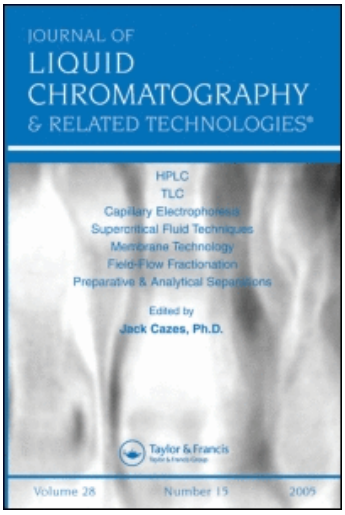

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USE OF NEW CHIRAL HYDROPHILIC GELS FOR THE DIRECT RESOLUTION α-AMINOACIDS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY::

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ABSTRACT

Total resolution of α-aminoacids was achieved by H P L C using porous gels based on acrylamide grafted with chiral substituents (L-α aminoacids) and complexed with metal ions.

We discuss the influence of the structure of the gel (porosity, hydrophilicity and coordination affinity), of the kinetics of the ligand exchange, of the nature of the complexing ion and of the chiral graft.

INTRODUCTION

Resolution of enantiomers has theoretical interest in the study of interactions between chiral molecules, as well as industrial importance.

Chromatography is well known as a very efficient technique of separation and the attempts to resolve enantiomers in this way are almost as old as chromatography itself ([1-5]).

For α-aminoacids their first complete resolution was performed by gas chromatography ([7-8]). Unfortunately this success, which undoubtely stimulated new investigations in this field, was possible only

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through a derivatization of the solutes (aminoacids are injected in the form of N-trifluoroacetylesters).

Also, the technique is not easily applied on a preparative scale. For this last aspect, liquid chromatography is advantageous. The best results are achieved by using synthetic optically active stationary phases. They are obtained by grafting a chiral substituent on cross-linked polymers, generally based upon polystyrene or acrylics.

Through asymmetrical crown ethers are potentially very efficient grafts for such stationary phases ([9-13]) other, less sophisticated, chiral substituents are generally used (for recent references see ([14-22]). As early as 1970 Bernauer ([26]), Rogozhin and Davankov ([24,25]) and almost simultaneously Angelici *et al.*([27]) suggested the use of a stationary phase where the graft (usually a L-α aminoacid) is complexed by a transition metal ion. For instance, L-proline is grafted by its reaction with chloromethylated polystyrene gels.

In this way, some total resolutions were observed. However, the efficiency of the system is not very high : for a given packing, only few racemates are completely resolved and the time for each experiment is long (typically ten hours). We have prepared new supports for high performance liquid chromatography. They are macromolecular gels grafted with asymmetric active aminoacids complexed by metal ions. These packings have a large efficiency and allow rapid resolution ([28-31]).

Four parameters play an important role in the efficiency of the system : the structure of the gel, the rate of exchange of ligands between the solution and the gel, the nature of the complexing ion and the nature of chiral graft.

EXPERIMENTAL

Nature of the packing

The packing is obtained in two steps ([28,29,31]) : 1) by pearl copolymerization of acrylamide and methylene bisacrylamide, we obtained a porous gel. The typical feature of the beads, *i.e.*granu-

lometry between ten and twenty microns, porosity of about 0.4.cm^3/cm^3 of wet resin and good mechanical properties, are suitable for use in H P L C. 2) An L-α aminoacid is easily grafted onto these pearls through a reaction with formaldehyde. An illustration of the structure is given in Fig.1 with proline, the amont of graft linked varies from 0 to 3 meq./g of dry resin.

Finally, the beads are shaken with a aqueous salt solution of the complexing metal (*i.e.* sulfate or nitrate), filtered and washed. The ion complexed on the packing is practically not washed off by pure water but sometimes to a slight extent by the elution of solutes. Pure solutes are then freed of metal ions by just adding, at the outlet of the chromatographic column filled with uncomplexed packing.

Chromatographic conditions

The supports obtained are slurry packed in stainless steel column 30 cm x 4.8 mm i.d. The efficiency of the columns is greatly dependent on the filling conditions. In Fig.2, the efficiency of the columns (measured by the total number, N, of theoretical plates) is plotted versus the packing flow rate (eluent : pure water).

For low flow rates, peaks tail and efficiency is low. A maximum is observed because, at high flow rates, beads are compacted, a steep back asymmetry appears, and the efficiency decreases.

