*Journal of Chromatography*, 316 (1984) 547–552
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 455

# OPTICAL RESOLUTION OF AMINO ACID ENANTIOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

NORIYUKI NIMURA*, ATSUKO TOYAMA and TOSHIO KINOSHITA
*School of Pharmaceutical Sciences, Kitasato University, 9-1 Shirokane-5, Minato-ku, Tokyo (Japan)*

## SUMMARY

Simultaneous analysis of common protein amino acid enentiomers was achieved by a chiral derivatization and a chiral mobile phase method. In the chiral derivatization method, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate was chosen as the reagent for the derivatization of enantiomeric amino acids to give diastereomeric thiourea derivatives. These derivatives were efficiently separated on a octadecylsilyl silica gel column by gradient elution and were detected by their absorbance at 250 nm. Derivatives of all common protein amino acid racemates, except cysteine, were resolved within about 2 h, although a few peaks were slightly overlapped. In the chiral mobile phase method, the optically active binary copper complex with N(*p*toluenesulphonyl)-D-phenylglycine was used as a chiral additive in the mobile phase for the ligand-exchange chromatographic resolution of underivatized D,L-amino acids. Simultaneous resolution of common protein amino acid enantiomers on a resversed phase was achieved by a column-switching technique, utilizing two ODS columns of different lengths, and by gradient elution with acetonitrile. The column eluate was monitored fluorometrically after reaction with *o*-phthalaldehyde.

## INTRODUCTION

Many attempts have been made to resolve amino acid enantiomers by liquid chromatographic techniques, using chiral derivatization reagents[1–4], chiral eluents[5–7] or chiral stationary phases[8–10]. However, no practical method capable of resolving simultaneously all common protein amino acid enantiomers has so far been reported. Only two systems[11,12] have hitherto been reported involving ordinary liquid chromatographic separation of amino acids followed by optical resolution of amino acid enantiomers. However, the two-step separation made these procedures tedious and time-consuming.

Previously, we reported a chiral derivatization method with the O-acetylated sugar isothiocyanates, such as 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC)[13,14], and a chiral mobile phase method with the N-tosyl amino acid copper complexes, such as N-(*p*-toluenesulphonyl)-D-phenylglycine-copper(II), Tos-PhG-Cu(II)[15].

In the present study, these methods were applied to the rapid and simple simultaneous analysis of common protein amino acid enantiomers.

0021-9673/84/$03.00 

## MATERIALS AND METHODS

Amino acids and other reagents were purchased from Wako (Osaka, Japan) and Tokyo Chemical Industry (Tokyo, Japan). Develosil ODS silica gel (Particle size 5 $\mu$m) was obtained from Nomura Chemical (Seto-shi, Japan). Prepacked reversed-phase columns (Fig. 1) were obtained from Erma Optical Works (Tokyo, Japan). Water was purified by passing through a Milli-R/Q system (Millipore Corp., Bedford, MA, U.S.A.). GITC was prepared as described previously[13], and TosPhG was prepared as described by Theodropoulos and Craig[16]. GITC is commercially available from Polysciences (Warington, PA, U.S.A.).

The first chiral mobile phase used for gradient elution consisted of 75 mg of $Na_2CO_3$, 125 mg of copper sulphate pentahydrate, 10 ml of a 100 m*M* solution of TosPhG in acetonitrile and water to make the volume up to 1 l. The second chiral mobile phase used for gradient elution was prepared by adding three volumes of acetonitrile to seven volumes of the first chiral mobile phase. The *o*-phthalaldehyde (OPTA) reagent comprised 17 g of boric acid, 15 g of potassium hydroxide, 2 ml of 2-mercaptoethanol, 5 ml of 8% methanolic solution of OPTA and 1.5 g of the di-sodium salt of ethylenediaminetetraacetate (EDTA). This reagent was degassed prior to use.

