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Note

Direct resolution of enantiomers by liquid affinity chromatography on albumin-agarose under isocratic conditions

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The ability of proteins, as highly chiral species, to exhibit enantioselectivity in their binding of certain ligands is well documented in the case of enzyme–substrate interactions[1], but much less information in this area is available for other proteins. It has been shown, however, by isotopic labelling experiments, that plasma albumin has a higher affinity for L-tryptophan of the D,L-pair[2] and that a corticosteroid-binding globulin selects (+)-aldosterone for similar reasons[3]. In 1973 it was demonstrated for the first time that D,L-tryptophan could be resolved by chromatography on a bovine serum albumin (BSA)-agarose column due to this difference in antipodal affinity[4]. As judged from two recently published reviews[5,6] on direct chromatographic resolution, this result seems to have been overlooked and never developed further, despite the potential value of this new technique.

In this paper, we demonstrate its use under optimized isocratic conditions with fluorimetric and continuous UV or electrochemical detection for the direct resolution of D,L-tryptophan, D,L-5-hydroxytryptophan, D,L-kynurenine and D,L-3-hydroxykynurenine.

EXPERIMENTAL

Racemic and optically active compounds investigated

D,L-, L- and D-tryptophan (TRP), D,L-, L- and D-5-hydroxytryptophan (5-HTP), D,L- and L-kynurenine (KYN), D,L-3-hydroxykynurenine (3-HKN), D,L-, L- and D-3,4-dihydroxyphenylalanine (DOPA) and D,L- and L-norepinephrine (NE) were obtained from Sigma (St. Louis, MO, U.S.A.).

Synthesis of BSA-agarose

Method 1. Activated CH-Sepharose-4B (Pharmacia, Uppsala, Sweden) (5 g of dry material) was swollen in 1 m*M* hydrochloric acid and washed on a glass filter with 1 m*M* hydrochloric acid followed by 0.1 *M* sodium hydrogen carbonate + 0.5 *M* sodium chloride (pH 8.0) used as the buffer for coupling. This amount of gel has a maximum binding capacity of 90 μmol for low-molecular-weight compounds.

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Defatted BSA (Sigma) (670 mg, 10 μmol) was dissolved in 30 ml of the coupling buffer, mixed immediately with the washed gel, and the suspension was ultrasonicated for 10 min and then placed in a mechanical shaker for 2 h at 25°C. The gel was filtered off, washed with coupling buffer and finally treated with 1 *M* ethanolamine at pH 8.5 for 30 min. Then the gel was washed thoroughly with 1 m*M* hydrochloric acid followed by 0.1 *M* borate buffer (pH 8.8).

Method 2. Epoxy-activated Sepharose 6B (Pharmacia) (7.5 g of dry material) was swollen in water and washed on a glass filter. BSA (1.5 g, 22 μmol) was dissolved in 50 ml of 0.1 *M* sodium hydrogen carbonate–sodium carbonate buffer/0.2 *M* sodium chloride (pH 9.75), and mixed immediately with the washed gel. After ultrasonication the suspension was placed in a shaker for 24 h at 25°C. The gel was then treated as described under method 1.

Chromatography

A 250 × 9 mm column (Pharmacia) was packed with the gel and equilibration of the system was carried out with the borate buffer of pH 8.8 for 2 h. No leakage of BSA could be detected. The bed height after equilibration was 185 mm unless stated otherwise. The column was connected to a peristaltic pump and the flow-rate determined. Flow-rates up to 50 ml/h could be used without unfavourable compression of the gel. Application of the sample was performed by means of a micropipette (for volumes >10 μl) and, more easily, by direct injection into the column bed with a Hamilton syringe (for volumes <10 μl).

In a second series of experiments a 500 × 3.0 mm glass column (Altex, Berkeley, CA, U.S.A.) was used. This was coupled to an HPLC pump (Altex Model 110A) and injections of the sample were made directly on to the column.

Detection of the species eluted from the column was carried out either by fluorescence measurements on collected fractions (used during the initial studies on D,L-TRP) or by continuous monitoring by means of a UV or an amperometric detector. In all instances aqueous buffers were used as the mobile phase.

