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Chromatographic properties of chemically bonded bovine serum albumin working as a chiral selector in alkaline mobile phases

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ABSTRACT

By chemical bonding of bovine serum albumin to a hydrolytically stable Separon HEMA matrix, a sorbent with an excellent lifetime in mobile phases with pH $\leq$ 11 is obtained. Maximum retentions of amino acids and separation selectivity of amino acids and monocarboxylic and dicarboxylic acids on chemically bonded albumin are attained around pH 9. By prolonged action of slightly alkaline mobile phases of pH < 10 to bonded albumin, the separation selectivity of the compounds with one carboxylic group (amino acids and monocarboxylic acids) decreases whereas that of dicarboxylic acids increases. The initial selectivity of albumin can be restored by methanol. At a given pH the rate of the changes can be increased by 1-propanol or caprylic acid. It was demonstrated that lowering of the efficiency of the column packed with Separon HEMA–BSA with respect to that packed with the matrix and the dependence of the solute retention on the injected amount of the solute are due to the bonded albumin.

INTRODUCTION

Systems with chemically bonded proteins belong to widely studied separation systems in liquid chromatography for the separation of chiral compounds. Compared with specially synthesized chiral stationary phases, protein stationary phases have two advantages: they are perfectly chirally stable and with a suitable selection of the protein they can be considerably cheaper.

Bovine serum albumin (BSA) is produced in large amounts, with sufficient purity and relatively cheaply. Moreover, albumin [1] interacts more specifically with many enantiomers than, *e.g.*, synthetic chiral stationary phases of Pirkle's type [1]. The enantioselective properties of BSA as a chromatographic chiral selector have been studied almost exclusively after bonding of albumin to silica gel. The unsatisfactory hydrolytic stability of currently available silica gels permits the application only of acidic and neutral mobile phases. Therefore, the properties of chemically bonded albumin acting as a chromatographic chiral selector are not known and have not been exploited over the whole range of pH were high selectivity of chemically bonded albumin may be reached [2,3].

High hydrolytic stability typical of porous organic sorbents is often observed with packings derived from them. For the wide-pore hydroxyethylmethacrylate polymer Separon HEMA the manufacturer guarantees perfect hydrolytic stability at pH 2–12 [4]. Investigations of the influence of temperature on the enantioselectivity of the sorbent obtained by bonding BSA to the matrix Separon HEMA [5] revealed perfect stability of the sorbent Separon HEMA–BSA at high temperatures in mobile phases up to pH 7.5. Hence the stability of this sorbent is

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also assumed to be sufficient for alkaline mobile phases.

This study attention mainly concerned the enantioselectivity of the sorbent Separon HEMA–BSA at pH > 7. The sorption capacity of the sorbent and the lifetime and efficiency of columns filled with the sorbent were also investigated. Experiments were performed in order to be able to differentiate the contribution of the matrix from that of bonded BSA.

EXPERIMENTAL

Two sorbents were used: Separon HEMA 1000–BSA (HEMA–BSA) and Separon HEMA 1000-BIO–BSA (HEMA BIO–BSA) (Tessek, Prague, Czechoslovakia). The sorbents were packed in stainless-steel columns (200 × 4 mm I.D.) and in glass CGC columns (150 × 3.3 mm I.D.) using techniques recommended by the manufacturer for unmodified matrices of Separon HEMA 1000 and Separon HEMA 1000 BIO. For comparative measurements with BSA bonded to the silica gel matrix, a commercial column (150 × 4 mm I.D.) containing the chiral stationary phase Resolvosil was used (Macherey–Nagel, Düren, Germany).

Mobile phase was supplied by an HPP 4001 syringe pump (Laboratory Instruments, Prague, Czechoslovakia). The samples were injected with a Hamilton (Reno, NV, USA) Model 1801 microsyringe or by the sampling valve. A PU 4021 diode-array spectrophotometer (Pye Unicam, Cambridge, UK) or an LCD 254 photometer (Laboratory Instruments) was used for detection. Between measurements (overnight) the columns were protected by a flow of mobile phase (0.1 ml/min) or stored in a refrigerator at 5°C. If the work was interrupted for a longer period, the columns were washed with phosphate buffer (pH 7.45) containing 0.1% (w/v) sodium azide.

