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Structural changes of human serum albumin immobilized on chromatographic supports: a high-performance liquid chromatography and Fourier-transform infrared spectroscopy study

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Abstract

Chiral stationary phases obtained by immobilization of HSA on [C8] and [C18] reversed-phases and on poly(1-vinylimidazole)-coated silica were tested to resolve DL-tryptophan, *N*-benzoyl-DL-phenylalanine, *RS*-oxazepam and *RS*-warfarin racemic mixtures. Parameters of enantioselectivity measured in HPLC are correlated to structural and solvation states for adsorbed HSA, evaluated by FTIR spectroscopy. HSA immobilized on [PVI]-anion-exchangers is highly selective. HSA molecules are not self-associated, only unfolded for a small hydrophobic helix. The HSA-coated reversed-phases have a lower selectivity. Unfolding is larger but the indole-benzodiazepine chiral site is preserved and remains accessible.

Keywords: Human serum albumin

1. Introduction

Problems related to the biological activity of proteins immobilized onto insoluble carriers or sensing surfaces are of practical and theoretical interest in biotechnology. The attachment of proteins to solid supports offers a convenient method of preparing water-insoluble derivatives of proteins. A number of papers [1,2] has dealt with enzyme carriers employed as heterogeneous specific catalysts and with immunosorbents [3,4] used for the specific adsorption of their corresponding antigen or antibody from a complex mixture. Moreover, chiral high-performance liquid chromatography (HPLC) stationary phases have been described, using mainly albumins [5–8] and alpha (1)-acid glycoprotein [9] as immobilized chiral selectors. However, the potential use of such immobilized proteins is obviously conditioned by their biological activity. The same problem is encountered in sensing technology with the development over the past decade of biosensors [10] such as biocatalytic sensors and immunosensors.

Especially, inactivation may occur when the binding of proteins to the matrix takes place near to the active site. This results in a decrease of the acces-

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sibility to the active center and/or in a modification of the protein structure [11]. Moreover, in some cases, the attachment of protein molecules to the surface may induce drastic conformational changes resulting in a poor biological activity. Therefore, suitable selection of the reactive matrix is essential to obtain useful active immobilized proteins.

In a previous work [12], chiral stationary phases obtained by the immobilization of HSA onto anion-exchangers showed interesting performances for HPLC purposes. In the present study other phases with immobilized HSA were tested to resolve racemic mixtures. The enantioselective supports were obtained by adsorption of HSA on C8 [C8] and C18 [C18] reversed-phases supports. They were compared to chiral phases resulting from the immobilization of HSA onto poly(1-vinylimidazole)-coated silica (PVI). We chose supports all with 300 Å pore diameter to allow HSA to enter the pores. With a view to understand the chiral properties of these phases, we attempted to correlate the parameters of enantioselectivity in HPLC, to the structure and the solvation of adsorbed HSA.

The techniques used to evaluate structural alterations of a protein in solution are generally not suited to adsorbed proteins, except for Fourier-transform infrared spectroscopy (FTIR). Indeed this spectroscopic method has already revealed significant structural differences for HSA adsorbed onto low porosity (80 Å) supports, either a C6 reversed-phase or an anion-exchange phase [13–15]. In this study, FTIR is used to compare the structural and solvation states of HSA adsorbed on the several enantioselective supports and to estimate the stability of the HSA-immobilized phases.

2. Experimental

2.1. Reagents

The C8 reversed-phase support [C8] (Nucleosil 300-10 C8, 10 μm particle size, 300 Å pore diameter and 100 $m^2\ g^{-1}$ specific area) was purchased from Macherey Nagel (Düren, Germany). The C18 reversed-phase support [C18] (LiChrospher 300 RP18, 10 μm particle size, 300 Å pore diameter and 60 $m^2\ g^{-1}$ specific area; no more available) and the silica used for the synthesis of the anion-exchangers (LiChrospher 300, 10 μm particle size, 300 Å pore diameter and 60 $m^2\ g^{-1}$ specific area) were obtained from Merck (Darmstadt, Germany). Deuterium oxide (100% ^2H_2O) was from CEA France. *N*-vinylimidazole (VI), azobis(isobutyronitrile) (AIBN), iodomethane and 1,4-butanedioldiglycidylether (BUDGE) were obtained from Aldrich-Chemie (Steinheim, Germany). Human serum albumin essentially fatty acid free (ref A-1887), DL-tryptophan, D- and L-tryptophan, *N*-benzoyl-DL-phenylalanine (DL-NBP), *N*-benzoyl-L-phenylalanine and *RS*-warfarin were purchased from Sigma (St. Louis, MO). *RS*-oxazepam was from Sanofi (Toulouse, France). Solvents and buffer constituents were of analytical-reagent grade.

2.2. Preparation of the quaternized PVI anion-exchanger ([PVI] support)

The anion-exchanger was synthesized as described previously [12]. PVI (average molecular weight: 40,000 g mol^{-1}) was adsorbed to silica particles (1 g) from a methanol solution (10 ml; 10% w/w). The immobilized polymer layer (44 mg of PVI per g of silica) was crosslinked and simultaneously quaternized with a solution of BUDGE (6 mmol) in methanol (10 ml). The suspension was heated at 60°C for 2 h. Further quaternization with iodomethane was carried out in the same conditions. The anion-exchange capacity of the supports was equal to 0.37 meq g^{-1}.

3. Chromatographic method

3.1. Equipment

The HPLC equipment consisted of two pumps (Waters, Milford, MA, USA, model 501 and Spectraphysics, San Jose, CA, USA, model SP 8810), a six port valve (Rheodyne, Berkeley, CA, USA, model 7010) to commute from one eluent to the other, a sample injector (Rheodyne, Berkeley, CA, USA, model 7125) with a 20 μl loop, a UV variable-

wavelength absorption detector (Kratos Analytical, Ramsey, NJ, USA, model 757) and a Kipp and Zonen recorder (type BD 41). The column temperature was controlled within ±0.1°C with a thermostated waterbath.

