Journal of Chromatography, 591 (1992) 65–73
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 796

Direct liquid chromatographic separation of enantiomers on immobilized protein stationary phases

IX. Influence of the cross-linking reagent on the retentive and enantioselective properties of chiral sorbents based on bovine serum albumin

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(First received May 22nd, 1991; revised manuscript received October 8th, 1991)

ABSTRACT

Three modifications of silica-bound, cross-linked bovine serum albumin (BSA) were evaluated as chiral sorbents for use in the liquid chromatographic separation of enantiomers. Glutaraldehyde, formaldehyde and di-(N-succinimidyl) carbonate were used as bifunctional reagents for the immobilization of BSA. The sorbents all contain the same loading of BSA (14.4 ± 0.1%, w/w) and differ only with respect to the cross-linker used for immobilization. Despite their apparent similarity, the sorbents show very different chromatographic propertics, not only with respect to retention of analyte enantiomers (k' and α), but also with respect to column efficiency (affecting R_s values). The data obtained indicate that the chemical structure of the cross-linking reagent affects to a large extent the accessibility of important chiral binding sites. Although the data obtained are difficult to interpret in any detail, certain generalizations concerning the different behaviour of the sorbents can be made.

INTRODUCTION

Chiral stationary phases (CSPs) based on bovine serum albumin (BSA) have been used for the reversed-phase separation of a variety of enantiomers [1–3]. A number of different techniques have been described for the immobilization of BSA to silica, *e.g.*, adsorption, covalent binding and entrapment by cross-linking of the protein [4–9]. The retention behaviour and the enantioselectivity of chiral compounds were found to be highly influenced by the choice of the immobilization technique; indeed, in some instances a total loss of chiral recognition was observed [5,9]. For the past few years we have been working with glutaraldehyde-cross-linked BSA sorbents which have shown high stability towards organic solvents. However, the increase in hydrophobicity of these sorbents due to incorporation of the cross-linking reagent was generally found to give undesirably longer retention times [6,8].

One of our aims has been to decrease the degree of achiral hydrophobic interaction generated by the cross-linking reagent, since theoretically this would lead to decreased capacity factors (k') and larger

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separation factors, provided that the chiral binding sites of BSA are unaffected. For this purpose, three different cross-linking reagents were used under otherwise equivalent conditions for the entrapment of BSA in 3-aminopropylsilica. The prepared sorbents were evaluated with respect to their retentive and enantioselective properties for a number of racemic compounds.

EXPERIMENTAL

Chemicals

The benzodiazepinones (**Ia–d**) were obtained from the Department of Drug Control, Biomedical Centre, Uppsala University. Albendazole S-oxide (SO.ABZ) and its structural analogues (**IIa–d**) were kindly supplied by Professor P. Delatour (Charbonnieres, France). Racemic tryptophan (**III**) and methaqualone {2-methyl-3-(*o*-tolyl)-4[3*H*]-quinazolinone} (**VI**) were obtained from Sigma (St. Louis, MO, USA). (±)-Benzoin and di-(N-succinimidyl) carbonate (DSC) were purchased from Fluka (Buchs, Switzerland). Both formaldehyde (FA) and glutaraldehyde (GLA) solutions (Merck, Darmstadt, Germany) were diluted with water to give 5% solutions before use. Bovine serum albumin (BSA) was obtained from Sigma. The spherical 3-aminopropylsilica (100 Å, 7 μm), was a gift from EKA Nobel (Surte, Sweden).

Structures of compounds **III–VII** are given in Fig. 1.

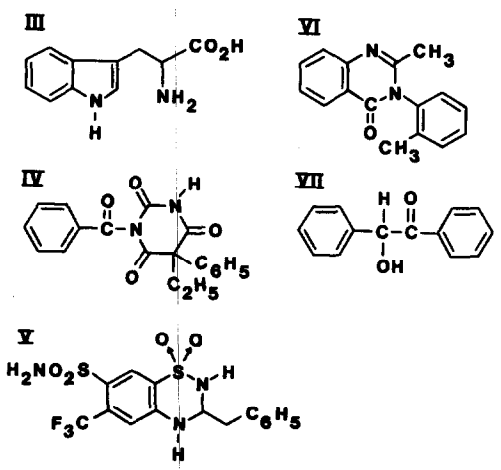


Fig. 1. Structures of compounds **III–VII**.

