, according to equation (9), it is dependent on the quotient between the products of the albumin concentration, C_p, and the constants $K_{XP(1)}$ for the two enantiomers.

When the detectability of the solutes is impaired owing to low molar absorptivity or interference by albumin, the solute cannot be applied in such a low concentration as to maintain a linear binding isotherm to albumin. As a consequence, the peaks will be deformed (leading) because the retention will be dependent on the concentration of the solute (cf. equation 8). However, it is often possible to also obtain complete resolution of

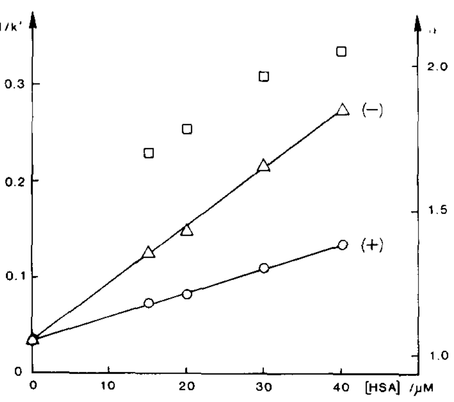

Figure 1
Influence of albumin concentration on retention and stereoselectivity. Plot of $1/k'$ vs C_p: ○ = (+)- and △ = (−)-enantiomer respectively, of 2-phenoxypropionic acid. Plot of separation factor α vs C_p; □ Solid phase: LiChrosorb RP-18. Mobile phase: HSA in phosphate buffer pH 6.5 (μ = 0.1). Solute concentration: 9.8×10^{-5} M.

Table 1
Dependence of chromatographic performance on solute concentration. Solid phase: LiChrosorb RP-2. Mobile phase: 20-μM HSA in phosphate buffer pH 6.5 (μ = 0.1)

Solute	Conc. M × 10^4	k'_-	k'_+	asf_1	asf_2	$\alpha_{+/-}$
Di-*p*-toluoyltartaric acid	23	5.92	21.2	0.24	0.40	3.57
	2.3	4.11	19.4	0.88	1.07	4.71
Di-benzoyltartaric acid	2.2	3.36	5.24	0.63	1.00	1.56
	0.5	3.07	5.00	1.73	1.88	1.63

asf = Asymmetry factor (see text).
$\alpha_{+/-} = k'_+/k'_-$.

enantiomers in these cases due to the high separation factors. The effect of solute concentration on retention, asymmetry and on the separation factor is illustrated in Table 1 for di-*p*-toluoyltartaric acid and di-benzoyltartaric acid when the binding isotherm is nonlinear.

Influence of pH

A change of pH can affect not only the protolysis of the solutes, and thereby their distribution to the adsorbent and the albumin, but also the binding properties of the albumin, since the charge distribution on the albumin changes owing to protolysis of its carboxylic and amino groups [11]. The separation of enantiomers by stereoselective complexation is usually assumed to require a three-point interaction between the solute and the complexing agent [12, 13] and a change in the charge distribution may then influence the stereoselectivity. The effect of a pH increase is illustrated in Table 2. For tryptophan there is a fairly small change in k' but a significant improvement of the stereoselectivity. For di-benzoyltartaric acid, there is a large decrease of k' while the stereoselectivity is almost unaffected.

Table 2
Influence of pH on retention and stereoselectivity. Solid phase: μ-Bondapak Phenyl. Mobile phase: HSA in phosphate buffer ($\mu = 0.1$). Solute concentration: 1.0×10^{-4} M

	HSA	pH 6.0			pH 6.7			pH 7.4		
Solute	$M \times 10^6$	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
Tryptophan	0	3.61	—	1.00	3.64	—	1.00	3.29	—	1.00
	20	3.47	3.29	1.05	3.49	2.79	1.25	3.16	1.78	1.78
Di-benzoyltartaric acid	0	27.0	—	1.00	8.17	—	1.00	2.89	—	1.00
	20	12.6	7.00	1.79	4.49	2.81	1.60	1.69	1.07	1.57

$\alpha = k'_2/k'_1$.

