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IN-SITU PREPARATION OF A CHEMICALLY BONDED CHIRAL STATIONARY PHASE FOR THE SEPARATION OF AROMATIC α-AMINO ACID ENANTIOMERS*

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SUMMARY

This paper describes an *in situ* preparation of a chiral stationary phase on silica gel for the resolution of amino acid racemates. A column prepared from a Waters Radial-Pak cartridge was useful for the rapid and efficient resolutions of some aromatic α-amino acids. Separation factors (α) of 2.8, 3.6, 4.2 and 4.6 were observed for phenylalanine, tyrosine, dopa and tryptophan, respectively. A plate count as high as 1700 plates/meter was observed at room temperature for D/L-dopa.

INTRODUCTION

Several publications have appeared recently on the separation of α-amino acid racemates by high-performance liquid chromatography (HPLC)[1–8]. In particular, the HPLC column developed by Gübitz *et al.*[1–4] is very effective in completely separating the optical isomers of a wide variety of α-amino acids. In this method an L-proline copper complex is chemically bonded to silica gel followed by packing into a stainless-steel column.

Given the difficulty and expense of packing HPLC columns[9] we decided to try an *in situ* column preparation of this same chiral stationary phase. We were encouraged to try this after having successfully repeated the *in situ* preparation of a propylamine column for carbohydrate analysis, as first reported by Aitzetmüller[10]. Furthermore, the synthetic method and the reagents and solvents required for the preparation of the chiral amino acid column are compatible with any common HPLC system. In effect, the method simply takes advantage of the high efficiency of prepacked HPLC silica columnss avoiding any physical manipulation of the stationary phase and its support.

We now describe the *in situ* preparation of the chiral amino acid stationary phase on silica gel using a Waters Radial-Pak cartridge and demonstrate its utility for the resolution of some aromatic α-amino acid racemates.

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EXPERIMENTAL

Materials

3-Glycidoxypropyltrimethoxysilane was obtained from Aldrich. L-Proline (Sigma grade, hydroxy-L-proline free) was obtained from Sigma. L-6-Fluorodopa [(*S*)-β-(2-fluoro-4,5-dihydroxyphenyl)alanine] was prepared by a modification of the method reported by Chirakal *et al.*[11]. Racemic 6-fluorodopa was prepared according to a method to be published by the authors. Racemic di-O-methyldopa [β-(3,4-dimethoxyphenyl)alanine] was prepared by the methylation of N-acetyldopa followed by acid hydrolysis. *m*-Fluorotyrosine [β-(3-Fluoro-4-hydroxyphenyl)alanine], *o*-fluorophenylalanine [β-(2-fluorophenyl)alanine], 3-O-methyldopa [β-(3-methoxy-4-hydroxyphenyl)alanine], 6-hydroxydopa [β-(2,4,5-trihydroxyphenyl)alanine], tryptophan, tyrosine, dopa, 5-fluorotryptophan and 5-hydroxytryptophan were purchased commercially.

Instrumentation

The HPLC chromatographic system consisted of a Spectro-Physics SP 8700 solvent delivery system, a Rheodyne 7126 injector fitted with a 0.5-ml sample loop (1.02 mm I.D.), a Waters radial compression module fitted with a Radial-Pak silica gel column (10 μm silica gel, 10.0 cm × 8 mm I.D.) and a Guard-Pak silica gel precolumn insert (3 mm × 8 mm I.D.), and a Waters 441 UV detector operated at 280 nm.

In situ column preparation

Methanol was pumped through the chromatographic system for 30 min (10 ml/min) followed by a solution of water–acetonitrile (1:4) for the same period of time and flow-rate. To the solvent reservoir containing 1 l of water–acetonitrile (1:4) was added 3-glycidoxypropyltrimethoxysilane (25 ml). The dissolution of the silane modifier was rapid and a clear colourless solution was obtained. The mixture was pumped through the system in the recycle mode (waste to reservoir) for 10 h at a rate of 4 ml/min. After this time the system was again flushed with methanol for 30 min (4 ml/min). During the recycle period with the silane modifier the detector was not in-line, in order to avoid the modification of its optical cell surfaces. The back-pressure of the system (determined at a flow of 10 ml/min) changed only moderately (+75 p.s.i.) during the process from an initial value of ≈850 p.s.i.

