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RESOLUTION OF TRITIUM-LABELLED AMINO ACID RACEMATES BY LIGAND-EXCHANGE CHROMATOGRAPHY

II*. L-HYDROXYPROLINE- AND L-PHENYLALANINE-MODIFIED RESINS FOR THE RESOLUTION OF COMMON α-AMINO ACIDS**

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SUMMARY

Preparations of racemic multiple tritiated valine, histidine and alanine with high specific activities were resolved into enantiomers using ligand-exchange chromatography on Cu^{2+}-saturated L-hydroxyproline-modified polystyrene and L-phenylalanine-modified polyacrylamide. These two resins allow the resolution of all common amino acids on a preparative scale and their optical and radiochemical purity to be established. The method does not require any chemical modification of the racemate to be resolved, does not impose any limitations on its specific activity and provides for the simultaneous radiochemical purification of the enantiomers.

INTRODUCTION

The range of methods for resolving racemates of α-amino acids into constituent optically active enantiomers is severely limited when labelled compounds of high specific activity are involved. All methods involving crystallization procedures are inapplicable because of rapid radiolysis of the radioactive compound in a condensed phase. Natural enzymes commonly used to modify selectively one of the enantiomers in solution are easily inactivated by radiation. Much more promising are chromatographic methods, particularly ligand-exchange chromatography on chiral chelating resins (for a review, see ref. 1). This chromatographic method does not require any chemical modification of the amino acid that would unavoidably lead to a decreased yield and loss of the radioactive compound.

In Part I[2] we described the resolution of D,L-[^{3}H]valine with a specific activity

* For part I, see ref. 2.

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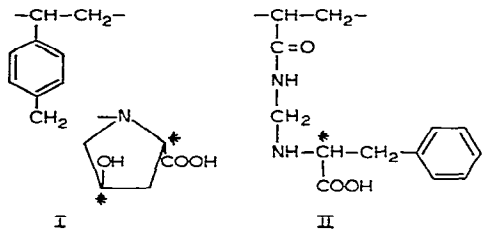
of $1.4 \cdot 10^{12}$ Bq/mmol on a column containing L-hydroxyproline-modified polystyrene resin. This paper is concerned with further optimization of the resolution procedure using this type of resin (I) and the synthesis of an L-phenylalanine-modified polyacrylamide gel (II) which easily resolves racemates of most common amino acids.



I II

EXPERIMENTAL

Sorbents

The synthesis of sorbent I by interaction of methyl L-hydroxyprolinate with chloromethylated cross-linked polystyrene was described earlier[3]. The starting copolymer contained 0.7% of divinylbenzene and was additionally cross-linked with monochlorodimethyl ether to give a total degree of cross-linking of 6 mol%. The water content of the swollen resin was 250% and the exchange capacity was 3.8 mmol of residues of L-hydroxyproline per gram of sorbent.

Sorbent II was obtained by treatment of Bio-Gel P-4 polyacrylamide beads (Serva, Heidelberg, G.F.R.)with formaldehyde and L-phenylalanine. The sorbent contained 1.4 mmol of groupings of L-phenylalanine per gram. The water uptake was 300%.

Before packing into columns, the sorbents were treated with an excess of copper(II)–ammonia solution and subsequently with a solution of potassium chloride in 1.0 *N* ammonia to achieve the desired content of Cu^{2+} ions in the sorbent.

Chromatography of racemates

The copper-loaded sorbents I and II suspended in 0.1 *N* ammonia or 1% ammonium phosphate solution (pH 9.2), respectively, were slurry-packed into glass columns and conditioned by passing the same eluents through them.

The racemic amino acids were introduced into the top of the column with the help of a micro-syringe. To detect the enantiomers resolved, the Radiochromatograph 2301 chromatographic system (U.S.S.R.) was used, equipped with a flow radioactivity detector cell of volume 170 μl and made of scintillating quartz. Another detector was a flow photometer operated at 210, 250 or 280 nm.