Influence of the solid phase

Retention can be regulated within a wide range by the hydrophobicity of the solid phase as shown in Table 3. The retention of tryptophan can thus be increased 30 times by changing from the hydrophilic DIOL phase to the hydrophobic RP-18 phase. The stereoselectivity is, however, unaffected by the change of solid phase as the stereoselective interaction takes place in the mobile phase. A demonstration of the chromatographic resolution of a racemic mixture of tryptophan using human serum albumin with LiChrosorb RP-2 as stationary phase is given in Fig. 2. The peak symmetry and the efficiency are good; the reduced plate height ($h = H/d_p$) is about 10. Even in cases with lower peak efficiency, chiral resolution due to high separation factors can be obtained, as illustrated in Fig. 3 for di-benzoyltartaric acid, where the reduced plate height is about 40.

Table 3
Solid phase influence on retention. Mobile phase: 20-μM HSA in phosphate buffer pH 6.5 ($\mu = 0.1$). Solute: D- and L-tryptophan 3.0×10^{-5} M

Solid phase	k'_L	k'_D	$\alpha_{D/L}$
LiChrosorb DIOL	0.83	0.94	1.13
LiChrosorb RP-2	4.65	5.34	1.15
Phenyl Hypersil	5.36	6.12	1.14
LiChrosorb RP-18	23.5	28.3	1.20

$\alpha_{D/L} = k'_D/k'_L$.

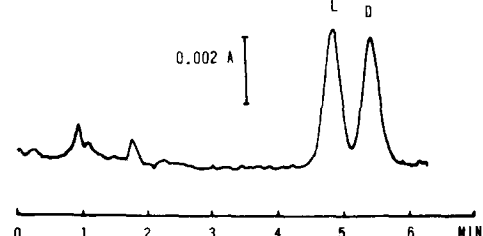

Figure 2
Resolution of D,L-tryptophan. Solid phase: LiChrosorb RP-2. Mobile phase: 20-μM HSA in phosphate buffer pH 6.5 ($\mu = 0.1$). Solute concentration: 3.0×10^{-5} M. Detection: 280 nm.

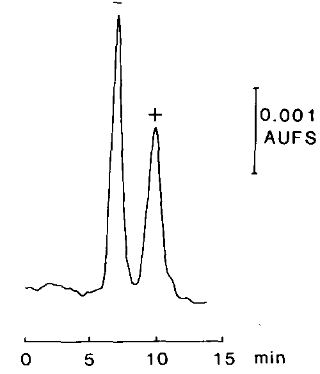

Figure 3
Resolution of (+/−)-di-benzoyltartaric acid. Solute concentration: 2.2×10^{-4} M. Solid phase: LiChrosorb RP-2. Mobile phase: 20-μM HSA in phosphate buffer pH 6.4 ($\mu = 0.1$). Detection: 280 nm.

Detection principle

The presence of albumin in the mobile phase will affect the detectability of solutes by UV-detection. The absorption spectrum of human serum albumin (HSA) is shown in Fig. 4. The concentration of albumin that can be used in the mobile phase is limited by the disturbing non-linearity of the response of the UV-detector and the increased noise level that appears when the background absorbance of the mobile phase exceeds 0.5–0.7 [14]. Direct UV detection at high concentrations of albumin is possible if the solute has an inherent chromophore absorbing at wavelengths above 310 nm, or if such chromophores are introduced by pre- or post-column derivatization.

