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Enantioselectivity properties of human serum albumin immobilized on anion-exchangers based on polyvinylimidazole-coated silica Effect of protein loading on separation properties

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Abstract

Chiral chromatographic supports were obtained by continuously applying solutions contained HSA to ion-exchange columns. The columns were packed with silica modified with polyvinylimidazole and a copolymer polyvinylpyrrolidone–polyvinylimidazole (75:25) respectively, quaternized and crosslinked. Small changes in the concentration of NaCl during immobilization of HSA lead to variations in the amount of HSA bound to the supports. These variations have consequences in terms of chromatographic retention (k'), selectivity (α) and resolution (R_s) of enantiomers. The effects of varying the pH and organic modifier of the mobile phase on the chromatographic properties were also examined. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Immobilized proteins; Chiral stationary phases LC

1. Introduction

In the last few years, chiral selectors have been developed to improve high-performance liquid chromatography (HPLC) separations of biologically active compounds. These include several protein-bonded stationary phases based on the stereoselective properties of proteins. Especially α_1-glycoprotein (AGP) [1], ovomucoid (OVO) [2], α-chymotrypsin [3], and various types of albumins [4–6], have been used successfully to resolve a wide variety of enantiomeric pairs.

There is a high similarity between human serum albumin (HSA) and bovine serum albumin (BSA) commercial stationary phases. They can be used to resolve the enantiomers of a wide range of chiral compounds and their selectivities are strongly affected by mobile phase parameters such as pH, temperature and organic modifier concentration [7]. However, there are some differences between these two albumin-based stationary phases, e.g., the elution order of warfarin enantiomers, have been observed and are the consequence of the influence of natural variations in the amino acid sequence of the two proteins [8].

Various methods were used for immobilizing BSA. It has been covalently bound to porous silica [9], directly adsorbed on silica [10] and ionically immobilized on ionic supports [11].

HSA has been also used successfully for chiral separations. Dominici et al. [7] have immobilized HSA on a commercially available diol column activated with 1,1-carbonyldiimidazole. Tittelbach and Gilpin have prepared chemically linked HSA via

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a three step procedure using a Schiff base [5]. While Aubry et al. have prepared a support with a 50:50 mixture of HSA and AGP [12]. HSA has also been adopted as a chiral additive in the mobile phase by Sébille and Thuaud [13].

In this work, HSA is immobilized on ion-exchange chromatography supports prepared with a silica modified respectively with polyvinylimidazole and a copolymer of polyvinylpyrrolidone and polyvinylimidazole (75:25), quaternized and crosslinked. These polymeric stationary phases developed in our laboratory have several advantages such as the simple and rapid preparation and the high stability observed with solvents commonly used for the HPLC of proteins. The amount of HSA bound to the anion exchanger is highly dependent on the mobile phase composition during the immobilization of the protein. This procedure allows the preparation of supports containing an increasing content of immobilized chiral selectors. Thus, the influence of the protein loading on the retention, selectivity and resolution of racemic mixtures was investigated. Moreover, these studies have permitted us to examine the influence of the charge density and the addition of hydrophilic character (polyvinylpyrrolidone) on the enantioselectivity properties of HSA.

2. Experimental

2.1. Materials

The porous silica (LiChrospher Si 300) support of particle diameter 10 μm and pore size 300 Å was purchased from Merck (Darmstadt, Germany). 1-Vinylimidazole (NVI), 1-vinyl-2-pyrrolidone (NVP), 1,4-butanediol diglycidyl ether (BUDGE), epichlorohydrin (EPI) and methyliodide were purchased from Aldrich (Steinheim, Germany). α,α′-Azoisobutyronitrile (AIBN) was purchased from Fluka (Buchs, Switzerland).

DL-Tryptophan, D- and L-tryptophan, N-benzoyl-DL-phenylalanine, N-benzoyl-L-phenylalanine, adenosine monophosphate and HSA (fraction V) were from Sigma (St. Louis, MO, USA). (*RS*)-Oxazepam was purchased from Sanofi (Toulouse, France).

2.2. Equipment

The chromatographic system consisted of two pumps (Waters, Milford, MA, USA, Model 501 and SpectraPhysics, San José, CA, USA Model SP8810), a 7125 sample injector (Rheodyne, Berkeley, USA) with a 20 μl loop and a UV 757 variable-wavelength absorption detector (Kratos Analytical, Ramsey, NJ, USA).

