Journal of Chromatography, 638 (1993) 255–261
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 924

Prospects of dissolved albumin as a chiral selector in capillary zone electrophoresis

Radim Vespalec*, Vladimír Šustáček and Petr Boček

Institute of Analytical Chemistry, Czech Academy of Sciences, Veveří 97, 611 42 Brno (Czech Republic)

ABSTRACT

It has been shown using as examples amino acids, mono- and dicarboxylic acids that the enantioselectivity of dissolved albumin may be employed with advantage in capillary zone electrophoresis (CZE) separations. Slow changes in the enantioselectivity of albumin dissolved in weakly alkaline aqueous solutions can be avoided by a short mild heating of the solution. The efficiency of a separation system for a given solute in CZE is at least one order of magnitude higher than that in LC based on bonded albumin. The good chiral separations obtained here and the high efficiency of CZE separation systems confirm that CZE separations based on dissolved albumin as a chiral selector can compete with analogous LC separations. Perfect qualitative agreement of both enantioselective properties of albumin and factors affecting its enantioselectivity in CZE and LC proves that the knowledge obtained in LC with bonded albumin may be transferred to CZE separation systems.

INTRODUCTION

The analysis of chiral compounds is of great importance in pharmacy, medicine and biological sciences as a rule. For liquid chromatography (LC) the is shown by the large increase in the number of communications devoted to the problem of enantioselective separations in recent years.

Because of a number of interactions contributing to the enantioselective discrimination, the specificity of particular interactions and further factors affecting the resulting separation selectivity in practice [1], a variety of chiral selectors should be available for routine analyses. Therefore, many chiral selectors of different kinds have been studied in LC [1,2]. Among them, macromolecules of biological origin, *e.g.* proteins, are of importance. Albumin chemically bonded to a proper wide-pore solid matrix is especially suitable for the separation of anionic and amphoteric solutes [3–5]. In LC theory, the enantioselectivity and other properties of the bonded albumin relevant to its functioning as a chiral selector are approximated by those of dissolved albumin [4,6]. The test on the ability of the dissolved albumin to act as a chiral selector in free solution capillary zone electrophoresis (CZE), done in this study is simply the consequence of such theory. In planning the CZE experiments, both experimental findings and ideas originated in LC were employed. Comparison of experimental results with those from LC served for evaluation of experiments as well as for considering the potential of CZE to compete with established LC analyses based on bonded albumin.

EXPERIMENTAL

Fused-silica capillaries of 80 and 100 μm I.D. (SGE, Austin, TX, USA and Polymicro Technologies, Phoenix, AZ, USA, respectively) with both laboratory-coated and non-coated inner walls, hanging in free air at room temperature, were used in the laboratory-made set-up with simple siphoning sampling. Non-coated capil-

* Corresponding author.

0021-9673/93/$06.00 © 1993 Elsevier Science Publishers B.V. All rights reserved

laries, pretreated with 10 mM sodium hydroxide and 100 mM nitric acid, were rinsed with 10 mM sodium hydroxide every day after experiments. Capillaries coated with linear polyacrylamide were prepared using the procedure described by Hjertén [7], but ethanol was used instead of water as a solvent for the silanizing agent.

A laboratory-made power supply unit delivering a constant voltage of ±9.5 kV was employed in experiments using 80 μm I.D. capillaries. In these instances, the current passing through the capillary never exceeded 10 μA. A Spellman CZE 1000 R power supply unit was employed in the experiment using 100 μm I.D. capillaries.