Methanol (analytical-reagent grade) for regeneration of the enantioselectivity was distilled. Phosphate buffers of pH 2.2–11.5, at a concentration of 50 mmol/l, occasionally with addition of 3% 1-propanol, were prepared from boiled distilled water. The solutes from various sources and the chemicals employed for preparation of the mobile phase were of analytical-reagent grade. The D- or L-enantiomers of the solutes being separated were identified by injecting the pure D- or L-form.

In all measurements the retention time of the peak maximum was considered as the retention time of the solute peak. The retention volume of water was regarded as the column dead volume.

RESULTS AND DISCUSSION

Selectivity

The selectivity of separation on chemically bonded BSA depends substantially on the pH of the environment. The buffer concentration and low-concentration mobile phase constituents (1-propanol, caprylic acid) especially influence retentions [6]. Therefore, the mobile phase pH was used as the main means for tuning the selectivity. Strong increases in both retention and separation selectivity for tryptophan enantiomers in weakly alkaline mobile phases with increasing pH have been reported [2]. At pH ⩽ 7 dissolved albumin very effectively retains solutes with negative charge [7,8]. For these reasons, we chose D,L-tryptophan, two of its analogues and several representatives of monocarboxylic and dicarboxylic acids as solutes.

The pH dependences of the retention and separation selectivity of D,L-tryptophan and its amphoteric analogues, D,L-hydroxytryptophan and D,L-kynurenine, on HEMA–BSA in the pH range 2–11 are summarized in Fig. 1. The marked increase in retention of the more retained form with respect to that of the less retained form in the neighbourhood of pH 9 is in agreement with the increase in the retention of D,L-tryptophan on a sorbent obtained by binding BSA to agarose [2]. This agreement, together with comparison of the retentions of D,L-tryptophan on the sorbent HEMA–BSA and D,L-tryptophan on the matrix HEMA (Fig. 2), indicates that both the course of the pH dependence of the retention and the separation selectivity of D- and L-enantiomers of tryptophan can be unequivocally ascribed to the bonded albumin. Addition of 1-propanol to the mobile phase reduced the retentions of both D- and L-tryptophan and the enantioselectivity of their separations. The pronounced difference in the retentions of D,L-tryptophan and its hydroxy derivative agrees with the earlier observation that even a relatively small change in the solute molecule can dramatically affect the retention of solutes or even their separation selectivity [9].