The column temperature was maintained within ±0.1°C of the desired temperature with a thermostatted waterbath.

2.3. Synthesis of anion-exchange supports

2.3.1. Polymers

Two polymers were used in this study: poly(1-vinylimidazole) (PVI) and a copolymer of 1-vinylimidazole and 1-vinyl-2-pyrrolidone (PVI–PVP).

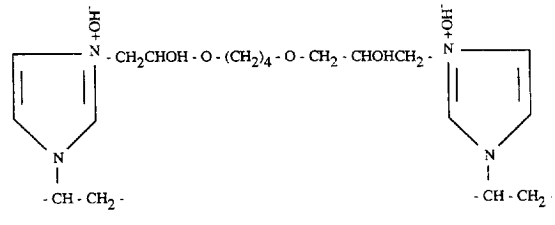
The PVI homopolymer was synthesized by radical polymerization of NVI at 60°C in methanol using AIBN as initiator [14]. The molecular mass determined by viscosimetry [15], was 40 000 g mol^{-1}.

The copolymer PVP–PVI (75:25) was synthesized by polymerization of NVI and NVP with AIBN as initiator, at 70°C in ethanol, for 48 h under a nitrogen atmosphere as previously described [16,17].

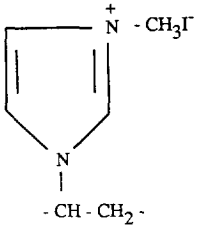
2.3.2. Preparation of PVI silica anion exchangers

The coating of the silica was carried out by putting the porous support in a 10% (w/w) polymer solution in methanol [14]. The amount of polymer deposited on silica was 44 mg per g of support.

Crosslinking of the coated polymer was performed with BUDGE (6 mmol per g of silica) (a).



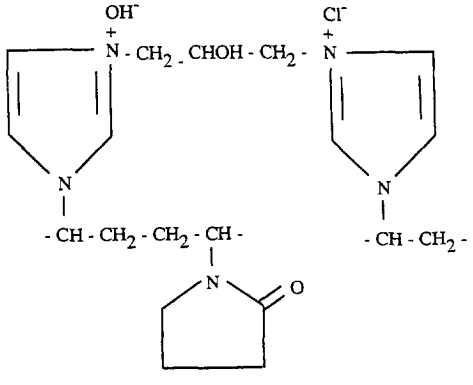
The material was then quaternized with methyl iodide (6 mmol per g of silica) (b) [14].



2.3.3. Preparation of PVP–PVI silica anion exchangers

The adsorption of the copolymer at saturation was achieved by putting the silica in a 10% (w/w) copolymer solution in methanol. The amount of polymer deposited on silica was 74 mg per g of support. The number of NVP units was 0.43 mmol per g of support, while, the number of NVI units was 0.27 mmol per g.

Quaternization and crosslinking of the coated copolymer were then performed with EPI (6 mmol per g of silica) (c)



in the conditions previously described [14].

2.4. Chromatographic conditions

The columns (100 mm×4.6 mm I.D.) were packed with the anion-exchange stationary phases by Colochrom (Gagny, France).

All studies were performed at 25±0.1°C. A flow-rate of 1 ml min^{-1} was used throughout this study. All mobile phases used in this work were prepared using 0.067 mol l^{-1} sodium phosphate buffer (pH 7.4).

2.4.1. HSA based chiral columns

HSA was immobilized in the column by pumping a 2 g l^{-1} HSA solution in 67 mmol l^{-1} phosphate buffer (pH 7.4) with various concentrations of NaCl (0 mol l^{-1} to 0.5 mol l^{-1}) into the column. The wavelength used for the detection of HSA was 280 nm. The amount of HSA immobilized on the supports was estimated first from the plot of frontal analysis and also, by an analysis of fractions collected during the percolation of albumin on a Li-Chrospher diol column (Merck).

2.4.2. Enantiomeric separations

The mobile phase used for the elution of the enantiomers was 67 mmol l^{-1} phosphate buffer (pH 7.4). The sample amount injected was 0.4 μg for each solute. The wavelength used for detection of the solutes was 280 nm except for N-benzoyl phenylalanine (250 nm).

The column void volume was determined by injecting adenosine monophosphate (AMP) in 67 mmol l^{-1} phosphate buffer with 0.5 mol l^{-1} NaCl (pH 7.4).

