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Determination of cyanine-labeled amino acid enantiomers by cyclodextrin-modified capillary gel electrophoresis combined with diode laser fluorescence detection

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Abstract

Enantiomeric amino acids were labeled with a cyanine derivative (Cy5) for selective determination by capillary electrophoresis combined with diode laser-induced fluorometry. Cyanine-labeled glutamic acid has a high negative charge so that it was difficult to detect by conventional capillary zone electrophoresis due to its electrophoretic mobility being greater than the electroosmotic mobility. Thus, cyclodextrin-modified capillary gel electrophoresis using replaceable poly(vinylpyrrolidone) (PVP), which effectively suppresses the electroosmotic flow, was employed to separate Cy5-labeled amino acid enantiomers. The separation of six amino acid enantiomers could be achieved by using 1% PVP solution containing 70 m*M* γ-cyclodextrin. It was also shown that PVP does not affect the selectivity of optical isomers. © 1998 Elsevier Science B.V.

Keywords: Enantiomer separation; Cyanine labelling; Derivatization, electrophoresis; Amino acids; Poly(vinylpyrrolidone)

1. Introduction

The enantiomeric separation of racemic mixtures has attracted many scientists because of its importance in the pharmaceutical and biological fields. One of the most powerful techniques for racemic separation is capillary electrophoresis (CE). There are several reports on the enantiomeric separations of amino acids by CE, in which a metal chelate [1,2], cyclodextrin [3,4], crown ether [5,6] and optical active detergents [7,8] were used as chiral selectors. On the other hand, sensitive detection in chiral separation is also an important subject since high optical purity is often needed in the field of biological study.

Laser-induced fluorescence (LIF) detection is one of the most sensitive detection technique in CE because of its low background signal and the small detection volume. Ueda et al. [4] have reported chiral separation of amino acids by cyclodextrin-modified micellar electrokinetic chromatography (MEKC) at attomole levels with LIF detection. However, almost all lasers are difficult to use due to their high cost and complex maintenance needed. We have reported the utilization of diode lasers as light sources for LIF [9,10]. Diode lasers are preferential for determining biological substances since the emission wavelength of diode lasers are in deep-red and infrared region, in which almost all the biological substances have no

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absorption band. We have already demonstrated highly sensitive detection of amino acids [11,12], polycyclic aromatic hydrocarbons [13,14] and oligonucleotides [15] by diode laser LIF combined with CE and MEKC.

Cyclodextrin-modified capillary gel electrophoresis (CGE), which was first introduced by Guttman et al. [16], is an excellent separation technique for chiral separation [16]. However, crosslinked poly(acryl amide) is not easy to use because of its poor reproducibility and short life time. Recently, replaceable non-crosslinked poly(acryl amide) [17,18] and poly(ethylene oxide) [19] have been used as a sieving matrix for DNA analysis. Schutzner et al. [20,21] reported the separation of diastereomers by CE with poly(vinylpyrrolidone) (PVP) as a polymer additive. They also demonstrated that PVP is the best polymer additive for the separation of diastereomers among three types of polymers, PVP, poly(ethylene glycol) and poly(acryl amide). This is due to the inherent low viscosity and strong stereoselectivity of PVP [22].

In this paper, we report the enantiomeric separation and detection of cyanine-labeled amino acid derivatives by cyclodextrin-modified CGE combined with diode LIF detection. Cyanine-labeled amino acids have highly negative charge so that they migrate toward a cathode even in the presence of electroosmosis. Thus, PVP and cyclodextrin were used as an additive to suppress the electroosmotic flow (EOF) and as a chiral selector to separate the enantiomers, respectively.

2. Experimental

2.1. Apparatus

CE apparatus has already been described elsewhere [23]. A diode laser emitting at 635 nm is used as an exciting light source. The laser beam is focused by a microscope objective lens into a capillary (GL Sciences, Tokyo, Japan; 50 μm I.D.×375 μm O.D.). Fluorescence is collected by an objective lens and is passed through interference and spatial filters. The signal from a photomultiplier tube is measured by a strip-chart recorder. The sample is injected by gravity. A high-voltage power supply, Model HCZE-30PN0.25 (Matsusada Precision Devices, Shiga, Japan), is used for applying the voltage. When the buffer solution containing PVP was used, the CE runs were carried out under reverse mode, in which samples were injected at the cathodic end.

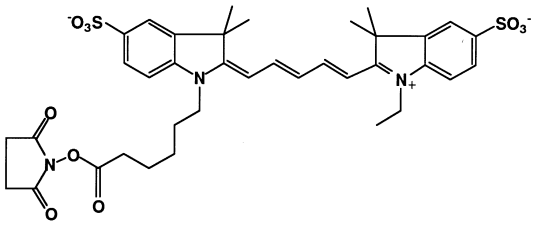

Fig. 1. Structure of cyanine derivative.