An indirect photometric detection technique can, however, be used in this case. It is based on the use of a mobile phase containing a UV-absorbing probe (charged or uncharged) in a concentration of 0.1–1 mM. The detector response is due to the fact that the distribution of the probe between the mobile and stationary phase changes in the presence of the solute [6]. 1-Ethylquinolinium was used as a UV-absorbing probe in this study for the detection of acidic compounds. It has an absorbance maximum at 320 nm and possesses the prerequisite condition of not affecting the stereoselective binding between the acidic solutes and the albumin to any great extent (Table 4). An example of a separation of the enantiomers of α-methoxy-α-trifluoromethylphenylacetic acid with

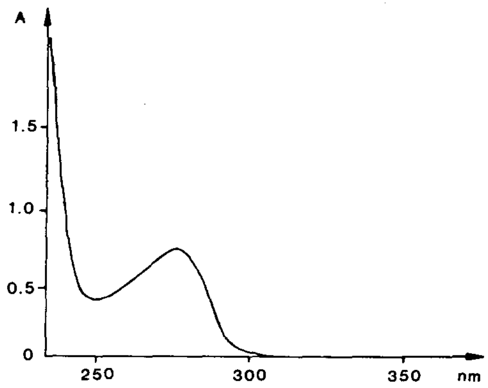

Figure 4
Absorption spectrum of albumin. Solution: 20-μM HSA in phosphate buffer pH 6.5 ($\mu = 0.1$).

Table 4
Influence of cationic probe on retention and stereoselectivity. Solid phase: Phenyl Hypersil. Solute: (+) and (−)-α-Methoxy-α-trifluoromethylphenylacetic acid

Mobile phase	k'_+	k'_-	k'_s*	$\alpha_{-/+}$
Phosphate buffer pH 6.5 ($\mu = 0.1$)	19.1	19.1	—	1.00
24-μM HSA in phosphate buffer pH 6.5 ($\mu = 0.1$)	5.00	9.17	—	1.83
200-μM 1-ethylquinolinium in phosphate buffer pH 6.5 ($\mu = 0.1$)	30.7	30.7	18.9	1.00
24-μM HSA and 200-μM 1-ethylquinolinium in phosphate buffer pH 6.5 ($\mu = 0.1$)	8.40	14.5	18.6	1.73

* k'_s = capacity factor of system peak (cf. [6]).
$\alpha_{-/+} = k'_-/k'_+$.

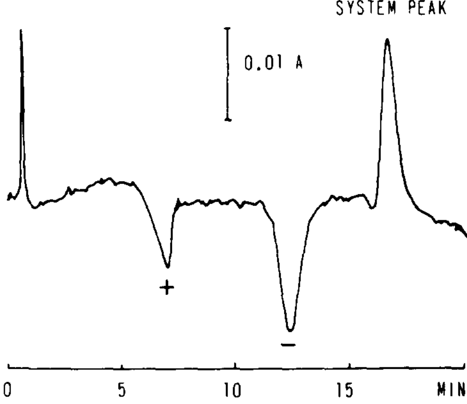

Figure 5
Resolution of (+/−)-α-methoxy-α-trifluoromethylphenylacetic acid. Solid phase: Phenyl Hypersil. Mobile phase: 24-μM HSA and 200-μM 1-ethylquinolinium in phosphate buffer pH 6.5 (μ = 0.1). Solute concentration: (+)-form 1.0×10^{-4} M; (−)-form 0.5×10^{-4} M. Detection: 328 nm.

HSA using 1-ethylquinolinium as probe is given in Fig. 5. The two negative peaks are the sample peaks, while the positive peak is the characteristic system peak [6].

Type of albumin

Albumin from different species differs partly in the content and/or sequence of amino acids. Previous studies of the stereoselective binding of small molecules have shown that albumin from different species can give not only different stereoselectivity, but also different retention order between the optical isomers [4, 15]. Studies with human, horse and bovine albumin in the mobile phase, presented in Table 5, indicate considerable differences in their stereoselective properties for these solutes. It might be possible to use these differences for optimization of chiral resolution in chromatographic procedures.