Background electrolytes (BGEs) were obtained by dissolving lyophilized human albumin (HA) or bovine serum albumin (BSA) for laboratory use (Imuna, Šarišské Michalany, Czech Republic) in a proper buffering electrolyte. Buffering electrolytes of pH 8–10 were prepared by dissolving chemicals of analytical grade in boiled deionized water. Details of buffer composition and pH as well as albumin treatment are given in the descriptions of individual experiments. Chemically pure 1:1 racemates D,L-kynurenine, D,L-trypthophan, D,L-3-indole lactic acid N-2,4-dinitrophenyl-D,L-glutamic acid (DNPG), dansyl-D,L-glutamic acid (DSG) as well as 2,3-dibenzoyl-D-tartaric acid and 2,3-dibenzoyl-L-tartaric acid without electrophoretically active admixtures served as samples.

A Jasco 875-UV spectrophotometer equipped with a laboratory-made capillary holder was used for on-line light-absorption detection. The detection window in the outer polyimide capillary coating was made by cutting off the coating with a razor blade. After introductory experiments, the capillary in the holder was sandwiched between quartz lenses having the focal distance of 5 mm at a wavelength of 254 nm. The lenses increased the radial luminous flux through the capillary 10–20 times, depending on the capillary diameter and adjustment of the capillary in the holder. As a result, the intensity of the light beam passing through the capillary of 80–100 μm I.D., where lighted capillary length was 0.6 mm, ranges from 20 to 50% of the intensity of the reference light beam.

Migration times, t_m, read from the strip chart record, were converted to apparent mobilities, $\bar{u}$, using the equation $\bar{u} = LlE^{-1}t_m^{-1}$, where L is the total capillary length, l is the distance to the detector and E is the voltage used. Electroosmotic mobility u_{osm} was calculated from the migration time of mesityl oxide or from the vacant peak caused by injection of a large sample volume of albumin-containing electrolytes at detection wavelength less than 330 nm. Separation selectivity is expressed as $|\Delta\bar{u}|$ for each pair of enantiomers. Units 10^{-9} m^2 V^{-1} s^{-1} were used for all the mobility data.

RESULTS AND DISCUSSION

Preliminary experiments with bovine serum albumin (BSA) revealed insufficient purity of the available BSA preparation —a ghost peak appeared on the record at wavelengths below 300 nm with detection time independent of the injected solute. No ghost peaks were observed with the human albumin (HA), therefore, only HA was used in the following experiments. Regarding minor differences between BSA and HA [8] and resemblance of BSA and HA enantioselectivities found in LC [9], the qualitative observations and conclusions made in this study with HA are valid for BSA as well.

A 10 mg/ml solution of HA (1% by weight) in 10 mM acetic acid–Tris buffer, pH 8, was used to test the applicability of the on-line light absorption detection. The light absorption curve of human albumin in the range 200–600 nm, corrected for the electrolyte background (Fig. 1), demonstrates that the albumin solution is optically transparent at wavelengths (λ) above 330 nm. At $\lambda < 330$ nm the on-line detection needs the compensation of the background absorption, which is no problem for modern spectrophotometers, such as Jasco 875-UV.

Albumin preferentially retains solutes with negatively charged groups [10]. Enantioselectivity of the bonded albumin to mono- and dicarboxylic acids as well as to amino acids is highest near pH 9 [11,12]. The test on enantioselectivity of the dissolved albumin at pH 9.6 showed a sufficient potential of the freshly dissolved albumin to separate enantiomers of amino acids