3. Results and discussion

3.1. Elution of HSA on PVI supports

The retention behavior of HSA on anion-exchange supports was evaluated using the stoichiometric displacement model [18,19]. When a monovalent salt is used as a displacing agent, this model is based on the following equilibrium:

$$P_m + ZA_{st} \Leftrightarrow P_{st} + ZA_m \quad (1)$$

Where P refers to protein, A refers to displacing ion, st and m correspond respectively to the stationary and mobile phases. In the present case (monovalent salt) Z represents both the number of monovalent ions required to displace the protein from the support, and the number of interaction sites on the protein.

Under isocratic ion-exchange conditions, this model describes the variation of protein retention expressed in terms of the capacity factor k', with the displacing ion concentration ($[A]_m$) according to the following relationship:

$$\log k' = \log K - Z\log [A]_m \quad (2)$$

were K is a constant proportional to the association constant for equilibrium 1.

The plots of log k' versus $\log(1/[A]_m)$ for the PVI and PVI–PVP stationary phases (data not shown) were linear suggesting that an anion-exchange mechanism really controlled the retention of HSA. The values of Z derived from the slopes of these lines were respectively equal to 4 and 1.9 for PVI and PVI–PVP supports. These results are consistent with the higher density of ionic sites on the PVI support.

3.2. Immobilization of HSA on PVI and PVP–PVI supports

In the present study where sodium chloride is used as a displacing salt, interactions between the protein and the support may be described by the following relationship:

$$Z[Cl^-]_{st} + [HSA^{Z-}]_m \Leftrightarrow [HSA^{Z-}]_{st} + Z[Cl^-]_m \quad (3)$$

Where, Z is the number of the accessible charges on the protein, determined before.

According to this equilibrium, minor diminution in the ionic strength tends to increase the amount of HSA bound to the support. Thereby, increasing amounts of HSA were immobilized on PVI and PVP–PVI supports by successive frontal analysis. This method was performed by continuously applying solutions of HSA at a given concentration (2 g l^{-1}) until equilibrium, with decreasing NaCl concentrations.

The capacity factor k' measured above during elution of HSA with 0.5 mol l^{-1} NaCl in the eluent was very low, showing that the protein was almost unretained under these conditions. Thereby immobilization of HSA on the anion-exchangers by frontal analysis was performed with lower salt concentrations.

Typical chromatograms corresponding to the binding of HSA to PVI supports are shown in Fig. 1. Similar results were observed for PVP–PVI supports.

It appears that, the successive breakthrough fronts started approximately at the same volume. However changes in the V_{50} and in the shape of the curves

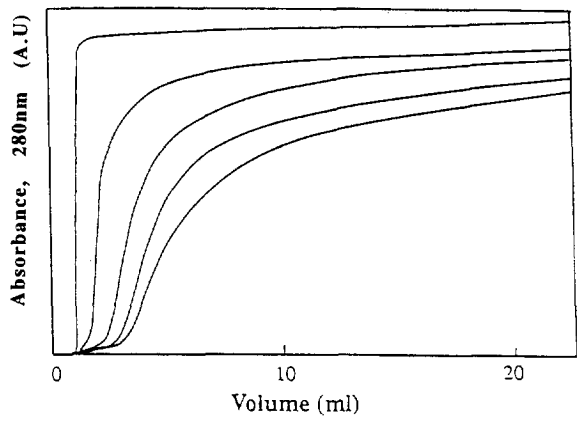


Fig. 1. Frontal analysis of HSA (2 g l^{-1}) at 25°C on PVI support. The NaCl concentrations (left to right) were: 0.2, 0.12, 0.08, 0.04 and 0 mol l^{-1}.

were observed, showing an increase in the amount of HSA fixed to the anion-exchanger with the decrease in salt concentration. The Fig. 2 shows the effect of NaCl concentration on the amount of HSA immobilized. The maximal loading capacity of the matrix

