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Separation of naphthalene-2,3-dicarboxaldehyde-labeled amino acids by high-performance capillary electrophoresis with laser-induced fluorescence detection

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ABSTRACT

Analysis of amino acids derivatized by reaction with naphthalene-2,3-dicarboxaldehyde (NDA) was investigated using high-performance capillary electrophoresis (HPCE) combined with laser-induced fluorescence detection. Of the HPCE modes, capillary zone electrophoresis, micellar electrokinetic chromatography and cyclodextrin (CD)-modified micellar electrokinetic chromatography (CD-MEKC) were applied to the separation of these amino acid derivatives. CD-MEKC allowed separation in less than 30 min and proved to be effective for chiral separation in some cases. A detection limit of 0.8 amol was obtained for NDA-labelled leucine at a signal-to-noise ratio of 2.

INTRODUCTION

Recent advances in biotechnology require highly sensitive analytical methods for the determination of biological compounds such as amino acids and polypeptides. This has led to the development of a variety of analytical instrumentation, including high-performance liquid chromatography (HPLC) and high-performance capillary electrophoresis (HPCE) with high resolving power. Compared with HPLC, HPCE seems to be more attractive in terms of the high column efficiency and the possibility of improved mass sensitivities for sample components due to smaller injection volumes [1]. Unfortunately, currently available commercial detectors limit the sensitivities actually obtainable.

Recently, several detection schemes including electrochemical [2,3], radiometric [4], mass spectrometric [5], indirect fluorescence [6] and laser-induced fluorescence [7–10] have been investigated to achieve improved sensitivity. To date, the most sensitive detector investigated for the capillary zone electrophoresis (CZE) of amino acids was based on the laser-induced fluorescence after precolumn deri-

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vatization with fluorescein isothiocyanate (FITC) [7]. Nickerson and Jorgenson [8] also demonstrated the utility of laser-induced fluorescence (LIF) detection for CZE by employing several tagging reagents, such as *o*-phthalaldehyde (OPA), naphthalene-2,3-dicarboxaldehyde (NDA) and FITC, for the pre-column derivatization of amino acids. The detection limits are at the attomole level, but the neutral amino acids migrate at similar velocities in the CZE mode, thereby resulting in poor separation of these amino acids.

Although lower detection limits can be obtained with LIF detection, it is necessary to find a greater variety of tagging reagents which favor the use of a laser, and whose derivatization product is compatible with the methods of separation in HPCE. Liu *et al.* [9] reported a new tagging reagent, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA), which gave isoindole derivatives of amino acids and peptides detectable in the low attomole range. CBQCA exhibited some desirable properties regarding the peak excitation wavelength coincidence of the derivatized compounds with the output wavelength of the helium–cadmium (He–Cd) laser (442 nm), the migration behavior of those compounds in HPCE and the reactivity with a variety of compounds such as peptides. However, the reagent is not yet commercially available. Therefore, we decided to examine NDA derivatization further, as only a limited exploration of HPCE has been made with this reagent, yet it is readily available commercially.

NDA reacts with primary amines in the presence of cyanide to form 1-cyano-2-substituted-benz[*f*]isoindole (CBI) derivatives, which exhibit improved stability compared with OPA derivatives and high quantum efficiencies [11,12], even in the aqueous buffer solutions usually required in HPCE. Further, the excitation maxima of these compounds are well matched to the output wavelength of the He–Cd laser. Although some of the CBI–amino acids have been used to reveal the advantages of LIF detection in CZE [7,13], the separation of these compounds has not been pursued by other electrophoretic methods, such as micellar electrokinetic chromatography (MEKC) or cyclodextrin (CD)-modified MEKC. In this study, the separation of CBI-amino acids was investigated using several modes of HPCE to characterize the migration profiles of these compounds within each mode. LIF detection of these compounds was performed to demonstrate the capability of the HPCE–LIF system as an analytical tool which can exhibit high resolving power and high sensitivity.