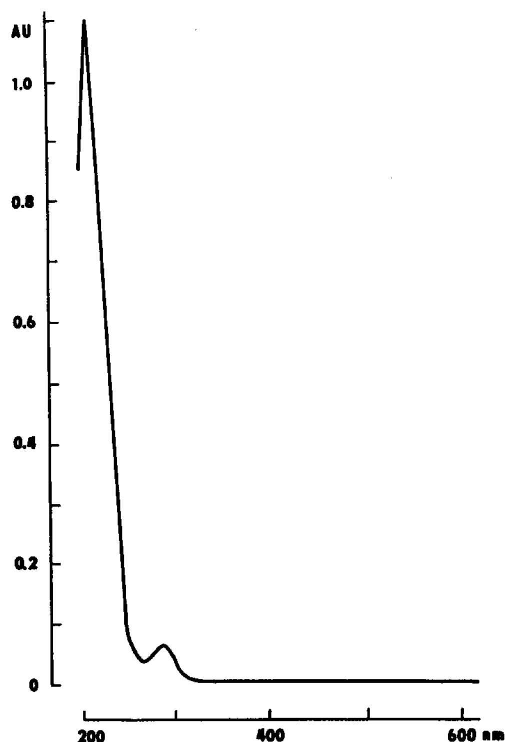

Fig. 1. Light absorption curve of 10 mg/ml human albumin solution, measured in a 100 μm I.D. fused-silica capillary and corrected for the background absorption of the 10 mM acetic acid–Tris buffer, pH 8.

and monocarboxylic acids (Fig. 2); separation of dicarboxylic acids was not satisfactory at an albumin concentration of 1 mg/ml. During a 3-h period, the ability of the albumin, dissolved in electrolyte of pH 9.6, to separate compounds with two carboxylic groups increased, and its enantioselectivity to monocarboxylic compounds decreased. This behaviour is qualitatively the same as that of bonded albumin in LC [12], but the rate of the enantioselectivity changes of the dissolved albumin is at least one order of magnitude higher than that of the albumin bonded in pores of a solid matrix. A decrease in albumin selectivity for amino acids with time in weakly alkaline solutions and its absence in weakly acidic solutions [12] suggests that dissolved albumin is not a proper chiral selector for CZE separations of amino acids.

The stabilization of the enantioselectivity of albumin dissolved in alkaline medium may be accelerated by a short mild heating of the solu-

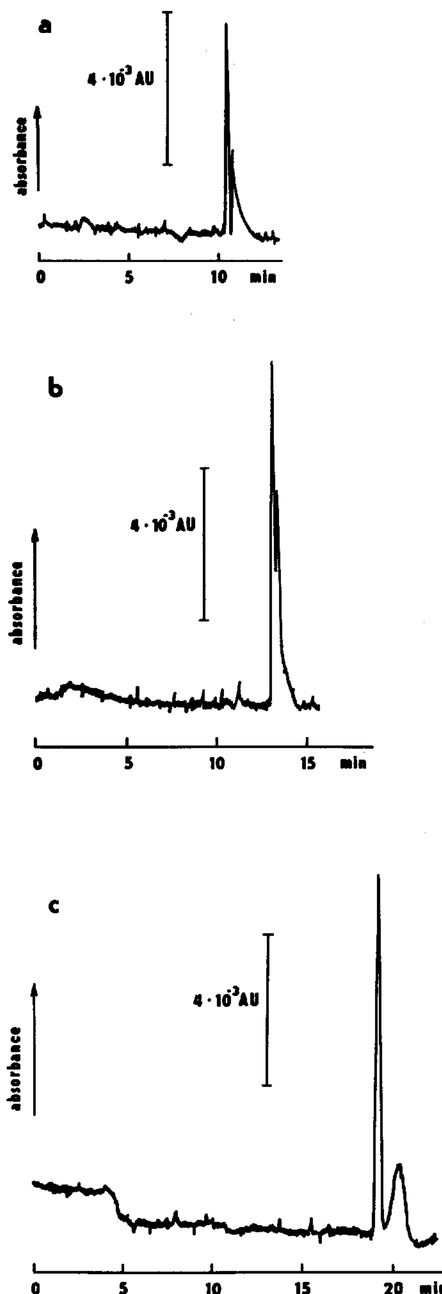

Fig. 2. Separation of (a) 11 pmol of D,L-kynurenine ($\Delta\bar{u}$ = 0.75), (b) 12 pmol of D,L-tryptophan ($\Delta\bar{u}$ = 0.18) and (c) 8 pmol of D,L-3-indole lactic acid ($\Delta\bar{u}$ = 1.3) in freshly prepared solution of 1 mg/ml HA in 10 mM boric acid having pH 9.6 adjusted by sodium hydroxide. Experimental details: Non-coated fused-silica capillary [70 cm (54 cm to detector) × 80 μm I.D.], voltage +9.5 kV, u_{osm} = 45, ambient temperature 24 ± 1°C. Detection: D,L-kynurenine 254 nm, D,L-tryptophan and D,L-3-indole lactic acid 280 nm, using capillary holder without lenses.