The C8 and C18 reversed-phase supports and the anion-exchangers were slurry packed into stainless steel columns (100×4.6 mm for the chromatographic part, 50×4.6 mm for the FTIR study).

3.2. Immobilization of HSA

Columns were first equilibrated with 0.067 *M* sodium phosphate buffer at pH=7.4 at a flow rate of 1.0 ml min^{-1}. Then a HSA solution in the same eluent with various concentrations of NaCl (0–0.5 *M*) was applied to the columns by switching the six port valve. The concentration of HSA used in all experiments was 2 g l^{-1}. Thereafter, columns were washed with 0.067 *M* sodium phosphate buffer at pH=7.4. The amounts of albumin immobilized on the supports were determined from the HPLC analysis of the fractions collected at the column outlet during the percolation of HSA and the washing step. HSA-coated supports were called respectively [HSA-PVI], [HSA-C8] and [HSA-C18].

3.3. Enantiomeric separations

The enantioselective properties of HSA immobilized on reversed-phase supports and anion-exchangers were evaluated from the chromatographic behavior of four racemic mixtures: DL-tryptophan, *N*-benzoyl-DL-phenylalanine (DL-NBP), *RS*-oxazepam and *RS*-warfarin. These enantiomers were not resolved in the absence of albumin on the stationary phases. Elution volumes V_1 and V_2 (indexes 1 and 2 corresponding respectively to the less and the most retained enantiomers), retention factors k_1 and k_2 and the selectivity α were measured. In order to determine these chromatographic parameters in the best conditions, small amounts (0.4 μg) of solutes were injected [12]. Compounds were detected at 280 nm except for *N*-benzoylphenylalanine at 250 nm.

The mobile phase used for enantiomeric separations was 0.067 *M* sodium phosphate buffer at pH=7.4. In some cases, 0.2 *M* sodium chloride was added to the eluent.

4. Infrared spectroscopy

4.1. Sampling

HSA solutions in deuterated phosphate buffer (0.067 *M*) were 5, 10, or 30 g l^{-1}. NaCl was added at 0.04 or 1 *M* in the 10 g l^{-1} HSA solution. HSA solutions were inserted into CaF_2 infrared cells, 50 μm thick. HSA loading on [C8] and [PVI] supports for FTIR analyses was performed using a HPLC pump (LKB 2150, Brama, Sweden). Solutions of HSA (2 g l^{-1}) in pure (for [C8]) or in saline 0.04*M* NaCl (for [PVI]) hydrogenated buffer with a flow rate of 1 ml min^{-1} were pumped. After loading of HSA on the [C8] and [PVI] supports, 97 or 78 mg of protein per g of support were respectively retained after washing the columns with 60 ml of deuterated buffer. Aliquots of HSA adsorbed on the supports in these chromatographic conditions were collected at the inlet or outlet of the columns and inserted into CaF_2 cells, 25 μm thick. The reference aliquots for these phases in suspension were extracted from a similar reference column, without HSA, also rinsed with the deuterated buffer. All these preparations, loading and sampling, were made in an Argon saturated glove-bag.

4.2. Spectral recording

Fourier-transform infrared (FTIR) spectra were recorded with a 1720 Perkin-Elmer spectrometer with 4 cm^{-1} resolution. The spectrometer was continuously dried before and during the measurements by a Balston air dryer (Whatman, UK). A spectrum for HSA in solution corresponds to a computed difference in the 1800–1350 cm^{-1} range between the spectrum of the solution and the spectrum of the buffer. A spectrum of adsorbed HSA corresponds to the difference between the spectrum of the phase with adsorbed HSA in suspension, and the spectrum of the phase without protein recorded in similar conditions. Several spectra were recorded for

the solution and for the adsorbed HSA from 10 min to 45 days. The times at which spectra were recorded are referenced to time zero when solid HSA was dissolved in the buffer.

4.3. Spectral computing and assignments

The analysis of HSA secondary structure relies on the spectral decomposition of the 1500–1750 cm^{-1} spectral range. The procedure is fully described in literature [13]. The infrared absorption in the 1615–1700 cm^{-1} range corresponds to the multicomponent Amide I band for the CON^2H peptide carbonyls. The relative intensities of each component are expressed in % of the sum of the seven components. Taking into account the repeatability of the intensities for samples prepared in strictly similar conditions, variations in intensity by $\pm 1\%$ are assumed to reflect significant solvation or structural changes along the polypeptide backbone [13–15]. HSA containing 584 peptide units a change of 1% should concern around 5–6 peptide units. Each component is ascribed to a set of structurally related peptide units [16–18]. The wavenumber of the Amide I′ vibrations depends on the environment of these peptide groups in HSA secondary structures and in random domains. A first set of wavenumbers corresponds to unbonded carbonyl groups related to random domains. The highest wavenumber (1681 cm^{-1}) is related to free carbonyls standing in hydrophobic domains of the protein while the component at 1670 cm^{-1} is ascribed to free carbonyls influenced by polar organic functions. A second set of the Amide I′ wavenumbers corresponds to hydrogen bonded peptide units. Peptide units involved in α-helices give rise to two absorption Amide I′ bands at 1660 and 1650 cm^{-1}. The Amide I′ band arises at a higher frequency (1660 cm^{-1}) when the α-helices are packed and distorted. The component at 1650 cm^{-1} is attributed to a more regular helical structure. The following amide I′ band at 1638 cm^{-1} is ascribed to carbonyls bonded to water molecules and then is related to the hydrated domains of the protein. HSA presents in its solid state a large number of loops which may involve more or less extended hydrogen bonded peptide strands. In such domains, the wavenumber of the Amide I′ band is located at 1630 cm^{-1}. The last hydrogen bonded carbonyl component at 1618 cm^{-1} is often observed during protein aggregation and denaturation, and corresponds to intermolecular peripheral peptide units. Assignments are summarized in Table 1.