Preparation of BSA–silica sorbents

Three different BSA-based stationary phases (CSPs I–III) were prepared under equivalent experimental conditions. CSP I was glutaraldehyde-cross-linked BSA entrapped in 3-aminopropylsilica (BSA–GLA sorbent); BSA/g silica, 143 mg (nitrogen analysis) and 147 mg (suphur analysis). CSP II was formaldehyde-cross-linked BSA entrapped in 3-aminopropylsilica (BSA–FA sorbent); BSA/g silica, 144 mg (nitrogen analysis) and 146 mg (sulphur analysis). CSP III was BSA cross-linked into 3-aminopropylsilica using N-succinimidyl carbonate (BSA–DSC sorbent); BSA/g silica, 143 mg (nitrogen analysis) and 146 mg (sulphur analysis).

CSPs I and II were obtained using the procedure described previously [8,9]. BSA–DSC sorbent was prepared as follows: BSA (1 g) dissolved in 0.1 *M* phosphate buffer (pH 7.0, 1% of (2-propanol) and 3-aminopropylsilica (2 g) were ultrasonicated for 3 min. Thereafter, the slurry was agitated overnight at room temperature for 24 h, 1 g of DSC suspended in buffer was added and the slurry agitated overnight at 35°C, isolated and washed thoroughly with 0.1 *M* phosphate buffer (pH 7) containing 40% of 2-propanol. The sorbents were then resuspended and packed into 125 × 4 mm I.D. columns as described previously [8].

Liquid chromatography

Chromatography at ambient temperature was performed using an LKB Model 2150 pump, a Rheodyne Model 7125 injection valve with a 20-μl loop and an LKB Model 2151 variable-wavelength UV detector. The mobile phase was a phosphate buffer containing 0–7% of 2-propanol. All chromatography was carried out under isocratic conditions. Retention times and peak areas were obtained using a Waters Model 740 electronic integrator interfaced with the detector.

Sorbent analysis

The amount of BSA incorporated into the silica was determined from elemental analysis data (NS) for the dried materials. The 3-aminopropylsilica used contained 1.84% (w/w) of nitrogen.

RESULTS AND DISCUSSION

The amount of BSA per gram of silica is approxi-

mately the same (14.4 ± 0.1%, w/w) in all the three BSA sorbents studied. This indicates that the steps in the preparation of the packing material prior to the addition of the cross-linking reagent determine to a large extent the amount of protein loaded into the silica. Hence the choice of the cross-linking reagent for the immobilization of BSA is of minor importance.

As the loading of BSA is the same, one can assume that the difference in retention and availibility of chiral binding sites for solutes chromatographed on the CSPs can be mainly attributed to the chemical structure of the cross-linker and to changes in protein conformation as a result of cross-linking. One would also expect the degree of achiral hydrophobic interaction generated by the cross-linking reagent to increase in the order DSC < FA < GLA.

Column performance

The column performance was evaluated using *p*-nitroaniline as the analyte. Asymmetry factors (*asf*) and plate heights (*h*) were calculated and plotted as shown in Fig. 2a and b. These results indicate that the BSA–DSC column has a lower column efficiency than the other two types of columns. Further, the BSA–FA sorbent shows the highest column load threshold. Similar results were found for racemic oxazepam (see Fig. 5), which will be discussed later.

The type of organic modifier added to the mobile phase was generally found to affect both the plate height and asymmetry factors. Hence the use of aprotic solvents such as acetonitrile results in better column efficiency and peak shape.