The highest separation selectivities in the neigh-

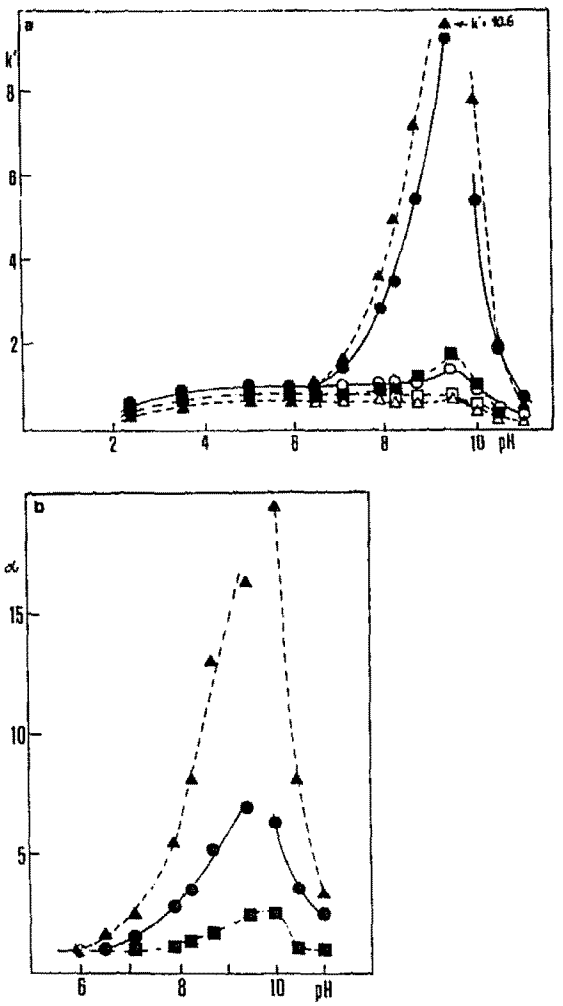

Fig. 1. pH dependence of (a) retention and (b) selectivities of amino acids. (a) ▲ = D-Tryptophan; ● = L-tryptophan; △ = D-kynurenine; ▲ = L-kynurenine; □ = D-hydroxytryptophan; ■ = L-hydroxytryptophan. (b) ● = D,L-Tryptophan; ▲ = D,L-kynurenine; ■ = D,L-hydroxytryptophan. Column, Separon HEMA 1000–BSA (200 × 4 mm I.D.); mobile phase, 0.05 mol/l phosphate buffer; flow-rate, F_m = 1 ml/min; amount injected, 5 μl (0.96 mmol/l).

bourhood of pH 9 were found also for the dicarboxylic compounds N-2,4-dinitrophenyl-D,L-glutamic, dansyl-D,L-glutamic and 3-dibenzoyl-D,L-tartaric acids and for the N-benzoyl derivative of D,L-phenylalanine carrying one carboxy group. Only the monocarboxylic D,L-3-indolelactic acid had a sufficient separation selectivity in the neutral and slightly acidic mobile phases also (Figs. 3 and 4).

In measurements requiring slightly alkaline mobile phases ($7 < pH < 10$) and lasting as long as several weeks, a decrease in the enantioselectivity of

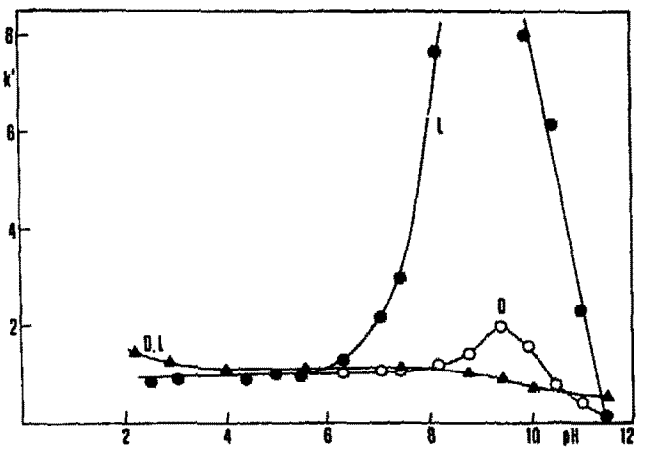

Fig. 2. Comparison of the dependence of the retention of D,L-tryptophan on the mobile phase pH for the sorbents (○, ●) Separon HEMA 1000–BSA and (▲) Separon HEMA 1000. Column, 200 × 4 mm I.D.; mobile phase, 0.05 mol/l phosphate buffer; flow-rate, F_m = 1 ml/min; amount injected, 5 μl (0.96 mmol/l).

D,L-tryptophan separation was observed. The rate of the decrease is strongly pH dependent. At pH 7.0–7.5 the enantioselectivity changed markedly only after several months, whereas at pH 9.5 it decreased

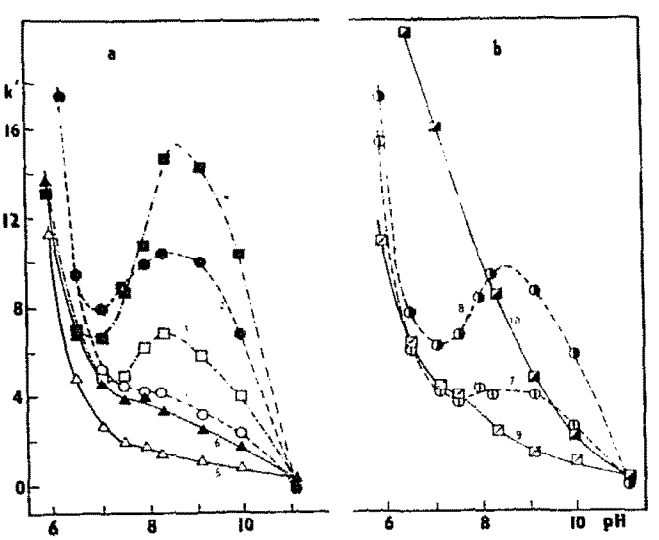

Fig. 3. Dependence of retentions of carboxylic acids on the pH of the mobile phase. Column, Separon HEMA 1000-BIO–BSA, CGC (150 × 3.3 mm I.D.); mobile phase, 0.05 mol/l phosphate buffer + 3% (v/v) 1-propanol. (a) 1, 2 = N-2,4-DNP-D,L-glutamic acid; 3, 4 = 2,3-dibenzoyl-D,L-tartaric acid; 5, 6 = N-benzoyl-D,L-phenylalanine. (b) 7, 8 = Dansyl-D,L-glutamic acid; 9, 10 = D,L-3-indolelactic acid.