Investigations of the influence of fatty acids on the binding properties of albumin have shown that the fatty acids may compete in binding to the protein and reduce the degree of binding of the solutes [16]. An example is given in Table 6. Only when using essentially fatty acid free albumin is there stereoselective retention of 2-phenoxypropionic acid. The addition of a strongly bound anion, such as 6-hydroxynaphthalene-2-sulphonate, can completely destroy the stereoselective effect of HSA. Ions intended for use as probes must obviously be tested with respect to effects like this before application.

Sample structure and stereoselectivity

The present study has focussed on the separation of enantiomers of carboxylic acids at pH values where the acids are mainly present in the anionic form. Capacity ratios and separation factors are given in Table 7A and 7B.

Albumin is a complex molecule with binding-sites of different kinds. The limited experimental data available from this study do not permit detailed discussion of the relationship between solute structure and stereoselectivity. Furthermore, due to detection difficulties the solutes sometimes have to be applied in such high concentrations that the separation factors are lower than the maximum values (see Table 1). Thus the conclusions drawn concerning the relation between stereoselectivity and solute structure must be considered as preliminary and relevant only under the conditions used here.

Both divalent and monovalent carboxylic acids can give stereoselective interaction with albumin. The introduction of a methyl group in the aromatic ring structure can have

Table 5
Stereoselectivity and retention with albumin from different species. Solid phase: LiChrosorb RP-2. Mobile phase: 20-μM albumin in phosphate buffer pH 6.5 ($\mu = 0.1$)

Solute	Conc. $M \times 10^4$	Type of albumin —	Human			Horse			Bovine		
		k'	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
Di-*p*-toluoyltartaric acid	1–2	85.6	4.11	19.4	4.71	2.62	9.00	3.44	4.04	23.6	5.85
Tryptophan	0.3	5.65	4.65	5.34	1.15	3.45	3.98	1.15	3.34	3.49	1.04

$\alpha = k'_2/k'_1$.

Table 6

Influence of anions on stereoselectivity and retention. Solid phase: μ-Bondapak Phenyl. Mobile phase: HSA in phosphate buffer pH 6.5 ($\mu = 0.1$) with and without 6-hydroxynaphthalene-2-sulphonic acid (HX) as additive. Solute (+/−)-2-Phenoxypropionic acid 1.0×10^{-4} M

Albumin quality	C_{alb} M × 10^6	C_{HX} M × 10^4	k'_+	k'_-	$\alpha_{-/+}$
Fraction V	30	—	5.76	5.76	1.00
Fraction V, essentially fatty acid free	30	—	2.68	4.84	1.81
Fraction V, essentially fatty acid free	20	2	5.09	5.09	1.00

$\alpha_{-/+} = k'_-/k'_+$.

Table 7(A)

Solute structure and stereoselectivity. Solid phase: LiChrosorb RP-2. Mobile phase: 20-μM HSA in phosphate buffer pH 6.5 ($\mu = 0.1$)

No.	Solute	Conc. M × 10^4	Structure	k'_1	k'_2	α
1.	Tryptophan	0.3		4.65	5.34	1.15
2.	*N*-Acetyltryptophan	0.2		3.63	4.24	1.17
3.	Di-*p*-toluoyl-tartaric acid	2.3		4.11	19.4	4.71
4.	Di-benzoyl-tartaric acid	2.2		3.36	5.24	1.56
5.	α-Methoxy-α-trifluoromethyl-phenylacetic acid	1.1		9.68	20.0	2.07
6.	N-(1-Phenyl-ethyl)-phthalamic acid	8.1		29.5	46.9	1.59

$\alpha = k'_2/k'_1$.

