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Note

Optical resolution of DL-proline by reversed-phase high-performance liquid chromatography using N-(*p*-toluenesulphonyl)-L-phenylalanine–copper(II) as a chiral additive

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High-performance liquid chromatographic (HPLC) resolution of amino acid enantiomers using a chiral chelate additive has been developed for several years[1–3]. However, few systems exhibiting both a highly efficient resolution and a highly sensitive detection for underivatized amino acid enantiomers have been established. We recently reported[4] a reversed-phase liquid chromatographic resolution method using a N-(*p*-toluenesulphonyl)-L-phenylalanine–copper(II) complex [TosPhe-Cu(II)] as chiral additive. Use of the *o*-phthalaldehyde (OPTA) reagent in this method facilitated sensitive detection of common α-amino acids down to pmole levels. However, the reagent could not be applied for the detection of imino acids such as proline. Although Gil-Av *et al.*[5] achieved a reversed-phase liquid chromatographic resolution of enantiomeric amino acids using a proline–copper(II) complex as chiral additive, it may not be possible to combine their system with any detection method effective for proline.

In the present study, DL-proline was resolved on a octadecylsilyl-bonded phase using TosPhe-Cu(II) as chiral additive, and monitored by a fluorimetric post-column derivatization using 7-chloro-4-nitrobenzofrazan (NBD-Cl).

MATERIALS AND METHODS

Proline and other reagents were purchased from Wako (Osaka, Japan). Chemically bonded octadecylsilyl silica gel, Develosil ODS (particle size 5 μm), was obtained from Nomura Chemical (Seto-shi, Japan). Glass-distilled water was used throughout the experiments.

TosPhe was synthesized as described by McChensney and Swann[6]. The mobile phase was prepared by dissolving 319 mg (1 mmol) of TosPhe and 125 mg (0.5 mmol) of $CuSO_4 \cdot 5H_2O$ in 1 l of water and adjusting the pH of the resultant solution with to 6.0 with 5% aqueous sodium carbonate solution. The NBD-Cl reagent was prepared by dissolving 600 mg of 7-chloro-4-nitrobenzofrazan (E. Merck, Darmstadt, G.F.R.) in 300 ml ethanol. Alkalinizing buffer for the post-column derivatization was prepared by mixing ethanol and 0.05 *M* borate buffer, pH 9.5, containing the disodium salt of EDTA (2 g/l) in a volume ratio of 1:1.

Fig. 1 shows the flow diagram of our chromatograph. The mobile phase was

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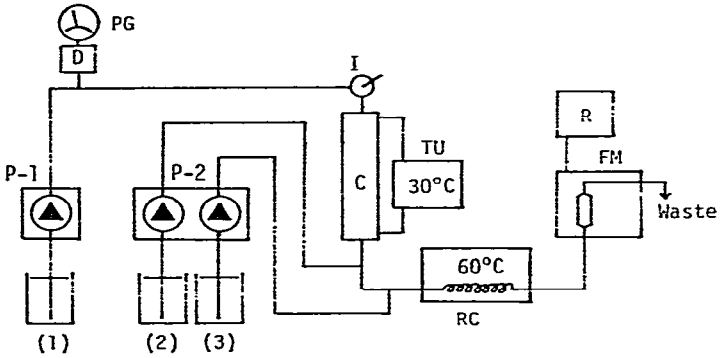

Fig. 1. Flow diagram of the chromatographic system. P-1 = Single plunger pump; P-2 = double plunger pump; D = damper; PG = pressure gauge; I = injector; C = column and column jacket; TU = thermo unit; RC = reaction coil; FM = fluoromonitor; R = recorder; 1 = mobile phase; 2 = alkalinizing buffer; 3 = NBD-Cl reagent.

delivered at a constant flow-rate of 1.0 ml/min using a single plunger pump (Sanuki Industry, Tokyo, Japan). Develosil ODS was packed in the stainless-steel column (10 cm × 4.0 mm I.D.) in our laboratory by the conventional slurry packing technique. The column was operated at 30°C utilizing a Taiyo thermo unit C-600 (Taiyo Scientific Industry, Tokyo, Japan). Both the alkalinizing buffer and NBD-Cl reagent were delivered at a constant flow-rate of 0.6 ml/min using a double plunger pump (Sanuki Industry).