tion. For example, heating for 30 min to 60°C at pH 9 is satisfactory. An example of the analysis using the preheated albumin is shown in Fig. 3. After heating the albumin in acetate–Tris solution of pH 9, the final pH 8 at 10 mM acetate concentration was adjusted by adding suitable amounts of acetic acid and Tris. The acetic acid–Tris buffer pH 8 was chosen to protect the capillary coating from the damaging action of the high-pH borate buffer. A separation selectivity of $\Delta\bar{u} = 2.3$, observed after injection of 12 pmol of 2,3-dibenzoyl-D,L-tartaric acid to the freshly prepared electrolyte at 29°C, was independent of time. Time-independent enantioselectivities were observed in this system with other tested dicarboxylic acids (DNPG and DSG) as well. Enantioselectivity for amino acids and monocarboxylic acids disappeared with heating, as for bonded albumin in LC [13]. With prolonged heating, *e.g.* for 24 h to 60°C, albumin completely lost its enantioselectivity.

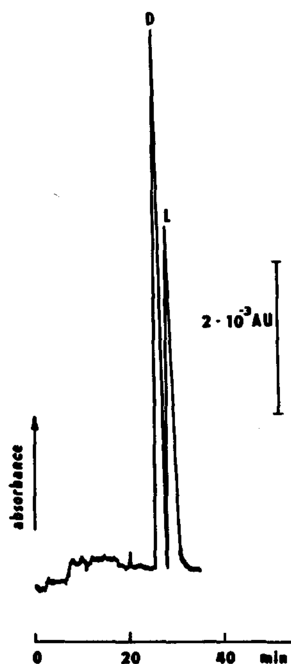

Fig. 3. Separation of 12 pmol of 2,3-dibenzoyl-D,L-tartaric acid in 10 mM acetate–Tris buffer, pH 8, containing 3 mg/ml human albumin, heated for 30 min at pH 9 and 60°C. Experimental details: Polyacrylamide-coated fused-silica capillary [76 cm (60 cm to detector) × 80 μm I.D.], voltage −9.5 kV, $u_{osm} < 5$, ambient temperature 29–30°C, detection at 238 nm using capillary holder with quartz lenses.

TABLE I

INFLUENCE OF HUMAN ALBUMIN CONCENTRATION ON SEPARATION SELECTIVITY OF N-2,4-DINITROPHENYL-D,L-GLUTAMIC ACID

Experimental details: Non-coated fused-silica capillary, 70 cm (54 cm to detector) × 80 μm I.D. BGE: 28 mM borate buffer, pH 9.0, human albumin heated for 30 min at 60°C. Injection: 0.6 pmol of N-2,4-dinitrophenyl-D,L-glutamic acid. Ambient temperature 20–30°C. Voltage +9.5 kV (0 and 0.5 mg/ml HA) or −9.5 kV (3 mg/ml HA) according to the magnitude of electroosmotic velocity

HA concentration (mg/ml)	$\|\Delta u\|$ (10^{-9} m^2 V^{-1} s^{-1})
0.0	0.0
0.5	0.8
3.0	4.0

The effect of the concentration of the preheated albumin on separation selectivity at pH 9.0 was studied in 28 mM borate buffer in non-coated capillary (Table I). Owing to the strong dependence of the electro-osmotic flow on the albumin concentration in non-coated capillaries, a polyacrylamide-coated capillary was used in