Influence of the cross-linking reagent on the retention and enantioseparation of different compounds on BSA sorbents

*Benzodiazepinones (**Ia–d**).* The benzodiazepinones (**Ia–d**) were generally found to be less retained on BSA–DSC columns, which was in accordance with expectation as this material was assumed to be the least hydrophobic. Further, the use of DSC as a cross-linker also leads to the highest separation factors (α) for three of the diazepinones studied (Table I). It is interesting that substitution of the amide hydrogen in lorazepam (**Ib**) with a methyl group results in a total loss of chiral recognition on the BSA–DSC and BSA–GLA sorbents, indicating participation of the amide hydrogen atom in the binding to BSA and that this is crucial for the enantiodiscrimination on these sorbents. This is not so, however, for the BSA–FA sorbent, on which lormetazepam (**Ic**) is well resolved, even if there is a decrease in the α value compared with lorazepam. One may therefore conclude that the enantioselective properties of the BSA–FA column differ substantially from those of the other two

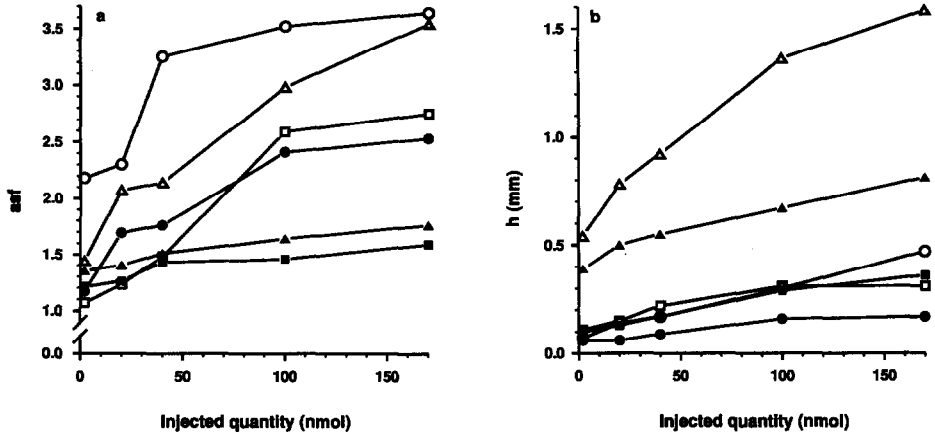
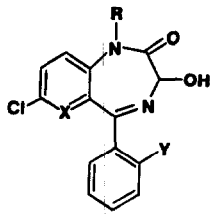


Fig. 2. Dependence of (a) peak symmetry and (b) plate height on column load and eluent composition on three different types of BSA-based columns. Mobile phase, 50 m*M* phosphate buffer (pH 7.2) containing 5% of organic modifier; flow-rate, 1.0 ml/min; UV detection, 254 nm; solute, *p*-nitroaniline. Organic modifier 2-propanol: △ = BSA–DSC; ○ = BSA–FA; □ BSA–GLA column. Organic modifier acetonitrile: ▲ = BSA–DSC; ● = BSA–FA; ■ = BSA–GLA column.

TABLE I

INFLUENCE OF CROSS-LINKING REAGENT ON RETENTION AND RESOLUTION OF BENZODIAZEPINONES ON BSA SORBENTS

Mobile phase, 50 m*M* phosphate buffer (pH 7.1) containing 7% of 2-propanol; flow-rate, 1.0 ml/min; column, 125 × 4 mm I.D.; amount injected, 4 nmol; UV detection at 230 nm. Solute structure:



No.	Compound	BSA–DSC			BSA–FA			BSA–GLA		
		k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
Ia	Oxazepam (R = H, X = CH, Y = H)	4.38	5.78	5.69	5.87	5.16	9.10	5.70	3.53	7.16
Ib	Lorazepam (R = H, X = CH, Y = Cl)	6.86	2.33	2.46	9.80	1.69	2.52	8.47	2.11	3.66
Ic	Lormetazepam ($R = CH_3$, X = CH, Y = Cl)	5.86	1.0	–	5.66	1.44	1.75	6.63	1.0	–
Id	Lopirazepam (R = H, X = N, Y = Cl)	1.36	3.33	2.53	1.75	2.54	3.71	2.07	2.30	3.72

types of columns. It is therefore reasonable to assume that accessibility to the various chiral binding sites is dependent on the type of cross-linking reagent used in the immobilization procedure.

Further, the low efficiency of BSA–DSC columns results in lower resolution (R_s) values. Lopirazepam (**Id**), for example, is least resolved on the BSA–DSC column even though it is best separated on this column (Fig. 3).

