

RESOLUTION OF TRITIUM-LABELLED AMINO ACID RACEMATES BY
LIGAND-EXCHANGE CHROMATOGRAPHY. PART 1.

Method for obtaining L- and D-[³H]valine using a
polystyrene resin with L-hydroxyproline groupings

Myasoedov N.F., Kuznetsova O.B., Petrenik O.V.

Institute of Molecular Genetics, USSR Academy of Sciences

Davankov V.A., Zolotarev Yu.A.

Institute of Organo-Element Compounds, USSR Academy of
Sciences

SUMMARY

Ligand-exchange chromatography is shown to be applicable for obtaining the optical isomers of [³H]valine. Data are presented showing the effect of the asymmetric sorbent's structure on its kinetic properties and the optimum conditions selected for the chromatographic resolution of D,L-[³H]valine. The method makes it possible to obtain both isomers of [³H]valine with an optical purity close to 100% in about 2 hours with a simultaneous radiochemical purification. The method does not require any preliminary chemical modification of the amino acid and does not impose any limitations to specific activity values.

INTRODUCTION

Tritium-labelled natural L-amino acids are usually obtained by resolving the appropriate racemates. The methods acceptable for isolating labelled optical isomers may roughly be classified into chromatographic and biological ones.

The numerous chromatographic methods [1] include: chromatography of diastereomeric compounds [2], the use of sorbents grafted with optically active crown-ethers [3], ion-exchange chromatography on sorbents containing L-arginine [4], and ligand-exchange chromatography [5]. The biological techniques are mostly based on the stereoselective action of enzymes [6].

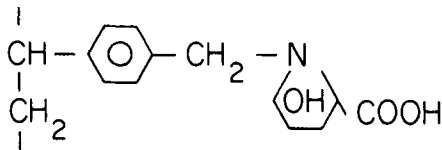
In spite of all this diversity, the range of methods for resolving racemic mixtures is rather limited where labelled compounds are concerned. For instance, all methods involving the introduction of reactive groups or their protection and thereby leading to a decreased yield, i.e. a loss of radioactive substances, become inapplicable. Any methods involving multi-stage separation processes are unsatisfactory for the same reason. Another important limitation is the requirement for using small quantities of the initial product, especially when dealing with preparations possessing a high specific activity.

These are the reasons why a combination of stereospecific enzymes and chromatographic methods is most often used for obtaining tritium-labelled L-amino acids [7-11]. This procedure, however, is time-consuming and cannot always be applied to amino acid preparations with high specific activities since enzymatic activity can under certain conditions be inhibited by radiation [11].

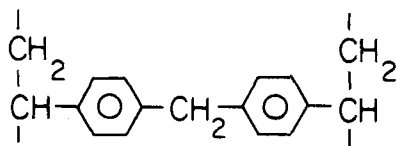
Ligand-exchange chromatography shows a high selectivity when separating substances with similar molecular structures, including optical isomers [12]. Asymmetric sorbents incorporating groups of L-amino acids in the Cu(II) form have been demonstrated to possess an extremely high enantioselectivity with regard to the racemates of many amino acids [13-15]. Proceeding from this, we selected the conditions for a chromatographic resolution of tritium-labelled racemates of amino acids with high specific activities into optically pure isomers. This paper describes the results of a research for the conditions of such a chromatographic separation. Data are cited for the resolution of D,L- $[^3\text{H}]$ valine with a specific activity of 38Ci/mmol into optically pure isomers.

MATERIALS AND METHODS

Sorbents. Asymmetric sorbents with L-hydroxyproline (see Table 1) were obtained by aminating chloromethylated macro-net polystyrene matrix with methyl-L-hydroxyprolinate hydrochloride according to the procedure previously described [16]. The active sites of the final sorbent have the following structure:



We synthesized sorbents 1,2,3 using the macro-net polystyrene containing 11 percent of cross-links of the structure



Sorbents 4 and 5 were obtained on a gel styrene copolymer containing 1% and 0.1% of divinylbenzene respectively. Sorbents 6 and 7 were obtained on a matrix including 0.7% divinylbenzene and 5.3% of additional cross-links of the diphenylmethylen type introduced as previously described [16].