A solution of sodium L-prolinate was prepared by the addition of sodium hydroxide pellets (3.5 g) to a stirred solution of L-proline (12 g) in methanol (500 ml). The resultant solution was pumped through the system in the recycle mode for 10 h at a rate of 10 ml/min. The system was flushed with 200 ml of water, the detector was connected in-line and then the mobile phase containing Cu^{2+} (0.05 *M* KH_2PO_4–1 m*M* $CuSO_4 \cdot 5H_2O$ adjusted to pH 4.0) was pumped through the system for 30 min at a rate of 9 ml/min (1900 p.s.i.). It was observed that the column sequestered cupric ions from the mobile phase for the first ≈200 ml of eluate. With the UV detector monitoring the absorbance at 280 nm the background reading of the eluate stabilized at 0.05 abs.

Analyses were performed after an additional 30 min of equilibration with the mobile phase. A flow-rate of 6 ml/min was found to be convenient for the purpose

of our analyses (*vide infra*). The performance of the column became consistent only after 3–4 injections onto the column.

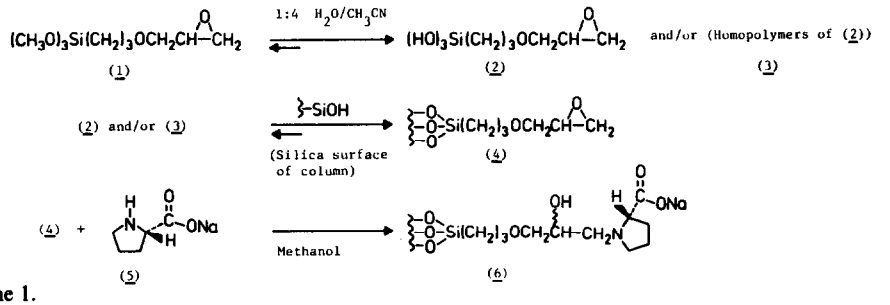
Characterization of the bonded phase

After two weeks of column use and six weeks of storage in water the modified Radial-Pak cartridge was sectioned into three equal lengths. The contents of each section were removed and dried at room temperature *in vacuo* for 96 h. Each dried portion weighed approximately 1 g. Microanalyses for C, H and N were performed by combustion whereas copper (column tail section only) was determined by atomic absorption spectroscopy on a sample digested in a mixture of nitric and hydrofluoric acids.

RESULTS AND DISCUSSION

Method

The details of the synthetic method used for the *in situ* column preparation are outlined in Scheme 1. The literature[10,12] suggests that in a partially aqueous medium an alkyl trialkoxysilane (*e.g.* 3-glycidoxytrimethoxysilane, 1) would be readily hydrolyzed to the corresponding alkyl trihydroxysilane (*e.g.* 2) and/or its homopolymers. These species would then extensively condense with the silica surface followed by crosslinking to deposit a chemically bonded polymeric network. In this case, the polymer bound epoxide functionalities are further transformed into the final stationary phase via nucleophilic ring opening of the epoxide moities with sodium L-prolinate in methanol, which fixes the chiral ligand to the stationary phase. In practice the method presented here requires approximately 24 h and the major portion of this time is devoted to unattended instrument pumping.



Scheme 1.

In essence, the column preparation presented here differs from the previously described method[1–4] only in the conditions under which the silica gel surface is modified to produce the initially bound glycidoxypropyl moities[3]. Regardless of whatever the actual course of chemical events in these two preparations are, the literature[10,12] does suggest that both processes are closely related and should afford near identical phases. This appears to be true since the separation factors (α) listed in Table I are practically identical to those previously reported for a conventionally prepared column. Unfortunately, since temperature control is not possible with our chromatographic system a direct comparison of the optimal performances of columns prepared by both methods cannot be made.

TABLE I

CHARACTERISTIC CHROMATOGRAPHIC VALUES (k' AND α) FOR SOME SELECTED AROMATIC α-AMINO ACIDS

Operating conditions: 0.05 M KH_2PO_4–1 mM Cu^{2+} at pH 4.0; ambient temperature; flow-rate, 6 ml/min; UV detection at 280 nm.

		k'_D	k'_L	α
1	Phenylalanine	2.0	5.5	2.8
2	*o*-Fluorophenylalanine	3.8**	6.2	1.6
3	Tyrosine	1.6	5.6	3.6
4	*m*-Fluorotyrosine	1.6**	5.2	3.2
5	Dopa	1.3	5.5	4.2
6	6-Fluorodopa	1.5	6.0	4.0
7	3-O-Methyldopa	–	10.5	–
8	Di-O-methyldopa	4.9**	21.1	4.3
9	6-Hydroxydopa	9.4**	15.1	1.6***
10	Tryptophan	4.8	22.3	4.6
11	5-Fluorotryptophan	4.6**	21.4	4.7
12	5-Hydroxytryptophan	2.9**	13.8	4.7