The intensity of the Amide II components in the 1560–1510 cm^{-1} range reveals the level of the $CONH/CON^2H$ exchange at a given time for the protein in the ^2H_2O buffer. This parameter indicates the water diffusion inside the protein core. For HSA with 100% peptide NH, the Amide II/Amide I′ area ratio is $\neq 0.5$ [13]. Only changes of the percentage in NH larger than 2%, concerning about twenty peptide units, can be reproducibly quantified.

5. Results

5.1. Retention data on reversed-phase supports

5.1.1. HSA immobilization

Experimental breakthrough curves obtained during the adsorption of HSA to Nucleosil 300 10 C8 ([C8]) and LiChrospher 300 10 RP 18 ([C18])

Table 1
Wavenumbers, assignments and intensities in (%) of the seven Amide I′ components for HSA in ^2H_2O buffered solution at pH 7.4

Frequency cm^{-1}	CO peptide groups assignments	% Amide I′ HSA in solution without salt	% Amide I′ HSA in solution with salt
1681	unbonded CO in hydrophobic environment	1.5	2
1670	unbonded CO in polar environment	10	9.5
1660	H-bonded CO in distorted α helices	12	13
1650	H-bonded CO in regular α helices	39.5	38.5
1638	hydrated CO	12.5	12.5
1630	H-bonded CO in extended or bent strands	20	20
1618	H-bonded CO in intermolecular HSA contacts	4.5	4.5

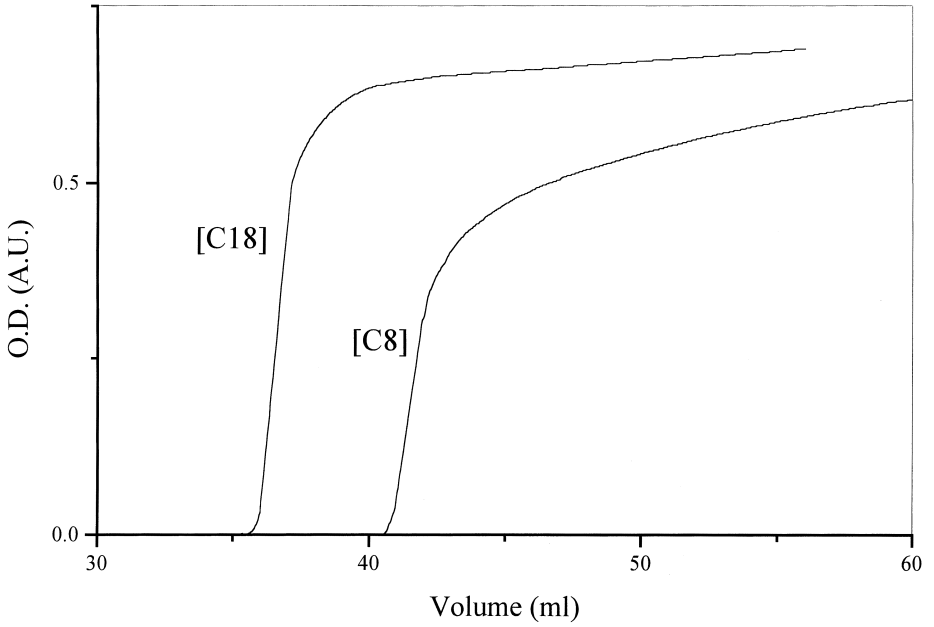

Fig. 1. Breakthrough curves for HSA adsorbed on [C18] and [C8] supports. Eluent: 67 m*M* phosphate buffer pH=7.4; flow-rate: 1 ml min^{-1}. C_{HSA}=2 g l^{-1}. Column length: 10 cm.

stationary phases are illustrated in Fig. 1. In the case of [C_{18}] supports, a sharp increase of the elution front was first observed, indicating a fast adsorption rate process, followed by a more diffuse elution profile. Curves with an even sharper front were reported in a previous study [14] dealing with the adsorption of human albumin to low porosity reversed-phase supports (Spherisorb RP6; 3 μm particle size, 80 Å pore diameter). In this last case, the faster kinetics of adsorption, compared to 300 Å [C18] supports, can be attributed to the lower pore diameter. HSA was mainly adsorbed to the external surface of 80 Å supports while it was able to enter into the pores of 300 Å [C18] phases. As shown in Fig. 1, breakthrough curves obtained with [C8] stationary phases displayed less abrupt elution fronts than with [C18] phases, indicating a slower adsorption rate process. However, pore diameters of both supports investigated in the present study were the same (300 Å). Thus, the slower adsorption rate observed with [C8] compared to [C18] supports may be attributed to structural differences between both grafted silica (Nucleosil and LiChrospher, respectively) and to chain length effects.

For the [C8] and [C18] columns, the total capacities (Q_{max}), the maximum surface concentrations (Γ_{max}) calculated from the Q_{max} values (Table 2), the specific areas and amounts of naked support in the columns (see the Experimental section), were compared. The maximum binding capacities of the [C18] columns were 20% lower than of the [C8] columns (Table 2), due to the lower specific area of the [C18] support. However higher Γ_{max} values were observed when HSA was adsorbed to [C18] phases. This result was consistent with the higher density of alkyl chains on these supports (0.65 μmol m^{-2} for [C18] and 0.3 μmol m^{-2} for [C8]). Approximated surface coverages are estimated by considering HSA molecule as a solid equilateral triangle with a length of 80 Å and a depth of 30 Å [19]. The surface coverages were found respectively equal to 50 and 32% for [C18] and [C8] supports.