*Albendazole sulphoxide and structural analogues (**IIa–d**).* The parent compound albendazole sulphoxide (**IIa**) and the free amine (**IIb**) were retained on all three types of columns but were resolved only on the BSA–FA column and then only partially. Fig. 4 shows the enantioseparation of *rac*-albendazole sulphoxide. Substitution of the propyl group for a phenyl group (**IIc** and **d**) has a marked effect on the enantioseparation of these compounds, the largest α values being obtained on a BSA–DSC column (Table II). However, the low column efficiency of the BSA–DSC column leads to low R_s values for these compounds. The BSA–FA column shows a drastic increase in retention for **IIc** and **IId**, indicat-

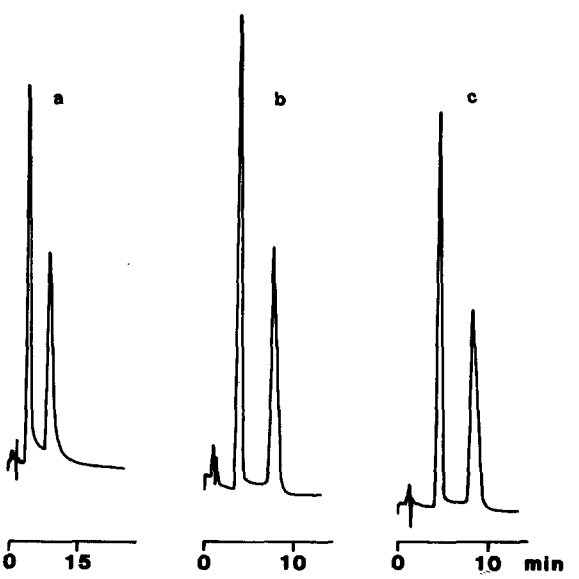


Fig. 3. Optical resolution of *rac*-lopirazepam (**Id**) on (a) BSA–DSC, (b) BSA–FA and (c) BSA–GLA columns. Mobile phase, 50 m*M* phosphate buffer (pH 7.1) containing 7% of 2-propanol; flow-rate, 1.0 ml/min; UV detection, 230 nm; amount injected, 4 nmol.

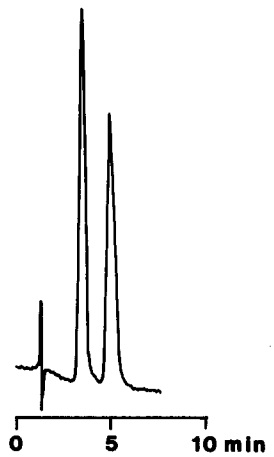


Fig. 4. Separation of enantiomers of *rac*-albendazole sulphoxide (**IIa**) on BSA–FA column. Mobile phase, 50 m*M* phosphate buffer (pH 7.7) containing 2% of methanol; flow-rate, 1.0 ml/min; detection UV 254 nm; amount injected, 4 nmol.

ing and the hydrophobic interaction between the solute and the CSP is strengthened; however, the enantioseparation is not significantly affected. One can therefore assume that the aromatic ring at the asymmetric centre is a prerequisite for chiral discrimination on the BSA–GLA and BSA–DSC sorbents but not on the BSA–FA sorbent. The free amines (**IIb** and **d**) were generally retained more strongly, indicating that hydrogen bonding is also involved in the retention process.

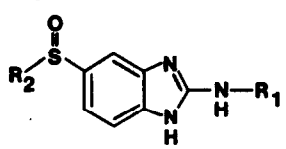
Miscellaneous compounds. As shown in Table III, under basic mobile phase conditions (pH 7.7), the BSA–DSC sorbent gives very large α values for compounds displaying acidic character, *i.e.*, D,L-tryptophan (**III**), benzonal (**IV**) and bendroflumethiazide (**V**). This indicates that the charged groups in the solutes can interact more easily with the protein when DSC is the cross-linking reagent, leading to an increase in enantiodiscrimination. Tryptophan, which is amphoteric, is best separated on the BSA–GLA column, however.

Earlier studies of methaqualone (**VI**) and benzoin (**VII**) have shown that the dominant interaction between the solute and BSA-based CSPs is hydrophobic in character [10]. The BSA–FA sorbent was found to interact more strongly with these compounds, which is reflected in both the k' and α values. The α values of **VI** and **VII** decrease in the series FA > DSC > GLA used as cross-linking reagents.