Table 7(B)
Solute structure and stereoselectivity. Solid phase: LiChrosorb RP-18. Mobile phase: 20-μM HSA in phosphate buffer pH 6.5 ($\mu = 0.1$)

No.	Solute	Conc. M × 10^4	Structure	k'_1	k'_2	α
1.	α-Methoxyphenylacetic acid	1.3	C_6H_5—C(OCH₃)(H)—COOH	4.07	5.13	1.26
2.	Tropic acid	1.2	C_6H_5—C(CH_2OH)(H)—COOH	4.25	4.81	1.13
3.	Mandelic acid	0.9	C_6H_5—C(OH)(H)—COOH	1.87	1.87	1.00
4.	Atrolactic acid	1.2	C_6H_5—C(OH)(CH_3)—COOH	2.41	3.52	1.46
5.	2-Phenylpropionic acid	1.1	C_6H_5—C(H)(CH_3)—COOH	5.45	5.45	1.00
6.	2-Phenoxypropionic acid	1.0	C_6H_5—O—C(H)(CH_3)—COOH	4.43	9.13	2.06
7.	2-Methoxymandelic acid	0.9	CH_3O—C_6H_4—C(OH)(H)—COOH	4.31	4.31	1.00
8.	3-Methoxymandelic acid	0.2	CH_3O—C_6H_4—C(OH)(H)—COOH	2.77	3.04	1.10
9.	3-Phenyllactic acid	1.0	C_6H_5—CH_2—C(OH)(H)—COOH	4.57	5.23	1.14

$\alpha = k'_2/k'_1$.

a drastic effect on the stereoselectivity, as illustrated by di-benzoyltartaric acid and di-*p*-toluoyltartaric acid (compounds 3 and 4, Table 7A). The position of a substituent in a ring can have a strong influence on the possibility to separate the enantiomeric forms. (+) and (−)-3-methoxymandelic acid can be separated but not the enantiomers with a substituent in the ortho position (compounds 7 and 8, Table 7B). In this case, the loss of stereoselectivity might be due to steric effects or to different hydrogen-bonding properties for 2- and 3-methoxymandelic acid.

All compounds that have shown stereoselective retention in this investigation have polar functions close to the asymmetric carbon atom. It seems that O or N in the vicinity

of the chiral center is needed for stereoselective interaction with albumin: stereoselective retention is obtained for 2-phenoxypropionic acid, but not for 2-phenylpropionic acid (compounds 6 and 5, Table 7B). Direct attachment of the polar functions to the chiral center does not seem to be required, cf. tropic and 3-phenyllactic acid (compounds 2 and 9, Table 7B). Stereoselective retention is achieved for *N*-(1-phenylethyl)-phthalamic acid, which indicates that it is not necessary for the charged group to be bound directly to the chiral center.

In summary: stereoselective retention seems to require the following features:
(i) one or two negatively charged groups;
(ii) an aromatic ring system;
(iii) a polar function in the vicinity of the chiral centre.

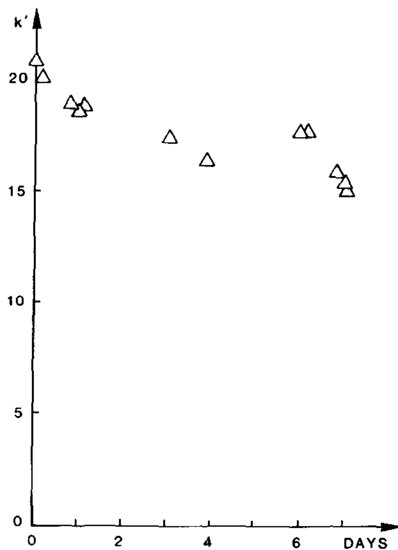

Figure 6
Stability of chromatographic system: capacity ratio (k'). Solid phase: LiChrosorb RP-18. Mobile phase: 20-μM HSA in phosphate buffer pH 6.4 ($\mu = 0.1$). Solute: D-tryptophan 2×10^{-5} M.