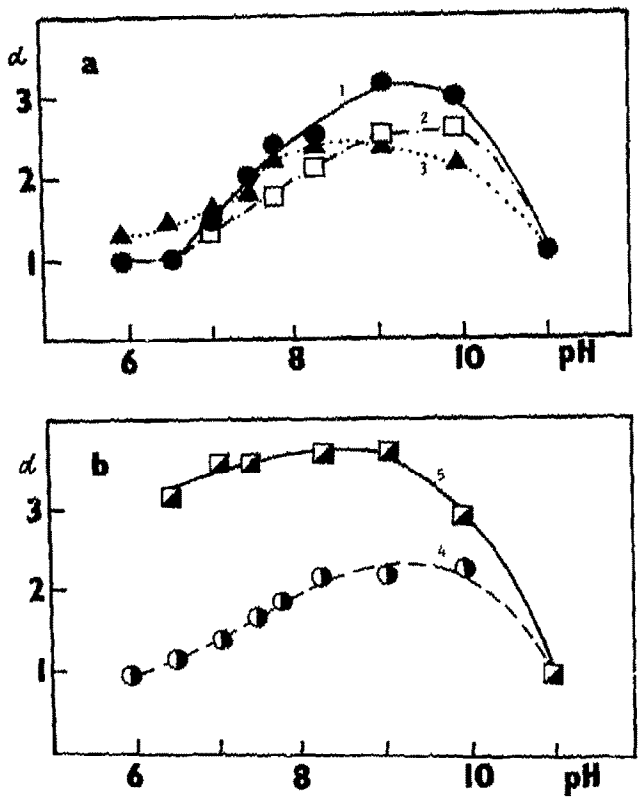

Fig. 4. Dependence of separation selectivity of carboxylic acids. (a) 1 = N-2,4-DNP-D,L-glutamic acid; 2 = 2,3-dibenzoyl-D,L-tartaric acid; 3 = N-benzoyl-D,L-phenylalanine. (b) 4 = Dansyl-D,L-glutamic acid; 5 = D,L-3-indolelactic acid.

markedly after 4 days (Fig. 5). Subsequent washing of the column exposed to pH 9 with mobile phase of pH 7.45 did not improve the enantioselectivity even after 5 days. Treatment with mobile phases of pH < 7 was also ineffective. The decreased enantioselectivity of the bonded albumin for D,L-tryptophan caused by the alkaline mobile phase was restored by washing the column with methanol for 24 h. Methanol acted similarly in restoring the decrease in the enantioselectivity of BSA for tryptophan caused by increased temperature [5].

Immediately after washing of the column with methanol, the enantioselectivity of the bonded BSA for D,L-tryptophan was higher than that of the bonded BSA tested after delivery of the column by the manufacturer (new column). During the separation of D,L-tryptophan in the mobile phase of pH 7.45 on the column treated with methanol, its retention and separation selectivity decreased markedly during the first 24 h. The rate of the decrease of both characteristics decreased with the time of action of the mobile phase pH 7.45. After 4 days the decrease in retention and selectivities caused by 24 h of action was comparable to the measurement error. The retention and selectivity were higher in this "stable" state than those of a new column.