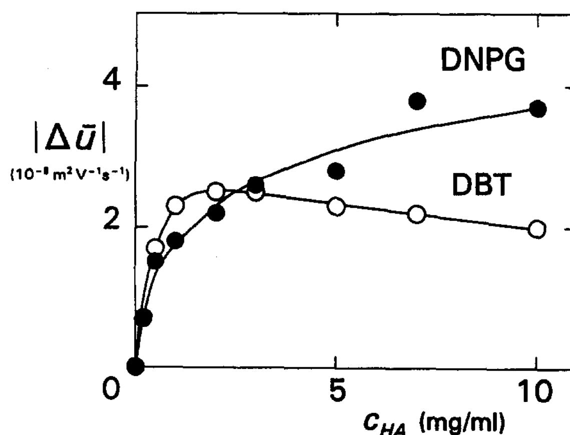

Fig. 4. Selectivity of enantiomeric separations $|\Delta\bar{u}|$ as a function of albumin concentration in 10 mM acetate–Tris buffer, pH 8 (DNPG = N-2,4-dinitrophenyl-D,L-glutamate; DBT = 2,3-dibenzoyl-D,L-tartrate). Experimental details: Polyacrylamide-coated fused-silica capillary [70 cm (55 cm to detector) × 100 μm I.D.], sample load 1 pmol, voltage −12 kV, current 10–25 μA, $u_{osm} < 2$, ambient temperature 21–22°C, detection at 380 nm (DNPG) or 254 nm (DBT) using capillary holder with quartz lenses.

the following experiment, shown in Fig. 4. Here the effect was measured in the concentration range 0.2–10 mg/ml albumin in 10 m*M* acetate–Tris buffer, pH 8.0. These two experiments prove the activity of albumin in chiral discrimination. Generally, the chiral selectivity of the system increases with increasing albumin concentration, regardless of pH, temperature, background electrolyte and type of solute. In Fig. 4, the dependence of separation selectivity passes through a maximum for DBT. An increase in the temperature inside the capillary as a result of greater Joule heat produced by the current increasing with albumin concentrations is one probable explanation. However, in addition to increasing enantioselectivity, a higher albumin concentration improves the peak shape and, consequently, the resolution as well. The resolution of peaks increases even if the apparent decrease in selectivity with increasing albumin concentration is observed (Fig. 5). The baseline drift at a concentration of 10 mg/ml HA is due to effects connected with the absorption of the UV light having $\lambda < 330$ nm in relatively concentrated albumin solution.

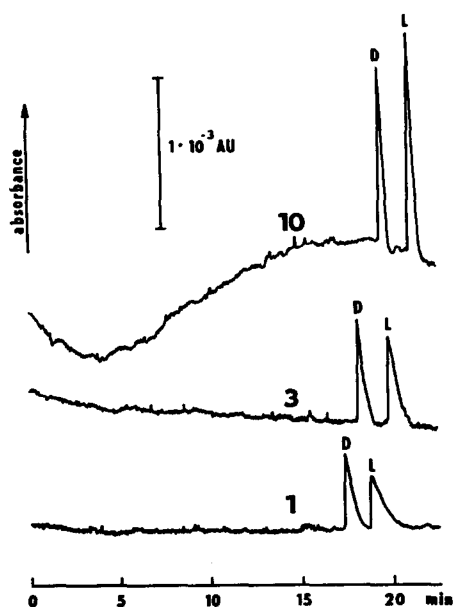

Fig. 5. Influence of albumin concentration (10, 3 and 1 mg/ml HA) on the separation of 1 pmol of 2,3-dibenzoyl-D,L-tartaric acid in 10 m*M* acetate–Tris buffer, pH 8. Experimental details: see Fig. 4.

Efficiency of the proper CZE separation systems based on albumin ranged in this study from 6000 (Fig. 6a) to 80 000 theoretical plates for a capillary of effective length of 60 cm and 20–40 min of analysis time, depending on solute, albumin concentration and its pretreatment as well as on the sample amount.

