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DIRECT LIQUID CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS ON IMMOBILIZED PROTEIN STATIONARY PHASES

VIII. A COMPARISON OF A SERIES OF SORBENTS BASED ON BOVINE SERUM ALBUMIN AND ITS FRAGMENTS

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SUMMARY

A mixture of three peptides (M_r 38 000), obtained by enzymatic cleavage of bovine serum albumin (BSA) was isolated, immobilized to silica and used as a chiral stationary phase in liquid chromatography. A comparison of sorbents containing these fragments and intact BSA, showed that the enantioselectivity is preserved for only a limited number of compounds. Under identical mobile phase conditions, retention on the columns based on BSA fragments was also much lower than on those containing intact BSA.

The method used for immobilization has a great influence on the retentive and enantioselective properties of the sorbent obtained. When sorbents based on BSA entrapped in silica and 3-aminopropylsilica, by cross-linking with glutaraldehyde were compared under identical mobile phase conditions, the latter were generally found to give larger capacity factors and often also larger α values.

These results indicate that the increased hydrophobicity, primarily caused by the aminopropyl groups, partly contributes to the overall retention and chiral discrimination process and that the situation may be different from that in a solution of the free protein.

INTRODUCTION

The enantioselectivity exerted by biopolymers,such as proteins, has been utilized in the development of chiral stationary phases (CSPs) for use in liquid chromato-

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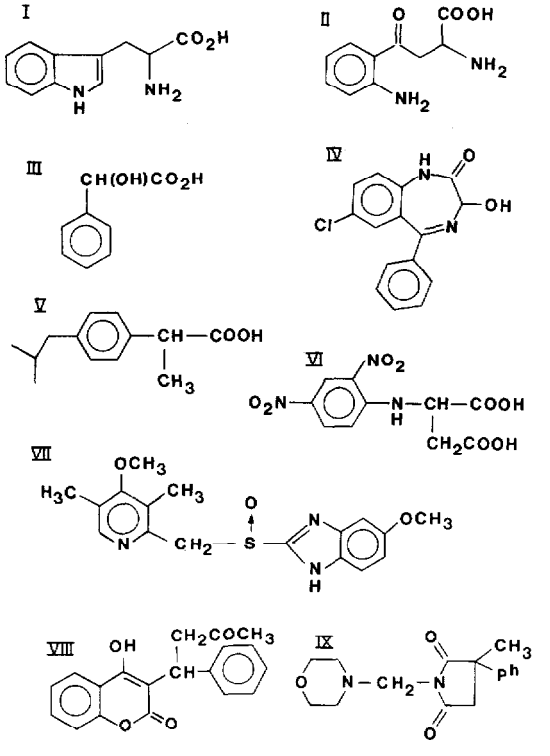
graphy[1]. Bovine serum albumin (BSA), immobilized to silica by various techniques[2–5], has been shown to act as a chiral discriminator for a variety of racemic organic compounds in aqueous buffers. A different approach for the immobilization of BSA has been developed, whereby derivatized silica is used for the entrapment of the protein within the silica pores by cross-linking with glutaraldehyde.

Recent investigations have indicated that only a limited number of ligand-binding sites are involved in the chiral discrimination process[6]. To gain further insight into the relationship between the solute structure and enantioselectivity, N-terminal peptic fragments (M_r 38 000) of BSA were also immobilized.

EXPERIMENTAL

Chemicals

Racemic tryptophan (**I**), kynurenine [3-(2-aminobenzoyl)-alanine] (**II**) and mandelic acid (**III**) were obtained from Sigma (St. Louis, MO, U.S.A.). Oxazepam (**IV**) and ibuprofen (**V**) were obtained from the Department of Drug Control, Biomedical Centre, Uppsala, Sweden. The synthesis of N-(2,4-dinitrophenyl)-(DNP-)aspartic acid (**VI**) was performed as described previously[7]. The sulphoxide omeprazol (**VII**) was a gift from Hässle (Mölndal, Sweden), as was warfarin (**VIII**) from Ferrosan (Malmö, Sweden). Morpholep [1-(morpholinomethyl)-3-methyl-3-phe-



Scheme 1. Structures of the compounds investigated.

nylsuccinimide] (**IX**) was kindly provided by Dr. J. Bojarski (Krakow, Poland). The structure of compounds **I–IX** are given in Scheme 1. Benzoin and the substituted benzoins (**Xa–f**) were kindly supplied by Dr. O. Weller (University of Hamburg, F.R.G.).