2.2. Chemicals

A cyanine derivative for labeling amino acids (Cy5) (FluoroLink Cy5 Mono Reactive Dye) was obtained from Amersham (Tokyo, Japan). The structure of Cy5 is shown in Fig. 1. D,L-Alanine, L-glutamic acid and PVP (molecular mass; 40 000) were purchased from Kishida (Tokyo, Japan). All other chemicals were obtained from Wako (Osaka, Japan).

2.3. Derivatization procedure

Amino acids were labeled with Cy5 as follows: 1 mg of Cy5 was dissolved in 500 μl of dried dimethylformamide, and then 10 μl of the solution was added to 50 μl of each 1 m*M* amino acid solutions containing phosphate buffer (pH 9.2). After standing for 1 h at room temperature, the solutions were mixed and diluted. The sample solution contains 10 μ*M* of D- and L-glutamic acid, 10 μ*M* of D- and L-alanine, 10 μ*M* of D- and L-valine, 11 μ*M* of D-phenylalanine, 12 μ*M* of L-phenylalanine, 11 μ*M* of D-tyrosine, 9 μ*M* of L-tyrosine, 17 μ*M* of D-tryptophan and 10 μ*M* of L-tryptophan.

3. Results and discussion

The excitation and the emission maxima of Cy5 are at 649 and 670 nm, respectively. Thus, a diode laser emitting at 635 nm was chosen as an exciting

light source to effectively exclude the scattering light. To evaluate the sensitivity for Cy5 in CE combined with diode LIF detection, 3 μ*M* of Cy5 aqueous solution and 10 m*M* phosphate buffer solution was used as a sample and running buffer, respectively. The electropherogram of Cy5 is shown in Fig. 2. The main peaks in Fig. 2 can be assigned as Cy5–NH_2 and Cy5 acid according to the literature [24]. The detection limit for Cy5 calculated for $S/N=3$ is $2\cdot10^{-8}$ *M*. Unfortunately, the detection limit is not better than the value obtained by other publications. Poor sensitivity in this study may be caused by large background due to scattering light. However, Chen et al. [24] obtained a detection limit of 10^{-10} *M* by using the combination of a narrow band filter at 670±10 nm and a long-pass filter to detect the Cy5-labeled oligonucleotide primer. This detection limit is smaller than that obtained by using a helium–cadmium laser for the detection of naphthalene-2,3-dicarboxyaldehyde-labeled amino acid ($3.6\cdot10^{-10}$ *M*) [4]. Thus, the sensitivity can be improved by changing optical filter from a long-pass filter to a dielectric mirror or a notch filter.

A cyanine derivative used as a labeling reagent is negatively charged so that amino acids labeled with the cyanine derivative have highly negative charge at the pH greater than its isoelectric point. Thus, a phosphate buffer solution of pH 4.55 was used to suppress the dissociation of amino acids. An electropherogram of amino acids obtained at pH 4.55 is shown in Fig. 3. Glutamic acid labeled with the dye has trivalent negative charge even at pH 4.55 so that it migrated toward the anode and could not be detected. In Fig. 3, the separation of optical isomers could not be achieved even in the presence of cyclodextrin because of large electroosmotic mobility. The detection limit, which was calculated for $S/N=3$, was $6\cdot10^{-8}$ *M* for tyrosine, alanine and valine in Fig. 3.

An easy way to detect all the amino acids is

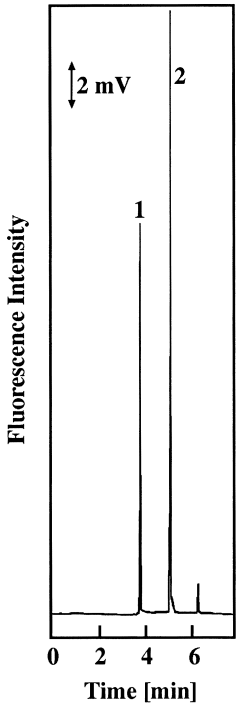

Fig. 2. Electropherogram of Cy5. (1) Cy5–NH_2, (2) Cy5–COOH. Buffer, 10 m*M* phosphate (pH 8.0); applied voltage, 20 kV (16 μA); sample concentration, 3 μ*M*.

