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Note

Milligram-scale separation of optical isomers of 2-pentafluoroethylalanine and 2-trifluoromethylalanine by medium-performance reversed-phase chromatography

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Reversed-phase separation of amino acid optical isomers using a copper(II)-amino acid mobile phase is now a standard analytical tool[1,2]. We have previously used this method for analytical-scale optical resolutions of several polyfluoro 2,2-dialkylglycines using a copper(II)–L-phenylalanine mobile phase[3]. We report here a preparative version of this method in which a column packed with commercially available 40 μm reversed-phase silica was used to resolve milligram quantities of 2-pentafluoroethylalanine (PEA) and 2-trifluoromethylalanine (TMA).

PEA and TMA are analogues of isovaline and 2-methylalanine. The latter are major constituents of alamethicin-type cytotoxic polypeptides produced by soil fungi[4] and are also substrates of the *Pseudomonas cepacia* 2,2-dialkylglycine decarboxylase[5-7]. The polyfluoro analogues, both racemic and optically active forms, are being investigated in our laboratory as potential inhibitors of the dialkylglycine decarboxylase or as components of synthetic peptides. The optical isomers of TMA have been prepared enzymatically in 98% enantiomeric excess by stereospecific hydrolysis of the N-trifluoroacetyl derivative by hog kidney aminoacylase[3,8]. However, other polyfluoro dialkylglycines cannot be resolved this way. For example, the N-acyl derivatives of PEA and 2-trifluoromethyl-2-aminobutanoic acid[3] are not hydrolyzed by hog kidney aminoacylase[9]. Thus we have developed a chromatographic method that resolves polyfluoro dialkylglycines on the preparative scale and requires only commonly available reagents and equipment.

MATERIALS AND METHODS

Racemic 2-pentafluoroethylalanine[3] and 2-trifluoromethylalanine[10] were prepared by literature methods.

Preparative chromatography was carried out with Baker octadecyl silica for flash chromatography (40-μm irregular particles). Bio-Rad Econocolumns (25 × 1.5 cm or 7.3 × 1.5 cm) equipped with a flow adaptor and a home-made constant-

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temperature jacket were slurry-packed (acetonitrile suspension, gravity flow), then equilibrated with mobile phase [2 mM L-phenylalanine, 1 mM copper(II) acetate, 3 mM potassium acetate adjusted to pH 4.4 with acetic acid, to which was added 2% (TMA) or 8% (PEA), v/v, acetonitrile]. Mobile phase was delivered with a Gilson Minipulse peristaltic pump through a Rheodyne Type 50 rotary injection valve. The eluate was monitored at 280 nm with a Gilson Holochrome detector.

During initial equilibration of the 7.3 × 1.5 cm column with mobile phase, phenylalanine concentrations in the eluate were determined spectrophotometrically after derivatization with o-phthaldialdehyde[11]. Copper(II) concentrations in the eluate were determined by atomic absorption spectrometry[12].

Copper and phenylalanine were removed from collected fractions by passage through Dowex 50 (10 × 1 cm, H$^+$), which does not bind the strongly acidic polyfluoro amino acids[3]. Eluate from the Dowex column containing the polyfluoro amino acid was dried *in vacuo* and the residue weighed.

Analytical high-performance liquid chromatography (HPLC) of fluoro amino acids was carried out at room temperature with a 250 × 4.6 mm C_{18} silica column (Alltech/Applied Science 60130, 5 μm) with eluate monitored as above. In some experiments the spectrophotometer output was digitized, stored, smoothed and plotted on an IBM-PC microcomputer equipped with a Data Translation DT-2805 A/D board and ASYST software.

RESULTS AND DISCUSSION

Reversed-phase columns must be loaded with copper by equilibration with the copper–amino acid mobile phase before they can be used for amino acid separation[1–3]. Since the degree of copper loading on the column can affect retention and resolution[1], the equilibration process was studied first. The glass chromatography column facilitated observation of this process. It was observed that as copper(II)–L-phenylalanine mobile phase was pumped through the newly packed column, a dark blue zone appeared at the top of the column separated from the white lower portion by a narrow (3 mm) transition zone. As the equilibration progressed, the narrow transition zone moved downward until the column was completely blue.

Chemical analysis of the eluate during the equilibration process suggests that the blue zone of the column is the portion saturated with Cu(L-Phe)$_2$. During column equilibration the position of the transition zone and the concentrations of copper and phenylalanine in the eluate were measured periodically. The data from equilibration of a 7.3 × 1.5 cm packed bed of C_{18} silica with copper(II)–L-phenylalanine mobile phase are shown in Fig. 1. These data indicate that, except for the first 100 ml, the eluate contained phenylalanine and copper in the nearly constant molar ratio of 2, suggesting that the major bound species is Cu(L-Phe)$_2$. The higher phenylalanine:copper ratio in the early part of the equilibration may have been caused by displacement of L-phenylalanine from Cu(L-Phe)$_2$ by higher-affinity silicate sites.