The efficiency of common LC columns, 15–25 cm in length, packed with sorbents prepared by bonding albumin to silica gel matrix ranges from 500 to 5000 theoretical plates at 10–40 min analysis time for various solutes [1,3–5]. The efficiency may be even lower for columns in which albumin is bonded to an organic polymeric matrix, *e.g.*, HEMA (Fig. 6b).

It is impossible to estimate from the experiments done so far how much the peak broadening in CZE is influenced by the albumin trapped on the capillary wall (albumin mobility is reported to be $-5.9 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} at pH 8.6 [8]). In non-coated capillaries, albumin adsorption is manifested by a strong decrease in electroosmosis with increasing albumin concentration. Even in polyacrylamide-coated capillaries, a slight albumin adsorption was observed at high albumin levels as a residual light absorption after replacing the albumin-containing BGE with the same electrolyte solution without albumin.

A typical feature of LC analysis based on bonded albumin is the increase in both solute retention and separation selectivity with decreasing solute amount, being the steeper the lower the solute amount [5,12]. In LC, the combination of a low sorption capacity of albumin for a particular solute [8,14] and an unfavourably low albumin/solute molar concentration ratio is supposed to be the reason. The albumin/solute ratio estimated from albumin content declared by manufacturers and reported sample amounts is usually of the order of 10^{-1} to 10^{0}.

For albumin concentrations of 0.5–1 mg/ml and injections exceeding 5 pmol, the albumin/solute molar concentration ratio was of the order of 10^{-3} to 10^{-2}. The dependence of detection times on sample amount was typical for such experiments at temperatures up to 30°C. The albumin/solute molar concentration ratio of the order of 10^{-1} was attainable at 3 mg/ml or higher albumin concentrations and injections of

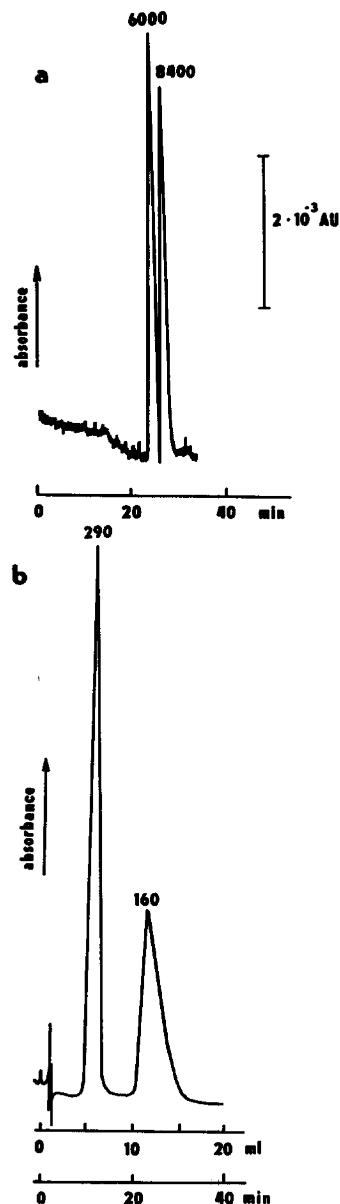

Fig. 6. Comparison of separation efficiencies, given as plate numbers at peak maxima, attained at separations of N-2,4-dinitrophenyl-D,L-glutamic acid in albumin-based systems in (a) CZE and (b) HPLC. Experimental details: (a) Polyacrylamide-coated fused-silica capillary [76 cm (60 cm to detector) × 80 μm I.D.]. BGE: albumin heated in acetate–Tris solution for 30 min at pH 9 and 60°C; final pH 8 at 10 m*M* acetate adjusted with acetic acid and Tris. Injection: 6 pmol of racemate. Voltage −9.5 kV. (b) Commercial 150 mm × 3.3 mm I.D. column packed with Separon HEMA-BIO-BSA (Tessek, Prague, Czech Republic), bonded albumin. Mobile phase: 50 m*M* phosphate buffer, pH 7.8 containing 3% (v/v) 1-propanol, flow-rate 0.5 ml/min. Injection: 5.3 nmol of racemate.