Saturation of sorbents with copper(II) ions. 2-3g of air dried sorbent was equilibrated with an excess of copper(II) ammonia solution. To obtain the required extent of saturation, the excess copper was removed by washing with 2N NH_4OH containing a certain amount of KCl [17]. After phase separation the sorbent was finally washed on a filter with 0.1N NH_4OH .

The kinetics of acid-base equilibration. Measurements were made in a four-necked vessel with a stirrer. The weighed amount of sorbent comprising 1.00 mmole of fixed groupings was introduced into the vessel. A pH-meter salt bridge and glass electrode were immersed into the solution. After equilibration for 3 hours, 5ml of 0.10 M KOH in a 1 M KCl solution were quickly added to the mixture and the changing acidity values were measured under continuous stirring.

The kinetics of establishment of the interphase ligand-exchange equilibrium.

80ml of 0.05 M proline solution with pH=11.2 were introduced into a four-necked vessel equipped with a stirrer. A batch of swollen sorbent in the copper form comprising 1.00 mmole of fixed groupings was quickly introduced into the vigorously stirred solution. About 2ml of the reaction solution were rapidly taken out with a syringe pipette and transferred into a special porous-bottomed column where rapid phase separation by compressed air was effected. The changing copper bis-prolinate content of the aqueous phase was evaluated from the absorption measurements at $\lambda_0 = 650\text{nm}$ on a Specol spectrophotometer. The final equilibrium solution was analysed for copper content spectrophotometrically with sodium diethyl carbamate on the same instrument at $\lambda_0 = 440\text{nm}$.

Chromatography of racemates. The copper-saturated sorbent suspended in 0.1 N NH_4OH was transferred into a glass column (7.8 x 140mm) and equilibrated with 0.1N NH_4OH for 1 hour. Amino acid samples in quantities of 0.1 to 1.0mg were introduced onto the column by a microsyringe into the sorbent layer through a special lateral inlet. Elution by 0.1N NH_4OH was carried out at a flow rate of 10 to 25ml per hour. The separation process was detected by Uvicord III, $\lambda_0 = 206\text{nm}$, or radio-metrically by liquid scintillation counting the radioactivity of individual fractions.

Peaks were assigned to the L- or D-isomer of the amino acid either by chromatography of individual isomers, or according to the effect of amino acid oxidases specific to a particular isomer [6].

Determination of the optical purity of amino acids. The optical purity of L- and D-amino acids was determined by oxidative de-amination under treatment with the L-amino acid oxidase from Crotalus adamanteus (Sigma) and the D-amino acid oxidase from pig kidney (Koch-Light) under standard conditions [6].

The products of the enzymatic reaction were analysed by means of thin layer chromatography on Silufol UV-254 plates in the system isobutanol: acetone: ammonia (12%) 15:9:6. Chromatograms were developed by spraying with a 0.5% solution of ninhydrin in acetone.

The preparation of DL-[³H]valine with a specific activity of 38Ci/mmol was synthesized in the Institute of Molecular Genetics, USSR Academy of Sciences.

Radiometry was carried out by means of a liquid scintillation counter with a tritium counting efficiency of approximately 12%.

Control of the radiochemical purity of the initial and final preparations was determined by means of thin-layer chromatography in different solvent systems.

Purification of amino acid isomers of trace amounts of copper was achieved on a column (0.6 x 80mm) with the Amberlite IRC-50 cation exchange resin by elution with water at a flow rate of 72ml per hour. The volume of fractions was 1.8ml. The amino acid was eluted in a volume of ca 20ml.

RESULTS AND DISCUSSION

The high enantioselectivity of ligand-exchange chromatography in respect of amino acid isomers and the possibility to avoid chemical modification of the initial racemate makes it a promising method for preparative separation of labelled racemic mixtures.

However data available by the time we began our study indicated that the high enantioselectivity of racemate sorption by chelating asymmetric sorbents in the Cu(II) form was not yet fully realized because of poor efficiency of ligand-exchange chromatography [18,19]. Hence the chromatographic process lasted for several hours and the separation of racemates was only partial.