Instrumentation

Fluorescence was measured with a Perkin-Elmer Model 204 spectrofluorimeter. The UV detector used was a variable-wavelength LDC Spectromonitor III. The amperometric detector connected to the column outlet consisted of a thin-layer flow cell equipped with a carbon paste anode and connected to a reference electrode compartment containing a silver–silver chloride electrode. The cell was provided with a 50-μm spacer and an anode potential of +0.75 V was used. The potentiostat/amplifier (Model LC-2A) and the other detector equipment were obtained from Bioanalytical Systems (West Lafayette, IN, U.S.A.). A Linear Model 264 potentiometric recorder was used to register the output signal as a function of time.

RESULTS AND DISCUSSION

Resolution of the enantiomers is highly dependent on the pH of the mobile phase. Thus, under the conditions used by Stewart and Doherty[4], L-TRP is strongly retained at pH 9.2 and 0.1 *M* acetic acid was used for its displacement. We were able to confirm their results completely and we also found that on decreasing the pH to 6.5

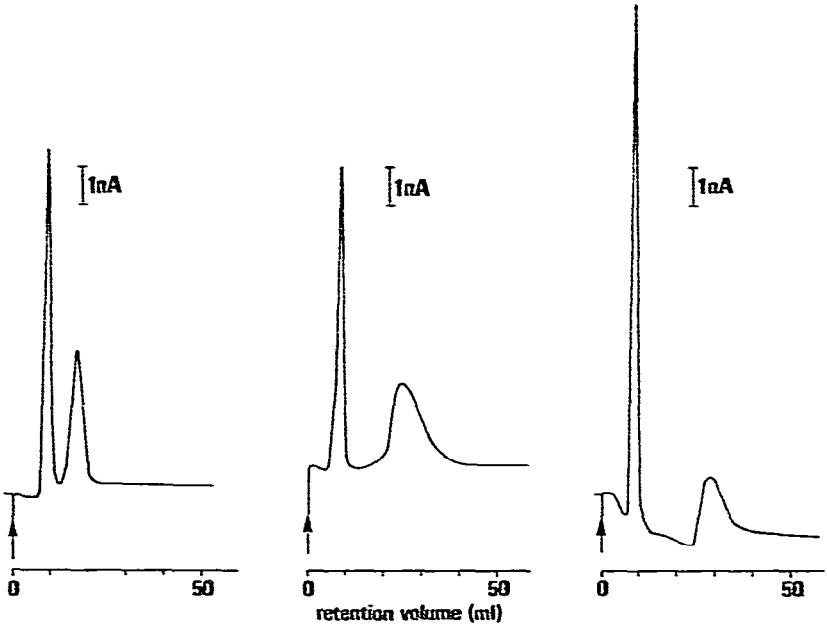

Fig. 1. Chromatograms showing the effect of the pH of the mobile phase. A 5-μl volume of a 4.46 mM solution of D,L-3-hydroxykynurenine was injected. Column, 140 × 9 mm; flow-rate, 44.5 ml/h; electrochemical detection at +0.75 V. Mobile phases (from left to right): 0.20 M phosphate buffer (pH 7.05); 0.05 M borate buffer (pH 8.00); 0.20 M Tris–HCl buffer (pH 9.00).

there was still a sufficient difference in retention between the antipodes to give a complete baseline separation under the isocratic conditions of elution used. Under the latter conditions, however, D,L-5-HTP was not resolved, but on increasing the pH to 7.9 complete resolution was achieved. Some results from a study of the effect of buffer pH on the chromatographic resolution of D,L-3-HKN are shown in Fig. 1. The

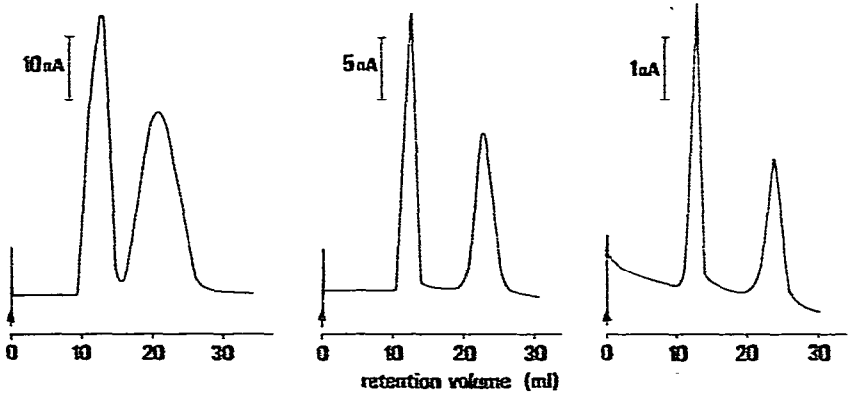

Fig. 2. Chromatograms showing the effect of the amount applied. Sample: 3.7 mM D,L-5-hydroxytryptophan. Volumes applied to the column (from left to right): 50, 5.0 and 0.50 μl. Column, 185 × 9 mm; flow-rate, 15 ml/h; electrochemical detection at +0.75 V. Mobile phase: 0.05 M borate buffer (pH 7.95).

