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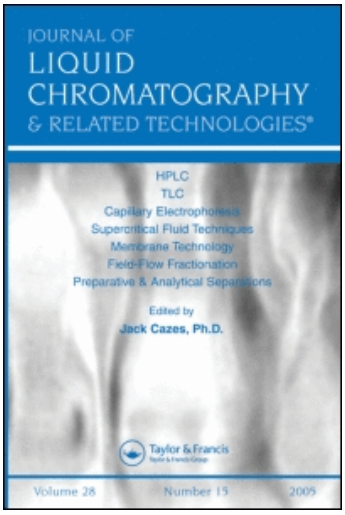

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Retention Mechanism in Ligand exchange Chromatography of α-Amino Acids: Improved Resolution of Racemates with A New Mutildentate Polymeric Packing

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RETENTION MECHANISM IN LIGAND EXCHANGE CHROMATOGRAPHY OF α-AMINO ACIDS: IMPROVED RESOLUTION OF RACEMATES WITH A NEW MUTILDENTATE POLYMERIC PACKING

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ABSTRACT

On the basis of the expected mechanism of chiral recognition in ligand exchange chromatography, a new chiral packing was prepared and tested. The optically active phase is a copolymer of acrylamide and of a vinylpyridine monomer substituted by an L-proline moiety. The packing is obtained by simple coating of a chromatographic silica followed by a convenient complexation with cupric ions. The selectivity factor, α, for such a packing is high : it is between 2 and 8 for most amino acids.

INTRODUCTION

Resolution of enantiomers by liquid chromatography reported during the past fifteen years proceeds through a chiral eluent or via an optically active stationary phase (3-5). With both approaches, systems involving ligand exchange are described as particularly efficient (3,4,6).

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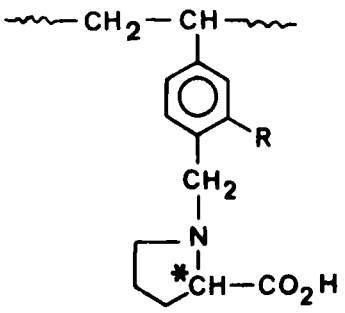
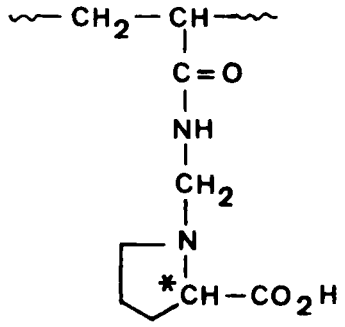
It immediately appears that, for chromatography on a chiral packing, the sorbent matrix plays a major role in the stereoselection mechanism. For a given chiral graft (L-proline) and the same complexing metallic ion, Cu(II), but based either upon a styrenic framework (I, R = H) or an acrylamide type polymer (II) the elution order is practically inverted (6) :



(I)



(II)

When, with most α-amino acids, the L-form is the less retained on type I packing, reverse order is observed with the other stationary phase.

In both systems, complexes solute/copper/graft (D or L) are involved (respectively structures III and IV). But with the polystyrenic backbone (III) the solute in the D-form is the more complexed due to hydrophobic interactions between its substitutent Z and the lipophilic part of the packing. Conversely, with a hydrophilic packing of the polyacrylamide type (IV), steric hindrance occurs between Z and the macromolecular chain when it may be involved directly in a complexation with copper in apical position (7-14).

Such a mechanism is supported by the results obtained with packings of type IV but with a hydroxyl group complexed in the apical position instead of the carbonyl group : the elution order is unmodified (15).