* $k'_i = (v_i - v_0)/v_0$; $v_0 = 35$ sec; $\alpha = k'_D/k'_L$

** The capacity factors k'_D and k'_L have been arbitrarily assigned.

*** The two optical isomers were resolved to approximately 0.3 peak height.

Applications

Based on our research interests in resolving racemates of phenylalanine and its analogues labelled with short-lived radionuclides (carbon-11, $t_{1/2}$ 20.4 min and fluorine-18, $t_{1/2}$ 110 min) the potential for using a column which could rapidly separate these types of compounds on an analytical or preparative scale was particularly attractive. For each of the entries listed in Table I, with the exception of 6-hydroxydopa, the racemate was resolved with the most mobile optical isomer being eluted within a small number of column volumes. A typical separation with the column is illustrated in Fig. 1. At pH 4.0 with a high flow-rate these separations were exceedingly rapid — as short as 2 min at 6 ml/min for D/L-dopa. With reference to Table II, we consider pH 4.0 to be the best compromise between column selectivity and chromatographic resolution if one considers the eluted peak volume to be of importance, as would be the case when using the column for the final purification of a radiopharmaceutical.

Because it is generally difficult to reduce HPLC injection volumes to less than 0.5 ml for semi-automated radiosyntheses we wished to evaluate the performance of the column (10 cm × 8 mm I.D.) under less than ideal conditions of sample volume injection. Fig. 2 shows the effect of progressively increasing the sample volume on the resolution. It can be seen that even with a sample injection volume as large as 0.5 ml the separation is nearly complete and it is thus possible to separate approximately 5 mg of the racemate on this column.

The presence of cupric ions within the mobile phase is a major concern when considering the use of this amino acid column for the production of pharmaceutical grade materials. With this in mind, we had hoped to use the column loaded with

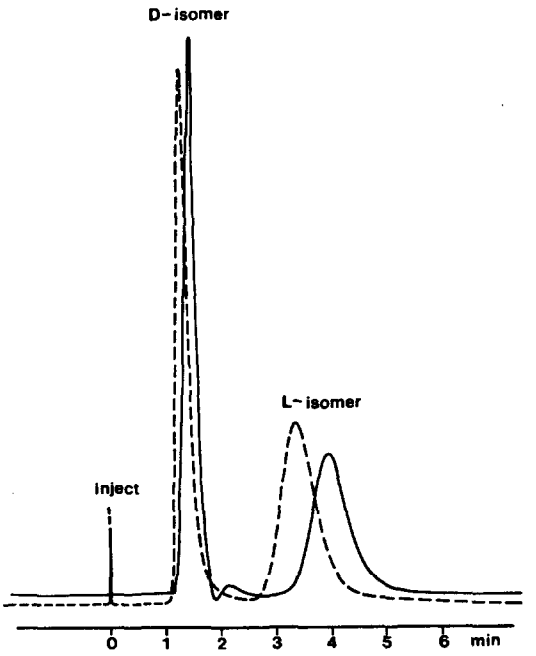


Fig. 1. Test chromatograms for D/L-dopa. For definitions, operating conditions and column dimensions see Tables I and II. Chromatogram A (———), freshly prepared column (α = 4.2, N = 1600 plates/m, R_s = 3.1); Chromatogram B (– – –), column after use and storage (α = 4.0), N = 1300 plates/m, R_s = 2.6).

cupric ions with a mobile phase consisting only of the phosphate buffer. Unfortunately, we observed that with the *in situ* prepared column it was essential to include the cupric salt in the mobile phase, albeit at low concentration, in order to maintain a high degree of performance. At both pH 3.5 and 4.0 when the column was thoroughly washed free of extraneous copper with the phosphate buffer the chromatographic resolution obtained with the column was degraded from that normally

TABLE II

CHROMATOGRAPHIC PERFORMANCE OF THE AMINO ACID COLUMN *vs.* pH FOR D/L-DOPA

pH	k'_D*	k'_L	α	N**	R_s***	*Eluted peak volume (ml)*§
3.5§§	0.4	1.4	3.5	100	1.8	8
4.0§§§	1.3	5.5	4.2	160	3.1	11
4.6§§§	4.2	18.1	4.3	170	3.7	36

* v_0 = 35 sec at 6 ml/min.