The equilibration data in Fig. 1 also allow the calculation of the ligand concentration on the copper-saturated column. Integration of the phenylalanine concentration curve in Fig. 1 reveals that a total of 1.1 mmol phenylalanine was bound during equilibration. Therefore, the ligand concentration in the packed column was 86 mM, which is significantly lower than that of amino acid ligands covalently linked to silica, which is typically 350–400 mM[13].
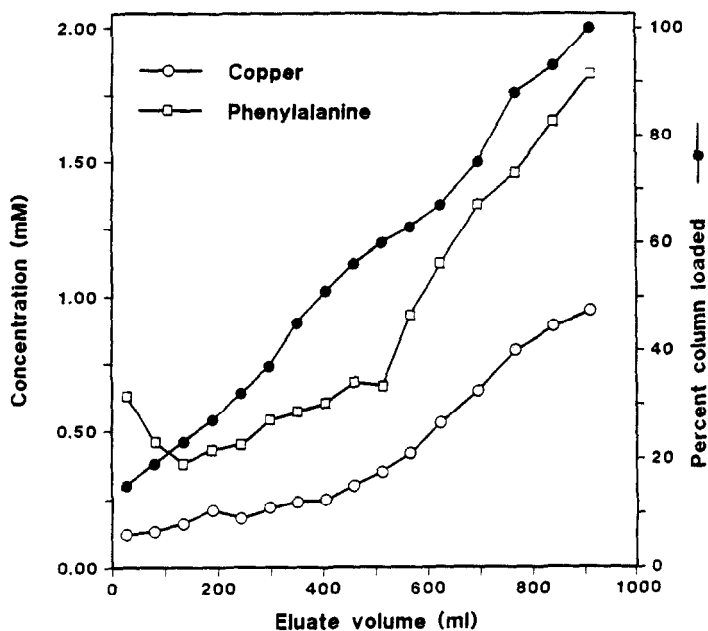
Fig. 1. Changes in eluate composition and column packing color during loading of a new 7.3×1.5 cm C_{18} column with copper(II)–L-phenylalanine mobile phase. Percent column loaded $= (L_b/L_t) \times 100$, where L_b = length of the blue portion and L_t = total length of the packed column.
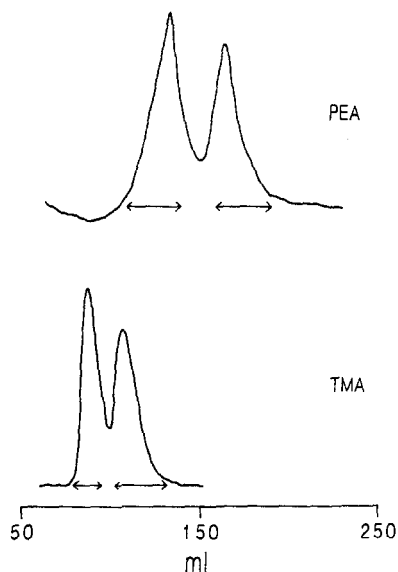

Fig. 2. Preparative chromatograms showing fractions combined for subsequent isolation. Samples: 4 mg of racemic PEA or TMA dissolved in 1 ml of mobile phase. Mobile phase: 2 mM L-phenylalanine, 1 mM copper(II) acetate, 3 mM potassium acetate to pH 4.4 with acetic acid containing 2% (TMA) or 8% (PEA) acetonitrile.

TABLE I

STRUCTURES, CAPACITY FACTORS (k'_1, k'_2) AND ENANTIOSELECTIVITIES ($\alpha = k/k'$) OF 2,2-DIAL-KYLGLYCINES ($R_1R_2CNH_2COOH$) IN COPPER(II)–L-PHENYLALANINE REVERSED-PHASE CHROMATOGRAPHY

Compound	R_1	R_2	Analytical			Preparative		
			k'_1, k'_2	α	Acetonitrile (%)	k'_1, k'_2	α	Acetonitrile (%)
2-Pentafluoro-ethylalanine (PEA)	CF_2CF_3	CH_3	5.0, 7.0	1.4	6	7.8, 9.7	1.2	2
2-Trifluoro-methylalanine (TMA)	CF_3	CH_3	12.9, 16.0	1.2	12	12.3, 15.5	1.2	8

Temperature and sample size parameters were tested in preliminary experiments with a 7.3 × 1.5 cm column. It was found that the resolution increased as the operating temperature increased from 25 to 50°C; however, significant column degradation was observed at 50°C, thus an operating temperature of 40°C was chosen. Resolution was adequate at this temperature and the column was stable for weeks of daily operation. As expected, resolution decreased with increasing sample size; therefore the largest sample size consistent with a near-baseline resolution, 4 mg of racemic amino acid in 1 ml of solution, was chosen.