EXPERIMENTAL

Apparatus

Fig. 1 shows a schematic diagram of the optical configuration of the LIF detector used. The laser is a Model 4207NB He–Cd laser (Liconix, Santa Clara, CA, USA), which can produce 7 mW of output power at 442 nm. The emitted light from the laser

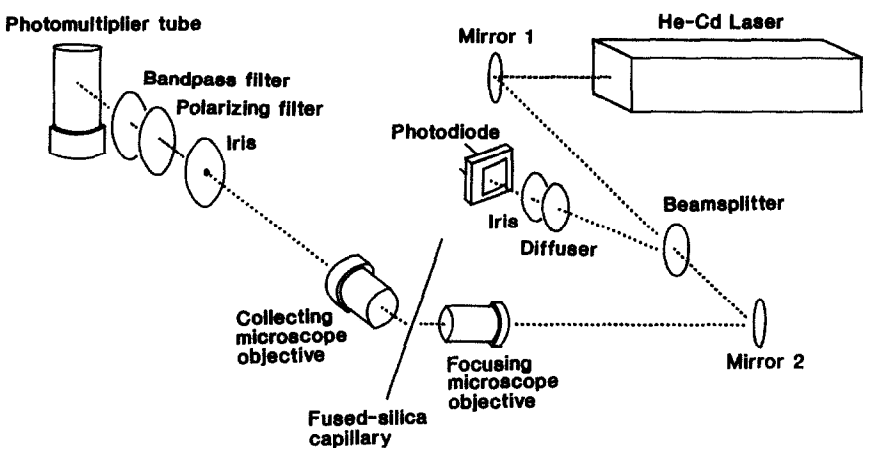

Fig. 1. Schematic diagram of laser-induced fluorescence detector for HPCE.

was reflected from a dielectric mirror (Model DM.6, 99% reflectivity at 442 nm) (Newport, Fountain Valley, CA, USA) and divided into two beams with a Model 44920 beam splitter (Oriel, Stratford, CT, USA). The laser output was monitored with a silicon photodiode (Hamamatsu, Bridgewater, NJ, USA) after passing the split portion of the laser beam through an optical diffuser (Oriel) and an iris diaphragm (Oriel). The output of the photodiode was converted to voltage and used as a reference voltage to compensate for the laser source noise with an analogue ratio circuit designed and constructed in this laboratory. The portion of the laser beam transmitted directly through the splitter was reflected from a second mirror and focused with a 10× microscope objective (Oriel) on the bared surface of a fused-silica capillary from which a 2-mm portion of the polyimide coating was removed at a point 50 cm from the inlet end. The capillary tubing of 50 μm I.D. and 290 μm O.D. (Code No. 062464; Scientific Glass Engineering, Ringwood, Victoria, Australia) was mounted axially at 45° to the laser beam in order to reduce the collection of scattered excitation light. The emitted fluorescence from the sample was collected with a 20× microscope objective (Oriel) at right-angles to the incident beam and passed through an iris diaphragm, a polarizing filter (Code No. 65.5050; Rolyn Optics, Covina, CA, USA) and a Model 490DF20 bandpass filter with a center wavelength of 490 nm and a half-bandwidth of 20 nm (Omega Optical, Brattleboro, VT, USA). As the numerical aperture of the microscope objective used in this study was 0.4, the calculated efficiency of collection of the emitted fluorescence from the analyte was *ca.* 4% [7]. A Model R1527 photomultiplier tube (Hamamatsu) operated at 900 V with a Model 227 high-voltage power supply (Pacific Instruments, Concord, CA, USA) was used to detect fluorescence. The current output from the photomultiplier was converted to voltage and divided by the reference voltage with the analogue ratio circuit. The resulting voltage ratio was displayed on a Model CR501 data processor (Shimadzu, Columbia, MD, USA).

Capillary electrophoresis was performed with an in-house designed instrument. A Model EH30R3 high-voltage power supply (Glassman High Voltage, Whitehouse Station, NJ, USA) was used to generate the electric field across a capillary of 70 cm total and 50 cm separation length. Each end of the capillary was immersed in a separate glass bottle filled with buffer solution. Platinum wire electrodes were inserted in the buffer solutions for electrical connection. The high-voltage end of the capillary was enclosed in a Plexiglas box with a safety interlock. For temperature control, the capillary was inserted in 40 cm × 0.8 mm I.D. Teflon tube, which was connected to two PEEK T-joints so that water could be circulated outside the capillary.