Now to obtain optically pure isomers of radioactive isotope-labelled amino acids, one needs a full separation of racemates to be completed in a short period of time. This requirement arises from the presence of high concentrations of radioactivity in the narrow chromatographic zone, causing the sorbent to be exposed to considerable radiation doses.

The problem of improving the efficiency of ligand-exchange chromatographic separation of amino acid enantiomers was tackled by looking for the optimum structure of the sorbent matrix. Such factors as the extent of cross-linking, the swelling capacity, the shape and size of resin particles were taken into account. Additionally, the rate of acid-base and ligand-exchange equilibrium was established and also the sorbent efficiency in the chromatographic process.

To investigate the kinetic properties we synthesized a number of hydroxypoline incorporated sorbents with different matrix structures. Their characteristics are presented in Table 1. Data on the rate of acid-base and ligand-exchange equilibration for these sorbents are shown in Figures 1 and 2.

Table I

THE PROPERTIES OF SORBENTS WITH L-HYDROXYPROLINE GROUPINGS

Sorbent	Extent of cross-linking %	Capacity mmol/g	Swelling capacity weight %	Size of particles μm
1	11	3,44	200	100
2	11	2,46	120	100
3	11	1,48	70	100
4	1	3,52	200	100
5	0,1	3,50	1000	100
6	6	3,86	250	300
7	6	3,86	250	50

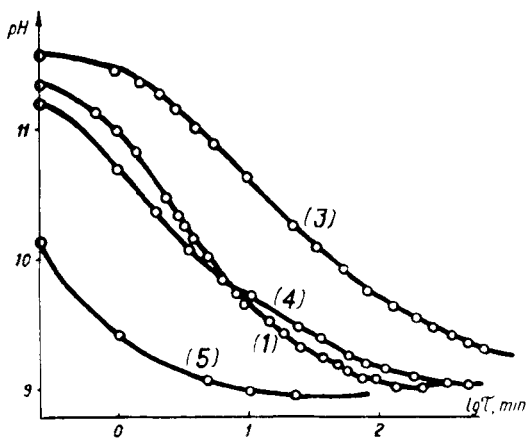


Figure 1 Kinetics of equilibration with alkali added to sorbents (1), (3), (4) and (5).

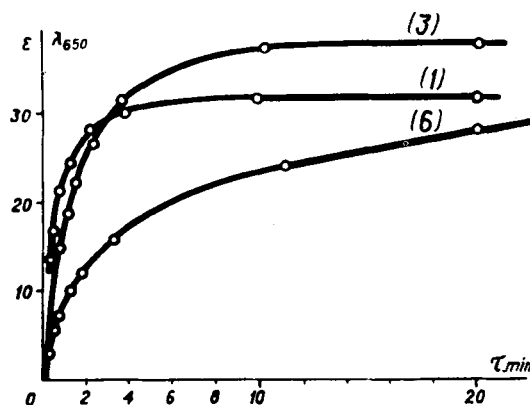


Figure 2 Kinetics of ligand-exchange equilibration with proline added to sorbents (1), (3) and (6) in the copper form

The sorbents on the macro-net type (1) and the standard gel-type (4) polystyrene matrix show a swelling capacity of about 200% in weakly alkaline solutions and, despite the substantial difference in their cross-linking (11% and 1% respectively), reach an acid-base equilibrium simultaneously in about 3 hours (Fig.1). Sorbent (3) with a lower exchange capacity shows a water uptake of 70% and its equilibration time is 48 hours. The swelling capacity of sorbent (5) (cross-linking of 0.1%) amounts to 1000% and, correspondingly, the equilibrium is reached in 10 minutes. The above data show that the rate of the interphase acid-base equilibration is mainly determined by the swelling capacity of sorbents.

Figure 2 presents data on the rate of ligand-exchange interphase equilibration. When the copper form of the sorbent interacts with an 0.05M hydroxyproline alkaline solution, the period of half-exchange is about 0.3 and 0.8 minutes for sorbents (1) and (3) respectively. Therefore an increase in the swelling capacity leads to a higher rate of ligand-exchange equilibration too.