5.1.2. Elution of racemic mixtures

The stereoselectivity of the binding of DL-tryptophan, DL-NBP, *RS*-oxazepam and *RS*-warfarin to human serum albumin was reported previously [20–22]. Oxazepam, tryptophan and NBP have a specific affinity for the indole-benzodiazepine site and warfarin interacts with the azapropazone site.

Table 2
Maximum HSA capacity (Q_{max}), maximum HSA surface concentration (Γ_{max}) and selectivity (α) for DL-tryptophan, DL-NBP and *RS*-warfarin of HSA-coated reversed-phase columns. Injection volume: 20 μl. Eluent: 0.067 *M* phosphate buffer. Flow rate: 1 ml min^{-1}. Detection at 280 nm (except for NBP: 250 nm). Column length: 10 cm

Chiral support	Q_{max}[a] (mg)	Γ_{max}[b] (mg m^{-2})	DL-Tryptophan	DL-NBP	*RS*-Warfarin
			α	α	α
[HSA-C8]	105	1.3	2.2	1.5	1.1
[HSA-C18]	85	2.0	3.1	1.7	1.2
[HSA-C18–NaCl]	90	2.1	1.0	1.4	1.0
			1.0[c]	2.3[c]	1.0[c]

[a] Q_{max}: maximum amounts of HSA immobilized in the columns (100×4.6 mm).
[b] Γ_{max}: maximum surface concentrations of HSA on supports.
[c] Eluent: 0.067 *M* phosphate buffer with 0.2 *M* NaCl.

The elution of the four racemic mixtures was performed on naked reversed-phase supports or on HSA-coated phases. Without HSA, tryptophan was highly retained on the [C18] columns while the other compounds were not eluted by using purely aqueous mobile phases. Hydrophobic forces are assumed to retain the organic substances on the grafted alkyl chains. With HSA-coated reversed-phase supports, the enantiomers of *RS*-oxazepam were not eluted. Broad peaks with high retention factors and poor resolution were observed for *RS*-warfarin (data not shown). The enantiomers of DL-tryptophan and DL-NBP were resolved on both chiral phases with similar elution profiles (Fig. 2). The elution on [HSA-C18] was faster than on [HSA-C8] supports, in accordance with lower amounts of HSA in [HSA-C18] columns.

5.1.3. Effect of NaCl

In an attempt to increase the hydrophobic interactions between HSA and reversed-phase support, salt (NaCl 0.2 *M*) was added to the HSA solution. The

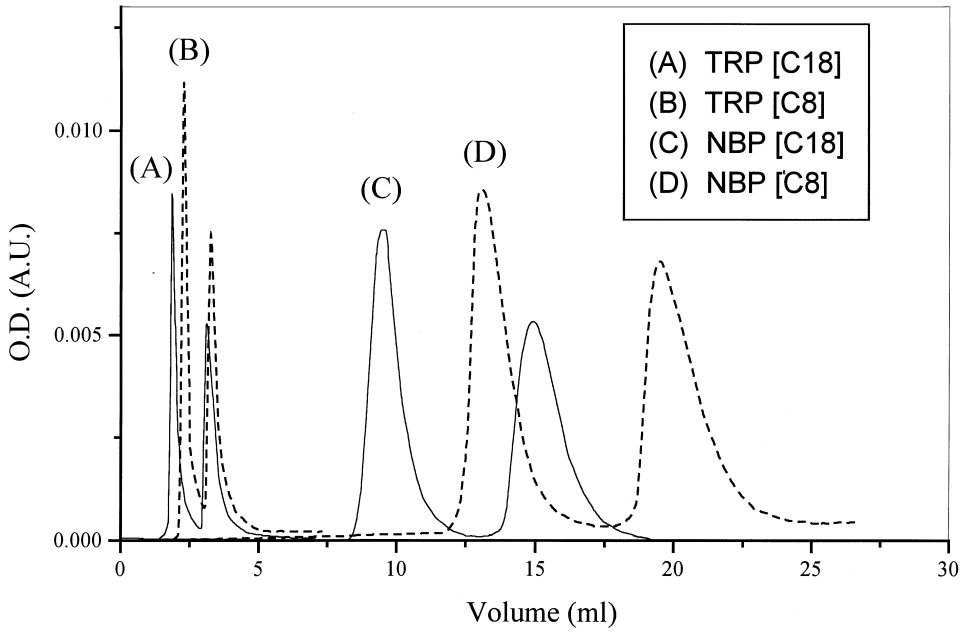

Fig. 2. Elution of DL-tryptophan (A and B) and DL-NBP (C and D) on [HSA-C8] (dotted line) and [HSA-C18] (full line). Elution conditions as in Table 2.

resulting supports were called ([HSA-C_{18}-NaCl]. The total capacities (Q_{max}) of these columns were almost unchanged (Table 2). However, their enantioselective properties were significantly decreased. As shown in Table 2, the enantiomers of DL-tryptophan and *RS*-warfarin were no more resolved and the separation of DL-NBP was performed with a lower selectivity. At this pH, tryptophan is zwitterionic while NBP and warfarin are negatively charged. It appears in Fig. 3 that DL-tryptophan was eluted with higher retention factors on [HSA-C_{18}–NaCl] than [HSA-C18]. On the contrary, the negatively charged enantiomers of DL-NBP and *RS*-warfarin were eluted faster. This suggests that HSA adsorbed in the presence of NaCl exhibits negatively charged side-chains accessible to the mobile phase.