### *Chromatographic system*

A microprocessor-controlled LC-4A liquid chromatograph (Shimadzu, Kyoto, Japan) was used for the chiral derivatization method. This high-performance liquid chromatographic (HPLC) apparatus was equipped with a ternary solvent delivery system, in which 0.1% aqueous phosphoric acid, methanol and acetonitrile were the three eluents. The gradient program is shown in Fig. 2. Develosil ODS was packed into a stainless-steel tube (200 × 6 mm I.D.) in our laboratory by the conventional

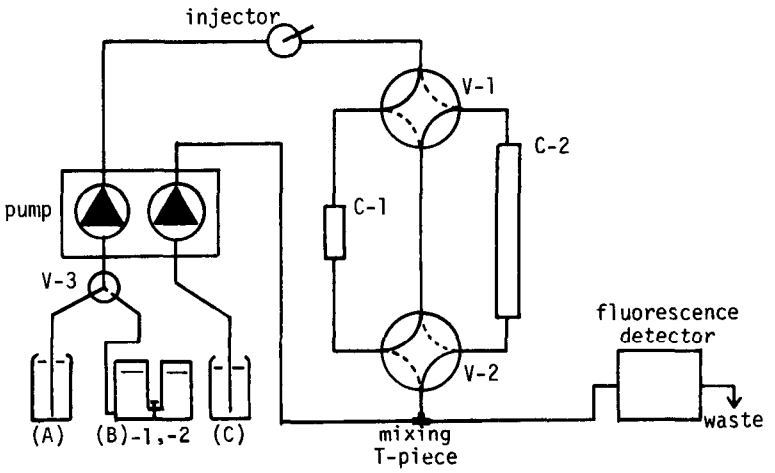


Fig. 1. Flow diagram of column-switching procedure for the simultaneous analysis of free amino acid enantiomers with the TosPhG-Cu(II) eluent system. C-1 column; ERC-ODS-1151 (50 × 6 mm I.D.). C-2 column; ERC-ODS-1171 (20 × 6 mm I.D.). (A) and (B)-1 = first chiral mobile phase; (B)-2 = second chiral mobile phase; (C) = OPTA reagent.

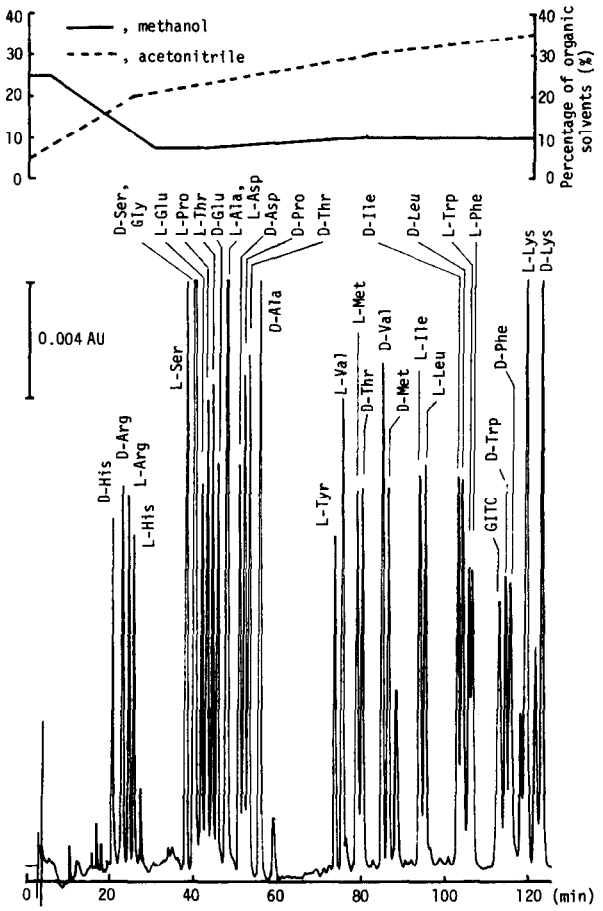


Fig. 2. Chromatogram and gradient program of the simultaneous analysis of GITC derivatives of all common protein amino acid enantiomers. Column: Develosil ODS (200 × 6 mm I.D., particle size 5 μm). Flow-rate; 1.3 ml/min. Each peak corresponds to 100 ng of free amino acid.

slurry-packing technique (about 12,000 theoretical plates per 20 cm). The column was eluted at room temperature and at a flow-rate of 1.3 ml/min. The column eluate was monitored at 250 nm by a Shimadzu SPD-2AS spectrophotometric detector.