TABLE II

CHANGES IN DETECTION TIMES, t_i, AND SELECTIVITIES, $|\Delta u|$, OF N-2,4-DINITROPHENYL-D,L-GLUTAMIC ACID WITH ALBUMIN/SOLUTE MOLAR RATIO AT THE CAPILLARY INLET

Experimental details: Human albumin concentration 3 mg/ml; other details as in Table I

Albumin/solute molar ratio	t_1 (s)	t_2 (s)	$\lvert\Delta u\rvert$ (10^{-9} $m^2 V^{-1} s^{-1}$)
$4.5 \cdot 10^{-2}$	4610	6160	2.62
$1.8 \cdot 10^{-2}$	5300	7540	2.70
$4.5 \cdot 10^{-1}$	5300	8040	3.10

2 pmol or lower. The independence of the detection time on injected amount was observed for the less retained enantiomer of N-2,4-dinitrophenyl-D,L-glutamic acid using heated albumin of 3 mg/ml concentration at 29°C (Table II). This suggests that there is chance to restrict or even eliminate in CZE the unwanted dependence of detection times on solute amount by a proper combination of albumin pretreatment, albumin concentration and elevated capillary temperature. Very long detection times in Table II are the result of a small difference between $u_{osm} = 30$ for 3 mg/ml albumin concentration in non-coated capillary at pH 9 and mobilities of DNPG enantiomers in the system.

ACKNOWLEDGEMENT

This research was supported by the Grant Agency of Czechoslovak Academy of Sciences, Grant No. 43106.

REFERENCES

1 S.G. Allenmark, *Chromatographic Enantioseparations, Methods and Applications*, Ellis Horwood, Chichester, 1988.
2 M. Zief and L.J. Crane (Editors), *Chromatographic Chiral Separations*, (*Chromatographic Science Series*, Vol. 40), Marcel Dekker, New York, 1988.
3 S. Allenmark, B. Bomgren and H. Borén, *J. Chromatogr.*, 316 (1984) 617.
4 S. Allenmark and S. Andersson, *J. Chromatogr.*, 351 (1986) 231.
5 J. Vindevogel, J. van Dijck and M. Verzele, *J. Chromatogr.*, 447 (1988) 297.

6 E. Domenici, C. Bertucci, P. Salvador, G. Félix, I. Cahagne, S. Motellier and I.M. Wainer, *Chromatographia*, 29 (1990) 170.
7 S. Hjertén, *J. Chromatogr.*, 347 (1985) 191.
8 T. Peters, Jr., Serum Albumin, in F.W. Putnam (Editors), *The Plasma Proteins*, Vol. I, Academic Press, New York, 2nd ed. 1975, pp. 133–181.
9 C. Pettersson, T. Arvidsson, A.-L. Karlsson and I. Marle, *J. Pharm. Biomed. Anal.*, 4 (1986) 221.
10 R.H. McMenamy, Albumin Binding Sites, in V. Rosenoer, M. Oratz and M. Rotshild (Editors), *Albumin Structure, Function and Uses*, Pergamon Press, Elmsford, NY, 1977, pp. 143–157.
11 K.K. Stewart and R.F. Doherty, *Proc. Natl. Acad. Sci. USA*, 70 (1973) 2850.
12 Z. Šimek and R. Vespalec, *J. Chromatogr.*, 629 (1993) 153.
13 Z. Šimek, R. Vespalec and J. Šubert, *J. Chromatogr.*, 543 (1991) 475.
14 J.F. Foster, in V. Rosenoer, M. Oratz and M. Rotshild (Editors), *Albumin Structure, Function and Uses*, Pergamon Press, Elmsford, NY, 1977, pp. 53–84.