TABLE II

CHROMATOGRAPHIC DATA OBTAINED ON DIFFERENT BSA COLUMNS FOR ALBENDAZOLE SULPHOXIDE AND STRUCTURAL ANALOGUES

Mobile phase, 50 m*M* phosphate buffer (pH 7.7) containing 2% of 2-propanol; flow-rate, 1.0 ml/min; column, 125 × 4 mm I.D.; amount injected, 4 nmol; UV detection at 254 nm. Solute structure:



No.	Compound	BSA–DSC			BSA–FA			BSA–GLA		
		k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
IIa	$R_1 = COOCH_3$ $R_2 = C_3H_7$	0.86	1.0	–	1.03	1.69	0.72	0.96	1.0	–
IIb	$R_1 = H$ $R_2 = C_3H_7$	1.33	1.0	–	1.28	1.42	1.08	1.46	1.0	–
IIc	$R_1 = COOCH_3$ $R_2 = C_6H_5$	5.06	1.85	1.11	23.1	1.20	0.87	6.42	1.57	1.44
IId	$R_1 = H$ $R_2 = C_6H_5$	6.59	1.70	1.46	19.3	1.40	2.02	7.47	1.55	2.52

TABLE III

OPTICAL RESOLUTION DATA FOR VARIOUS COMPOUNDS ON DIFFERENT BSA SORBENTS

Mobile phase, 50 m*M* phosphate buffer (pH 7.7) containing 2-propanol; flow-rate, 1.0 ml/min; column, 125 × 4 mm I.D.; amount injected, 4 nmol.

Compound	2-Propanol (%)	BSA–DSC			BSA–FA			BSA–GLA		
		k'_1	α	R_s	k'_1	α	R_s	k'_1	α	R_s
III	2	1.34	6.71	4.34	0.50	2.02	1.96	0.90	9.24	5.27
IV	5	25.8	1.47	1.85	28.3	1.40	1.73	32.5	1.12	0.56
V	5	8.19	1.79	1.63	10.1	1.16	1.07	9.04	1.47	2.04
VI	2	3.93	1.29	0.67	4.08	1.50	1.68	3.42	1.20	0.74
VII	5	3.85	2.60	2.62	5.05	3.09	3.56	3.93	2.23	3.70

TABLE IV

INFLUENCE OF ORGANIC MOBILE PHASE ADDITIVES ON RETENTION AND COLUMN EFFICIENCY

Mobile phase, 50 m*M* phosphate buffer (pH 7.7) containing 2% of organic modifier; flow-rate, 1.0 ml/min; column, 125 × 4 mm I.D., amount injected, 4 nmol.

CSP	Solute	Organic modifier	k'_1	α	R_s	h_1 (mm)	h_2 (mm)
BSA—DSC	Methaqualone	Methanol	5.83	1.18	0.40	1.02	0.88
		Acetonitrile	4.58	1.24	0.49	0.87	1.08
		2-Propanol	3.93	1.29	0.67	0.63	0.61
	SO.ABZ.NH_2 **(IIb)**	Methanol	8.15	1.47	1.15	0.62	0.51
		Acetonitrile	6.92	1.50	1.17	0.53	0.70
		2-Propanol	6.59	1.70	1.46	0.52	0.64
	D,L-Tryptophan	Methanol	1.21	14.0	5.03	0.24	0.42
		Acetonitrile	0.69	11.2	4.21	0.32	0.29
		2-Propanol	1.34	6.71	4.34	0.24	0.57
BSA–FA	Methaqualone	Methanol	7.41	1.31	1.09	0.40	0.30
		Acetonotrile	5.17	1.39	1.39	0.29	0.24
		2-Propanol	4.08	1.50	1.68	0.30	0.23
	SO.ABZ.NH_2 **(IIb)**	Methanol	28.7	1.62	2.71	0.15	0.16
		Acetonitrile	18.0	1.54	2.56	0.14	0.14
		2-Propanol	19.3	1.40	2.02	0.16	0.14
	D,L-Tryptophan	Methanol	0.56	2.54	2.08	0.23	0.98
		Acetonitrile	0.48	2.10	2.03	0.13	0.24
		2-Propanol	0.50	2.02	1.96	0.13	0.60
BSA–GLA	Methaqualone	Methanol	5.48	1.19	0.88	0.19	0.40
		Acetonitrile	4.66	1.18	1.09	0.11	0.15
		2-Propanol	3.42	1.20	0.74	0.26	0.35
	SO-ABZ.NH_2 **(IIb)**	Methanol	9.46	1.44	2.19	0.13	0.21
		Acetonitrile	7.01	1.46	2.63	0.09	0.16
		2-Propanol	7.47	1.55	2.52	0.13	0.18
	D,L-Tryptophan	Methanol	1.30	11.1	5.73	0.17	1.19
		Acetonitrile	0.76	6.93	5.75	0.12	0.33
		2-Propanol	0.90	9.24	5.27	0.18	0.66