** Column dimensions: 10.3 cm × 8 mm I.D.; $N = 5.54\,(t'/w'_{0.5})^2$.

*** $R_s = 1.177\,[(t'-t)/(w'_{0.5} + w_{0.5})]$.

§ Volume of eluate containing L-dopa.

§§ 0.05 *M* KH_2PO_4–1 m*M* Cu^{2+}, 6 ml/min, ambient temperature.

§§§ 0.05 *M* KH_2PO_4–0.4 m*M* Cu^{2+}, 6 ml/min, ambient temperature.

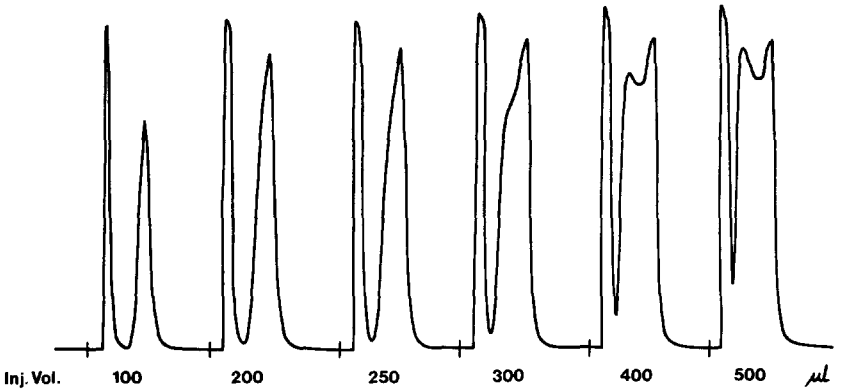


Fig. 2. Effect of sample loading on column performance. Injection of a 10 mg/ml solution of D/L-dopa. Operating conditions as listed in Tables I and II.

observed using a copper containing mobile phase. If fact, we were surprised to observe that no resolution could be effected after only a few (maximum of three) successive injections onto the column. Clearly under these conditions, the injected racemate was causing a loss of bound copper from the stationary phase. We suggest that this is a result of the injected racemate competing strongly, in a reversible ligand exchange process, for cupric ions held by the L-proline containing stationary phase.

Use of the Waters Radial-Pak plastic cartridge provided an opportunity to correlate the overall loading of the stationary phase on the silica support with column performance and to examine the distribution of the stationary phase along the length of the column after use. Fig. 1 (chromatograms A and B) compares the performance of the freshly prepared column to that after two weeks of continual use followed by six weeks of storage in water. There is not a great deal of difference in the two chromatograms and thus the stationary phase is reasonably stable with this type of treatment. Table III lists the analytical data obtained for each of the three equal length sections of the column. The data suggest the loading of the stationary phase

TABLE III

ANALYTICAL DATA FOR THE SECTIONED AMINO ACID COLUMN

The column dimensions were 10.0 × 0.8 cm (which excludes the guard column). The entire column of packed material was sectioned into three equal lengths. Each section weighed approximately 1.0 g. The values quoted are % (w/w) for the respective element to that of the sample.

Section	*Analyses**			
	C	*H*	*N*	*Cu***
1	1.28	<0.1	0.08	***
2	1.44	<0.1	0.10	***
3	2.05	<0.1	0.13	0.24

* Combustion analyses for C, H and N.

** Determined by atomic adsorption spectroscopy on a sample digested in a mixture of nitric and hydrofluoric acids.

*** Colour indicated copper was present but sample was not analyzed.

on the packing is not uniform, with the head section of the column holding roughly half the quantity of the phase held by that of the tail section. It is suspected that this uneven distribution is a result of the normal leaching action of the mobile phase with use. It was interesting to observe that the *in situ* prepared column holds, on the average, only one fifth the loading of the phase which is possible for a conventionally prepared column[1–4]. Even with this low level of phase loading the *in situ* prepared column provides for good peak symmetry and selectivity with a reasonable degree of efficiency (maximum of 1700 plates/meter).

CONCLUSION

Many of the technical difficulties associated with the preparation of the chiral amino acid column by the conventional method can be overcome by the *in situ* or on-column synthesis of the stationary phase using a commercially available HPLC silica column. A column of suitable efficiency may be prepared by this method for the practical micro-scale resolution of a series of aromatic α-amino acids of biological interest.

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