Therefore, another analysis of the racemic mixtures was carried out on the same [HSA-C_{18}–NaCl] columns but with NaCl (0.2 *M*) in the elution buffer. As illustrated in Fig. 3, DL-tryptophan was eluted faster without NaCl while the other compounds (DL-NBP and *RS*-warfarin) were more retained. All the changes observed with NaCl suggest the involvement of salt competitive effects during the interactions of the enantiomers with their specific chiral centers in the protein, since these sites as well as the enantiomers are electrically charged.

5.2. Retention data on PVI anion-exchangers

5.2.1. HSA immobilization

As shown in a previous study, HSA negatively charged at pH=7.4, may be immobilized on the PVI anion-exchangers by ionic interactions [12]. The amount of HSA bound to the supports could be adjusted by varying the salt concentration during the protein immobilization. In the presence of 0.5 *M* NaCl, the amounts of albumin on the stationary phase ([HSA-PVI]) were negligible, while the total column capacities up to 130 mg could be obtained by decreasing the salt concentration (0.02 *M*).

5.2.2. Elution of racemic mixtures

Chiral stationary phases obtained by this method were much more selective than [HSA-C8] and [HSA-C18] supports since efficient chiral separations were carried out with [HSA-PVI] columns containing only 9 mg of HSA. As an illustration, chromatograms obtained for DL-tryptophan on [HSA-PVI] columns with different HSA loadings are shown in

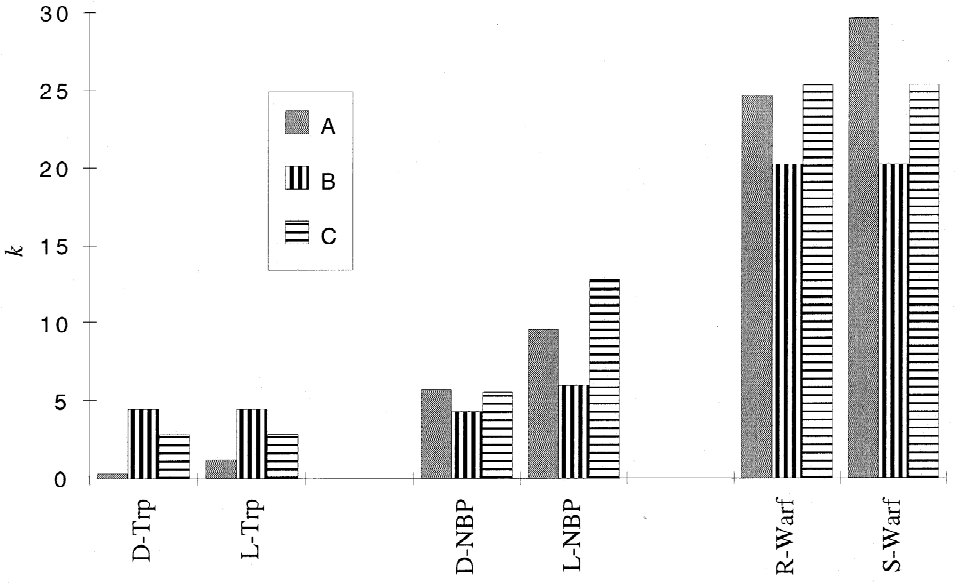

Fig. 3. Retention factors of D and L tryptophan (Trp), D and L NBP, *R* and *S* warfarin (Warf) on [HSA-C18] (A) and [HSA-C_{18}–NaCl] (B and C) columns. Eluents: A and B: 67 m*M* phosphate buffer, pH=7.4; C: 67 m*M* phosphate buffer, pH=7.4 with 0.2 *M* NaCl. Flow rate: 1 ml min^{-1}. Column length: 10 cm.

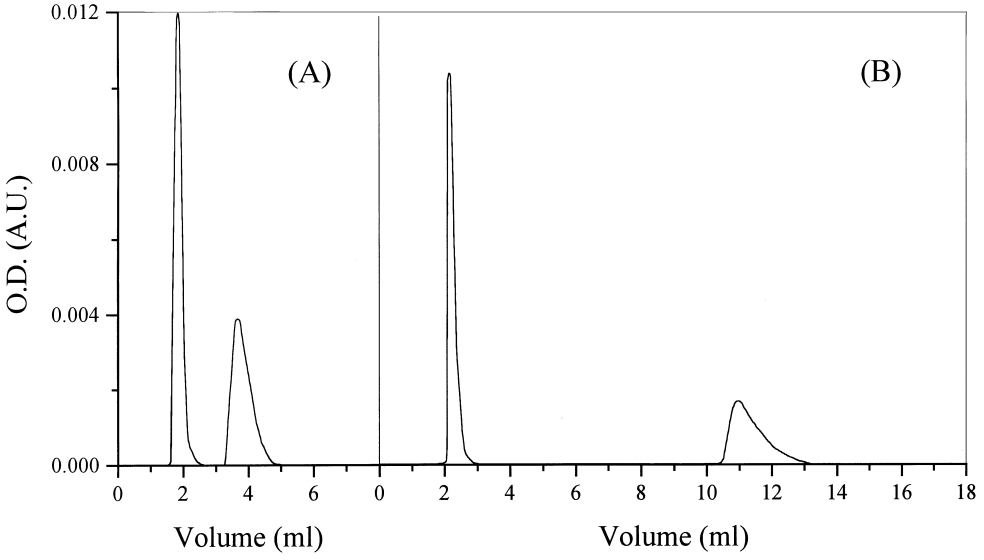

Fig. 4. Elution of DL -tryptophan on [HSA-PVI] with HSA loading of 9 mg (A) and 22 mg (B). Elution conditions as in Table 2.

Fig. 4. Better enantioselective properties are obtained on [HSA-PVI] (Fig. 4) than on [HSA-C8] and [HSA-C18] (Fig. 2) although the amounts of HSA immobilized on the [PVI] supports (9 and 22 mg) are much lower than loadings on reversed-phase supports (105 and 85 mg).