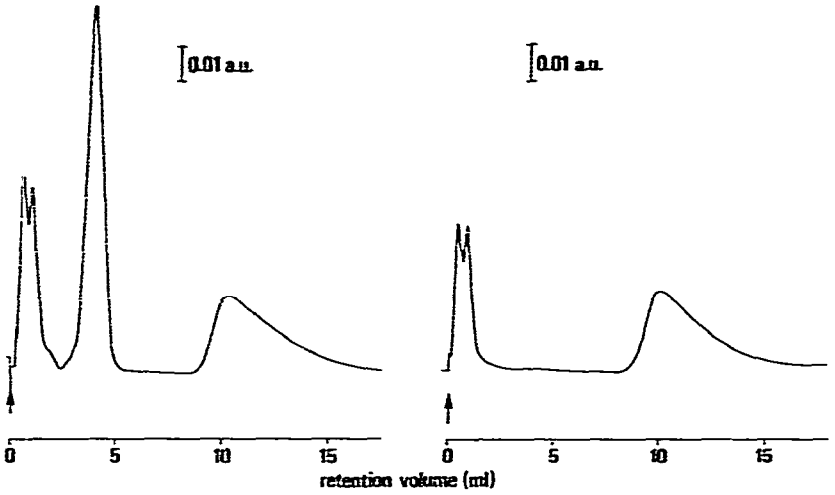

Fig. 3. Enantiomer identification. Chromatograms of D,L-kynurenine (30.6 nmol; left) and L-kynurenine (15.3 nmol; right) obtained under identical conditions. Volume injected: 4 μl. Column, 500 × 3.0 mm; flow-rate, 6.0 ml/h; UV detection at 264 nm. Mobile phase: 0.05 *M* borate buffer (pH 7.95).

progressive increase in k' for the L-enantiomer with increasing pH is readily observed.

Owing to the relatively low capacity of these gels, the amount of sample applied to the column is of great importance, as shown in Fig. 2. Two effects of a decrease in the sample load are obvious: (a) an increase in the column efficiency and (b) an increase in the separation factor, α, due almost exclusively to an increased k' value of the second component (the L-form).

Of the compounds resolved so far, the naturally occurring L-enantiomer was always found to be that most retained on these columns. Identification was performed by chromatography of one of the optically active forms, as shown in Fig. 3 for the case of kynurenine. Preliminary experiments with two catechol derivatives with different types of chirality, *viz.*, D,L-DOPA and D,L-NE, were unsuccessful with respect to resolution under the conditions used. Some of the chromatographic data obtained are summarized in Table I.

TABLE I

SEPARATION FACTORS (α) OBTAINED BY CHROMATOGRAPHY OF VARIOUS D,L-PAIRS ON ALBUMIN-AGAROSE UNDER DIFFERENT CONDITIONS

Compound	*Buffer*	*pH*	α
TRP	Borate, 0.05 *M*	7.95	2.0
5-HTP	Phosphate, 0.20 *M*	7.05	1.3
	Borate, 0.05 *M*	9.25	1.7
3-HOK	Phosphate, 0.20 *M*	7.05	4.5
	Borate, 0.05 *M*	8.00	11.5
	Tris–HCl, 0.20 *M*	9.00	14.4

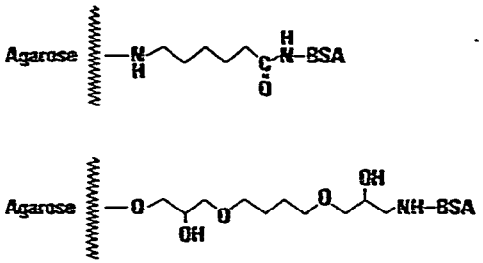

Fig. 4. Assumed structures of BSA-agarose synthesized by method 1 (above) and method 2 (below).

The gels prepared according to method 1 were found to give a better resolution than those prepared by method 2. Fig. 4 shows the expected chemical structures of the gels. However, because the degree of albumin substitution on these gels has not yet been determined, the reason for this effect is still unknown. Further work is in progress.

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