For the chiral mobile phase method, a column-switching system (Fig. 1) was assembled. Both the mobile phase and OPTA reagent were delivered at a constant flow-rate of 0.8 ml/min by means of a double-plunger pump (Samuki Industry Co., Tokyo, Japan). The laboratory-built gradient device was designed for a linear gradient from 1 to 30% acetonitrile. The eluent flow was switched by using two valves (V-1 and V-2) connected to the two ODS columns. The column eluate was mixed with OPTA reagent in a mixing T-Piece and a PTFE-tubing reaction coil (50 cm × 0.5 mm I.D.). The fluorescence intensity of the effluent was measured at the excitation and emission maxima of 340 and 455 nm, respectively, in a Shimadzu RF-500LC spectrofluoromonitor, equipped with a xenon discharge lamp.

*GITC derivatization procedure*

To 10 μl of 0.1 *M* HCl, containing 100 μg/ml of each amino acid, were added 10 μl of acetonitrile, containing 50 mg/ml of triethylamine and 20 μl of GITC reagent, prepared by dissolving 10 mg of GITC per ml of acetonitrile. The resulting mixture was allowed to stand at room temperature for about 20 min. An aliquot of 3–5 μl of the reaction mixture was directly injected into the chromatograph.

## RESULTS AND DISCUSSION

*Chiral derivatization method*

GITC is a widely applicable chiral derivatization reagent for the reversed-phase liquid chromatographic resolution of enantiomeric amino compounds such as amino acids[13,14] and catecholamines[17]. It reacts readily with a racemic free amino acid to form a pair of diastereomeric thiourea derivatives. Previously, these diastereomers, prepared from racemates of common protein amino acids, were completely resolved on an ODS column, eluted with methanol–phosphate buffer (pH 2.8). The resolution obtained may be due to the lipophilic nature of the tetraacetylglycosyl residue as well as conformational rigidity.

The present study accomplished simultaneous resolution of GITC derivatives of all common protein amino acid enantiomers. A highly efficient column and a ternary solvent delivery system were required. Recently, a column of particle size 3-μm became commercially available. However, since chromatography was performed at lower pressures (below 200 kg/cm$^2$), a 5-μm particle size ODS column (200 × 6 mm I.D.) was used. A typical chromatogram of the simultaneous resolution of GITC derivatives and the gradient program used in this case are shown in Fig. 2. The methanol content in the mobile phase had a significant effect on the separation of mono-GITC derivatives of arginine and histidine enantiomers. Complete separation of these derivatives was effected by eluting with a methanol-rich mobile phase. However, the viscosity of the methanol increased the column pressure. Therefore, other derivatives were eluted with an acetonitrile-rich mobile phase. GITC derivatives of all common protein amino acid racemates, except cysteine, were separated within about 2 h, although a few peaks of the derivatives were overlapped.

*Chiral mobile phase method*

Resolution with a mobile phase containing a chiral additive is simple and permits the sensitive detection of enantiomeric amino acids by post-column derivatization with various reagents. We have previously reported the direct resolution of underivatized amino acid racemates with a chiral mobile phase containing the copper(II) complex of N(*p*-toluenesulphonyl)-L-phenylalanine, TosPhe-Cu(II)[15,18,19], or that of N(*p*-toluenesulphonyl)-D-phenylglycine, TosPhG-Cu(II)[15], by reversed-phase HPLC. These methods provided excellent resolution of each of the protein amino acids which were sensitively detected with the OPTA reagent.