The pH range in which the enantioselectivity of

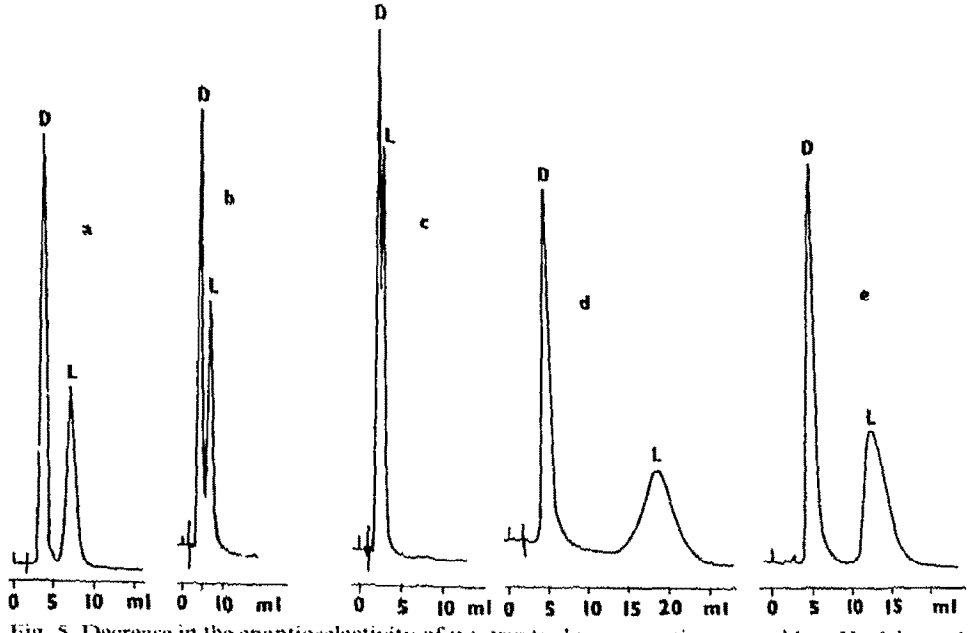

Fig. 5. Decrease in the enantioselectivity of D,L-tryptophan separation caused by pH of the mobile phase > 7 and its regeneration with methanol. Column, Separon HEMA 1000–BSA (250 × 4 mm I.D.); mobile phase, 0.05 mol/l phosphate buffer (pH 7.45); flow-rate, F_m = 1 ml min; amount injected, 5 μl (0.96 mmol l). (a) Fresh column; (b) after 6 months in mobile phase of pH 7–8; (c) after 4 days in mobile phase of pH 9.5; (d) immediately after regeneration with methanol; (e) 4 days after methanol-induced regeneration, washed out with mobile phase of pH 7.45.

the column with bonded BSA for D,L-tryptophan separation decreases is identical with that in which albumin in aqueous solution creates a special covalently stabilized conformation labelled the A (aged) form [10,11]. Under the assumption that the chemical binding of albumin to a support does not fully rule out a capability of the bonded molecules to respond to alterations in the liquid environment by changes in spatial arrangement (conformation), one can explain the observed change in the enantioselectivity of D,L-tryptophan separation by the formation of a conformation of the bonded BSA analogous to the A form ("ageing").

For control of the retentions of some types of solutes, 1-propanol and caprylic ions are used. Their influence on the ageing of bonded BSA were tested at pH 8.22 and it was found that 3% (v/v) of 1-propanol accelerates the ageing about twofold. If caprylic acid at a concentration of 5 mmol/l is added to a mobile phase of pH 8.22 containing 3% (v/v) of 1-propanol, after treatment with this mobile phase for 8 days the chemically bonded BSA completely loses its ability to separate D,L-tryptophan. In the same mobile phase without caprylic anions $R_s = 4$ after 8 days. Caprylic anions, therefore, act similarly to 1-propanol or even they exhibit a synergic effect. Such an influence of caprylic anions is surprising as caprylic acid is generally used as an additive stabilizing albumin against changes induced by increased temperature [12–14].

The enantioselectivity of bonded BSA decreased by mobile phase containing 1-propanol or 1-propanol and caprylic anions may be restored by methanol. Similarly to the effect of phosphate buffers of $7 < pH < 10$, these cycles, even if repeated, did not induce irreversible changes or the permanent loss of the enantioselectivity of the sorbent. From the chromatographic point of view, bonded BSA always returned to the same state.

The "ageing" of bonded BSA in alkaline medium and subsequent treatment of the column with methanol did not have the same effect on the separation selectivity of all the solutes applied. The separation selectivity of dicarboxylic acids increased owing to the "ageing", whereas the separation selectivity of monocarboxylic acids decreased (Table I). Because of the action of methanol, the separation selectivity increased for the monocarboxylic acids (similarly as for D,L-tryptophan), whereas it decreased below the initial value for the dicarboxylic acids. No separation at all took place with dibenzoyltartaric acid after the treatment with methanol. When, however, the mobile phase of pH 7.45 was used for conditioning of the column treated with methanol, the enantioselectivity became gradually re-established (Fig. 6).

