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Enantiomeric properties of human albumin immobilized on porous silica supports coated with polymethacryloyl chloride

M.C. Millot*, B. Sébille, C. Mangin

Laboratoire de Physico-Chimie des Biopolymères, UMR 27, CNRS, Université Paris XII, 2–8 Rue Henri Dunant, 94 320 Thiais, France

Abstract

Human serum albumin (HSA) was bound to porous silica, using a reactive polymer derived from polymethacryloyl chloride. Two different procedures were used for coating silica with the polymer. In the first method, the polymer was deposited onto amino-silica by reaction between its reactive functions and NH_2 groups on silica. In the second method, the monomer was first linked to the amino-silica and copolymerization with the excess of monomer initiated thereafter. The enantiomeric properties of the resulting supports after the coupling of HSA were compared using different mobile phases. The higher amount of HSA bound using the later method, resulted in higher retention of the enantiomers and better enantioselectivity. © 1997 Elsevier Science B.V.

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1. Introduction

The separation and analysis of chiral compounds is an area of increasing interest and high-performance liquid chromatography columns containing immobilized protein supports have a great potential for optical resolution of such compounds. Chiral stationary phases based upon immobilized human serum albumin (HSA) have been used to resolve a wide number of chiral solutes [1–4]. This protein is a plasmatic protein (M_r 66 500) involved in the binding of various small organic and inorganic compounds [5,6]. Different procedures have been investigated for the immobilization of HSA to porous supports. The protein has been bound covalently onto diol-bonded silica particles by the Schiff base method [7] and by another technique involving an activation step with 1,1-carbonyldiimidazole [8]. More recently, HSA was ionically immobilized on silica particles coated with a polymeric anion exchanger [9]. In this latter method, the masking of silanol groups was effective. However, only mild elution conditions could be used for the separation of enantiomers, since the protein was bound to the support in a non covalent manner.

In this work, a reactive polymer derived from polymethacryloyl chloride (PMAC) was used as an intermediate for the covalent binding of HSA to porous silica supports. The use of a polymer layer permits a stable binding of HSA to silica particles and decreases possible interactions between samples and silanol groups. Two different procedures were investigated for coating silica with the polymer. In the first method, the polymer layer was deposited onto silica by reaction of porous amino-silica with the reactive polymer itself. However, grafting meth-

*Corresponding author.

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ods [10,11] in which the whole polymeric layer would be accessible to the protein should improve the contact between HSA and the immobilized polymer. Thereby, another method was investigated in which the monomer (methacryloyl chloride) was bound covalently to the porous amino-silica and copolymerization with the excess of monomer initiated thereafter. The enantioselective properties of the resulting materials after the binding of HSA were investigated, using DL-tryptophan, N-benzoyl-DL-phenylalanine, (*RS*)-warfarin and (*RS*)-oxazepam as model compounds.

2. Experimental

2.1. Reagents

Nucleosil-NH_2 silica (d_p=7 μm; porosity 300 Å) was purchased from Macherey–Nagel (Düren, Germany). The LiChrospher Diol column (250×4.6 mm) was from Merck (Darmstadt, Germany). Methacryloyl chloride (MAC), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), DL-tryptophan, (*RS*)-warfarin were obtained from Aldrich (St. Quentin Fallavier, France). Human albumin (fraction V, fatty acid free) and N-benzoyl-DL-phenylalanine (NBP) were from Sigma (St. Quentin Fallavier, France). α,α′-Azoisobutyronitrile (AIBN) and ethanolamine were purchased from Fluka (St. Quentin Fallavier, France). All solvents were from Prolabo (France) and mineral salts from Merck.

2.2. Polymerization of methacryloyl chloride

Polymethacryloyl chloride was synthesized by radical polymerization of MAC (0.1 mol) in dioxane (10 ml) in presence of α,α′-azoisobutyronitrile (10^{-3} mol) [12,13]. Polymerization was carried out at 55°C, for 40 h, in a nitrogen atmosphere. The polymer was purified by precipitation in hexane. Its weight-average mass determined by size-exclusion chromatography, was near 75 000 g mol^{-1}.

2.3. Coating of nucleosil-NH_2 with PMAC (method 1)

The coating of the amino-silica (dried overnight at 50°C) was performed for 5 h under nitrogen, using a solution of PMAC (5%, w/w) in dry dioxane. The amount of polymer bound to silica, from elemental analysis data, was approximately 100 mg g^{-1}.