Figure 2 also shows the effect of the size of sorbent particles on ligand-exchange kinetics. The equilibration time for sorbent (6) is about an order of magnitude longer than that for sorbent (1) (swelling capacities 250% and 200%, particle size 300 μ m and 100 μ m respectively). This pronounced dependence of the ligand-exchange equilibration time on the size of particles could be explained if the gel diffusion were the rate limiting stage of the process. In this case the equilibration time would be proportional to the square of the particle size [20].

Thus the above study has shown that the particle size should be decreased and the swelling of sorbents increased in order to achieve an efficient chromatographic process.

We synthesized resin (7) which best conformed to the above requirements and used it for all further research. Indeed, as shown in Figure 3, by passing over from sorbent (3) to sorbent (7) we achieved a substantial gain in efficiency. Using sorbent (7) we managed to carry out a quantitative separation of valine isomers in an acceptable period of time.

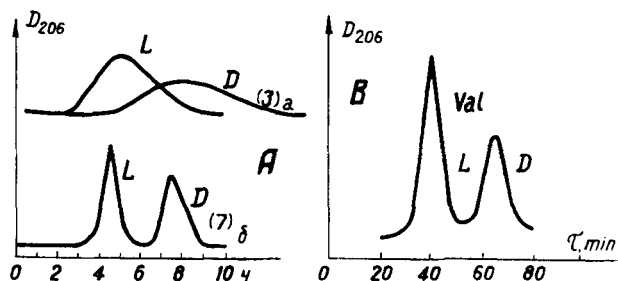


Figure 3 Chromatography of valine enantiomers on asymmetric sorbents (3) and (7), with 100% (A) and on sorbent (7) with 65% (B) saturation with Cu(II) ions.

Chromatography time may also be shortened by changing the degree of saturation of sorbent with Cu(II) cations. The sorbent's copper content strongly influences the parameters of amino acid retention. A decrease in the copper content causes rapid decrease in the amino acid retention time. The optimum saturation by Cu(II) cations depends on the nature of the amino acid concerned. In the case of valine isomers, saturation of 65% of the maximal theoretical Cu(II) content and elution by 0,1N ammonia proved to be optimum. Under these conditions we achieved a full separation of D,L-valine into optically pure isomers in about 2 hours, which allowed us to effect a preparative-scale separation of tritium-labelled D,L-valine.

Figure 4 shows the elution curve for 54.5mCi of D,L- $[^3\text{H}]$ valine with a specific activity of 38Ci/mmol and a radiochemical purity of 90%. About 170 μg of tritium-labelled D,L-valine in a volume of 250 μl were introduced

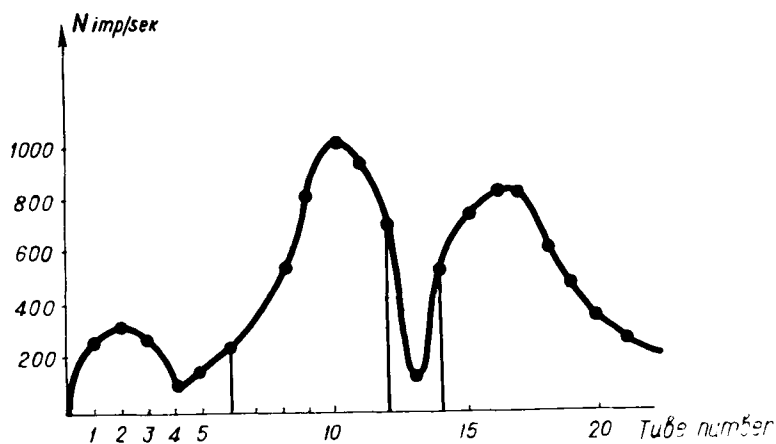


Figure 4 Elution curve for the chromatography of D,L- $[^3\text{H}]$ valine (38Ci/mmol) on a column (7.8 x 140mm). Sorbent (7) had a Cu(II) saturation degree of 65%. Elution by 0.1N NH_4OH at a flow rate of 13ml per hour.

into the column (7.8mm I.D., height 140mm) filled with sorbent (7). Unlike the situation in Figure 3, the elution curve for the labelled D,L-valine shows three peaks with respect of radioactivity. The first peak (4.4mCi), eluting immediately after the void volume, comprises non-amino acid products. L-Valine (20.6mCi) was collected in fractions 6-12 and D-valine (24.8mCi) in fractions 14-25. During the purification of the solutions from traces of copper no more than 3% of the product were lost.