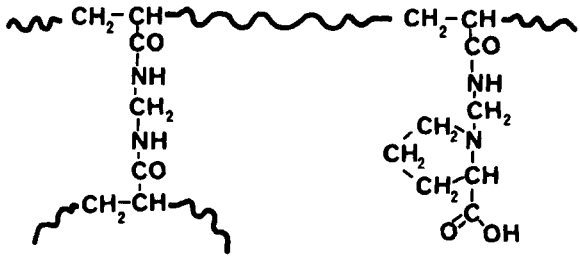

Figure 1 : Structure of the gel. After copolymerization of acrylamide and methylenebisacrylamide an L-α aminoacid is grafted through formaldehyde (proline, in this case).

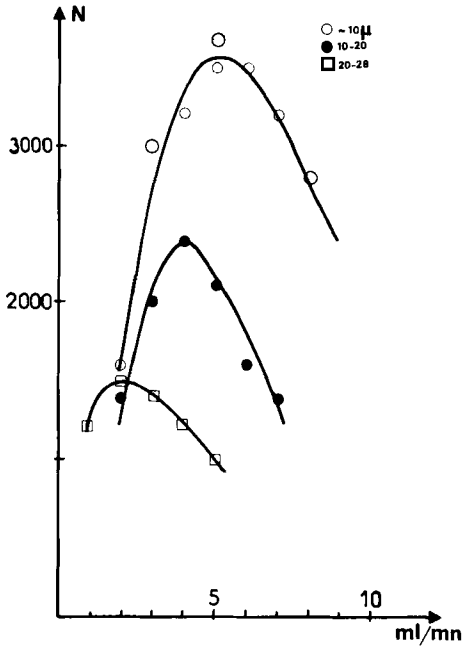

Figure 2 : Number of theoretical plates, N, versus the flow rate of filling (30 cm x 0.48 cm ID Column). N is calculated from the ethyleneglycoldimethylether
granulometrie : □ 20-28µ ; ● 10-20µ ; ○ ▫ 10µ.

The same shape of curves - with a maximum - is also observed by plotting N, versus the dilution of the slurry before filling. The existence of such optimum conditions for the dilution and the packing flow rate is probably a general rule for semi-rigid beads.

Chromatographic apparatus

We used a Waters liquid chromatograph (ALC 201) and added to the classical refractometer a polarimetric detector (Perkin Elmer 241 MC) which continuously measures the rotatory power of the eluates.

RESULTS AND DISCUSSION

Influence of the structure of the gel

The packing grafted with L-proline and complexed by cupric ions is efficient for the resolution of most of the α-aminoacids ([30]). All the racemates with relative retention (α value) greater than 1.5 are completely resolved with a 30 cm long column (or shorter) in less than one hour.

In the case described in Fig.3, six compounds are simultaneously injected : two polyoxyethylenes, D,L-alanine and D,L-threonine. The polyoxyethylene $\bar{M}_n$ = 6 000 (first peak) and the ethyleneglycoldimethylether (second peak) have no coordination affinity for the support, their fractionation is due to the size exclusion effect and shows the porosity of the beads.

Threonine is totally resolved as checked by the polarimetric detector. This detector shows that, though alanine is not completely resolved under these conditions, an excess of the D form is observed in the front of the peak and an enrichment of the L form in its tail.

Fig.4 shows the resolution of a mixture of D,L-phenylalanine and D,L-tryptophan with a 5 cm long column. These results can be compared with those published by Rogozhin and Davankov ([32,34]) and recently by Josefonvicz *et al.*([35]) which used the same graft (L-proline) and the same complexing ion (Cu^{++}). The k' and the α-values are not only largely different but in some cases the order of elution of the isomers is reversed. This suggests that the macromolecular framework is involved in the separation mechanism.

Russian workers proposed ([36,37]) the formation of stereoselective complexes in which the benzene ring of macromolecular support might participate. In our case, the amide function of the gel may play a part in the formation of the complexes graft/ion/D or L solute, involved in the separation of the solutes. Structures like those schematized in the Fig.5 might be expected.