Selectivity values measured for the four racemic mixtures determined under the same elution conditions with [HSA-C8] and [HSA-PVI] chiral phases, are compared in Table 3. It clearly appears that human albumin was more efficient when bound to anion-exchangers, showing the influence of the stationary phase on the enantioselective properties of the immobilized protein.

In the case of PVI-anion-exchangers, all studied enantiomers were eluted with low retention factors on the naked supports showing that interactions between solutes and the PVI-coated silica itself were negligible. The retention of the enantiomers increased significantly with the protein loading. Some of the experimental retention data obtained with DL-tryptophan and DL-NBP on [HSA-PVI] columns with 9, 22 and 37 mg of HSA are shown in Fig. 5A, B. For higher HSA loadings, only one enantiomer of both aminoacids was eluted. Likewise, the capacity factors of *RS*-warfarin and *RS*-oxazepam increased with the amounts of HSA on the chiral phase (results not shown).

Table 3
Comparison between selectivity values obtained on [HSA-C8] and [HSA-PVI] columns. Same elution conditions as in Table 2

Racemic mixture	[HSA-C8][a]	[HSA-PVI]
DL-Tryptophan	2.2	12.7[b]
DL-NBP	1.5	2.0[b]
RS-Oxazepam	No elution	21[c]
RS-Warfarin	1.1	1.3[b]

[a] Amounts of HSA in the column: [a]105 mg, [b]37 mg, [c]9 mg.

For a better understanding of the chromatographic results, a structural analysis of HSA bound to [C8] reversed-phase supports and [PVI] anion-exchangers was undertaken.

5.3. Structural analysis of HSA

Structure and solvation parameters measured for HSA in buffered solution are used as reference values (Tables 1 and 4) for a quantification of the structural changes drawn by adsorption, either on [C8] or on [PVI] support.

5.3.1. Reference data in solution

The structural results are given in Table 1 for HSA at 10 g l^{-1}, at 24 h. Intensities for each Amide I′ component and for the Amide II band very slightly

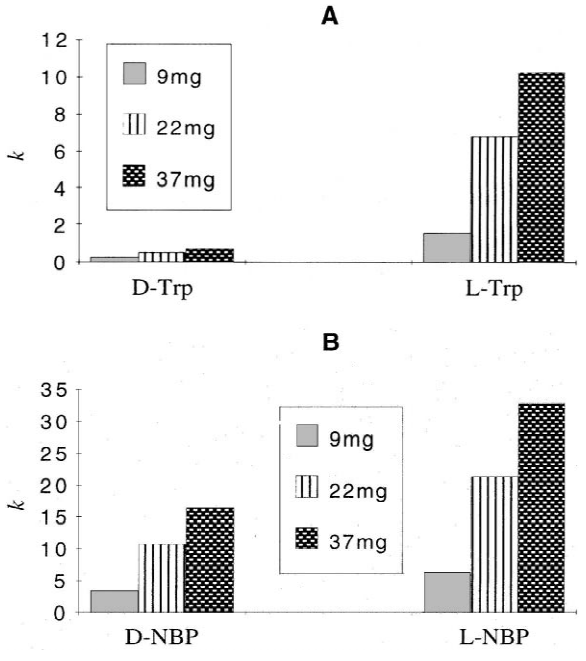

Fig. 5. Retention factors of D- and L-tryptophan (A), D and L NBP (B) on [HSA-PVI] supports as a function of the amount of HSA immobilized in the columns. Elution conditions as in Table 2.

depended on protein concentration from 5 to 30 g l^{-1}, with less than 3% variation for the overall peptide backbone structure and 4% variation for the residual peptide NH (results not shown). At 24 h, the residual amount of peptide NH accounts for about 14% of the overall backbone. NaCl addition does not affect much HSA structure and solvation. However, with 1*M* NaCl, the exchange was reduced by 5%, meaning that about 25–30 internal peptide units become protected from ^2H_2O contacts. In solvating charged side chains, the salt might reduce repulsive electrostatic forces, that constrain the secondary structures.

5.3.2. Structural changes for HSA adsorbed on [C8] reversed-phase

Adsorption on reversed-phase supports of HSA molecules has been shown to entail a significant conformational transition for this protein [13,14]. This is illustrated for HSA adsorbed on the [C8] support (Fig. 6A). Intensity differences measured for each Amide I′ component for an equilibration time of 24 h show that adsorption draws unfolding in both irregular and regular helical domains concerning about 10% of the backbone, meaning 50–60 peptide units. Unfolding in hydrophilic helical domains induces direct peptide hydration, while unfolding in hydrophobic helical regions rather favors HSA self-association on the solid surface. As expected, the so-called self-associated domains of the protein include H-bonded (1618 cm^{-1}) and non H-bonded (1670 cm^{-1}) peptide carbonyls. In agreement with the structural transition the NH/N^2H exchange increased by 6% (30–35 peptide units) compared to that for the solution at the same time (Table 4). Adsorption entails water diffusion inside the protein core. The drift of external/internal hydrophobic amino-acid side chains of HSA towards the [C8] surface induces structural changes that are very similar to those found for HSA adsorbed on an RPC6 phase with a lower porosity (80 Å) [13].

5.3.3. Structural changes for HSA adsorbed on [PVI] supports

The adsorption of negatively charged HSA molecules on the electropositive [PVI] phase in presence of NaCl at 0.04 *M*, does not affect the structure of

Table 4
Amount of residual peptide NH evaluated in (%) of the overall peptide backbone from 6 h after HSA dissolution in the ^2H_2O buffer to 7 days, for HSA in solution and for adsorbed HSA

% NH in HSA peptide backbone				
Time	Solution without salt	Adsorption on [C8]	Solution with salt	Adsorption on [PVI]
6 h	16	10	18	14
24 h	14	7	17	7
3 days	–[a]	7	14	7
7 days	–[a]	7	–[a]	7

[a]: results not available.