The IR spectra (spectra not shown) of resulting materials [PMAC-Sil 1] showed three bands with a poor resolution in the carbonyl region, assigned respectively to the acyl chloride carbonyl ($\nu_{C=O}$= 1788 cm^{-1}) and to the anhydride doublet ($\nu_{C=O}$= 1765 and 1805 cm^{-1}).

2.4. Grafting of PMAC to nucleosil-NH_2 (method 2)

Amino-silica (1 g) dried overnight at 50°C was immersed in a dry solution of MAC (10 ml) in dioxane (10 ml). After 1 h at room temperature, in a nitrogen atmosphere, AIBN (1.2 mmol) was added to the suspension and the mixture heated at 55°C for 18 h. After polymerization, unbound polymer was separated from the modified silica [PMAC-Sil 2] by repeated washings with dioxane. The amount of polymer bound to the silica surface was near 120 mg g^{-1}. IR spectra revealed, as in the first method, the presence of acyl groups and of anhydride functions.

2.5. Activation with NHS of the polymer coated silica

Acyl chloride and anhydride functions of polymer coated silicas ([PMAC-Sil 1] and [PMAC-Sil 2]) were first transformed into carboxylic groups by hydrolysis at 50°C in phosphate buffer (100 mmol l^{-1}, pH 8.2) for 24 h. Thereafter, carboxylic functions were activated into N-hydroxysuccinimide ester groups with NHS (1.4 mmol g^{-1}) and EDC (1.4 mmol g^{-1}) in 2-propanol following the procedure described by Jarrett [14]. These N-hydroxysuccinimide ester functions are known to react rapidly with amino groups of proteins in aqueous media [15]. Stationary phases obtained after activation with NHS, were called respectively [NHS-Sil 1] and [NHS-Sil 2].

2.6. Binding of HSA to [NHS-Sil] columns

Columns (150×4.6 mm) were slurry packed with [NHS-Sil] (1.6 g), washed with 2-propanol, then with water. Thereafter, a HSA solution (2 g l^{-1}) in

phosphate buffer (50 mmol l^{-1}) with NaCl (0.5 mol l^{-1}, pH 7.4) was circulated over the activated support. Simultaneously, fractions collected at column outlet were analyzed on a LiChrospher Diol column. Percolation of HSA was stopped when HSA concentrations at the inlet and outlet of the [NHS-Sil] column were the same. The amounts of HSA bound to the supports were determined from the mass balance.

After binding of HSA to the reactive stationary phases, a solution containing ethanolamine (0.2 mol l^{-1}, pH 7.9) was passed through the column in order to block residual reactive functions on the polymer.

2.7. Equipment

The liquid chromatography system comprised a Kontron (Model 422) solvent pump, a Rheodyne (Model 7125) injection valve equipped with a 20-μl loop, a 150×4.6 mm column, a Shimadzu (Model SPD-6A) variable-wavelength UV detector and a Kipp-Zonen peak integrator.

2.8. Chromatographic conditions

All chromatographic experiments were performed under isocratic conditions at a flow-rate of 1 ml min^{-1}, using phosphate buffer (10 to 400 mmol l^{-1}) at pH values from 6.0 to 8.0. In some cases, 1-propanol was added to the mobile phase, especially for the elution of oxazepam and warfarin. The amount of solute injected on [HSA-Sil] columns was 0.4 μg for oxazepam and warfarin, 0.1 μg for tryptophan (detection at 280 nm), 0.1 μg for NBP (detection at 250 nm). The column void volume was determined using $NaNO_3$.

3. Results and discussion

3.1. Binding of HSA to the reactive stationary phases

The reaction of proteins with NHS-ester functions was usually carried out in basic conditions [15], amino groups of proteins being implicated in the reaction. In this study, pH values lower than 8.5 had to be used, since reaction took place at the surface of polymer coated silica particles. Moreover, in basic buffers an hydrolysis of NHS-ester functions resulting in a spontaneous release of NHS and loss of reactivity was observed and became predominant at pH values higher than 7.5.

Therefore, a pH of 7.4 was chosen for the binding of HSA to the reactive supports. Sodium chloride (0.5 mol l^{-1}) was added to the protein solution in order to prevent ionic repulsion between the negatively charged HSA and ionised carboxylic groups on the stationary phase.