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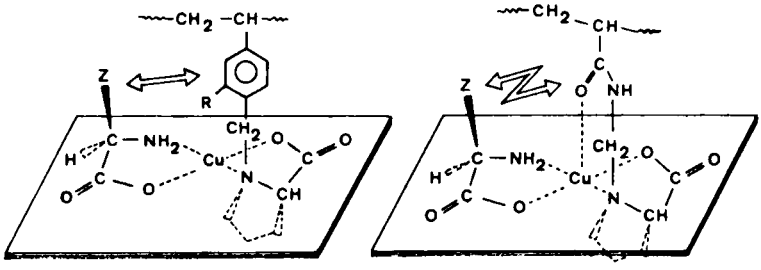
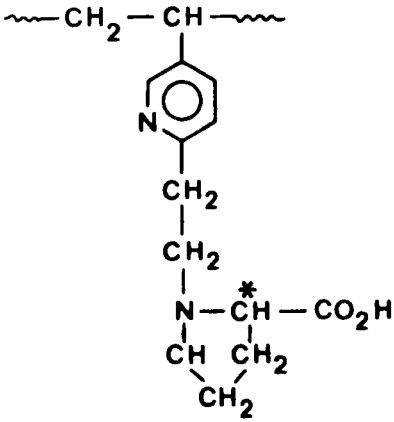
On the other hand, the use of hydrophobic substituents for R in type III packing was shown recently to increase the hydrophobic interaction for the substituents of D-α-amino acids and consequently the resolution as expected.

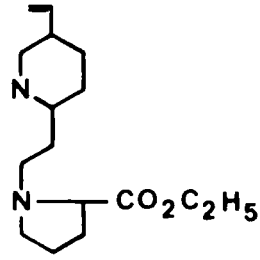


III D-solute/copper/graft IV D-solute/copper/graft

At the same time, according to an idea also based on these mechanisms we tried (11) to improve the efficiency of type IV packing by substituting the carbonyl group with a pyridinic site, leading to a stronger complexation and greater steric hindrance. Packings with structure V were prepared. They had the same elution order as packings IV and the strong improvement in selectivity that was expected.



V



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SYNTHESIS OF THE PACKING

The complete synthesis, in five steps, is described elsewhere (11,16). A vinylic ethyl ester of L-proline is obtained (VI) ; it is homopolymerized or copolymerized with acrylamide according to the classical radical technique before the hydrolysis leading to the structure V. Each product is characterized by the molar ratio, x, of the chiral units in the copolymer. According to gel permeation chromatography measurements, the molecular weight of these polymers is in the range 30,000 - 60,000 with a polydispersity of about 2.

COMPLEXATION BY COPPER

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With polymers of type IV, in absence of solute, the copper is involved in "sandwich" type complexes graft/copper/graft and the maximum rate r = [copper]/[graft] is 0.5. In the case of our new polymers, because of the bulkiness of the pyridinc substituent, the formation of such complexes is not favored. Titration of Cl^-, Cu^{++} and H^+ associated with ultrafiltration of polymer solutions shows that, whatever the comonomer and the rate of copolymerization x, the complexes formed are mainly of 1-1 type (1 proline for 1 cupric ion) ; less than 10% of copper is involved in 2-1 complexes. As for complexes of type IV (8,9) the level of the copper fixation is pH dependent (see fig. 1).

For pH = 6, a tridentate complex involving the pyridinic nuclei (VII) is supported by spectroscopic data : the maximum of U.V. adsorption spectra of copper/polymer system is 655nm, value between the observed maximum for tetracoordinated systems anminoacid/Cu/amino acid (600-620nm) and the di-ccordinated complexes Cu/amino acid (720-730nm) (17). In addition, an E.P.R. study was performed (Varian apparatus 9140 GHZ) with a sample obtained by lyophilization of a polymer solution with a low load of copper (r = 0.05) (11). It confirms a coordination in apical position in agreement with the structure VII.