Bovine serum albumin (BSA) (Art. A7030) was obtained from Sigma (St. Louis, MO, U.S.A.). The spherical silica used was from Macherey–Nagel (Düren, F.R.G.) and had a pore diameter of either 100 or 300 Å and a particle size of 5 or 7 μm.

All other chemicals and solvents were of analytical-reagent grade.

Column preparation

Four different types of BSA–silica sorbents were used: type I, Resolvosil-BSA-7 (100 Å, 7 μm; Macherey–Nagel); type II, glutaraldehyde-cross-linked BSA entrapped in silica (100 Å, 7 μm); the preparation procedure has been described previously[3]; type III, BSA cross-linked into 3-aminopropylsilica (100 Å, 7 μm) by glutaraldehyde; and type IV, BSA adsorbed on silica (300 Å, 5 μm). The last two types were also used for the immobilization of the BSA fragments (IIIF and IVF).

The protein content of the sorbents I–III was determined by elemental analysis of nitrogen and sulphur. For sorbents containing 3-aminopropylsilica only sulphur elemental analysis was performed. The analyses were performed by Mikro Kemi (Uppsala, Sweden).

Preparation of BSA–3-aminopropylsilica sorbent (type III)

Spherical silica (100 Å, 7 μm) (4.0 g) was suspended in dry toluene (80 ml) and the silane reagent 3-aminopropyltriethoxysilane (4.3 ml) was added. The reaction mixture was refluxed for 4 h, during which time the ethanol formed was continuously removed. The derivatized silica was isolated by filtration and washed successively with 100 ml each of toluene and diethyl ether before being dried at room temperature overnight *in vacuo*.

Entrapment of BSA was carried out in 0.1 *M* phosphate buffer (pH 7.0, 1% 1-propanol) by the same procedure as used for type II sorbents.

BSA fragments–aminopropylsilica sorbent (type IIIF)

Fragments of serum albumin obtained by limited proteolysis such as peptic hydrolysis suffer little chemical damage, *e.g.*, amino acid side-chains are not altered[8]. Peptides isolated from a peptic digest were purified on an ion-exchange resin (Mono Q). A fraction showing a single band with M_r 38 000, as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was further purified by gel permeation (Sephadex); the detailed procedure is described elsewhere[9,10]. An amino acid sequence analysis showed that three sequences were present in the final peptide solution[10]. The three peptides were found to originate from the N-terminal half of BSA and contained mainly the same overlapping sequence. The peptide composition was found to consist of *ca.* 50% of a peptide starting with amino acid 1 (Asp) and *ca.* 25% each of peptides starting with amino acid 11 (Phe) and 49 (Phe). SDS-PAGE showed that also a small amount (*ca.* 5%) of a larger peptide (M_r 45 000) was present in the peptide solution. The peptides were collected in 50 m*M* ammonium hydrogencarbonate buffer (pH 7.8). The peptide solution was concentrated to a volume of 5 ml by ultrafiltration using a Filtron Novacell-Omegacell, diluted with 100 m*M* phosphate

buffer (pH 7.0; 1.0% 1-propanol) and reconcentrated to a volume of 3 ml (*ca.* 9 mg/ml). 3-Aminopropylsilica (200 mg) was added to the solution and the fragment immobilized by the same procedure as used for the other cross-linked sorbents.

Column packing

The columns were packed by the upward-flow packing method at a pressure of 300 bar using a stainless-steel tube *ca.* 18 ml in volume as the slurry reservoir. The slurry and packing medium was a 50 m*M* phosphate buffer containing 40% 1-propanol. The columns were then used in the liquid chromatographic system without further conditioning.