After saturation of the columns, the amounts of HSA bound to the coated silica [NHS-Sil 1] and to the grafted silica [NHS-Sil 2] were respectively equal to 27 and 42 mg. These values are low compared to other covalent HSA-stationary phases [1] and could be attributed to the competitive hydrolysis reaction observed during the coupling of HSA to the immobilized polymer.

Nevertheless, the higher amount of HSA bound to [NHS-Sil 2] column (compared to [NHS-Sil 1]) could indicate that the accessibility of reactive functions to the protein was better in the case of grafted silicas ([NHS-Sil 2]) and could result from an improved mobility of the polymer chains promoting a tentacle effect.

After the coupling of human albumin, unreacted N-hydroxysuccinimide ester groups were blocked with ethanolamine (0.2 mol l^{-1}; pH 7.9) in order to avoid any covalent binding of racemic mixtures to the stationary phase. Fractions collected at column outlet did not contain any HSA, showing that human albumin was strongly bound to the support.

3.2. Separation of enantiomers on conventional and grafted supports

Four racemic mixtures were analyzed on chiral stationary phases described above: (*RS*)-oxazepam, (*RS*)-warfarin, DL-tryptophan and N-benzoyl-DL-phenylalanine.

It must be outlined that racemic pairs were not resolved on the naked supports (without HSA) after deactivation of the reactive functions. All compounds were eluted near the void volume, except oxazepam ($k'=7$) showing that interactions between this solute and the support were taking place. A similar phenomenon was observed on silica supports coated with copolymers of N-vinylimidazole and

N-vinylpyrrolidone which decreased as soon as small amounts of protein were bound to the support [9].

The enantiomeric properties of the HSA-stationary phases ([HSA-Sil 1] and [HSA-Sil 2]) were first compared using phosphate buffer (50 mmol l^{-1}, pH 7.4). Oxazepam and warfarin being not eluted from the columns in these conditions, 2% of 1-propanol were added to the mobile phase for their elution.

As shown in Table 1, enantiomeric pairs were resolved on both supports (k'_2 and k'_1 refer respectively to the more and the less retained enantiomers). This result demonstrates that the immobilization of human albumin onto silica via reactive polymers did not alter its enantioselective properties, showing that drug binding sites on the protein were still accessible after its coupling.

On the other hand, it appears that retention factor k' increased when chiral separations were carried out using the grafted support. Small effects (36%) were observed in the case of D-tryptophan, resulting from its well known low affinity for human albumin [16]. More substantial effects (from 80 to 160%) were noted with other enantiomers. The increased retention on the grafted support may be attributed to the higher amount of HSA on this stationary phase as reported previously [9,17].

Moreover, selectivity values (α) were different on both stationary phases. These variations depended on the investigated racemic pair (Table 1) and were conditioned by the relative increase in retention of each enantiomer ($\alpha = k'_2/k'_1$). For instance, the separations of DL-tryptophan, warfarin and oxazepam were characterized by higher enantioselectivity values on grafted supports, while the separation of NBP was similar on both stationary phases. This later result could be predicted, since increases in retention measured for the D and L enantiomers were respectively equal to 98 and 91%.

The comparison of resolution values on both columns was more disputable since resolution depended on the homogeneity of the packing inside the column. However, according to results reported in Table 1, it appears that variations in resolution and in selectivity were very similar.

As shown on Fig. 1, some tailing effects were observed on chromatograms obtained with both supports. Peak asymmetry defined by b/a, b and a being peak half-width at 10% of peak height, depended on the sample, on the amount of solute injected onto the column and on elution conditions. b/a values between 3.5 and 4.3 were measured with the [HSA-Sil 1] column, while b/a values were between 1.8 and 3.2 with the [HSA-Sil 2] column (phosphate buffer 50 mmol l^{-1}, pH 7.4; amounts of solute as in Section 2.8). This slight difference could result from a better diffusion of solutes within [HSA-Sil 2] supports.