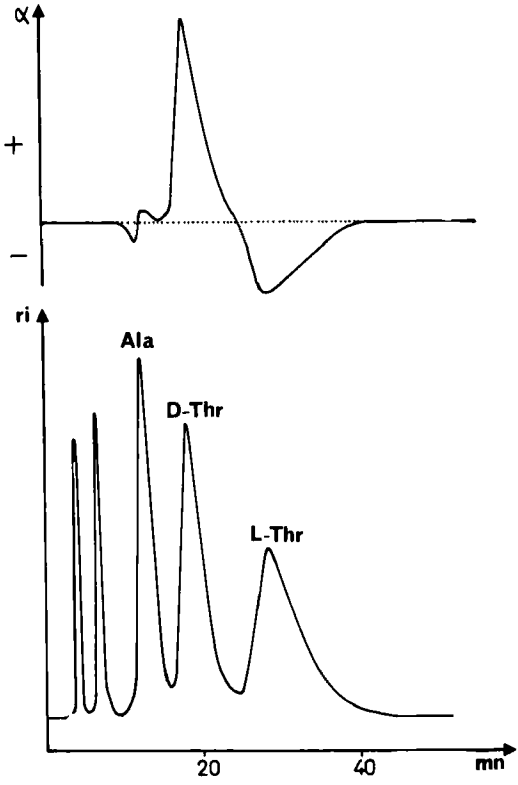

Figure 3 : Upper curve : polarimetric detector
Lower curve : refractometric detector
Elution order : 1) polyoxyethylene $\bar{M}_n$ = 6 000 ;
2) ethyleneglycoldimethylether ;
3) D and L alanine (partial resolution) ;
4) D threonine ;
5) L threonine ;
Stainless steel column 30 cm x 0.48 cm ID. Pressure drop of about 8 bars for a flow rate of 0.5 ml/m.
Gel : the graft is L proline, complexing ion Cu^{++} (0,4 cupric ion for one proline grafted). Capacity (in graft) 2.5 meq/g.
Eluent : water, room temperature.

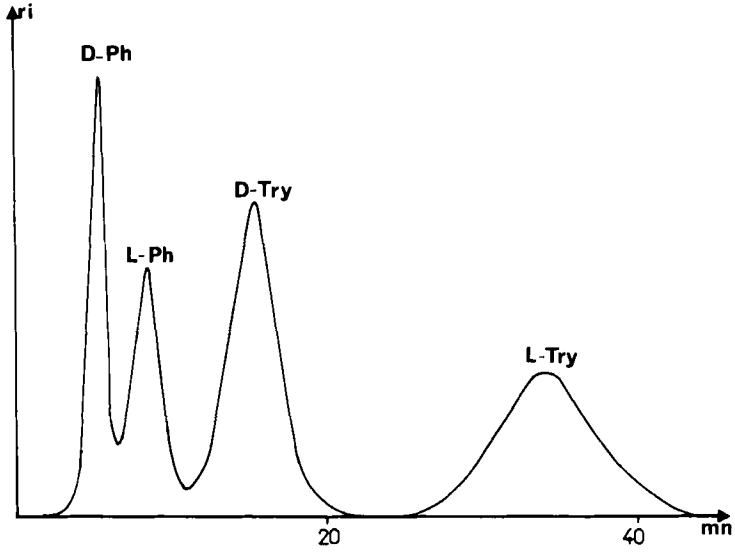

Figure 4 : Elution order : 1) D phenylalanine ;
2) L phenylalanine ;
3) D tryptophan ;
4) L tryptophan.
Gel : graft is L proline and complexing ion Cu^{++}(r = 0.2).
Capacity 0.95 meq/g.
Column : 5 cm x 0.48 cm ID
Eluent : water (0.4 ml/mn).

On the other hand, the porosity of our beads allows a high rate of exchange of the solute between the gel and the mobile phase. This benefit disappears by elution with a poorly swelling solvent (methanol/water mixtures for instance) and the efficiency decreases as a result of the porosity.

Finally, the use of a hydrophilic macromolecular network also improves the rate of exchange of ligands between the solution and the gel. This kinetic aspect is known to be one of the most important in the apparent "reactivity" of functions grafted on a polymeric structure. For instance, a catalyst, effective for a given reaction