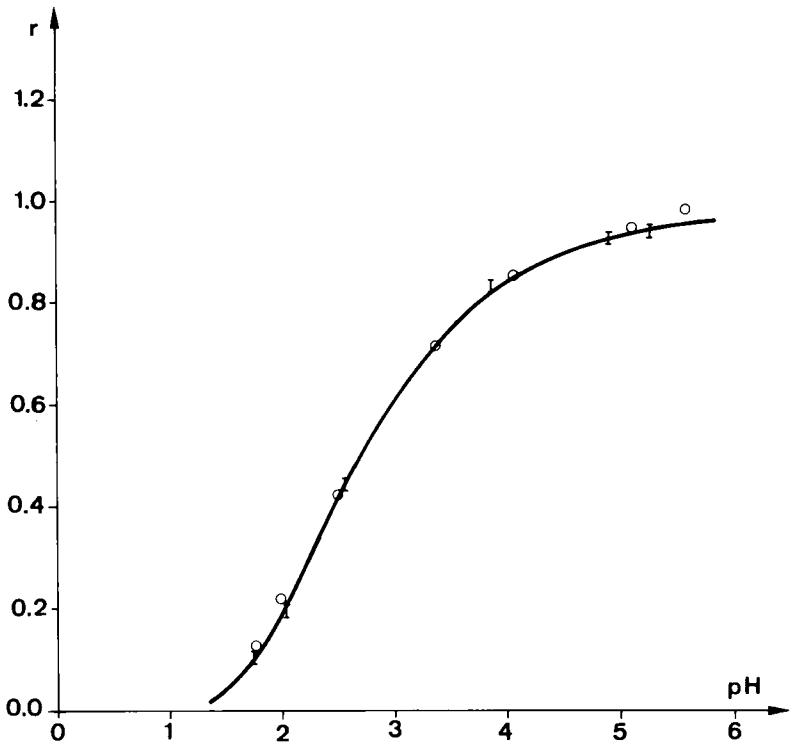
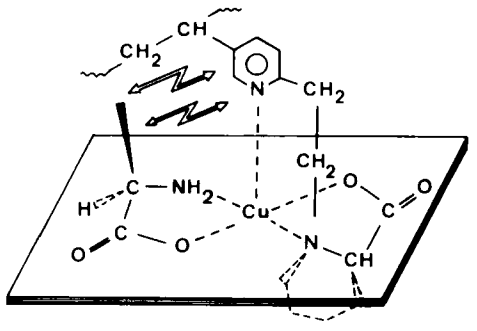


Figure 1 : Evolution of the ratio r = [copper]/[proline] versus the pH of surronding cupric solution ○ homopolymer type V

I copolymer with acrylamide

x = 0.39



VII

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Hydroxylated species like graft/Cu/OH occurred progressively at pH's higher than 5. This trend to hydrolysis of copper-α-amino acid complexes has been previously pointed out (18-20).

PREPARATION OF THE PACKINGS

Packings are easily prepared by adsorption of chiral polymers onto a chromatographic silica. Typically, 2g of silica (Partisil-5, Whatman, Inc. Clifton, NJ, USA) are added to 20ml of a 2% w/w solution of polymer and agitated for two hours. The beads are then filtered and rinsed with water. The titration of polymer in the filtrate gives, by difference, the load of polymer adsorbed. Under these conditions the saturation of silica is obtained, that is 11-12% in weight of polymer/weight of silica as well for homopolymer (type V) as for copolymers with acrylamide units. Beads are then treated with a solution of 0.05N $CuCl_2$ in a convenient buffer to adjust the r value (see fig.1) and avoid direct fixation of Cu^{++} on silica which occurs at pH's higher than 5.5(22) (for most of our experiments we used the formic/formate buffer pH = 3.8). The column (15cm long, 0.47cm of internal diameter) is filled by classical slurry packing and rinsed with the eluent.

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CHROMATOGRAPHIC CONDITIONS

Experiments were performed with a Waters ALC 201 apparatus (Waters Assoc., Milford, Mass., USA) equipped with a U6K injector and, for detection, a UV spectrometer (Waters MC-440) and a differential refractometer (Waters R401). Occasionally a spectrophotopolarimeter (Perkin Elmer 241 MC) was used. The eluent is a 0.02 molar solution of KNO_3 and the flow rate 1ml/min. The level of copper loading (r) and the molar ratio of proline units in the copolymer (x) are given for each experiment.

RESULTS AND DISCUSSION

Influence of the ratio of chiral units in the copolymer

With polymers based on acrylamide (type II), a maximum of the capacity factor k' and of the selectivity $\alpha = k'_D/k'_L$ is observed versus the molar ratio of chiral units in the polymer (9). This observation is coherent with the formation of graft/Cu/graft complexes; it is not found here (fig.2) where this kind of complex is always in very low concentration. However, there is no linear relation between k' and the capacity of proline of the packing (or x). This suggests a possible participation of the amide group of acrylamide comonomer in the stereo-complex which gives only a slight negative effect on the selectivity (fig.2). From a practical standpoint, the duration of the experiment can be greatly reduced by using a copolymer with x in the range 0.3-0.6 without significant loss of selectivity.