Columns with adsorbed BSA and BSA fragments (types IV and IVF)

Nucleosil (300 Å, 5 μm) was suspended in chloroform–methanol (2:1) and poured into a 75-ml packing reservoir. The upward-flow technique was used to pack the column at 300 bar. The columns were then rinsed with water followed by 50 m*M* phosphate buffer solution (pH 5.0).

A solution of the BSA (0.12 mg/ml) in 0.5 *M* phosphate buffer (pH 5.0) was pumped through the column until breakthrough was detected at 280 nm[5]. The amount of protein adsorbed on the column was found to be 60 mg/g (*ca.* 11 mg). The column was then equilibrated with the pure phosphate buffer (50 m*M*, pH 5.0).

The preparation of the adsorbed BSA fragment column was performed using the same procedure. The concentration of BSA fragments in the 50 m*M* phosphate buffer was 0.33 mg/ml and the amount of peptide immobilized was *ca.* 98 mg/g (*ca.* 18 mg)[10]. The method of calculation of the amount of BSA adsorbed has been described previously[5].

Chromatography

The chromatographic system consisted of an LKB Model 2150 pump and an ISCO Model V[4] variable-wavelength UV absorbance detector equipped with 3.5-μl (5-mm) cell. The column dimensions were either 200 × 2.1 mm I.D. or 100 × 1.6 mm I.D. and the injections were made by means of a Rheodyne Model 7125 injection valve using a 5-μl loop.

A different instrumental setup was used for chromatography on adsorbed BSA columns. A Philips PU 4003 pump and PU 4025 variable-wavelength UV detector (1 μl, 5 mm cell) together with a Rheodyne Model 7520 sample injector (0.5-μl loop) were used.

Elution was performed isocratically using 25 100 m*M* phosphate buffer containing 0–4% of 1-propanol and 0–4 m*M* octanoic acid as retention modifiers. Retention times and peak areas were determined with a Waters Model 740 electronic integrator or a Philips PM 8252 recorder interfaced with the detector.

RESULTS AND DISCUSSION

Column performance

The performances of the two columns (100 × 1.6 mm I.D.) packed with either immobilized BSA (III) or BSA fragments (IIIF) are shown in Fig. 1, which illustrates the resolution of *rac*-oxazepam (**IV**). The columns show good efficiency and peak

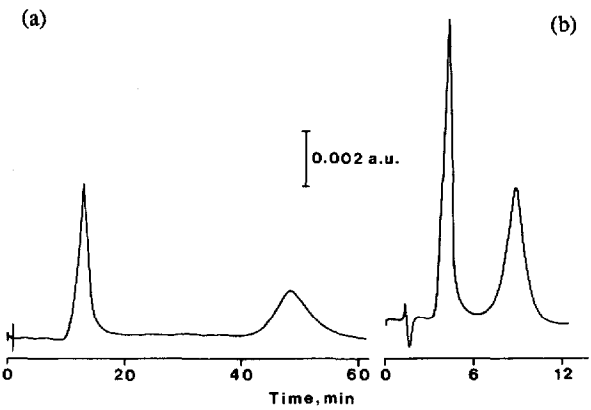


Fig. 1. Optical resolution of *rac*-oxazepam on (a) immobilized BSA (III) (k'_1 = 7.3, k'_2 = 31.7) and (b) on BSA fragment (IIIF) column (k'_1 = 1.4, k'_2 = 4.6). Mobile phase, 50 m*M* phosphate buffer (pH 7.6) containing 4% 1-propanol; flow-rate, 0.2 ml/min; UV detection 230 nm. A 25 μM solution was injected.

symmetry and the separation corresponds to a resolution factor of R_s = 5.0 and 2.6, respectively. The performance of the BSA fragment column deteriorated significantly after the use of buffers with a high alkanol content. The reason for this behaviour is not known, but one can assume that the peptides, obtained by cleavage of BSA, are less stable towards organic solvents.