Effect of organic modifiers in the mobile phase on retention and resolution on BSA-based CSPs

The use of glutaraldehyde as a cross-linking reagent for the immobilization of BSA results in a more "hydrophobic" sorbent compared with BSA–DSC and BSA–FA sorbents. To gain further insight into the behaviour of the CSPs, a study of the hydrophobic interaction between structurally different solutes and BSA sorbents was conducted by using different organic mobile phase additives.

The chirality of metaqualone (**VI**) is generated by restricted internal rotation in the molecule. As already mentioned, the dominant interaction between this compound and BSA is hydrophobic, which can be seen in that the lowest k' values are obtained for the most hydrophobic mobile phase additive, 2-propanol. With the BSA–FA column, 2-propanol as mobile phase additive also leads to the highest enantioseparation and resolution of methaqualone, mainly owing to the faster elution of the first enantiomer (Table IV). A similar behaviour is seen on the BSA–DSC column.

This is not the case on the BSA–GLA column, where the α values remain basically the same irrespective of the hydrophobicity of the mobile phase additive. However, there is a marked increase in resolution when acetonitrile is used as the retention modifier, mainly owing to a large increase in column efficiency. It is interesting that although the enantioseparation of methaqualone on the BSA–DSC column is greater than or equal to that on the BSA–GLA sorbent, the low column efficiency of the former leads to unexpectedly low R_s values on the BSA–DSC column.

As mentioned previously, both the free amino group and the aromatic phenyl group at the chiral centre participate in the retentive process of the basic compound **IId**, a structural analogue of albendazole sulphoxide. Retention on the BSA–DSC sorbent decreases with increasing eluting power of the organic modifier in the mobile phase, especially for the first-eluted enantiomer. Consequently, α increases with increasing hydrophobicity of the mobile phase modifier (Table IV). The more hydrophobic sorbents, BSA–FA and BSA–GLA, show an interesting retention behaviour, namely that both enantiomers of **IId** are less retained when acetonitrile is used instead of 2-propanol as the mobile pase modifier. However, the effect on enantioselectivity is different on the two sorbents. On the BSA–GLA column, α increases with increasing hydrophobicity of the mobile phase additive whereas α decreases on the BSA–FA column. The latter is mainly due to faster elution of the last-eluted enantiomer.

We have reported previously the enantioseparation of D,L-tryptophan on BSA sorbents [11]. The interaction between tryptophan and the protein is highly pH dependent and separation is afforded at basic pH (>7.5). The large α value attained (Table IV) reflects the presence of a specific indole binding site on BSA, which has a very high affinity for the D-enantiomer [12]. In contrast to the two earlier compounds (**IIb** and **VI**), the DSC-cross-linked BSA sorbent displays the largest enantioselectivity towards tryptophan ($\alpha \geqslant 6.7$). All three columns show a similar retention behaviour, *i.e.*, D,L-tryptophan is least retained on BSA sorbents when acetonitrile is the organic modifier in the mobile phase. However, the enantioseparation of tryptophan is affected differently on these sorbents; on BSA–DSC and BSA–FA columns, α decreases with increasing hydrophobicity of the mobile phase additive whereas on the BSA–GLA column α decreases on using acetonitrile as a mobile phase modifier (Table IV). Further, there is a drastic decrease in the enantioseparation of tryptophan on the BSA–FA column ($\alpha \approx 2$) compared with the other two types of sorbents. One can therefore assume that the availibility of the indole binding site is restricted when formaldehyde is used as a cross-linking reagent.