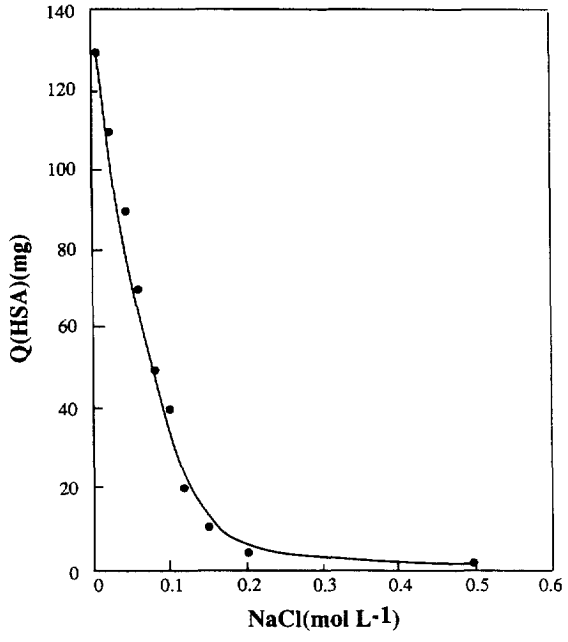


Fig. 2. Variations of the amount of HSA immobilized with NaCl concentration used during the column loading. Eluent: phosphate buffer 67 mmol l^{-1} with NaCl, pH 7.4. Flow-rate: 1 ml/min. Support: PVI coated silica.

was approximately 130 mg HSA per g of silica for PVI supports and 60 mg HSA per g for PVP–PVI supports. These values were low as compared to the theoretical total exchange capacity of the anion-exchangers (respectively 470 and 270 μmol g^{-1}), and probably resulted from the partial accessibility of the anion-exchange sites to the protein. This observation is in accordance with previous results reported by Whitley et al. [20].

3.3. Enantioselective properties of HSA immobilized on PVI and PVP–PVI supports

The enantioselectivity of HSA immobilized on ionic phases was evaluated by studying the retention of three racemic mixtures: DL-tryptophan, (*RS*)-oxazepam and N-benzoyl-DL-phenylalanine.

The influence of solute concentration on retention and peak symmetry was investigated for tryptophan and benzoyl phenylalanine racemates. In the case of tryptophan, it can be seen from Fig. 3A that the capacity factor of the first eluted enantiomer was relatively unchanged while retention of L-enantiomer decreased slightly. Similarly the asymmetry factor of the first peak changed slightly in opposite to the second which was more affected by the effect of sample size (Fig. 3B). For N-benzoyl-phenylalanine (data not shown) significant changes in the peak shape from symmetrical to asymmetrical were observed when amounts greater than 0.5 μg where injected, due to the existence of a non linear isotherm. In the same way, with increasing N-benzoyl phenylalanine concentration, the retention time of the enantiomers decreased. In order to measure the chromatographic parameters in the best conditions 0.4 μg of solutes were injected for the determination of capacity factors k', selectivity α and resolution factor R_s. This study was carried out after each frontal analysis and the results are presented in Fig. 4A–D.

The mobile phase used was prepared using 0.067 mol l^{-1} sodium phosphate buffer (pH 7.4). Under these conditions, HSA immobilized onto the matrix was stable even in the presence of NaCl.

Relationships between capacity factors k' and protein content on PVI and PVP–PVI solid-phase are shown in Fig. 4A and C, respectively. It appears that the retention of solutes is highly influenced by the

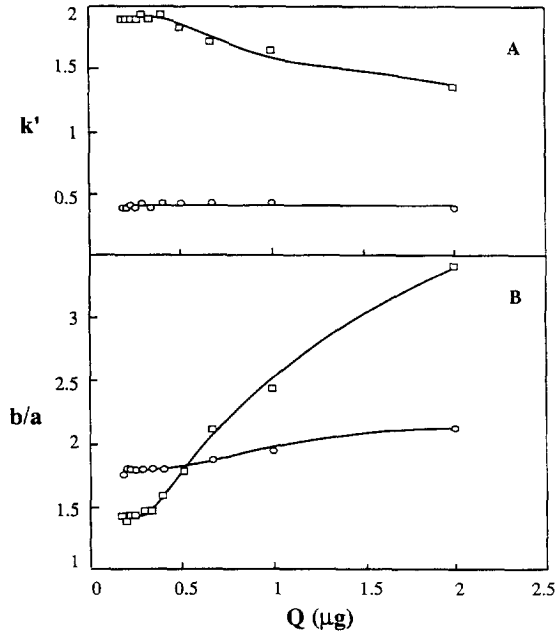


Fig. 3. Variations of capacity factors (*A*) and peak asymmetry (*B*) with amount of tryptophan injected. (Peak asymmetry definite by *b/a*, *b* and *a* being peak half-width at 10% of peak height). Amount of HSA immobilized: 15 mg. ○=D-Tryptophan, □=L-tryptophan. Eluent: phosphate buffer 67 mmol l^{-1} pH 7.4. Flow-rate: 1 ml/min. Support: PVI–HSA coated silica. 20 μl injection loop.