Influence of the immobilization technique on retention and enantioselectivity

The existence of specific binding sites on albumin is well known, *e.g.*, for tryptophan. A specific benzodiazepine binding site on albumin has recently been postulated[6]. It is therefore plausible that the availibility of the stereospecific binding site can be affected by the immobilization technique used.

The optical resolution of oxazepam (**IV**) and omeprazol (**VII**) was performed on three different types of columns prepared under identical conditions. A selection of chromatographic data is given in Table I. It can be seen that both the resolution and

TABLE I

RESOLUTION OF RACEMIC OXAZEPAM AND OMEPRAZOL ON BSA–SILICA COLUMNS

Column dimensions, 200 × 2.1 mm I.D.; amount injected, 0.5 nmol.

Column type	*Amount of BSA*	*System*[a]	k'_1	k'_2	α	R_s
I	110	a	4.50	5.69	1.3	0.8
		b	2.69	4.00	1.5	1.4
II	190	a	9.00	28.4	3.2	4.1
		b	9.37	10.7	1.1	0.5
III	210	a	9.68	41.1	4.2	4.6
		b	7.25	17.0	2.3	3.7

[a] (a) Solute, (±)-oxazepam; mobile phase, 50 m*M* phosphate buffer (pH 7.6) containing 4% 1-propanol; flow-rate, 0.5 ml/min; UV detection at 230 nm. (b) Solute, (±)-omeprazol, mobile phase, 50 m*M* phosphate buffer (pH 5.8) containing 2% 1-propanol; flow-rate, 0.5 ml/min; UV detection at 225 nm.

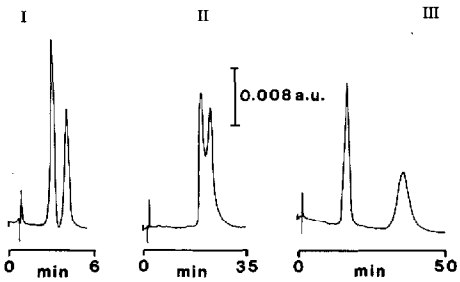


Fig. 2. Influence of the immobilization technique as shown by the optical resolution of *rac*-omeprazol on three different types (I–III) of BSA columns. Mobile phase, 50 m*M* phosphate buffer, (pH 5.8), containing 2% 1-propanol; flow-rate, 0.5 ml/min; UV detection at 225 nm. A 100-μM solution was injected.

the retention of the enantiomers of oxazepam correlate with the increased amount of entrapped BSA and increased hydrophobic character of the sorbents. However, the resolution of omeprazol shows a marked dependence on the type of column used, as illustrated in Fig. 2.

The difference between the BSA–silica type II and III sorbents is particularly well shown by their retention of acidic analytes (Table II).

The marked increase in the resolution of compounds **I** and **II** is largely due to the increased retention of the last-eluted enantiomer. The other acidic compounds and compound **VIII** show increased retention of both antipodes, resulting in less pronounced effects on the resolution.

Capacity and ligand binding of immobilized BSA fragments

The capacity of BSA–silica columns is low owing to the small proportion of the protein actually involved in the chiral recognition process. We therefore assumed that immobilization of a fragment of the protein containing some of the binding regions

TABLE II

CHROMATOGRAPHIC DATA OBTAINED WITH GLUTARALDEHYDE-CROSS-LINKED BSA COLUMNS (II AND III)

Column dimensions, 200 × 2.1 mm I.D.; amount injected, 0.5 nmol.