3.3. Retention behaviour of enantiomers as a function of mobile phase composition

3.3.1. Effects of ionic strength

The effect of phosphate concentration on the retention of enantiomers was investigated with conventional supports (Fig. 2). It appears that the retention behaviour of NBP and warfarin was quite similar, since k' values of the less retained enantiomers increased continuously with buffer concentration, while a minimum in retention factor was reached for the more retained enantiomers. This latter result suggested that two antagonist effects were involved in the mechanism of retention of the

Table 1
Retention factors (k'), selectivity (α) and resolution (R_s) of racemic mixtures chromatographed on conventional [HSA-Sil 1] and grafted [HSA-Sil 2] supports

Compound	HSA-Sil 1				HSA-Sil 2			
	k'_1	k'_2	α	R_s	k'_1	k'_2	α	R_s
Tryptophan	0.63	2.00	3.2	2.8	0.86	4.9	5.7	4.2
NBP	2.77	5.5	2.0	2.4	5.5	10.5	1.9	2.2
Warfarin[a]	4.2	10.0	2.4	1.8	9.1	26.3	2.9	2.3
Oxazepam[a]	4.4	6.8	1.54	1.9	7.9	15.6	2.0	3.2

Eluent: phosphate buffer (50 mmol l^{-1}; pH 7.4), [a] with 2% of 1-propanol for warfarin and oxazepam. Flow-rate: 1 ml min^{-1}.

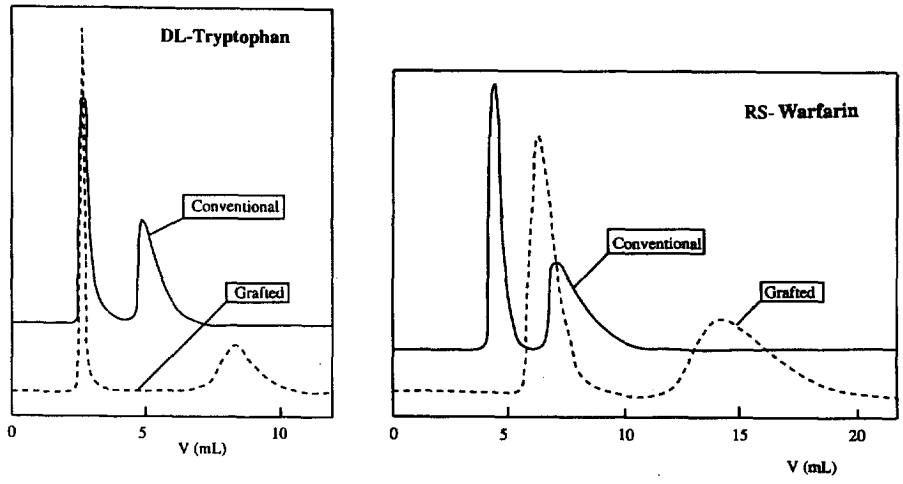

Fig. 1. The results of the chromatography of DL-tryptophan and (*RS*)-warfarin on conventional (———) and grafted supports (– – –). Eluent: 50 mmol phosphate buffer pH 7.4, with 4% of 1-propanol for warfarin. Flow-rate: 1 ml min^{-1}.

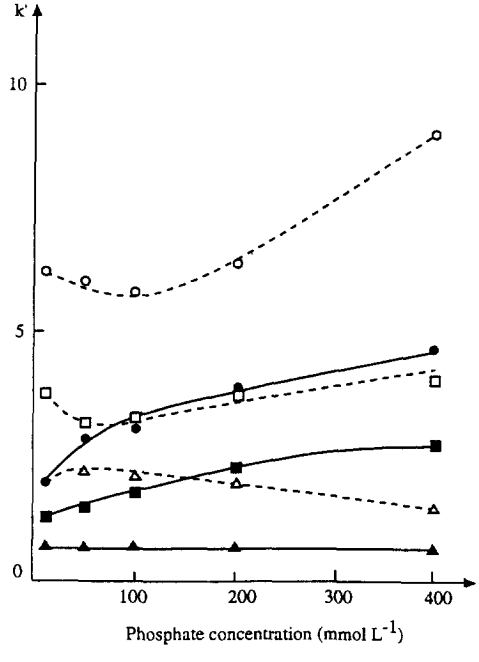

Fig. 2. Effect of buffer concentration on the retention factors (k') of tryptophan (▲ and △), N-benzoylphenylalanine (● and ○) and warfarin (■ and □). Less retained enantiomers (———); more retained enantiomers (– – –). Column: [HSA-Sil 1], 150×4.6 mm. Eluent: phosphate buffer pH 7.4, with 4% of 1-propanol for warfarin. Flow-rate: 1 ml min^{-1}.

more retained enantiomers: first, coulombic interactions at low ionic strength resulting in more pronounced tailing effects on the elution peak and next, hydrophobic interactions which became predominant at phosphate concentrations higher than 100 mmol l^{-1}. Similar results were observed by Allenmark et al. [19] on bovine serum albumin chiral stationary phases.