Influence of column load on peak symmetry and column efficiency

The binding capacity of protein columns is relatively low owing to the small part of the molecule actually involved in the chiral recognition process [13]. At small amounts of solute injected (<10 nmol) there is no significance difference in the extent of deterionation of the peak symmetry of the enantiomers of oxazepam and plate height on the three different columns (Fig. 5a and b). However, as the column load increases, the peaks show increasing tailing and at injected solute quantities $\geqslant 40$ nmol a tendency towards fronting and thereby an overall lower peak asymmetry factor is found for all the three types of BSA columns. The last-eluted peak on the BSA–GLA column, however, shows

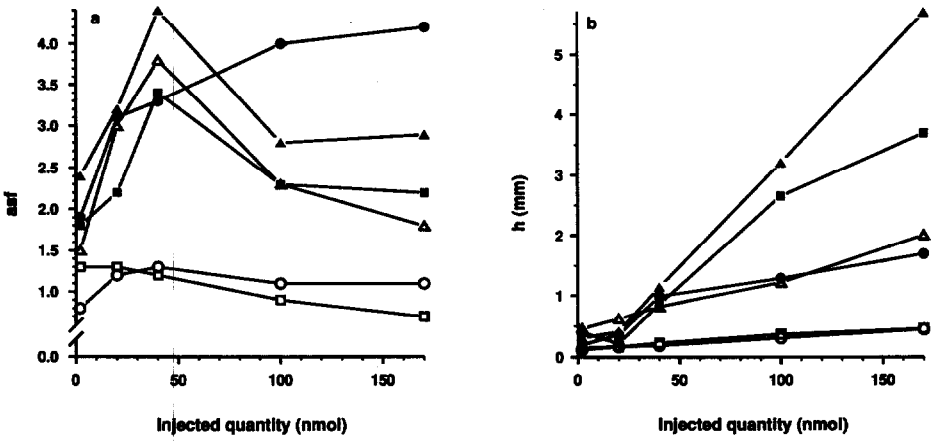


Fig. 5. Influence of column load on (a) peak symmetry and (b) plate height of *rac*-oxazepam. Column: △,▲ = BSA–DSC; ○,● = BSA–FA; □,■ = BSA–GLA. Open symbols represent the first-eluted enantiomer. Mobile phase, 50 m*M* phosphate buffer (pH 7.1) containing of 7% of 2-propanol; flow-rate, 1.0 ml/min; UV detection, 230 nm.

increasing tailing with increasing amounts of injected solute. These results are in general agreement with the binding model suggested by Guiochon and co-workers [13,14], *i.e.*, there are two types of binding sites on BSA, one of which is chiral selective and the other a non-selective binding site having a roughly ten times larger capacity. Hence, as the column load increases, the stereoselective site is saturated and the non-selective binding sites begin to contribute in the retention process. This phenomenon could also explain the peak fronting observed as the column load increases.

CONCLUSIONS

The hydrophobicity of the cross-linking reagent used for immobilization of BSA is reflected by the retentive properties of the BSA-based sorbents. The least hydrophobic sorbent, BSA–DSC, was generally found to show the lowest k' values and high enantioselectivity, particularly for acidic compounds. However, the low column efficiency displayed by this type of sorbent results in broader peaks and consequently low R_s values. Further, the enantioselective properties of BSA sorbents are greatly affected by the choice of the cross-linking reagent used during the immobilization procedure. The BSA–FA sorbent displays substantially different enantioselectivity to the BSA–DSC and BSA–GLA columns. One can assume that during the immobilization procedure the protein attains different conformations with different cross-linking reagents and as a result availibility of some of the chiral binding sites is affected.

The type of organic modifier in the mobile phase was found to influence not only retention and enantioselectivity but also column efficiency and peak shape. Generally, an aprotic solvent such as acetonitrile was found to give better peak symmetry and higher column efficiency. Further, of the three cross-linked sorbents, the BSA–FA column had the highest capacity.

ACKNOWLEDGEMENT

This work was supported by the Swedish Natural Science Research Council (K-KU 2508-300).

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