amount of HSA immobilized and increased with the amount of protein. It is observed that the capacity factor k' for a given amount of affixed HSA is different with the two supports. For example with the more retained enantiomer of oxazepam $k'=25$ with PVI–HSA support, whereas, under the same elution conditions $k'=10$ with PVP–PVI–HSA support. Similar results were observed with the other compounds. These observations could suggest that HSA orientation and site accessibility depended on the contact between the protein and the synthetic polymer. In the case of PVI and PVI–PVP supports, the occurrence of hydrophobic contacts (see Section 3.4) added to electrostatic interactions could be invoked to predict a slight change in HSA conformation. This modification could induce an increase of the number of accessible chiral sites, selectivity (α) and resolution (R_s) parameters.

An incurvation of the curves $k'=f[Q(HSA)]$ was observed at high protein loadings. This was due

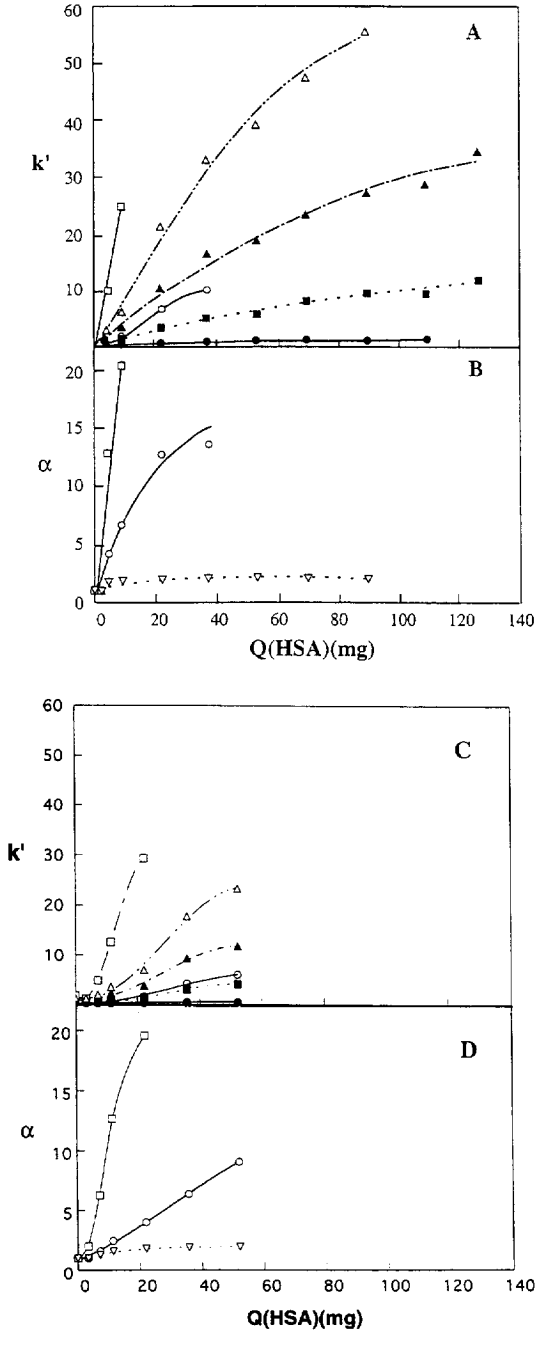


Fig. 4. Variation of capacity factors (k') and selectivity (α) with protein loading on PVI–HSA (A and B) and on PVI–PVP–HSA (C and D) supports. ●=k'_1 tryptophan, ○=k'_2 tryptophan, ■=k'_1 oxazepam, □=k'_2 oxazepam, ▲=k'_1 N-benzoyl phenylalanine, △=k'_2 N-benzoyl phenylalanine, ⊙=α tryptophan, square with dot=α oxazepam and ▽=α N-benzoyl phenylalanine. Eluent: phosphate buffer 67 mmol l^{-1} pH 7.4. Flow-rate: 1 ml/min. 20 μl injection loop. Amount injected: 0.4 μg.