The column eluate was first mixed with the alkalinizing buffer in a T-piece, and then with NBD-Cl reagent in another T-piece. The mixture was then allowed to flow through a PTFE-tubing reaction coil (4 m × 0.5 mm I.D.) immersed in a water-bath at 60°C. The fluorescence intensity of the effluent was measured at 530 nm; excitation of fluorescence was achieved at 470 nm, using an RF-500 LCA spectrofluoromonitor (Shimadzu, Kyoto, Japan) equipped with a xenon discharge lamp.

RESULTS AND DISCUSSION

NBD-Cl was first described by Ghosh and Whitehouse[7] as a fluorigenic reagent for amino acids and amines, and it has been shown[8] to provide a much more intense fluorescence on reaction with imino acids than with α-amino acids. Roth[9] applied NBD-Cl for the specific detection of proline and hydroxyproline in liquid chromatography. In the present study, NBD-Cl was found to be suitable for the micro-detection of D- and L-proline after resolution. TosPhe did not interfere with the fluorimetric detection of proline since NBD-Cl gives only a slow colour reaction with sulphonamides[10]. Precipitation of copper(II) ions in the mobile phase by the action of the alkalinizing buffer was prevented by adding EDTA. With this system, the quantitation of proline down to 1 nmol was achieved.

The conditions for the resolution were essentially those used previously for common amino acids[4]. Fig. 2 shows the resolution of DL-proline when 20 nmol of the racemate were injected into the chromatograph shown in Fig. 1. L-Proline was eluted before the D-isomer, as with the racemates of neutral amino acids described pre-

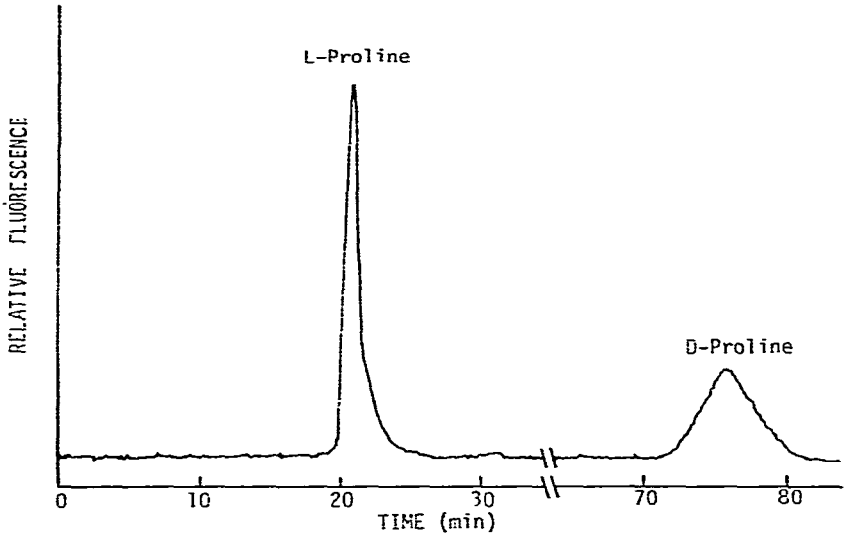

Fig. 2. Separation of DL-proline with TosPhe-Cu (II) eluent. Mobile phase; aqueous solution containing 1 m*M* TosPhe and 0.5 m*M* $CuSO_4 \cdot 5H_2O$. pH 6.0. Approximately 10 nmol of each isomer were injected.

viously[4]. In addition, DL-proline showed a relatively large separation factor (α = 3.92), corresponding to a difference in the free energies[11] between the solutions of the two enantiomers ($\Delta\Delta G^0 = -RT \ln \alpha$) of 820 cal/mol, whereas other common α-amino acid enantiomers gave α values of less than 2.90[4].

In the mobile phase, TosPhe and copper(II) ion are assumed to form a binary complex, $(TosPhe)_2Cu(II)$ in which TosPhe is in the form of its mono anion. D- and L-proline injected into the column are believed to undergo ligand exchange with the binary complex on the reversed phase, and the corresponding diastereomeric ternary complexes, TosPhe–Cu(II)–D-proline and TosPhe–Cu(II)–L-proline, may be formed. The excellent resolution of DL-proline may be due to the difference in stability between the two diastereomeric species caused by the fixed conformation of the pyrrolidine ring.

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