Reagents

The amino acid standard solution (A2161) used as a stock solution was purchased from Sigma (St. Louis, MO, USA). According to the manufacturer, it is prepared as a standard for the fluorescence detection of amino acids from protein hydrolysates, and contains $2.5 \cdot 10^{-5}$ *M* of each amino acid, except L-cystine at $1.25 \cdot 10^{-5}$ *M*. The standard solution was tested to ensure that each component was within 4% of its stated concentration. Individual L-amino acids for spiking for peak identification and DL-amino acids for chiral separation were also purchased from Sigma (Code No. LAA-21 and DLAA). NDA (Code No. A5594) was obtained from Tokyo Kasei (Tokyo, Japan). Sodium cyanide from Fluka Chemical (Ronkonkoma, NY, USA) was used as received. Sodium hydroxide of semiconductor grade, boric acid, sodium dodecyl sulfate (SDS), CDs (Code No. 85608-8 for β-CD and 86141-3 for γ-CD) were purchased from Aldrich (Milwaukee, WI, USA). All solutions were prepared in NANOpure (Sybron Barnstead, Boston, MA, USA) and filtered through a 0.2-μm pore size membrane filter before use.

Stock solutions

Stock solutions of 100 m*M* borate buffer were prepared weekly by dissolving 1.316 g of boric acid and an appropriate amount of sodium hydroxide in 200 ml of NANOpure water. NDA was dissolved in HPLC-grade acetonitrile (Fisher Scientific, Pittsburgh, PA, USA) at 4.6 mg per 25 ml to give a 1 m*M* solution every month. Stock solutions of 10 m*M* cyanide were prepared every 2 weeks by dissolving 49 mg of sodium cyanide in 100 ml of NANOpure water. For the preparation of amino acid solutions, serial dilutions with 0.1 *M* hydrochloric acid were made on a weekly basis from the stock solution to

give the desired concentration. All the solutions were stored at 4°C. For the experiments in which samples of less than 2.5 μM of each amino acid were used, all glassware was well rinsed with fresh NANOpure water just before use.

Derivatization procedure

To 700 μl of borate buffer solution (100 mM, pH 9.5) in a 1.5-ml vial, 100 μl of sodium cyanide solution (10 mM) and 100 μl of amino acid solution were added and mixed. Next, 100 μl of NDA solution (1 mM) were added to the vial and the vial was capped. After gentle shaking, the reaction was allowed to proceed at 25°C for 30 min. The standard sample solutions were prepared in concentrations ranging from 1 nM to 1 μM for each amino acid (except for L-cystine at half those concentrations).

Procedure

Sample solutions were introduced into the capillary with a hydrodynamic injection method. The injection volume was calculated according to the equation described by Rose and Jorgenson [14]. After the introduction of sample solution, 15 kV were applied across the capillary. Peak identification was performed by spiking the mixture with the individual amino acids (internal addition method).

RESULTS AND DISCUSSION

Separation of CBI-mino acids

Although the sample solutions used in this study contained sixteen amino acids with primary amine groups, multi-derivatized compounds, such as lysine and cystine, cannot be detected with this method