Compound	*Mobile phase*[a]	*Column II*			*Column III*		
		k'_1	k'_2	R_s	k'_1	k'_2	R_s
D,L-Tryptophan (**I**)	a	0.76	1.88	1.9	1.41	7.53	5.6
D,L-Kynurenine (**II**)	a	0.47	2.24	3.3	0.59	11.8	9.3
(±)-Mandelic acid (**III**)	b	3.67	6.56	1.7	11.7	18.8	3.2
Ibuprofen (**V**)	c	12.4	26.5	3.1	25.3	57.4	5.1
2,4-DNP-D,L-aspartic acid (**VI**)	a	5.85	19.0	3.7	21.1	47.1	3.5
(±)-Warfarin (**VIII**)	a	12.4	18.4	1.3	18.2	24.3	1.4

[a] Flow-rate, 0.5 ml/min; (a) 50 m*M* phosphate buffer (pH 7.6) containing 4% 1-propanol; (b) 50 m*M* phosphate buffer (pH 5.6); (c) 50 m*M* phosphate buffer (pH 8.0) containing 3% 1-propanol and 4 m*M* octanoic acid.

TABLE III

COMPARISON OF THE CAPACITY AND RESOLUTION OF (±)-BENZOIN ON COLUMNS PACKED WITH IIIF AND III

Column dimensions, 100 × 1.6 mm I.D.; solute, (±)-benzoin; mobile phase, 50 m*M* phosphate buffer, (pH 7.6) containing 4% 1-propanol; flow-rate, 0.2 ml/min; UV detection at 247 nm.

Amount injected (nmol)	*Packing*	k'_1	k'_2	η_1	η_2	α	R_s
0.125	IIIF	0.9	2.1	1.1	0.9	2.3	1.9
	III	4.1	10.2	1.2	1.0	2.5	2.8
0.50	IIIF	0.9	1.9	1.2	1.2	2.1	1.6
	III	4.0	9.7	1.0	1.4	2.4	2.5
1.00	IIIF	1.0	1.9	1.3	1.4	1.9	1.0
	III	4.0	9.6	1.1	1.4	2.4	2.4
2.50	IIIF	1.0	1.8	1.6	1.9	1.8	0.5
	III	3.9	9.0	1.2	1.7	2.3	1.7

would result in a sorbent with increased capacity owing to a higher density of chiral recognition sites. For this purpose, a study of the loading capacity of columns based on BSA (type III) or BSA fragments (type IIIF) was performed for (±)-benzoin (**Xa**). The sorbents were prepared under identical conditions.

The amount of entrapped peptide was estimated from sulphur analysis of the dried sorbent and was *ca.* 140 mg/g silica, *i.e.*, 4 μmol/g, whereas the amount of entrapped BSA was 210 mg/g (3 μmol/g).

At low concentrations there is no significant difference in the chiral recognition and overall performance of the two columns. However, as the solute concentration increases there is a marked decrease in the resolution of benzoin on the BSA fragment column (Table III). This is mainly due to the decrease in the k' value of the last-eluted isomer while the retention of the first-eluted isomer remains approximately constant. The BSA column shows a decrease in the retention of both isomers, the effect being more pronounced for the last-eluted isomer. Hence the resolution of benzoin decreases

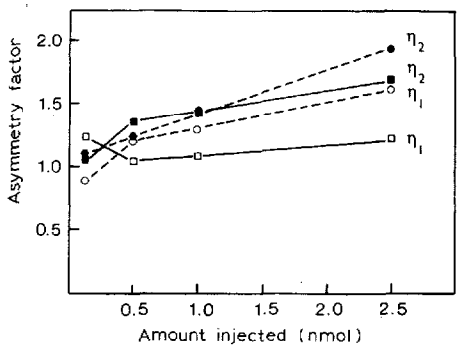


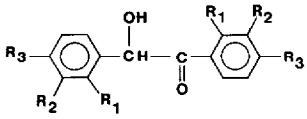
Fig. 3. Influence of sample loading on peak symmetry (η). Column: (■) type III; (●) type IIIF. Solute: (±)-benzoin. Mobile phase, 50 m*M* phosphate buffer (pH 7.6) containing 4% 1-propanol; flow-rate, 0.2 ml/min; UV detection at 247 nm.