Isolation and characterization of enantiomers

Using the hydrolytical stable resin of type I and ammonia solutions as the eluent, the resolved amino acid enantiomers can be easily obtained by evaporation of the corresponding eluate fractions, which should be previously purified to remove trace amounts of Cu^{2+}. The purification consists in filtering the eluate through a small column (15 × 8 mm I.D.) with ANKB-50 chelating resin (polystyrene bearing iminodiacetate groups).

To obtain directly D- and L-enantiomers of [^{3}H]histidine in a copper-free state, the lower part (20 mm) of the chromatographic column was packed with copper-free resin I and the remainder of the resin (100 mm) was saturated with Cu^{2+} ions to 45% of the theoretical capacity. In contrast to many other amino acids, histidine enantiomers can be detected photometrically without being complexed with Cu^{2+}.

With resin II and phosphate-containing eluents, the fractions of resolved enantiomers have to be purified to remove mineral salts. Therefore, the enantiomers were sorbed on the Cu^{2+} form of the ANKB-50 iminodiacetate resin, washed with water and desorbed with 0.3 *N* ammonia solution.

The purity of the isolated enantiomers was tested by thin-layer chromatography (TLC) on Silufol UV-254 plates and by treatment with specific amino acid oxidases in a standard manner.

RESULTS AND DISCUSSION

When dealing with multiple tritiated amino acids of very high specific radioactivity, chromatography seems to be the method of choice for separating the optical isomers and purifying them simultaneously to remove the contaminating radiolysis products. One of the chiral resins suitable for this purpose is L-hydroxyproline incorporated in a macronet polystyrene with active sites of type I. When saturated with Cu^{2+}, this resin displays high enantioselectivity towards optical isomers, which allows one to resolve racemates of several amino acids[4].

In order to shorten the time of exposure of the sorbent to irradiation, measures should be taken to optimize the chromatography process. As gel diffusion is the rate-determining factor in establishing the ligand-exchange equilibrium between the resin phase and solution[2,5], enhancement of the chromatographic efficiency is achieved by using a sorbent of enhanced swellability and small particle size. Figs. 1 and 2 show the results of the preparative chromatographic resolution of enantiomers of DL-[^{3}H]-valine and DL-[^{3}H]histidine under optimized conditions. Here, resin of type I was used with particle diameter 25–32 μm (irregularly shaped particles). The degree of saturation of the resin with Cu^{2+} and the ammonia concentration in the eluent were selected so as to complete the process in 1–2 h. Fig. 1 clearly indicates the presence

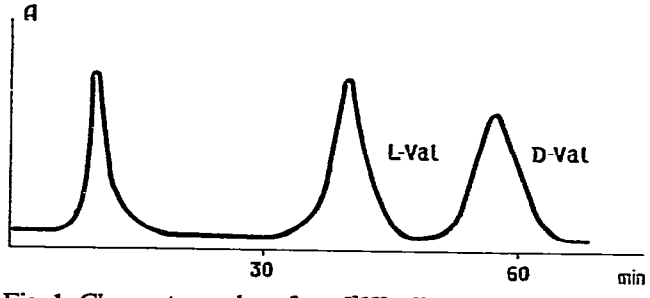


Fig. 1. Chromatography of DL-[^{3}H]valine (40 μg in 0.1 ml of water; activity $7.4 \cdot 10^8$ Bq; specific activity $2.1 \cdot 10^{12}$ Bq/mmol) on the L-hydroxyproline-containing resin I (particle diameter, d_p = 25–32 μm; saturation with Cu^{2+} 70%). Column, 300 × 4 mm I.D.; eluent, 0.15 *N* ammonia solution; flow-rate, 16 ml/h.