In contrast, a different behaviour was observed for tryptophan (Fig. 2). The effect of phosphate concentration on the retention of the D-enantiomer was negligible, while a maximum in k' values of the L-enantiomer was observed. Moreover, by addition of sodium chloride (0.5 mol l^{-1}) to the mobile phase (50 mmol l^{-1} phosphate buffer), the retention of the L-enantiomer decreased ($k'=1$) resulting in a poor resolution of the racemic mixture ($R_s<0.5$). These results could indicate that significant ionic effects were involved in the retention mechanism of this compound.

Minor variations in the retention of oxazepam with ionic strength were noted (data not shown), resulting in an almost constant enantioselectivity. On the contrary, separations of tryptophan, NBP and warfarin were characterized by decreased enantioselectivity values with increasing buffer concentrations. Thereby, a phosphate concentration equal to 50 mmol l^{-1} was used in this study.

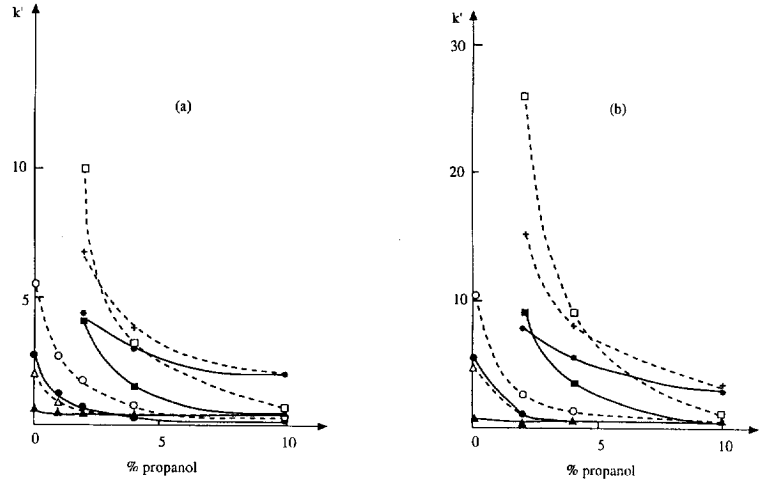

Fig. 3. Effect of 1-propanol added to the mobile phase on the retention factors of tryptophan (▲ and △), N-benzoylphenylalanine (● and ○), warfarin (■ and □) and oxazepam (* and +). Columns: [HSA-Sil 1] (a); [HSA-Sil 2] (b). Eluent: phosphate buffer (50 mmol l^{-1}), pH 7.4. Flow-rate: 1 ml min^{-1}.

3.3.2. *Effects of an organic modifier*

As shown in Fig. 3, the retention of enantiomers on conventional (Fig. 3a) and grafted (Fig. 3b) stationary phases decreased upon addition of 1-propanol to the mobile phase. This phenomenon was in agreement with previous findings [1,19] and indicated the importance of hydrophobic interactions in chiral chromatography separations, especially for warfarin and oxazepam which could not be eluted from the columns without addition of an organic modifier. Generally more pronounced effects were observed for more retained enantiomers resulting in a decrease of selectivity (Fig. 4).

However, for NBP, selectivity of both supports was found to increase slightly on addition of 1-propanol up to 3%. This result compared to the effects of ionic strength, could further support the role played by ionic interactions in the retention

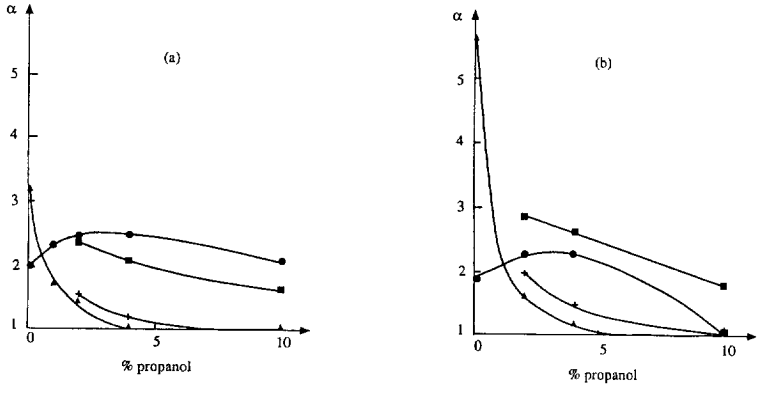

Fig. 4. Effect of 1-propanol added to the mobile phase on the enantioselectivity of HSA-stationary phases for tryptophan (▲), N-benzoylphenylalanine (●), warfarin (■) and oxazepam (+). Columns: [HSA-Sil 1] (a); [HSA-Sil 2] (b). Eluent: phosphate buffer (50 mmol l^{-1}), pH 7.4. Flow-rate: 1 ml min^{-1}.

mechanism of the L-enantiomer of NBP. At higher propanol contents, α values decreased showing that hydrophobic interactions were involved too.