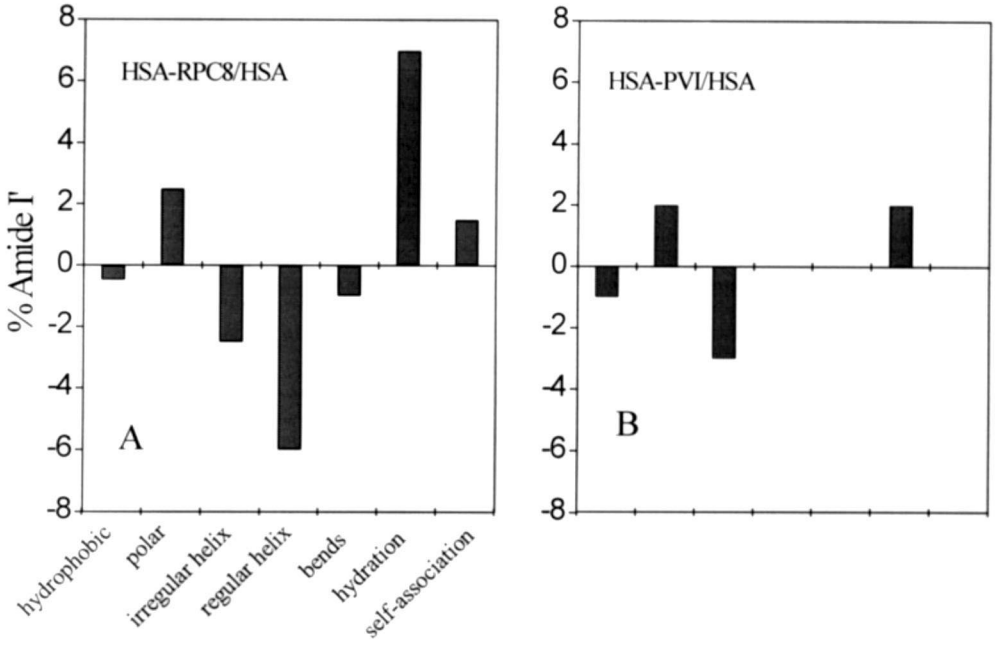

Fig. 6. Differences of the areas of the Amide I′ components (%) between adsorbed HSA and HSA 10 g l^{-1} in ^2H_2O phosphate buffer, p^{2}H 7.4. Amide I′ areas for [HSA-C8] minus Amide I′ areas for HSA solution at 24 h (A); Amide I′ areas for [HSA-PVI] minus Amide I′ areas for HSA solution with 0.04 *M* NaCl at 24 h (B).

the protein much (Fig. 6B). The same conclusion rose already from the adsorption of HSA on [PVI] phases without any additional salt [15]. Adsorption of HSA on [PVI] unfolds an irregular helix for only 3% of the backbone, corresponding to 15–20 peptide units probably located in a relatively internal hydrophobic domain. The unfolded helix turns into hydrated or polar domains. The decrease of residual NH amounts concerned 10% of the backbone or about 50–60 peptide units (Table 4). The solvation in internal domains is in that case linked to a weak structural change of the protein.

5.3.4. Structural evolution of HSA adsorbed on chromatographic supports

The quantification of the results obtained as a function of time from 6 h to several days, informs us on the structural stability of the protein adsorbed on HPLC phases (Fig. 7).

HSA is fast affected by the hydrophobic [C8] surface then the system is stable in time (Fig. 7A). The level of the NH/N^{2}H exchange was remarkably constant from 6 h to twenty days (Table 4).

In solution, with salt (0.04*M* NaCl), a slow solvation process slightly increases self-association during the first day at the expense of an internal helical domain (Fig. 7B). Then, until 3 days, another internal helical unfolding turns into more regular helix and bent structures with unbonded peptide carbonyls. A significant enhancement of the NH/N^{2}H exchange corroborated this structural transition (Table 4).

HSA undergoes weak structural changes when adsorbed on [PVI]. The evolution in time of the adsorbed system (Fig. 7C) is similar to the evolution of the protein in solution (Fig. 7B), except for the self-association. HSA self-association on [PVI] is reduced from 6 h to 24 h. At that time washing the column with the buffer at 1 *M* NaCl did not affect the structure of adsorbed HSA. After a while, the system remains very stable until 10 days or even more (45 days; data not shown).