The experiments on the effect of bonded albumin "ageing" and regeneration of its selectivity by methanol show that in addition to the regulation of the separation enantioselectivity by changing the

TABLE I

EFFECT OF "AGEING" OF THE SORBENT SEPARON HEMA 1000-BIO-BSA IN PHOSPHATE BUFFER (pH 8.22) CONTAINING 3% (v/v) 1-PROPANOL AND ITS REGENERATION WITH METHANOL ON RETENTIONS AND SELECTIVITIES

Column, Separon HEMA 1000-BIO-BSA (150 × 3.3 mm I.D.). Mobile phase, 0.05 mol/l phosphate buffer (pH 7.45) + 3% (v/v) 1-propanol.

Acid	Fresh column		"Aged" column		Regenerated column	
	k'_1	α	k'_1	α	k'_1	α
N-2,4-DNP-D,L-glutamic	4.09	2.00	4.28	2.69	4.33	1.62
Dansyl-D,L-glutamic	3.69	1.80	3.07	2.19	3.92	1.53
2,3-Dibenzoyl-D,L-tartaric	4.94	1.77	3.80	3.35	6.36	1.00
D,L-Indolelactic	3.84	4.19	0.98	2.58	6.55	4.50
N-Benzoyl-D,L-phenylalanine	2.00	1.93	1.70	1.61	2.58	2.20
D,L-Tryptophan[a]	0.78	8.03	0.54	4.59	0.86	9.65

[a] Mobile phase without 1-propanol, pH 7.39.

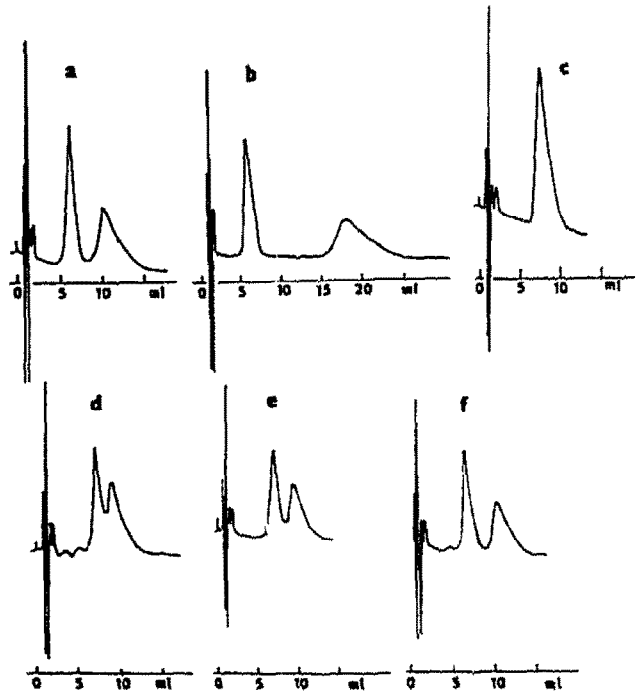

Fig. 6. Separation of 2,3-dibenzoyl-D,L-tartaric acid in 0.05 mol/l phosphate buffer (pH 7.45) with addition of 3% (v/v) of 1-propanol. (a) Fresh column; (b) after "ageing" of the column in mobile phase of pH 8.22 + 3% (v/v) of 1-propanol; (c) 48 h after regeneration of the column; (d) 72 h after regeneration of the column; (e) 96 h after regeneration of the column; (f) 192 h after regeneration of the column. Column, Separon HEMA 1000-BIO BSA CGC (150 × 3.3 mm I.D.).