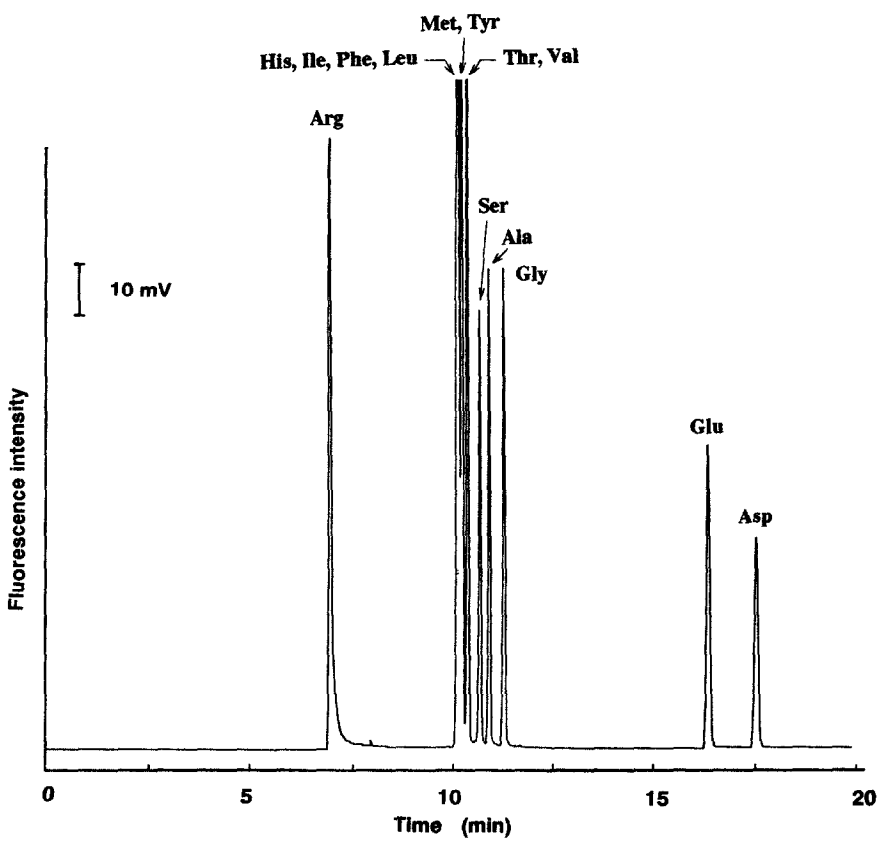

Fig. 2. Electropherogram of CBI-amino acids obtained by CZE. Electrolyte, 100 mM borate buffer (pH 9.0); capillary, 50 μm I.D. (290 μm O.D.), 70 cm in total length (50 cm to the detector); applied voltage, 15 kV; current, 27 μA; estimated injection volume, 2.9 nl; concentration for each CBI-amino acid, $2.5 \cdot 10^{-7}$ M.

because of fluorescence quenching [15]. The quantum efficiency of the di-derivatized lysine is only 0.02, compared with 0.5–0.8 for other amino acids. Even though NDA and OPA display common functionalities, *i.e.*, dicarboxaldehyde, it was reported that the addition of a surfactant such as Brij did not reduce the fluorescence quenching as it did with the OPA system [15]. However, it may be possible to enhance the fluorescence intensity for di-derivatized compounds with the addition of SDS or CDs to the electrolyte which worked well in the OPA system [16]. Therefore, three modes of HPCE, *i.e.*, CZE, MEKC and CD-MEKC, were applied to the separation of these CBI-amino acids with LIF detection.

In CZE, migration of each component in the sample is dependent of the ionic nature of the component. Usually, electroosmotic mobility through a fused-silica capillary at a neutral pH is larger than the electrophoretic mobility of the sample component. Hence the CBI-amino acids, which are negatively charged at pH 9.0 because of the remaining carboxylate, would migrate toward the detector. As shown in Fig. 2, these compounds are not well resolved in the CZE mode owing to the similarity of the molecular structure. However, the migration order of these compounds can be explained on the basis of the theory of CZE; CBI-Arg appeared first, then the CBI derivatives of neutral amino acids and finally CBI-Glu and CBI-Asp. As the isoelectric points (p*I*) are 10.76 for Arg, 3.08 for Glu and 2.98 for Asp, the observed migration order seems reasonable at least for these amino acids. The separation of other derivatives seemed to be difficult in the CZE mode because of their close p*I* values.

MEKC, which was first proposed by Terabe *et al.* [17], has been investigated by many researchers as a separation technique for both neutral and ionic compounds while retaining the high resolving power of HPCE. In this separation mode, an ionic surfactant such as SDS is added to the electrolyte at concentrations exceeding the critical micellar concentration. The separation of the solutes occurs on the basis of the partition mechanism between an aqueous buffer and a micelle. Therefore, MEKC would be expected to expand the application of HPCE to a variety of compounds. Fig. 3 illustrates the electropherogram of the CBI-amino acids obtained by MEKC. Compared with Fig. 2, the separation of the amino acids was dramatically improved by the addition of SDS to the electrolyte. CBI-Ser and CBI-Thr migrated faster than other amino acids, presumably owing to their hydrophilic