In the present study, simultaneous direct resolution of the protein amino acids was investigated by ligand-exchange chromatography, with TosPhG-Cu(II) as a chiral additive in the mobile phase.

Gradient elution with an organic solvent, such as methanol or acetonitrile, is generally required for the separation of a complex mixture of substances having

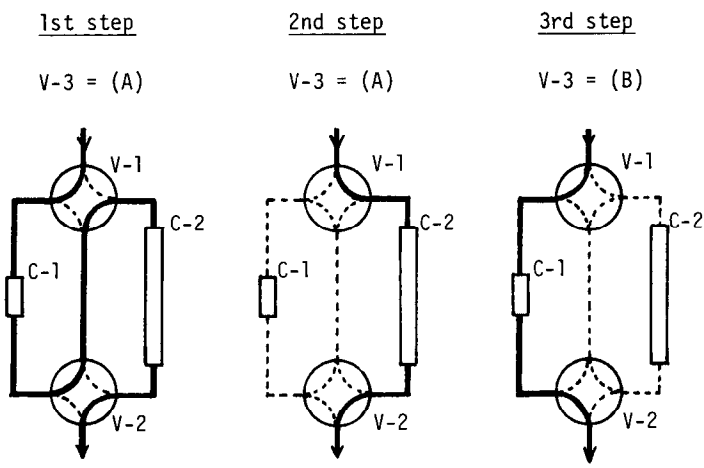


Fig. 3. Mode of column switching in the method represented by Fig. 1 for the simultaneous analysis of free D,L-amino acids with the TosPhG-Cu(II) eluent system. The solid line shows the flow of the chiral mobile phase.

similar chemical properties. However, gradient elution causes a delay in the equilibration of the column with the chiral additive in the mobile phase. The stereoselective retention was previously proposed[15] to be based on a dynamic ligand-exchange mechanism involving the labile immobilization of TosPhG-Cu(II) on the stationary phase. First, the binary complex $(TosPhG)_2Cu$ in the mobile phase is adsorbed on the surface of the chemically bonded phase through hydrophobic interaction, and equilibrium is attained between the free and immobilized chelate complexes. When gradient elution with acetonitrile is performed after the equilibrium was established, the immobilized chelate complexes are removed from the surface of the column support. These chelate complexes accidentally deposited in the stainless-steel line between the column and the detector. Furthermore, reequilibration of the column with TosPhG-Cu(II) after gradient elution was time-consuming. These problems could be overcome by using a short column packed with ODS-silica gel of a low carbon content. On the other hand, relatively polar amino acids, such as the acidic amino acids, were not well retained on such a short column. Therefore, a column-switching technique with two ODS columns, 5 cm and 20 cm long, was adopted in the HPLC system described in Fig. 1. Changes in the mobile phase flow-rate were accomplished in three steps (Fig. 3. In the first step, the two columns are connected in series, and the mobile phase passes through columns C-1 and C-2 in succession. Amino acids injected into the chromatograph were first resolved on the column C-1. After the faster-moving, relatively polar amino acids (Asp, Glu, Ser, Thr and Ala) had been eluted from this column, valve V-1 was switched to the position in the second step, as shown in Fig. 3. Then the relatively polar amino acids were more finely resolved on column C-2. After this resolution was over, both valves V-1 and V-2 were turned as shown in Fig. 3, and the linear gradient elution through column C-1 was started by turning valve V-3. The less-polar amino acids, which had been retained on column C-1 were then resolved on column C-1 alone. A chromatogram of the simultaneous resolution of acidic and neutral protein amino acids by the column-switching pro-