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Influence of the copper/proline ratio

A complete knowledge of all the equilibria involved in the system must, theoretically, lead to a relation between k and r. For instance, for packings II, where complexes proline/Cu/proline only are involved, the capacity factor is proportional to r/(1-2r), which is effectively observed (9). If only complexes proline/graft exist, one can predict a linear relation between k' and r. In fact, with our new packings both 1-1 and, for a minor part, 2-1 complexes are involved and the effective evolution of k' versus r is given in the fig.3. On this same figure, the resolution for racemic valine is plotted. The shapes of the curves are the same for other amino acid solutes, so the best practical range of copper complexation for the packings is $0.3 < r < 0.8$, that is, a pH in the range 2-4 for the copper complexation of the packing.

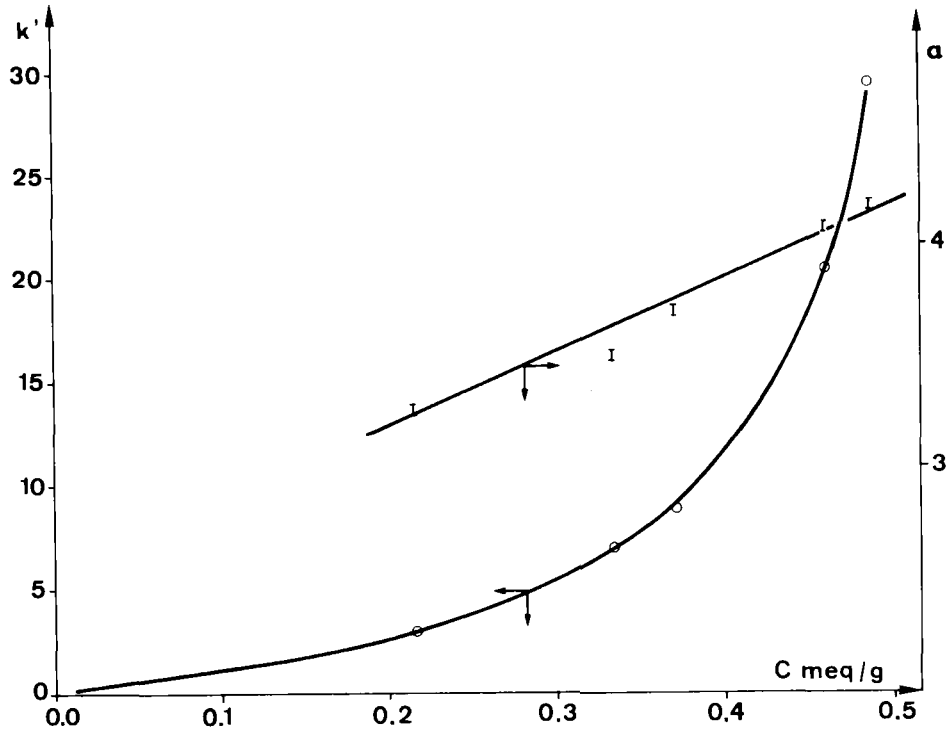

Figure 2 : Influence of the L-proline capacity, C, of various copolymers on the capacity factor k' measured with D-valine and the selectivity factor $\alpha = k'_D/k'_L$ determined with D,L-valine. The packings were prepared in a formic buffer.

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Chromatographic results

Retention data for about twenty amino acids are given in Table I for a pellicular packing based on copolymer with pyridinic groups (type V, x = 0.4, r = 0.8, eluent : KNO_3 0.02M). They are compared with our results obtained with a gel based on acrylamide partly grafted with L-proline moieties (ref. 8, type II, x = 0.3, R = 0.4, eluent : pure water).