to a lesser extent on the BSA column than on the BSA fragment column. From the asymmetry factors (η), one can also see that the column based on the intact protein has a higher column load threshold than the BSA fragment column (Fig. 3). Further, at low solute concentration, an increase in peak asymmetry is seen on the BSA column for the first-eluted enantiomer of benzoin. The reason for this behaviour is not clear, but a similar effect has been noted for N-benzoyl-D,L-alanine[3]. Theoretically, one would expect the stereoselectivity and capacity to increase owing to the increased density of stereoselective sites on the BSA fragment column; our results, however, were not in agreement with this prediction. A plausible explanation may be that the number of intact or accessible stereoselective sites in the BSA fragment column may have been reduced in a number of ways, *e.g.*, the sites may have been affected during the enzymatic cleavage or purification procedure, the stereoselective properties of the individual peptides may differ because of the small variation in the amino acid

TABLE IV

OPTICAL RESOLUTION DATA OBTAINED FOR A SERIES OF BENZOINS (**Xa–f**) ON SORBENTS IIIF AND III

Column dimension, 100 × 1.6 mm I.D.; flow-rate, 0.2 ml/min; amount injected, 125 picomol; UV detection at 254 nm.



Compound	R_1	R_2	R_3	*Column type*	*Mobile phase*[a]	k'_1	k'_2	α	R_s
Xa	H	H	H	IIIF	A	1.5	3.9	2.6	2.0
				IIIF	B	1.1	2.6	2.4	1.9
				III	B	5.1	13.1	2.6	2.8
Xb	F	H	H	IIIF	A	2.6	2.6	1.0	
				IIIF	B	1.5	1.5	1.0	
				III	B	6.0	8.0	1.3	0.9
Xc	H	F	H	IIIF	A	4.2	5.8	1.4	0.6
				IIIF	B	2.0	2.7	1.4	0.4
				III	B	10.9	16.7	1.5	1.6
Xd	H	H	F	IIIF	A	2.3	2.9	1.3	0.4
				IIIF	B	1.7	1.7	1.0	
				III	B	6.7	10.7	1.6	1.6
Xe	Cl	H	H	IIIF	A	11.0	11.0	1.0	
				IIIF	B	7.8	7.8	1.0	
				III	B	23.2	28.1	1.2	0.8
Xf	H	H	Cl	IIIF	A	19.5	23.3	1.2	
				IIIF	B	10.1	10.1	1.0	
				III	B	55.1	83.7	1.5	2.0
				III	C	4.8	5.9	1.2	1.0

[a] Mobile phase: 50 m*M* phosphate buffer (pH 8.3) containing (A) 0%, (B) 4% and (C) 20% 1-propanol.

sequence of the three peptides, or the immobilization procedure is not optimal for the peptides.

The resolving power of the immobilized BSA fragments was lost for a number of compounds, *e.g.*, tryptophan, kynurenine and warfarin, which indicates that the binding sites for these compounds have been lost or otherwise affected. This is consistent with the proposed location of the binding site for tryptophan and warfarin reported by Peters[11], *i.e.*, in the C-terminal part of the BSA molecule. However, it was possible to separate certain uncharged, aromatic compounds, *i.e.*, oxazepam (**IV**), morpholep (**IX**) and benzoins (**X**), on the BSA fragment column. The large α-value for oxazepam is in agreement with the highly stereospecific benzodiazepine binding site postulated by Müller[6]. A decrease in the retention was found for all compounds, indicating the elimination of interactions not contributing to the chiral recognition.

A series of benzoins were resolved on the two different types of columns (Table IV). Introduction of substituents on the aromatic rings has an unfavourable effect on the enantioseparation of benzoins, particularly with the peptide column. Further, the position of the substituents in the isomers has a significant effect on the resolution and the smallest α-values were found for the *ortho*-substituted isomers. Fig. 4 illustrates the resolution of compound **Xd**. The large increase in the retention of benzoins on changing the halogen atom from F to Cl is difficult to explain, but these results are consistent with our earlier studies on the resolution of a series of racemic barbiturates[12].

To investigate the possible adverse effect of cross-linking on the availibility of the enantiomer-discriminating binding sites, a comparison of sorbents prepared by cross-linking and by adsorption of the protein or peptide was performed (Table V). It was found that the same compounds were resolvable on both types of peptide-containing sorbents.