probably to a more limited access to the drug binding sites, resulting in a significant loss of specific activity of the immobilized HSA. In both cases, D-tryptophan retention was almost unaffected by the amount of protein in the column. This relatively low affinity was reported previously for this isomer [21]. On the contrary, high capacity factors were observed for the more retained enantiomer of oxazepam. Very broad peaks were obtained and consequently, the determination of the elution volume became very imprecise at high protein content. It must be outlined that we used here an eluent devoid of any organic solvent in order to keep the HSA content as constant as possible. Moreover, the capacity factor of oxazepam with the PVI–PVP support without HSA was slightly higher than in presence of HSA. This phenomenon may be explained as the consequence of the interactions between the OH group of oxazepam and the PVP polymer since there was no effect with PVI supports. This interaction was decreased in presence of HSA molecules which masked the PVP polymer.

Retentions of both D- and L-isomers of benzoyl phenylalanine were affected similarly by the amount of protein immobilized on the supports. It may be assumed that both enantiomers are retained at high affinity sites on human albumin. Thereby, the selectivity α of D,L-benzoyl phenylalanine was almost unaffected by the HSA content (Fig. 4B–D). Whereas, the selectivities of tryptophan and oxazepam were dependent on the protein content on the solid-phase, since the second eluted enantiomer was much more affected by the amount of HSA than the first one. (Fig. 4C and D). The influence of the amount of protein immobilized on the resolution R_s was also investigated. This parameter increased with the protein loading. However, the precision on R_s determination at high amounts of HSA is poor because of the great width of the peaks under the chosen elution conditions.

Moreover, we have compared in Table 1 our results with those obtained on a column where HSA have been covalently immobilized on an activated silica with 3-glycidoxy propyltriethoxysilane prepared by Felix et al. [12]. It can be seen that the covalent support gave higher retention of the racemates for a same HSA content than our column. As explained above, the covalent fixation of HSA on the support could enable modifications of the sites

Table 1
Capacity factors (k') and selectivity (α) on the PVI–HSA and Diol–HSA supports

Solutes	HSA covalently grafted support			HSA–PVI support		
	k'_1	k'_2	α	k'_1	k'_2	α
Tryptophan	1.9	27.5	14.3	0.8	10.3	13.7
Oxazepam	19.4	not eluted	–	5	not eluted	–
N-Benzoyl phenylalanine	98	not eluted	–	16.5	33	2

Eluent: phosphate buffer 67 mmol l^{-1} pH 7.4. Flow-rate: 1 ml/min. 20 μl loop injection. Amount injected: 0.4 μg. Amount of HSA immobilized: 30 mg.

accessibility and improve the enantioselectivity properties.

3.4. Effect of the mobile phase composition

The effects of varying the pH and organic modifier content of the mobile phase on the retention of the enantiomers were examined. Due to the instability of the columns towards high ionic strength, the influence of phosphate concentration was not studied.

The retention was affected by the pH of the mobile phase (Fig. 5). An increase in the pH from 6 to 8 resulted in a decrease in the k' of N-benzoyl phenylalanine. In contrast, the k' values of tryptophan were found to increase under the same conditions. These results are consistent with previous work performed with tryptophan on immobilized HSA-silica [22], and with N-benzoyl phenylalanine on immobilized BSA-silica [4] and was attributed to an increase in total negative charge of albumin with the pH.