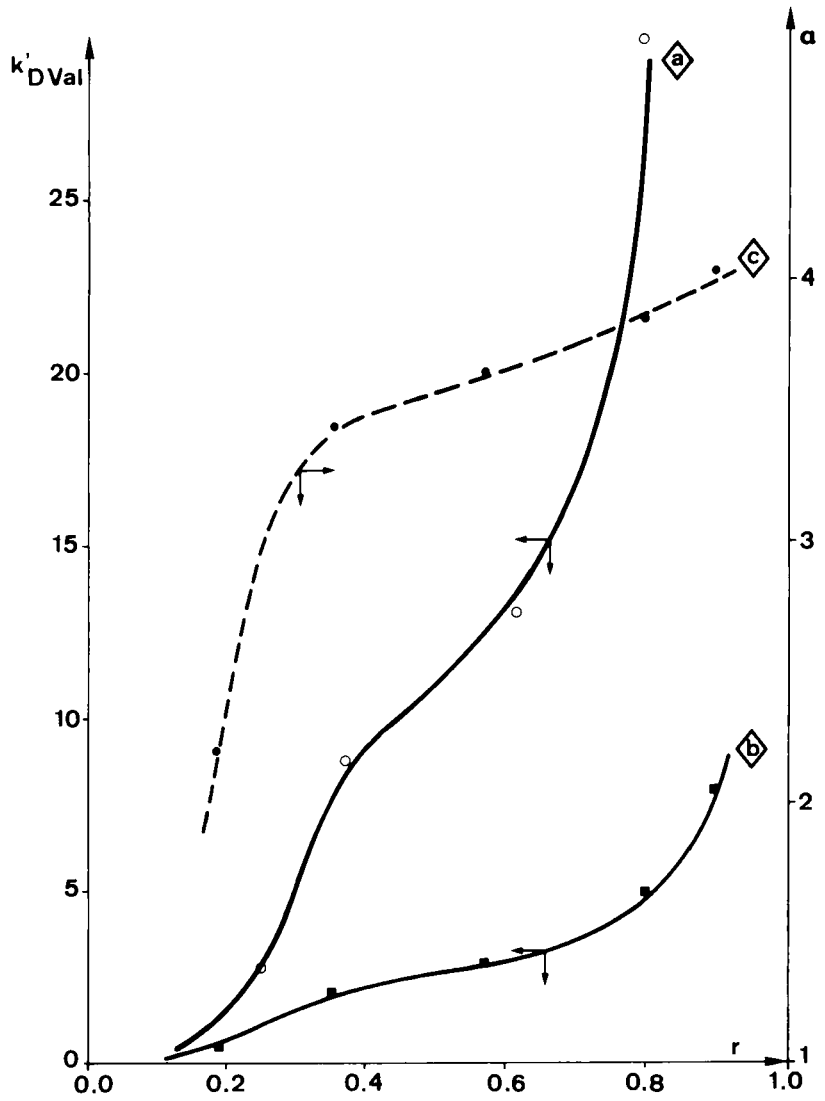

Figure 3: Influence of the copper ratio r = [copper[/[proline] on the capacity factor k' measured with D valine and rhe selectivity factor α determined with D,L-valine.

a : homopolymer of type V

b and c : copolymer with acrylamide

x = 0.4

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For the two kinds of packings, the D isomer is the first eluted (except for the cyclic solutes like proline or pipecolic acid) which support the idea that the stereoselection mechanism is similar for both.

On the other hand, in accordance with the idea of an increasing selectivity with the bulkiness of the copper complexing site in apical position, the selectivity is better for the pyridinic packing (except for serine) than for our type II packing. Even for a solute with a small substituent like alanine, α is always higher than 2 for other amino acids. However acidic solutes like aspartic acid or glutamic acid are not eluted from the new basic packing.

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Semi preparative assays

A major problem in chromatographic resolution through multidentate ligand exchange chromatography is the poor chromatographic efficicency generally observed with such packings, due to the slowness of ligand exchange. Practically, although the diameter of the silica beads coated by the pyridinic polymer is 5µm, the observed number of theoretical plates for the column is only about 400/m (measured for D-valine at room temperature). The use of a ternary eluent may lead to classical values (10 000-20 000 plates/m) of efficiency (11,16), but the system is not convenient for preparative purposes because of the very low solubility of the solutes in the eluent. On the other hand, 5µm silica beads are generally considered to be too expensive, for use in a preparative column.