Typical chromatograms obtained when 4-mg samples of racemic PEA or TMA were applied to a copper-saturated 25 × 1.5 cm column are shown in Fig. 2 (see also Table I). As observed in HPLC separations, the PEA isomers were more strongly retained than the TMA isomers, probably because of the increased hydrophobicity of the pentafluoroethyl substituent[3]. By analogy to TMA, the early-eluting PEA isomer
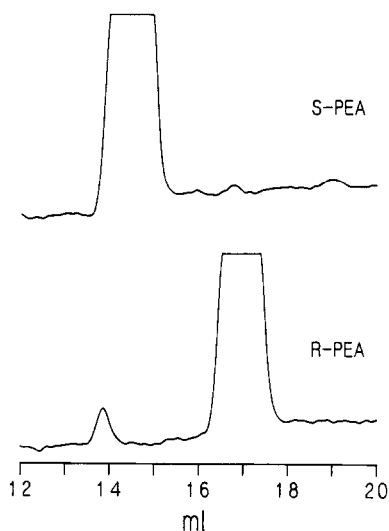

Fig. 3. Analytical HPLC of chromatographically resolved PEA isomers. Only the lowest 10% of the major peak is shown. Sample: 20 μg; mobile phase as in Fig. 2 but with 12% acetonitrile.

is predicted to have the S configuration since this one has the smaller, less hydrophobic group in the pro-S position[3,8,14].

Nine PEA-samples (total 35 mg) were chromatographed; 17 and 14 mg respectively of the early- and late-eluting peaks were recovered from fractions combined as shown in Fig. 2. Optical-purity analysis of these samples by HPLC showed that the amino acid recovered from the early-eluting peak contained no measurable amounts of the late-eluting component; probably 0.1% of the minor isomer could have been observed above the baseline noise (Fig. 3). The late-eluting sample contained 5% of the early-eluting isomer.

Eight TMA samples (total 30 mg) were chromatographed, with 9 and 16 mg respectively recovered from the early (S) and late (R) eluting peaks (Fig. 2). HPLC analysis showed that the early (S) peak contained 0.5% of the R isomer and the late (R) peak contained 2.9% of the S isomer (Fig. 4).
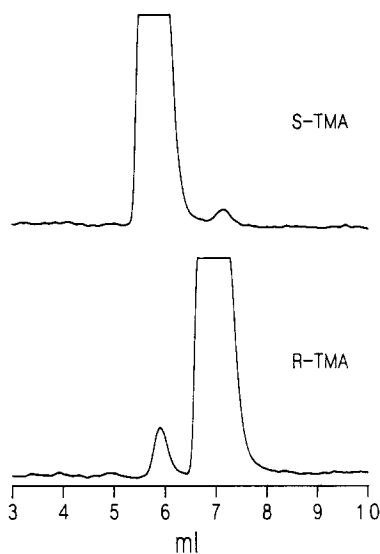

Fig. 4. Analytical HPLC of chromatographically resolved TMA isomers. Only the lowest 10% of the major peak is shown. Sample: 20 μg; mobile phase as in Fig. 2 but with 6% acetonitrile.

This chromatographic method significantly improved the optical purity of the early-eluting (S) isomers of PEA and TMA compared to the S-TMA produced enzymatically, which contained 1% of the minor (R) isomer[3,8]. However, the late-eluting (R) isomers of PEA and TMA both had lower optical purity (5.0% and 2.9% S isomer contamination, respectively) than the enzymatically produced R-TMA (1% S-TMA[3,8]), although they were isolated from fractions taken from the apparent peak centers. This was probably caused by tailing of the early-eluting isomer. Therefore maximum optical purity of R-PEA or R-TMA could be obtained by collecting the early-eluting peak from chromatography of the racemic amino acid on a Cu(D-Phe)$_2$-loaded column.

Thus we have demonstrated the use of reversed-phase chromatography for preparation of milligram quantities of polyfluoro amino acids in high optical purity. This method should be useful for small-scale resolutions of some amino acids or for improving the optical purity of amino acids which have been partially resolved enzymatically. Successful application of the method requires subsequent separation of the resolved amino acid from phenylalanine.

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