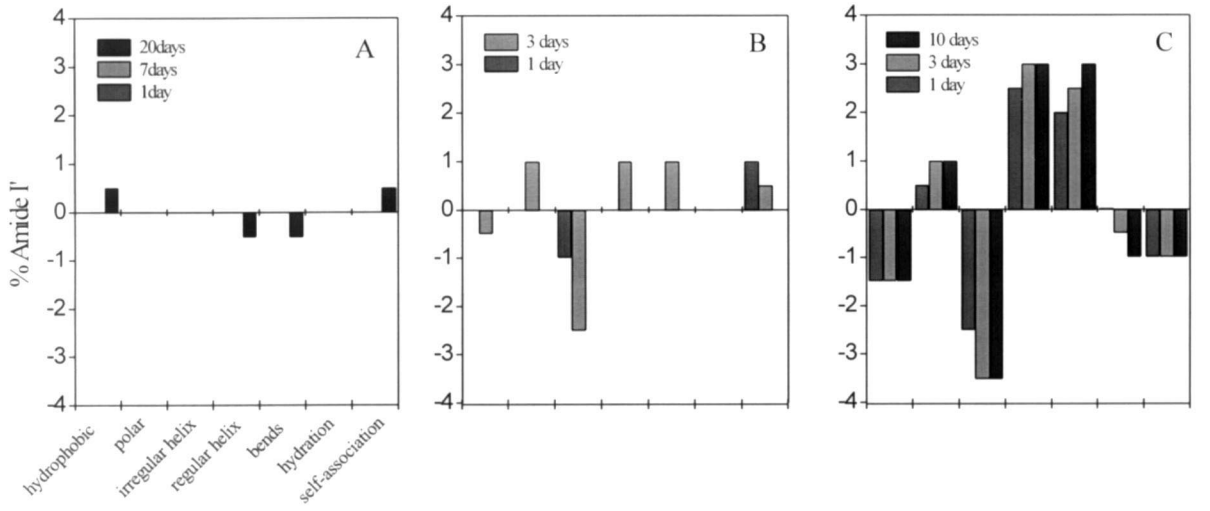

Fig. 7. Time dependence of the Amide I′ intensities for [HSA-C8], solution and [HSA-PVI]. Differences between Amide I′ areas for [HSA-C8] at 1, 7 and 20 days, minus Amide I′ areas for [HSA-C8] at 6 h (A); differences between Amide I′ areas for HSA in solution at 1 and 3 days, minus Amide I′ areas for HSA in solution at 6 h (B); differences between Amide I′ areas for [HSA-PVI] at 1, 3 and 10 days and Amide I′ areas for [HSA-PVI] at 6 h (C).

6. Discussion

Several chiral stationary phases using proteins as a chiral selector have been described previously [5–9,23,24]. However, some problems may arise from the immobilization of biomolecules, leading to supports with a poor enantioselectivity. Especially, the binding of proteins to the matrix may induce conformational changes that inhibit chiral discrimination. Moreover, the accessibility of the chiral center to the enantiomers as well as its three-dimensional structure may be modified if contact between the protein and the support is located in the neighborhood of this site. In both cases, interactions between enantiomers and chiral centers could be reduced. Hence, chiral stationary phases with poor enantioselectivity should be also characterized by low retention factors, unless non-specific interactions are involved.

In the case of [PVI]-anion-exchangers, interactions between the solutes and the PVI-coated silica were negligible. After the immobilization of HSA high retention factors that increased with the protein loading were observed for the racemic mixtures (Fig. 5). The separation of the four enantiomers was generally performed with a high selectivity (Table 3). It must be noted that the binding of warfarin was slightly less stereoselective ($\alpha=1.3$) than for the other compounds, but such a result has been reported previously [20]. Consequently, both chiral centers of HSA, the indole-benzodiazepine for oxazepam, tryptophan [20–22] and NBP [data not published] and the azapropazone site for warfarin, are accessible. HSA immobilization on [PVI]-anion-exchangers probably results in an incomplete protein monolayer in which most of the adsorbed molecules remain able to interact selectively with the enantiomers. Since the elution times increase with the amount of HSA, the sites are not hindered by further HSA adsorption, meaning that no protein stacking occurs. This assumption is in complete agreement with the results deduced from the FTIR study. Indeed for such column the FTIR results indicate that at the equilibrium, the adsorbed HSA molecules are only changed by 20–25 peptide units but no more self-associated. The weak local unfolding should not disturb the sites that specifically interact with the studied enantiomers.

As shown in previous reports, adsorption of proteins on reversed-phase supports [13,14] as well as separation processes on such stationary phases [25] can induce protein denaturation. However, with [HSA-C8] and [HSA-C18] phases, a chiral recognition was observed for the amino-acids enantiomers DL-tryptophan and DL-NBP (Table 2 and Fig. 2). The binding site could be in contact with the hydrophobic surface since it has been suggested it was located in a hydrophobic pocket [19,20]. However, the remaining chirality is not consistent with this hypothesis. Stereoselective interactions between these amino-acids and immobilized HSA dominate the hydrophobic effects that retained them on the naked reversed-phases. The adsorption of HSA on reversed supports may have released charged side chains towards the eluent leading to less hydrophobic supports. From FTIR investigations, the increase of the solvation for adsorbed HSA compared to HSA in solution suggests that the polar interface between immobilized HSA and the eluent is increased. Furthermore, if it is clear that a large internal hydrophobic unfolded domain comes into contact with the solid-phase, apparently it does not directly involve the indole-benzodiazepine site. Thus chiral discrimination at this binding site concomitant with non specific electrostatic interactions would be involved in the retention mechanism of these enantiomers. Ionic effects are confirmed by the HPLC study, especially when salt was added to the buffer during the adsorption process.

Although *RS*-oxazepam is known to interact at the same indole-benzodiazepine site [21,22] it was not eluted from [HSA-C8] and [HSA-C18] columns. For this neutral compound the hydrophobic interactions with naked domains of the matrix would dominate the possible interactions with the sub monolayer adsorbed HSA. The *R*- and *S*-warfarin were highly retained (Fig. 3) but badly resolved demonstrating that non-specific interactions were also involved. Since warfarin is more hydrophobic than amino-acids, the higher retention for warfarin could result from an adsorption on non-coated alkyl chains. Moreover, the structural changes detected by FTIR could have partially denaturated the azapropazone site.

On the other hand the enantioselectivity is lower with [HSA-C8] and [HSA-C18] than with [HSA-PVI]. Especially, with tenfold less immobilized HSA [HSA-PVI] columns have the same performance as the reversed-phase supports. This result is consistent with HSA structural changes evaluated by FTIR-spectroscopy, which indicate that adsorption on reversed-phases unfolded about 50–60 peptide units. They were involved in solution in regular and irregular helical domains. The structural change is much weaker for HSA adsorbed on PVI. At structural equilibrium, unfolding only concerns 15–20 peptide units. A hydrophobic helix becomes hydrated.

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