With a silica packing "Lichroprep Si-100", 40-60µm (Merck) coated according to the same process as our analytical packing (pyridinic copolymer x = 0.47, r = 0.9), we prepared a 90cm long by 2.7cm internal diameter column. The eluent was a 0.02 molar solution of KNO_3. The poor efficiency is balanced by the high selectivity. An example of resolution (D,L ornithine) is given in fig.4. The system was tested for many α-amino acids and the maximum sample

Table 1 : Comparison of type II and type V packings : retention data

Solute	Copolymer (type V)		Gel based upon acrylamide (type II) (6)	
	k'_2	α	k'_2	α
Alamine	21	1.85	0.8	1.1
Amino butyric acid	21	2.85	1	1.4
Valine	27	3.7	1.5	1.9
Norvaline	22	2.50	1.2	1.4
Leucine	27	2.0	1.7	1.0
Norleucine	29	2.30	1.7	1.3
Serine	41	1.35	3.1	1.9
Methionine	40	2.50	2.6	1.0
Aspartic acid		-	1.2	1.0
Glutamic acid		-	0.5	1.0
Diamino butyric acid	7.3	4.0	4.5	1.3
Ornithine	7.1	4.3	3	1.3
Lysine	3.5	4.2	2.4	1.3
Arginine	4.4	3.0	4.5	1
Phénylalanine	30-3.4*	3.04-2.2*	9.8	2.3
Tyrosine	21*	2.7*	24	2.50
Tryptophan	23.6	2.25	54	2.50
Proline	16	1/8.1	1.1	1/1.50
Pipecolic acid	2.6	1/1.4	0.2	-

* r = 0.25

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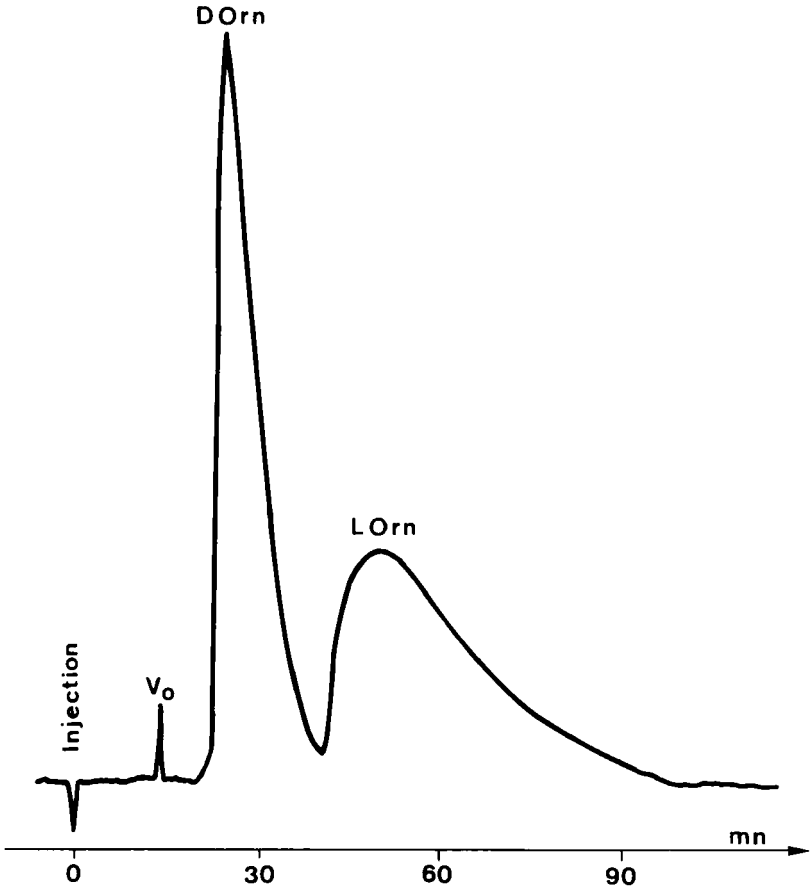

Figure 4: Semi-preparative resolution of D,L-ornithine (40mg).
Column L = 90cm, 2.7cm I.D.
Packing silica "Lichroprep" Si-100 coated 5% w/w with a copolymer
x = 0.47, r = 0.9
Eluent KNO_3 0.02M - 25°C.

Table 2 : Evaluation of type V packing in preparative chromatography

Solute	Weight injected (mg)	Weight of solute / Weight of stationary phase
DL Proline	250	0.029
DL Valine	80	0.01
DL Ornithine	40	0.005
DL diaminobutyric acid	40	0.005
DL Lysine	40	0.005

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load which can be completely separated in one run is given in the Table II.

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