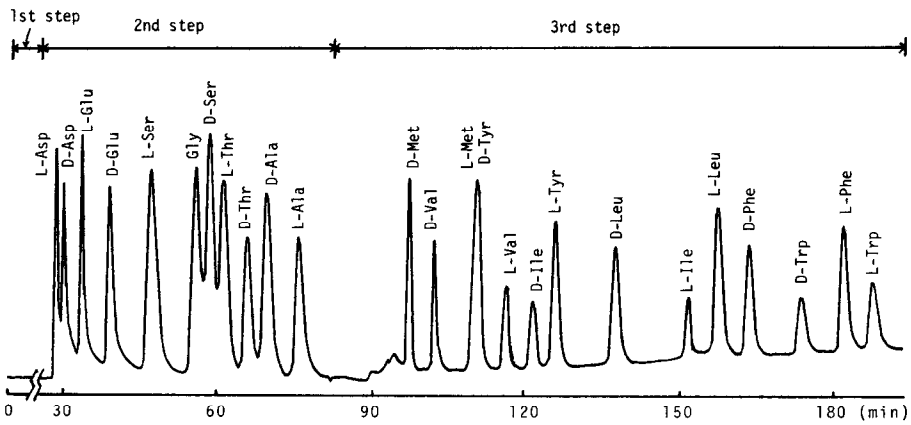


Fig. 4. Chromatogram of the simultaneous resolution of free D,L-amino acids with the TosPhG-Cu(II) eluent system and column-switching technique. The chromatographic conditions and the mode of column switching are described in the text and shown in Fig. 3. Injection: 100–250 pmol of each amino acid.

cedure is shown in Fig. 4. The imino acids, such as proline, could be detected by post-column derivatization with 7-chloro-4-nitrobenzofrazan (NBD-Cl)[19].

The methods described here are expected to be useful for the detection of enantiomeric amino acids in previous samples, only small amounts of which can be spared for analysis.

## REFERENCES

1 T. Tamegai, M. Ohmae, K. Kawabe and M. Tomoeda, *J. Liquid Chromatogr.*, 2 (1979) 1229.
2 T. Nambara, S. Ikegawa, M. Hsegawa and J. Goto, *Anal. Chim. Acta*, 101 (1978) 111.
3 J. Goto, N. Goto, A. Hikichi and T. Nambara, *J. Liquid Chromatogr.*, 2 (1979) 1179.
4 J. Goto, N. Goto, A. Hikichi, T. Nishimaki and T. Nambara, *Anal. Chim. Acta*, 120 (1980) 187.
5 P. E. Hare and E. Bil-Av, *Science*, 204 (1979) 1226.
6 E. Gil-Av, A. Tishbee and P. E. Hare, *J. Amer. Chem. Soc.*, 102 (1980) 5115.
7 S. Weinstein, *Angew. Chem., Int. Ed. Engl.*, 21 (1982) 218.
8 V. A. Davankov and S. V. Rogozhin, *J. Chromatogr.*, 60 (1971) 280.
9 G. Gubitz, W. Jellenz and W. Santl, *J. Chromatogr.*, 203 (1981) 377.
10 J. Boué, R. Audebert and C. Quivoron, *J. Chromatogr.*, 204 (1981) 185.
11 Y. Tapuchi, N. Miller and B. L. Karger, *J. Chromatogr.*, 205 (1981) 325.
12 S. Weinstein, M. H. Engel and P. E. Hare, *Anal. Biochem.*, 121 (1982) 370.
13 N. Nimura, H. Ogura and T. Kinoshita, *J. Chromatogr.*, 202 (1980) 375.
14 T. Kinoshita, Y. Kasahara and N. Nimura, *J. Chromatogr.*, 210 (1981) 77.
15 N. Nimura, A. Toyama, Y. Kasahara and T. Kinoshita, *J. Chromatogr.*, 239 (1982) 671.
16 D. Theodropoulos and L. C. Craig., *J. Org. Chem.*, 21 (1956) 1376.
17 N. Nimura, Y. Kasahara and T. Kinoshita, *J. Chromatogr.*, 213 (1981) 327.
18 N. Nimura, T. Suzuki, Y. Kasahara and T. Kinoshita, *Anal. Chem.*, 53 (1981) 1380.
19 N. Nimura, A. Toyama and T. Kinoshita, *J. Chromatogr.*, 234 (1982) 482.