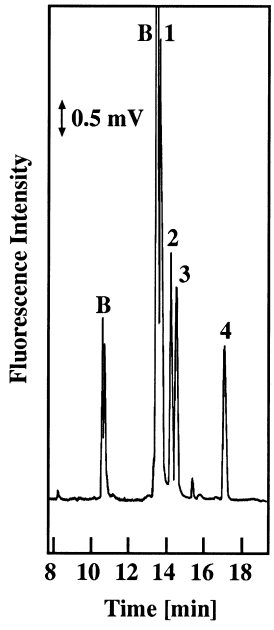

Fig. 3. Electropherogram of Cy5-labeled D,L-amino acids. (1) Tryptophan and phenylalanine, (2) tyrosine, (3) alanine, (4) valine, (B) blank. Buffer, 10 m*M* NaH_2PO_4 containing 50 m*M* γ-cyclodextrin (pH 4.55); applied voltage, 20 kV (3 μA); sample concentrations, 9.9 μ*M* D,L-alanine, 9.5 μ*M* D-glutamic acid, 11 μ*M* L-glutamic acid, 11 μ*M* D-phenylalanine, 12 μ*M* L-phenylalanine, 17 μ*M* D-tryptophan, 10 μ*M* L-tryptophan, 11 μ*M* D-tyrosine, 9.2 μ*M* L-tyrosine, 9.9 μ*M* D-valine, 10 μ*M* L-valine.

suppression of the EOF. Polymers have been used as additives to suppress the EOF, e.g., hydroxyethyl cellulose, poly(ethylene oxide) and poly(acryl amide). Generally, polymer solutions are difficult to inject into the capillary due to high viscosity needed for sufficient resolution. PVP, which is used for the separation of diastereomers by Schutzner et al. [22], has low viscosity compared to poly(ethylene glycol) and poly(acryl amide). In fact, it was ascertained in a preliminary study that PVP solution could be easily injected into the capillary. Thus, PVP was used to suppress electroosmosis in the present study.

The chiral separation of cyanine-labeled amino acids was shown in Fig. 4. Six amino acids could be separated in all the electropherograms which were obtained by using different PVP concentrations. While increasing the PVP concentration, migration times of amino acids became smaller, but resolution between optical isomers could not be improved. Thus, the change in the migration of amino acids was caused by insufficient suppression of the EOF. The difference in the electrophoretic mobilities of optical isomers can be written as

$$\Delta\mu = \mu_L - \mu_D \quad (1)$$

where μ_L and μ_D are electrophoretic mobilities of L and D isomeric forms, respectively. In the presence of the EOF, $\Delta\mu$ is calculated from the migration times of amino acids as

$$\Delta\mu = \frac{L}{t_L E} - \frac{L}{t_D E} = (\mu_L + \mu_{eo}) - (\mu_D + \mu_{eo}) \quad (2)$$

where t_L and t_D are migration times of L and D isomeric forms, μ_{eo} is the electroosmotic mobility, E is the strength of the electric field, and L is the effective length of the capillary. The values of $\Delta\mu$ calculated from the migration times in Fig. 4 are listed in Table 1. It is noted in Table 1 that PVP does not change the selectivity between D- and L-amino acids. Thus, PVP is effective only for the suppression of the electroosmosis. However, the EOF is not completely suppressed in Fig. 4 since all the migration times of amino acids decreased with increased concentration of PVP.

The effect of varying cyclodextrin concentration is shown in Fig. 5. The ratio of the migration times, t_L/t_D, decreased with increased concentration of γ-cyclodextrin. The selectivity is improved only for tyrosine and tryptophan at the concentration of 70

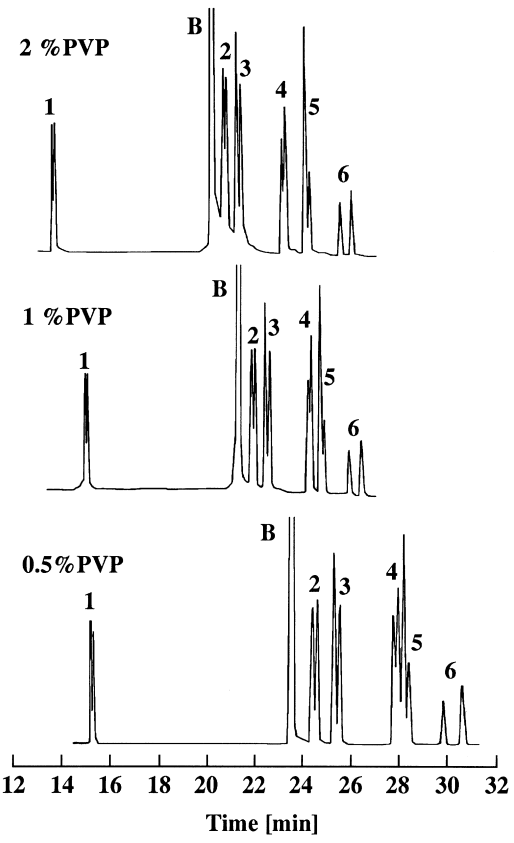

Fig. 4. Chiral separation of Cy5-labeled amino acids. PVP concentrations, 2%, 1%, and 0.5%. (1) Glutamic acid, (2) alanine, (3) valine, (4) phenylalanine, (5) tyrosine, (6) tryptophan, (B) blank. Buffer, 10 m*M* phosphate buffer (pH 7.0) containing PVP and 50 m*M* γ-cyclodextrin; applied voltage, 25 kV (8 μA). Other conditions as in Fig. 3.