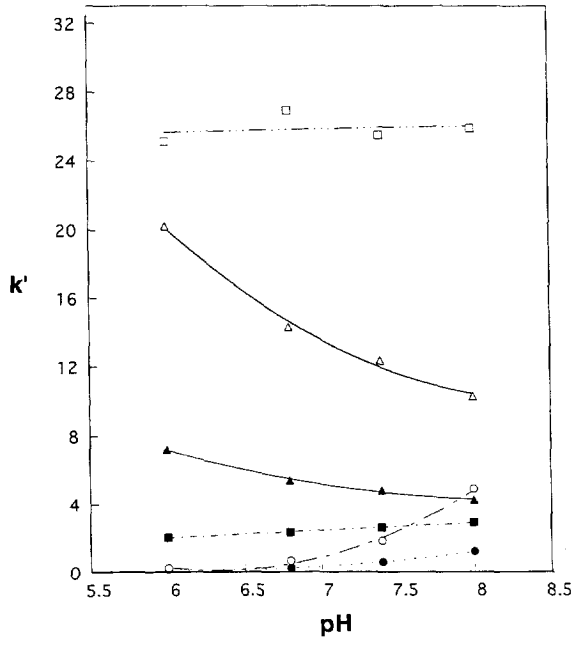


Fig. 5. Effect of mobile phase pH on retention on the PVI–HSA support. Eluent: phosphate buffer 67 mmol l^{-1} pH 7.4. Flow-rate: 1 ml/min. 20 μl injection loop. Amount injected: 0.4 μg. Amount of HSA immobilized: 15 mg. Solute symbols as in Fig. 4.

The influence of the concentration of 1-propanol in the mobile phase on the capacity factors of the racemates was studied. It can be seen in Fig. 6, that the alcohol had a significant effect on the retention of the enantiomers. Indeed, the k' values were lowered by addition of the organic modifier to the mobile phase. In the cases of tryptophan and oxazepam, this influence was more important on the second peak than on the first. These results are in agreement with observations of Yang and Hage [22] for tryptophan and of Dominici et al. [7] for oxazepam hemisuccinate and outlined the importance of hydrophobic interactions during separation of enantiomers on HSA chiral stationary phases.

A slight decrease in the retention and selectivity of the enantiomers was observed after using the organic modifier. These results can be attributed to the loss of HSA from the column and were previously observed by Jacobson and Guiochon [23].

3.5. Stability

The stability of the columns depended essentially on the composition of the mobile phase used during the binding of HSA to the supports. The durability of supports in phosphate buffer (0.067 mol l^{-1} pH 7.4) was reasonable as far as NaCl salt concentration higher than 0.04 mol l^{-1} was added to the phosphate

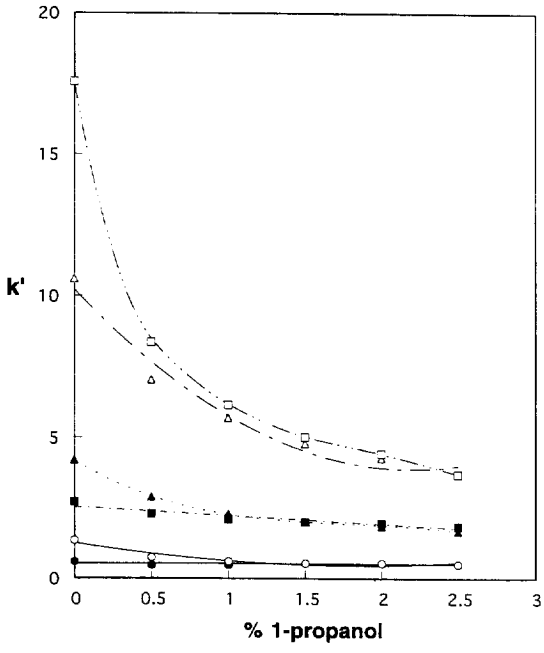


Fig. 6. Effect of mobile phase 1-propanol content on retention on the PVI–HSA support. Eluent: phosphate buffer 67 mmol l^{-1}. Flow-rate: 1 ml/min. 20 μl injection loop. Amount injected: 0.4 μg. Amount of HSA immobilized: 15 mg. Solute symbols as in Fig. 4.

buffer during fixation of HSA. However, when the immobilization was carried out without any additional ionic strength, the protein slightly "bled" from the columns resulting in a continuous drift of the baseline. The same observation was reported previously for BSA immobilized on a quaternary ammonium anion exchanger [23].

In order to improve the stability of the stationary phases obtained at low ionic strengths and to extend the applicability of the columns, several attempts of crosslinking by glutaraldehyde were made. All tests were followed simultaneously in a test-tube. The column was equilibrated with a solution of glutaraldehyde during 15 min and stored until the HSA in contact with the same solution of glutaraldehyde in the test-tube crosslinked. The column was then immediately washed with phosphate buffer 67 mmol l^{-1} pH 7.4. After this treatment, a complete loss of enantioselectivity of HSA fixed onto the supports was observed even with diluted solutions of glutaraldehyde (0.5%, v/v) showing that crosslinking induced drastic changes in the conformational structure of HSA immobilized on PVI and PVP–PVI supports.