3.3.3. Effects of mobile phase pH

As shown in Fig. 5, the effects of pH on the retention of racemic mixtures were similar on conventional (Fig. 5a) and grafted (Fig. 5b) stationary phases. It appears that k' values decreased with increasing pH, except for tryptophan. Similar results were observed by previous workers for tryptophan [18] and N-substituted-amino acids [19] and were attributed to an increase in the negative net charge of the protein with pH (isoelectric point, pI of albumin=4.6). However, in the case of NBP, the effects of ionic strength, propanol content and mobile phase pH could also suggest that a positively charged residue was involved in the binding site for this compound (pK_a=2.58 for phenylalanine). The retention behaviour of warfarin (pK_a=6) could be explained in a similar way, although other workers using HSA bound to diol-silica found that the retention of warfarin increased with pH [4].

The effects of pH on the retention of racemic mixtures did not result in any significant changes in selectivity, except for tryptophan. For this compound, a better separation was observed at pH=8 (α=3.8 on conventional support; α=6.9 on grafted support), while a total loss of enantioselectivity took place at pH=6.

3.4. Stability of the columns

The binding of HSA to the immobilized polymers was stable. Fractions collected at the column outlet at different times did not contain any protein. Moreover, elution volumes remained unchanged when phosphate buffer (50 mmol l^{-1}) was used as a mobile phase. However, a slight decrease in retention (10 to 15%) was observed in some cases when high salt concentrations or high propanol contents were used. This could result from changes in the conformation of the immobilized protein under these conditions.

4. Conclusion

Reactive polymers are convenient intermediates which can be used to bind proteins to porous silica supports. The resulting materials are stable and the enantioselective properties of human albumin are preserved after its reaction with the immobilized polymer. Grafted stationary phases are more attractive than supports obtained by conventional coating methods: the amounts of protein which can be bound to the supports are higher, resulting in most cases in an improved selectivity and resolution.

As shown by previous workers [1,4,18,19], the retention properties and enantioselectivity of HSA-chiral stationary phases described in this paper depend on the elution conditions. It appears clearly

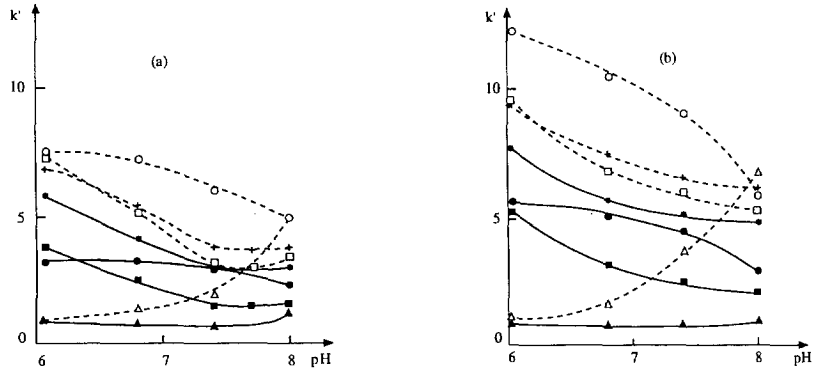

Fig. 5. Effect of mobile phase pH on the retention factors of the enantiomers, represented by the same symbols as in Fig. 3. Columns: [HSA-Sil 1] (a); [HSA-Sil 2] (b). Eluent: phosphate buffer (50 mmol l^{-1}), with 4% of 1-propanol for warfarin and oxazepam. Flow-rate: 1 ml min^{-1}.

that hydrophobic interactions are involved in the mechanism of retention of the investigated enantiomers, especially for warfarin and oxazepam. Moreover, according to the effects of pH and ionic strength, electrostatic interactions are involved too and it seems that the retention of L-enantiomers of tryptophan and N-benzoylphenylalanine is significantly influenced by these interactions.

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