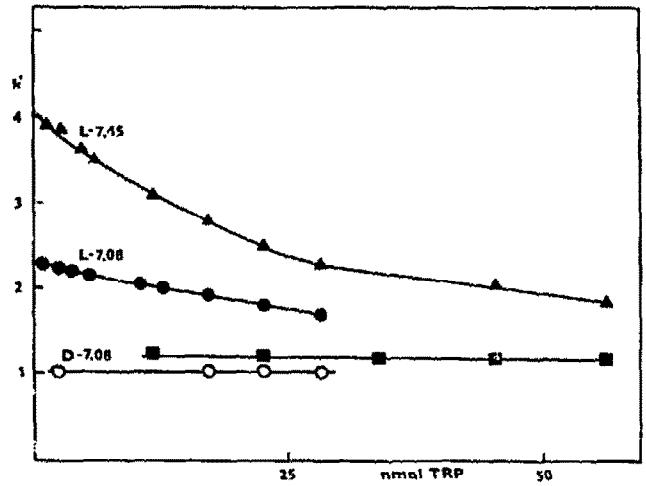

Fig. 7. Dependence of capacity factors on the amount of D,L-tryptophan injected. Sorbent, ■ = Separon HEMA 1000 (pH 7.4); ○, ● = Separon HEMA 1000–BSA (pH 7.08); ▲ = Separon HEMA 1000–BSA (pH 7.45). Mobile phase, 0.05 mol/l phosphate buffer with pH as indicated. ○ = D-Tryptophan; ● = L-tryptophan.

albumin charge by means of the pH of the environment, there exists an additional possibility, probably the influence of the bonded albumin conformation. Our ideas on the role of BSA charge and conformation, permitting a consistent qualitative explanation of the effects and dependences reported here, will be the subject of a separate paper.

Sorption capacity and efficiency

A characteristic feature of analyses on sorbents prepared by binding BSA to a silica gel support is the dependence of the retention volume of the peak maximum and the asymmetry of the peak on the amount injected [1,2]. Similar behaviour was observed using D,L-tryptophan as a solute chromatographed on columns packed with sorbents with BSA bonded to the hydroxyethylmethacrylate matrix (Fig. 7). As can be seen from the comparison with dependences measured on the matrices HEMA and HEMA BIO, the non-linearity and the increase in the non-linearity of the L-isomer with increasing retention must be ascribed to the interactions between the L-isomer and the bonded albumin.

The binding of BSA to the silica gel matrix reduces the efficiency of the silica gel-based columns [15]. Our measurements show a significant decrease in column efficiency caused by the binding of BSA to the HEMA matrix also (Fig. 8). The column efficiency is low also when the linear velocity of the mobile phase is very close to that representing the optimum linear velocity for silica gel-based sorbents. A comparison with Resolvosil (BSA–silica) shows that the efficiency of the silica gel-based sorbent is substantially higher. With a reduced linear velocity below 5, the efficiency of the Resolvosil column for the D-isomer is even better than the efficiency of columns packed with HEMA or HEMA-BIO supports. The efficiency of columns packed with HEMA-BIO–BSA and HEMA–BSA did not differ within the limits of accuracy of the measurements.

The dependences of H/d_p on the reduced linear velocity of the mobile phase for D-tryptophan measured on the Resolvosil and HEMA-BIO–BSA are parallel (Fig. 8). The same holds for the depen-