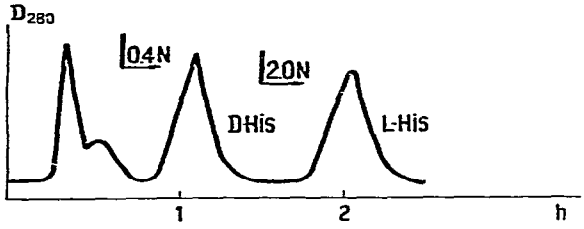


Fig. 2. Chromatography of DL-[^{3}H]histidine (500 μg in 0.1 ml of water; activity $7.4 \cdot 10^9$ Bq; specific activity $2.4 \cdot 10^{12}$ Bq/mmol) on the L-hydroproline-containing resin I (d_p = 25–32 μm; saturation with Cu^{2+} 45%). Column, 120 × 8 mm I.D. (lower 20 mm of the resin bed free of copper); eluent, 0.1, 0.4 and 2.0 *N* ammonia solution; flow-rate, 40 ml/h.

of radioactive contaminants in the starting racemate and the efficiency of the radiochemical purification of the enantiomers.

Fig. 3 demonstrates the analytical possibilities opened up by the ligand-exchange chromatography of racemates. The enantiomeric composition of an amino acid sample can be determined within 15–20 min on a 10-cm column of 2 mm I.D. filled with *ca.* 10-μm particles of resin I. Another approach for evaluating both the optical and radiochemical purity of the resolved enantiomers is the chromatography of, *e.g.*, $3.7 \cdot 10^7$ Bq of a labelled L-[^{3}H]amino acid in the presence of 1 mg of DL-amino acid. The distribution of radioactivity between the L- and D-fractions reflects the optical purity of the labelled product, and the total radioactivity yield in the two amino acid fractions represents its radiochemical purity. According to this test, the optical purity of enantiomers obtained by the proposed preparative resolution is not less than 99%. This is in agreement with the results of standard tests using specific amino acid oxidases. The radiochemical purity of the enantiomers exceeds 95% according to both the above chromatographic method and the standard method using TLC on Silufol UV-254 with different eluting mixtures.

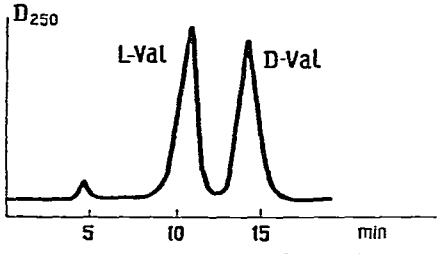


Fig. 3. Chromatography of DL-valine (15 μg) on the L-hydroproline-containing resin I ($d_p \approx 10$ μm; saturation with Cu^{2+} 70%). Column, 100 × 2 mm I.D.; eluent, 0.25 *N* ammonia solution; flow-rate, 5 ml/h; pressure, 20 bar.

Although easy to use, the L-hydroxyproline-containing polystyrene resin I displays insufficient enantioselectivity in the chromatography of aspartic and glutamic acids, asparagine, ornithine, lysine, methionine, alanine and α-aminobutyric acid[4]. These racemates, and many other amino acids, can best be resolved by using sorbent II obtained by treatment of Bio-Gel P-4 beads (particle diameter <64 μm) with formaldehyde and L-phenylalanine. This type of sorbent has been mentioned

elsewhere[6], but no enantioselectivity was found in resolution tests with proline and valine. In our experiments, sorbent II showed enantioselectivity of at least $\alpha = 1.25$ in all systems tested (see Table I) and a high enough efficiency to resolve all common amino acids. L-Enantiomers of amino acids are always eluated ahead of the D-isomers.

TABLE I

PARAMETERS OF AMINO ACID ELUTION ON THE L-PHENYLALANINE-CONTAINING POLYACRYLAMIDE RESIN II SATURATED WITH Cu^{2+} IONS TO 60%

Eluent, 2% ammonium phosphate solution, pH 9.2. k' = Capacity factor; α = separation factor.