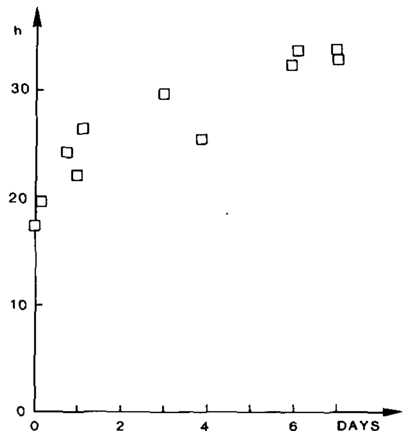

Figure 7
Stability of chromatographic system: efficiency (reduced plate height, h). Conditions as in Fig. 6.

Stability of the chromatographic system

The stability of retention is demonstrated in Fig. 6, which shows the change in capacity factor of D-tryptophan in a system with recirculating mobile phase. The retention initially decreases but a constant value is usually reached after 3 to 4 days.

The separating efficiency also decreases slowly with time (Fig. 7). However, the stereoselectivity is often so high that complete resolution of the enantiomers can still be obtained as illustrated above (Fig. 3). As a rule useful separations can be made with the same column for at least 2–3 weeks.

A slight increase in the column back-pressure has been observed in systems with albumin in the eluent. The increase is usually well below the pressure limit of the pump. Washing of the filters in the bed support with 5 M nitric acid reduces the column back-pressure to almost the initial value.

Acknowledgements: We are grateful to Professor G. Schill, Professor D. Westerlund and Dr K.-G. Wahlund for their interest in this work and for valuable discussions on the manuscript.

References

[1] T. Alebic-Kolbah, S. Rendic, Z. Fuks, V. Sunjic and F. Kajfez, *Acta Pharm. Jugoslav.* **29**, 53–70 (1979).
[2] B. Sébille and N. Thuaud, *J. Liq. Chromatogr.* **3**, 299–308 (1980).
[3] K. K. Stewart and R. F. Doherty, *Proc. Nat. Acad. Sci. U.S.A.* **70**, 2850–2852 (1973).
[4] C. Lagercrantz, T. Larsson and I. Denfors, *Comp. Biochem. Physiol.* **69C**, 375–378 (1981).
[5] S. Allenmark, B. Bomgren and H. Boren, *J. Chromatogr.* **264**, 63–68 (1983).
[6] L. Hackzell and G. Schill, *Chromatographia* **15**, 437–444 (1982).
[7] G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660–672 (1949).
[8] A. Tilly-Melin, Y. Askemark, K.-G. Wahlund and G. Schill, *Anal. Chem.* **51**, 976–983 (1979).
[9] K. G. Wahlund and T. Arvidsson, *J. Chromatogr.* **282**, 527–539 (1983).
[10] G. Schill and K.-G. Wahlund, in *Trace Organic Analysis: A New Frontier in Analytical Chemistry*, pp. 509–523, Proceedings of the 9th Material Research Symposium, 10–13 April 1978, Gaithersburg, Md. National Bureau of Standards Special Publication 519, 1979.
[11] J. Steinhardt and J. A. Reynolds, in *Multiple Equilibria in Proteins*, pp. 176–213. Academic Press, New York (1969).
[12] R. Audebert, *J. Liq. Chromatogr.* **2**, 1063–1095 (1979).
[13] W. F. Lindner, *Chemical Derivatisation in Analytical Chemistry*, Volume 2, pp. 145–190 (R. W. Frei and J.F. Lawrence, Eds). Plenum, New York (1982).
[14] L. Hackzell, T. Rydberg and G. Schill, *J. Chromatogr.* **282**, 179–191 (1983).
[15] R. I. Nazareth, T. D. Sokoloski, D. T. Witiak and A. T. Hopper, *J. Pharm. Sci.* **63**, 203–211 (1974).
[16] D. Rudman, T. J. Bixter and A. E. Delrio, *J. Pharmacol. Exp. Ther.* **176**, 261–272 (1971).

[*Received for review 24 August 1984*]