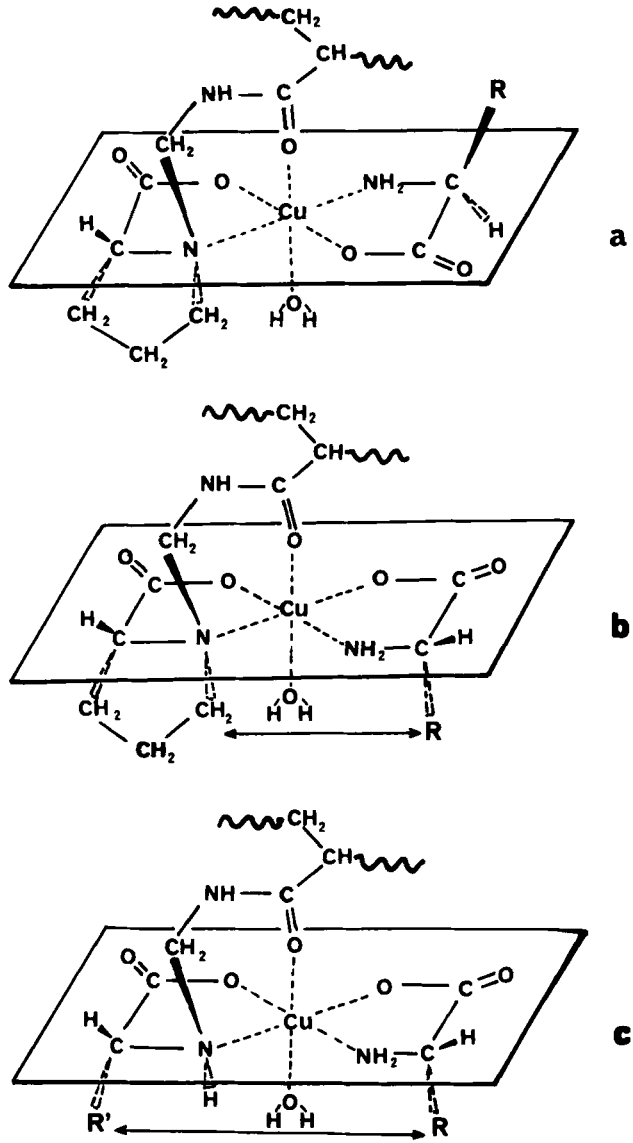

Figure 5: Possible structures of complexes. Grafted aminoacid/ Cu^{++}/eluted aminoacid. 5a and 5b: Trans and Cis form. The grafted aminoacid is proline. 5c: the grafted aminoacid is not cyclic.

in a polar medium, may present an activity when grafted onto a polymer, only if the framework is hydrophilic.

Influence of the kinetics of ligand exchange

If the ligand exchange is slow, chromatographic peaks are abnormally broad and the resolution is bad. Cupric ion, known to be one of the most mobile ions, is also one of the most efficient for resolution. This kinetic aspect probably plays a part in determining the influence of the temperature, as previously described ([30]). For valine, for instance, retention time is practically not perturbed by increasing the temperature in the range 0-50°C, but the resolution increase from 0.5 to 2. Even with copper as complexing metal, the number of theoretical plates of our columns is abnormally low, when calculated from an α-aminoacid peak ; it is about ten times lower than for a non-complexing solute ([30]). In connection with Horvath's theory ([39]), the slowness of the ligand exchange explains this result and also the broadening and probably the tailing of the peaks when k' increases. Nevertheless, if this mechanism of peaks broadening is important, it is not the only one.

Influence of the nature of the complexing ions

A dozen different ions were checked with different solutes([30]). Results are rather difficult to interpret. α-Aminoacids and N - substituted α-aminoacids are known to give complexes of different structures, depending both upon the nature of the ion and upon the α-aminoacid nature ([6,22,36-38]). The variety of the complexes which occur may explain the diversity for the results observed for the retention data and for the elution order of D and L solutes.

In the absence of a solute, the metal ion is probably kept in a "sandwich" complex between two grafts. If we suppose that all the metal ions are either in such a complex graft/metal/graft (MG_2) or in complexes schematized in Fig.5 graft/metal/solute (G M S), we can predict the incidence, on the retention times, of the rate, r, of complexation of the resin (r is the number of metal atoms by graft, its maximum value, referring to a "sandwich" form complex, is 0.5). It can be adjusted according to the pH value of the salt solution used in the preparation of the resin (see Fig. 6). Assuming

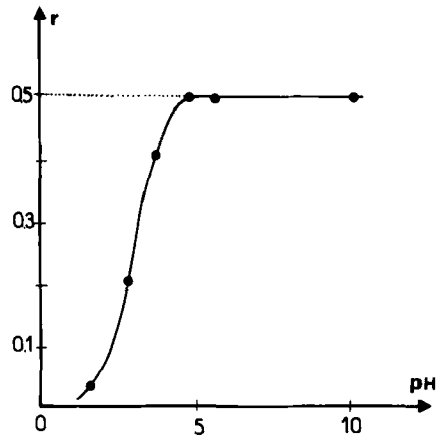

Figure 6 : Number (r) of cupric ion for one proline graft versus the pH value of water around the gel.