Although, the immobilization of HSA on the supports was mainly due to electrostatic interactions between positively charged imidazole rings and the negatively charged protein as demonstrated above (stoichiometric displacement model and effects of the ionic strength during the frontal analysis) desorption attempts using a high ionic strength in the eluent were not probative since albumin was not completely desorbed from the columns. Moreover, when acetonitrile was used as an organic modifier, a loss of albumin was observed but without a complete extraction of the protein: the presence of remaining protein was revealed by enantioselective separation of tryptophan. Accordingly, it could be assumed that the fixation of HSA on the supports was due to electrostatic interactions in addition to hydrophobic interactions resulting in very strong interaction. This aspect is presently under further investigation.

4. Conclusion

The results here reported show the interest of using a strong anion-exchange support for binding HSA. In most cases, the enantioselectivity of the protein is maintained and the selectivity values increase with increasing amounts of protein in the column. This type of protein immobilization is, indeed, highly dependent on the nature of the eluent. Minor changes in concentration of NaCl during the immobilization of HSA lead to variations in the amount of protein bound to the support. This procedure has been developed for coating an ionic phase support with increasing amounts of HSA. However, it is not necessary to immobilized very important amounts of HSA in order to obtain a good resolution. The method permits us to adjust the retention properties and the selectivity of the support. These parameters are useful for comparing the various methods of HSA immobilization.

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References

[1] J. Hermansson, J. Chromatogr. 269 (1983) 71.
[2] T. Miwa, M. Ichikawa, M. Tsuno, T. Hattori, T. Miyakawa, M. Kayano, Y. Miyake, Chem. Pharm. Bull. 35 (1987) 682.
[3] I.W. Wainer, P. Jadaud, G.R. Schombaum, S.V. Kadodkar, M.P. Henry, Chromatographia 25 (1988) 903.
[4] S. Allenmark, B. Bomgren, J. Chromatogr. 264 (1983) 63.
[5] V. Tittelbach, R.K. Gilpin, Anal. Chem. 67 (1995) 44.
[6] T.A.G. Noctor, G. Felix, I. Wainer, Chromatographia 31 (1991) 55.
[7] E. Dominici, C. Bertucci, P. Salvadori, G. Félix, I. Cahagne, S. Motellier, I.W. Wainer, Chromatographia 29 (1990) 170.
[8] C. Lagercrantz, T. Larsson, I. Denfors, Comp. Biochem. Physiol. 69C (1981) 375.
[9] S. Allenmark, B. Bomgren, H. Born, J. Chromatogr. 237 (1982) 473.
[10] P. Erlandsson, L. Hansson, R. Isaksson, J. Chromatogr. 370 (1986) 475.
[11] S.C. Jacobson, G. Guiochon, J. Chromatogr. 600 (1992) 37.
[12] A.-F. Aubry, N. Karkoglon, V. Descorps, I.W. Wainer, G. Felix, J. Chromatogr. A 685 (1994) 1.
[13] B. Sébille, N. Thuaud, J. Liq. Chromatogr. 3 (1980) 299.
[14] B. Sébille, B. Boussouira and J. Piquion, Fr. Pat., 85-176 98 (1985).
[15] J.S. Tan, A.R. Sochor, Macromolecules 14 (1981) 1700.
[16] B. Sébille, J. Piquion and B. Boussouira, Eur. Pat. Appl., EP 225829 A2870616 (1987).
[17] R. Lemque, C. Vidal-Madjar, M. Racine, J. Piquion, B. Sébille, J. Chromatogr. 553 (1991) 165.
[18] W. Kopaciewicz, M.A. Rounds, J. Fausnaugh, F.E. Renier, J. Chromatogr. 266 (1983) 3.
[19] R.R. Drager, F.E. Regnier, J. Chromatogr. 359 (1986) 147.
[20] R.D. Whitley, R. Wachter, F. Liu, N.-H.L. Wang, J. Chromatogr. 465 (1989) 137.
[21] R.H. McMenamy, J.L. Oncley, J. Biol. Chem. 233 (1958) 1436.
[22] J. Yang, D.S. Hage, J. Chromatogr. 645 (1993) 241.
[23] S.C. Jacobson, G. Guiochon, Anal. Chem. 64 (1992) 1496.