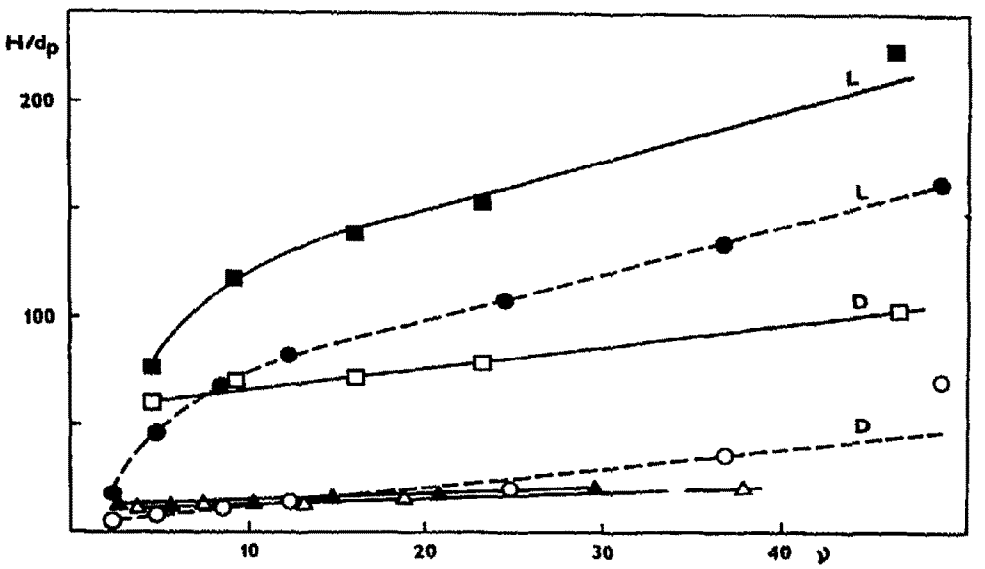

Fig. 8. Dependence of efficiency of D,L-tryptophan separation on the reduced linear flow-rate of the mobile phase. Mobile phase, 0.05 mol/l phosphate buffer (pH 7.45). Column, △ = Separon HEMA 1000; ▲ = Separon HEMA 1000-BIO; □, ■ = Separon HEMA 1000-BIO–BSA; ○, ● = Resolvosil.

dences of L-isomer at reduced velocity $\nu > 18$. In this range, (for any $\nu > 18$) $\Delta H/d_p$ measured on the sorbents Resolvosil and HEMA-BIO–BSA for the L-isomer also agrees with $\Delta H/d_p$ for the D-isomer found on the same sorbents. This indicates that the matrix HEMA contributes to the low efficiency of the columns. During efficiency measurements the optimum linear mobile phase velocity for HEMA-based columns was never approached. This suggests that the reduced optimum linear mobile phase velocity of the HEMA matrix may be well below that of the silica gel.

The matrix HEMA gives a sorbent of high hydrolytic stability and lifetime. During running of one of the columns for 18 months no loss of enantioselectivity was found. The pH of the mobile phase varied in the range 2–11 and as much as 5% of 1-propanol was added to the mobile phase. The decreased enantioselectivity was repeatedly restored by washing with methanol. The efficiency of the column decreased by about 30% in the course of this period.

CONCLUSIONS

Comparison of the enantioselectivity, efficiency and sorption capacity of columns packed with sorbents prepared by bonding of BSA to the organic polymeric matrix with those measured on columns packed with the matrix, demonstrated on the example of D,L-trytophan, enabled the influence of bonded BSA and the matrix on the measured dependences and characteristics to be resolved. It has been demonstrated that bonded BSA has a high selectivity for D,L-tryptophan in alkaline medium and that with bonded BSA a high enantioselectivity can be obtained not only for some other amino acids but also for monocarboxylic and dicarboxylic acids. The shape of the pH dependences of the retentions and the separation enantioselectivity was characteristic for each group of compounds. This indicates the dominant influence of ionized functional groups on the enantioselective retention of the solutes.

Changes in separation enantioselectivity induced by long-term action of slightly alkaline mobile phases showed that the influence of pH on the enantioselective properties of chemically bonded albumin is complex. Rapid (immediate) changes are undoubtedly caused by the influence of the pH of the medium on the albumin charge. Slow changes can be explained only as consequences of conformational changes having the same course as with dissolved albumin [11], but probably with a lower rate. The influence of methanol and the following slow changes in both the retentions of different types of compounds and the enantioselectivity of albumin

for these compounds in mobile phases with pH > 7 can be considered to be a consequence of conformational changes also. Experiments explicable only if conformational changes of bonded albumin are considered demonstrate the importance of the conformation of albumin for its action as a chiral selector. There is no reason to presume that conformation will be important only for the enantioselectivity of BSA. On the contrary, it must be assumed that the conformation and conformational variability of any biomacromolecule will be important properties in any analytical applications.

For 1-propanol and caprylic anions as minor components of the mobile phase, it is impossible to establish on the basis of the described experiments whether they affect only the rate of conformational changes or if they have any further influence. However, it has been demonstrated that the dependence of the retentions of solutes on the volume injected and the low efficiency of the columns are inseparably connected with bonded BSA.

There are three possibilities for the construction of the BSA-bonded sorbents having both an efficiency comparable to that of present-day silica gel sorbents and acceptable durability up to pH 10: (i) to improve the mass transport in the particles of the matrix HEMA, (ii) to select a more suitable organic matrix than HEMA and (iii) to test the durability of sorbents prepared by bonding BSA on the matrix from high-purity silica gel; such a matrix provides reversed-phase sorbents of remarkable stability in mobile phases up to pH 9 [16]. An interesting alternative is particles of alumina protected by a cross-linked polymer [17].

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