Table 1
Difference in the electrophoretic mobilities between D- and L-amino acids

Solute	PVP concentration (%)		
	0.5	1	2
Glu	$1\cdot10^{-6}$	$1\cdot10^{-6}$	$2\cdot10^{-6}$
Ala	$4\cdot10^{-7}$	$6\cdot10^{-7}$	$6\cdot10^{-7}$
Val	$9\cdot10^{-7}$	$9\cdot10^{-7}$	$8\cdot10^{-7}$
Phe	$5\cdot10^{-7}$	$5\cdot10^{-7}$	$4\cdot10^{-7}$
Tyr	$5\cdot10^{-7}$	$7\cdot10^{-7}$	$7\cdot10^{-7}$
Try	$2\cdot10^{-6}$	$1\cdot10^{-6}$	$1\cdot10^{-6}$

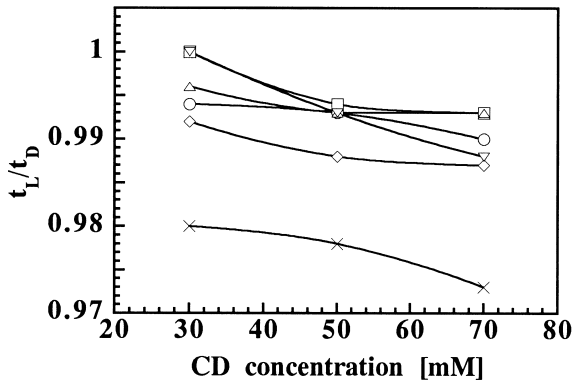

Fig. 5. Dependence of ratio of migration time between D- and L-amino acids on the concentration of γ-cyclodextrin. Buffer, 10 m*M* phosphate buffer (pH 7.0) containing 1% PVP. Symbols: ○=glutamic acid, □=alanine, ◇=valine, △=phenylalanine, ▽=tyrosine, ×=tryptophan. Other conditions as in Fig. 4.

m*M*. The electropherogram obtained by using 1% PVP solution containing 70 m*M* γ-cyclodextrin as a running buffer is shown in Fig. 6. As described above, the resolution between enantiomers is enhanced by increasing the concentration of γ-cyclodextrin though the peaks of alanine and valine are somewhat overlapped to that of the blank.

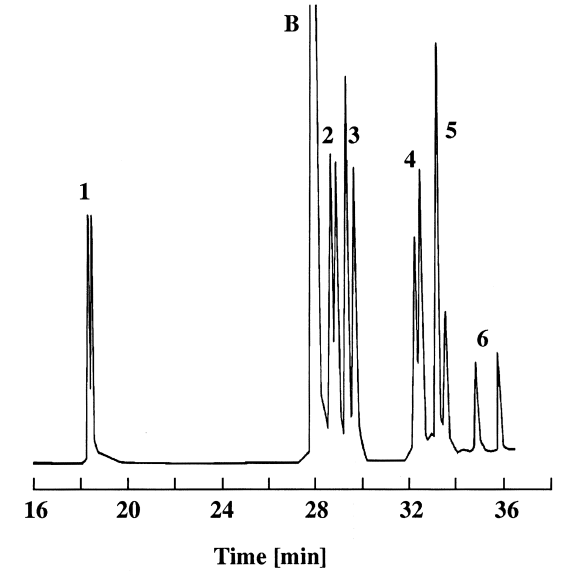

Fig. 6. Chiral separation of Cy5-labeled amino acids. (1) Glutamic acid, (2) alanine, (3) valine, (4) phenylalanine, (5) tyrosine, (6) tryptophan, (B) blank. Buffer, 10 m*M* phosphate buffer (pH 7.0) containing 1% PVP and 70 m*M* γ-cyclodextrin; applied voltage, 25 kV (9 μA). Other conditions as in Fig. 3.

4. Conclusions

Cyanine-labeled amino acids were elucidated with diode LIF detection after the separation by cyclodextrin-modified CGE using replaceable PVP solution. The detection limit calculated for $S/N=3$ was $6\cdot10^{-8}$ *M* for tyrosine, alanine and valine. Further improvement in sensitivity can be expected by replacing optical filters to exclude the scattering laser light. Increasing the concentration of PVP efficiently suppressed the EOF, but the difference in the electrophoretic mobilities of the optical isomers was not changed. It was demonstrated that the resolution of optical isomers could be enhanced by increasing the concentration of γ-cyclodextrin. Diode LIF detection is a powerful technique for highly sensitive determination of amino acid enantiomers.

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