Amino acid	k'_L	k'_D	α
Aspartic acid	1.02	1.34	1.31
Glutamic acid	1.13	1.50	1.32
Asparagine	3.04	4.12	1.35
Glutamine	1.47	2.20	1.50
Ornithine	2.84	3.78	1.33
Lysine	6.85	9.34	1.36
Serine	2.04	2.67	1.31
Threonine	2.52	3.36	1.33
Methionine	3.15	4.98	1.58
Alanine	1.68	2.31	1.36
Valine	1.53	2.35	1.55
Leucine	2.29	3.26	1.42
Norleucine	2.33	3.68	1.58
Isoleucine	1.74	2.79	1.60
Proline	3.19	5.33	1.65
allo-Hydroxyproline	5.25	6.59	1.25
Tyrosine	5.17	7.58	1.37
Phenylglycine	1.51	2.54	1.66
Phenylalanine	3.61	4.85	1.34
Tryptophan	8.95	12.7	1.42

Figs. 4 and 5 demonstrate the resolutions of racemic lysine and methionine and Fig. 6 the preparative resolution of DL-[^{3}H]alanine. The resolution of DL-[^{3}H]-glutamic acid on sorbent II was described earlier[7]. In all experiments with sorbent II, ammonium phosphate solution of pH 9.2 was used as the eluent. The purification of the resolved enantiomers to remove mineral salts and trace amounts of Cu^{2+} was effected using chelating resins saturated with Cu^{2+} and free of copper, respectively, as is described under Experimental.

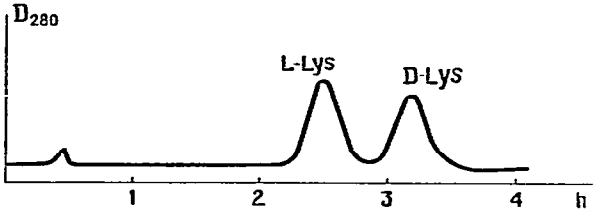


Fig. 4. Chromatography of DL-lysine (300 μg) on the L-phenylalanine-containing resin II ($d_p \leqslant 64$ μm; saturation with Cu^{2+} 60%). Column, 300 × 9 mm I.D.; eluent, 2% ammonium phosphate solution, pH 9.2; flow-rate, 30 ml/h.

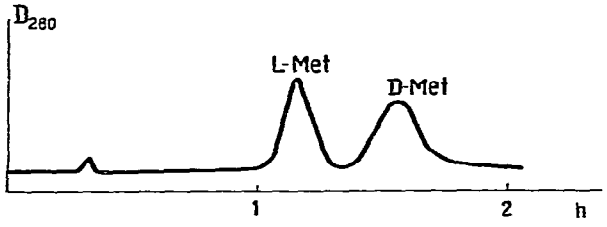


Fig. 5. Chromatography of DL-methionine (300 μg) on the L-phenylalanine-containing resin II (d_p < 64 μm; saturation with Cu^{2+} 70%). Column, 190 × 8 mm I.D.; eluent, 2.5% ammonium phosphate solution, pH 9.2; flow-rate, 25 ml/h.

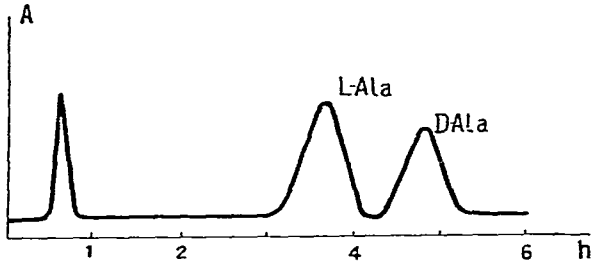


Fig. 6. Chromatography of DL-[^{3}H]alanine (315 μg in 0.1 ml of water; activity $5.5 \cdot 10^9$ Bq; specific activity $1.7 \cdot 10^{12}$ Bq/mmol) on the L-phenylalanine-containing resin II (d_p < 64 μm; saturation with Cu^{2+} 70%). Column, 300 × 9 mm I.D.; eluent, 0.1% ammonium phosphate solution, pH 9.2; flow-rate, 25 ml/h.

CONCLUSION

The reliability and efficiency of the ligand-exchange chromatographic resolution of racemates make it possible to obtain optically and radiochemically pure amino acids from a racemic stock solution shortly before they are required for use.

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