The optical purity of both isomers, determined by use of the appropriate stereoselective oxidases, was over 99%. According to the results of repeated ligand-exchange chromatography, the value was no worse than 99%. Hence we had obtained optically pure isomers of [³H]valine.

The radioactivity eluted within the 1 peak of ligand-exchange chromatography coincides with the value of radiochemical purity of the initial preparation, obtained by thin-layer chromatography. This means that the ligand-exchange chromatography of racemic amino acids radiochemically purifies the preparations also from all non-amino acid impurities.

The specific activity of [³H]valine preparations remains unchanged after the chromatographic separation of enantiomers.

The sorbent used showed practically no non-specific sorption: the balance of the collected radioactivity amounted to 96.3%.

Thus a method has been developed for obtaining both optically pure isomers of [³H]valine with a high specific activity and a high radiochemical purity by a simple chromatographic procedure.

REFERENCES

1. LOCHMULLER C.H., SOUTER R.W. *J.Chromat.*, 113, (1975), 283
2. VALENTINE D., CHAN K.K., SCOTT C.G. - *J.Org.Chem.*41 (1976), 62
3. DUTSEVI G., SOGAK Y., CRAM D.J. - *J.Amer.Chem.Soc.*, 98 (1976), 3038.
4. BACZUK R.J., LANDRAM G.K., DUBOIS R.J. - *J.Chromat.*, 60 (1971), 351.
5. ROGOZHIN S.V., DAVANKOV V.A. *Dokl.Acad.Sci. USSR*, 192 (1970), 1288.
6. GREENSTEIN J.P., WINITZ M. *Chemistry of the amino acids*. N.Y. - London. John Wiley & Sons, inc., 1961.
7. GORDAY Ch., VERLY W.G. - *J.Lab.Comp.*, 4 (1968), 334.
8. BRUNDISH D.E., ELLIOT D.F., WADE R. - *J.Lab.Comp.*, 7 (1971), 473.
9. JOHANNSEN B., SYHRE R., BERGER R. - *J.Lab.Comp.*, 8 (1972), 475.
10. RAMAMURTHY T.V., PICHAT L. - *J.Lab.Comp.*, 9 (1973), 325.
11. FIGOREVA N.S. *Candidate thesis, Leningrad*, 1974.
12. DAVANKOV V.A., SEMECHKIN A.V. - *J.Chromat.*, 141 (1977), 313.
13. DAVANKOV V.A., ZOLOTAREV Yu.A. - *J.Chromat.*, 155 (1978), 285.

14. DAVANKOV V.A., ZOLOTAREV Yu.A. - J.Chromat., 155 (1978), 295.
15. DAVANKOV V.A., ZOLOTAREV Yu.A. - J.Chromat., 155 (1978), 303.
16. ZOLOTAREV Yu.A., KURGANOV A.A., DAVANKOV V.A. - Talanta, 25 (1978), 493.
17. DAVANKOV V.A., ZOLOTAREV Yu.A., TEVLIN A.B. - Bioorg.Khim. (USSR), 4, (1978), 1164.
18. DAVANKOV V.A., ROGOZHIN S.V., SEMECHKIN A.V., BARANOV V.A., SANNIKOVA G.S. - J.Chromat., 93 (1974), 363.
19. SEMECHKIN A.V., ROGOZHIN S.V., DAVANKOV V.A. - J.Chromat., 131 (1977), 65.
20. BOYD G., ADAMSON A., MARYNSKY L. - J.Amer.Chem.Soc., 69 (1947), 2836.