that the amount of the solute injected is small, if K is the partition coefficient of the free solute (not complexes) between the gel and the mobile phase and K^{*} the stability constant of the equilibrium :

$$MG_2 + S \rightleftharpoons MGS + G$$

(G = graft, S = free solute in the gel) ; under chromatographic conditions (high dilution of the solute), assuming that the retention on the non-complexed resin is very weak ($K^{*} >> K$) and considering the mass-balances of ligand, solute and metal we can write ([31]) that the capacity factor is proportional to $KK^{*} \frac{r}{1-2r}$ for any value of r not too close to 0.5 (*e.i.* when $|G| >> |MGS|$). Fig.7 shows that the theoretical results (dotted line) reasonably fit the experimental results.

Influence of the chiral graft

Results previously described are relative to L-proline graft. Data presented in Table I were obtained with other grafts. Some of them are inefficient or weakly efficient. The best results are observed with cyclic aminoacids, the efficiency increasing with the size of the ring.

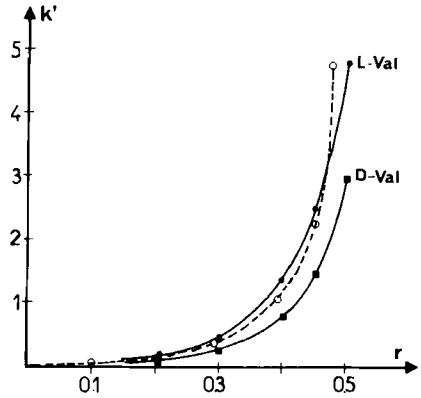

Figure 7 : Calculated (dotted line) and experimental value of k' (measured for D and L valine) versus the rate of saturation (r) of the gel (graft is L proline) in cupric ion.

The stereoselectivity of the complex is probably connected with a repulsion between the substituent of the solute R, the substituent R' of the graft of the macromolecular framework and, eventually, a water molecule in apical position (as suggested in Fig.5). This repulsion seems most important when R' is cyclic (compare Fig.5b and 5c) and the larger the ring size, larger appears to be steric hindrance and the higher the efficiency.

CONCLUSION

Some general rules for the chromatographic resolution of α-aminoacids on chiral gels have been emphasized and discussed. They can be extended to unusual aminoacids or other complexing solutes like diamines, diacids, acidalcohols etc..., if they are highly complexed by metal ions. For instance dihydroxyphenylalanine (DOPA), o-thyrosine, malic acid are completely resolved on short columns.

The mechanism of the separation is not yet completely understood and its establishment will require the study of the precise

Table I

Influence of the nature of the chiral graft on the retention data of some aminoacid enantiomers. The complexing ion is copper. Chromatographic conditions are given in Fig.3. In some cases (*) the peak broadness prevented precise measurements.

Solute / Graft	Proline k'_2	Proline α	Valine k'_2	Valine α	Serine k'_2	Serine α	Phenyl-alanine k'_2	Phenyl-alanine α	Asparagine k'_2	Asparagine α	Leucine k'_2	Leucine α
Alanine	0,74	0,7	0,65	1	1,7	1	2,7	1	2,9	1	0,77	1
Valine	0,9	1,5	1,4	1,6	2,1	1,4	3,8	1,2	1,9	1	1,2	1
Threonine	1,4	1,3	1,5	1,4	3	1,3	7,3	1,5	*		2,5	1
Phenylalanine	1,8	1	1,4	1	*		*		*		*	
Phenylglycine	0,2	1	0,25	1	*		1,6	1	*		*	
Histidine	0,35	<1	0,4	1	0,9	1	1,8	0,8	1,3	1	0,6	1
Azetidine carboxylic acid	2,5	0,6	3,5	1,6	5,8	1,5	1 7	2,1	3,5	1,5	4	1
Proline	1,1	0,65	1,5	1,9	3,1	1,9	9,8	2,3	3,4	1,5	1,7	1
Pipecolic acid	1,2	0,75	1,2	2,0	3	2,8	9,1	2,8	4,1	1,8	1,2	1
Hydroxyproline	1,7	0,47	1,4	1,4	5,1	1,9	1 6	2,1	7,8	1,3	2,7	1

structure of the complexes involved. Systematic investigations are planned on soluble models of the asymmetric grafts.

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