The higher BSA content and the increased hydrophobicity of the cross-linked sorbents gave an increased retention of the solutes investigated and, in most instances, increased the enantioseparation. Columns III and IIIF show a similar trend in the k' values when the pH of the mobile phase is changed from 6.0 to 7.5. However, compared with the BSA column (IV), the adsorbed fragment column (IVF) shows the opposite retention behaviour in the case of oxazepam and warfarin on increasing the pH. The reason for this chromatographic behaviour is not known.

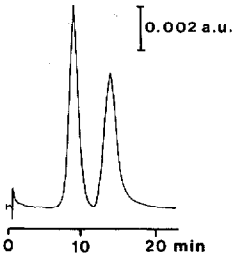


Fig. 4. Enantiomers of fluoro-substituted benzoin (**Xd**) separated on type III sorbent. Mobile phase, 50 m*M* phosphate buffer (pH 8.3) containing 4% 1-propanol; flow-rate, 0.2 ml/min; UV detection at 254 nm. A 25-μM solution was injected.

TABLE V

INFLUENCE OF IMMOBILIZATION TECHNIQUE FOR BSA FRAGMENTS ON THE OPTICAL RESOLUTION OF A SERIES OF COMPOUNDS

Column dimensions, 100 × 1.6 mm I.D.; mobile phase, 50 m*M* phosphate buffer; flow-rate, 0.2 ml/min amount injected, 125 pmol.

Compound	*pH*	*Chiral stationary phase*							
		III		*IV*		*IIIF*		*IVF*	
		k'_1	α	k'_1	α	k'_1	α	k'_1	α
D,L-Tryptophan (**I**)	6.0	2.1	1.6	0.28	1.2	1.3	1.0	0.23	1.0
	7.5	2.3	7.8	0.32	7.2	1.4	1.0	1.17	1.0
D,L-Kynurenine (**II**)	6.0	1.2	1.9	0.24	1.3	0.89	1.0	0.24	1.0
	7.5	1.6	14.5	0.29	13.0	1.09	1.0	0.26	1.0
Oxazepam (**IV**)	6.0[a]	7.4	4.9	3.1	2.0	1.9	3.1	1.9	1.3
	7.5[a]	10.6	4.8	2.6	2.0	2.7	3.0	2.1	1.5
(±)-Warfarin (**VIII**)	6.0[a]	24.9	1.5	3.3	1.5	6.4	1.0	1.0	1.0
	7.5[a]	22.7	1.6	3.4	1.5	5.1	1.0	0.29	1.0
Morpholep (**IX**)	6.0	1.7	2.0	0.91	1.3	1.2	1.4	0.75	1.3
	7.5	1.3	1.1	0.54	1.0	0.93	1.0	0.32	1.0
(±)-Benzoin (**Xa**)	6.0[a]	3.6	2.6	1.2	1.3	1.8	2.5	0.66	1.2
	7.5[a]	6.4	2.5	1.5	1.4	4.0	2.4	0.82	1.7

[a] Mobile phase contained 2% 1-propanol.

The retention behaviour of oxazepam was also dependent on the technique used for the immobilization of BSA. The cross-linked sorbent (III) showed increasing k' values with increasing pH whereas the opposite was true for the adsorbed BSA column (IV). A possible reason for this behaviour could be that the orientation of the adsorbed protein molecule is such that the binding site for oxazepam is affected in a different manner on increasing the pH. Earlier results using adsorbed BSA columns show certain differences in the chiral discrimination of sulphoxides. Omeprazol and 2-methylsulphinylbenzoic acid could not be resolved on adsorbed BSA–silica columns, which indicates that the binding sites for these compounds are no longer available[5]. No resolution of omeprazol was achieved on columns containing BSA fragments (IIIF and IVF).

An advantage of the adsorption immobilization technique, however, lies in its simplicity, which makes it suitable for the preparation of CSPs for semi-preparative purposes.

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