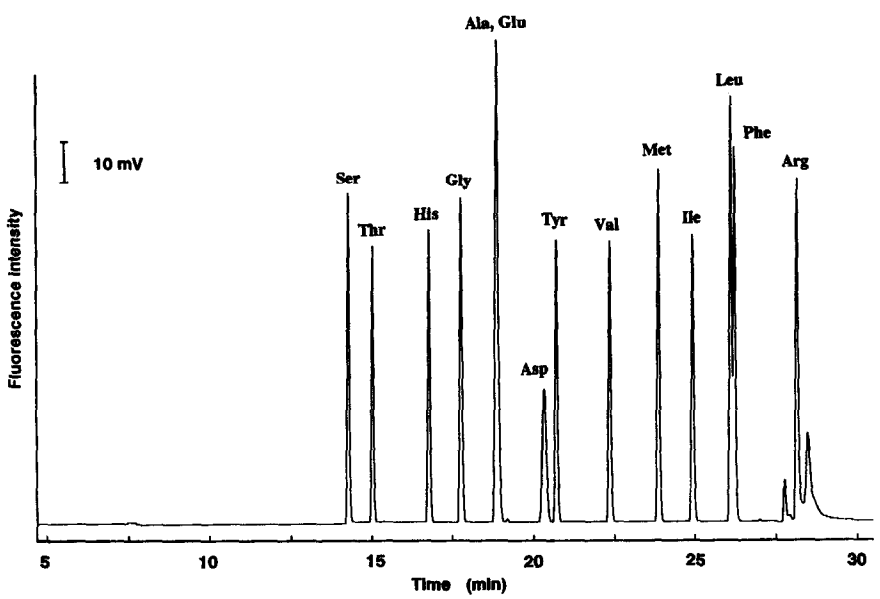

Fig. 3. Electropherogram of CBI-amino acids obtained by MEKC. Electrolyte, 50 m*M* SDS–100 m*M* borate buffer (pH 9.0); applied voltage, 15 kV; current, 35 μA; estimated injection volume, 2.5 nl; other conditions as in Fig. 2.

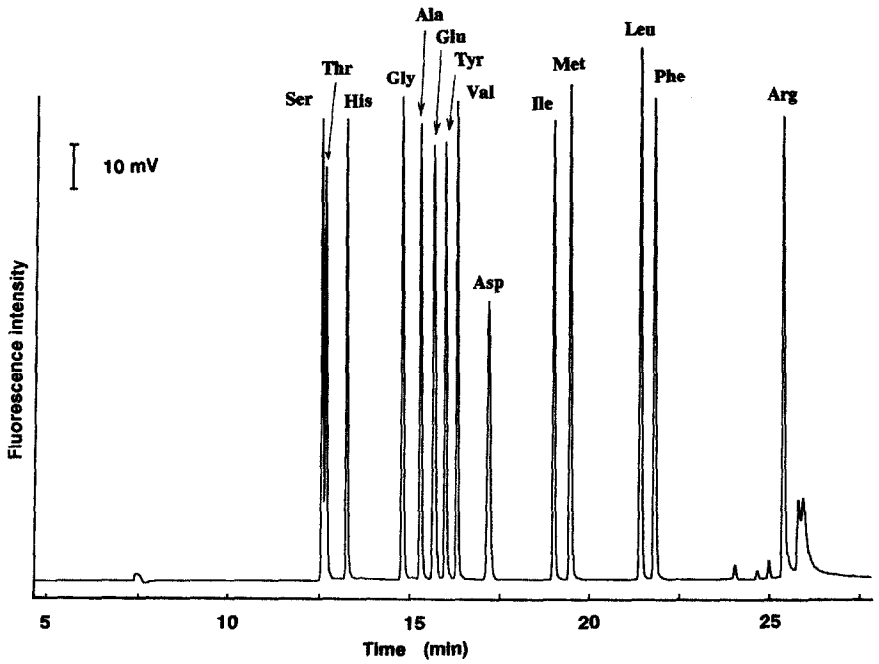

Fig. 4. Electropherogram of CBI-amino acids obtained by β-CD-MEKC. Electrolyte, 10 m*M* β-CD–50 m*M* SDS–100 m*M* borate buffer (pH 9.0); applied voltage, 15 kV; current, 36 μA; estimated injection volume, 2.5 nl; other conditions as in Fig. 2.

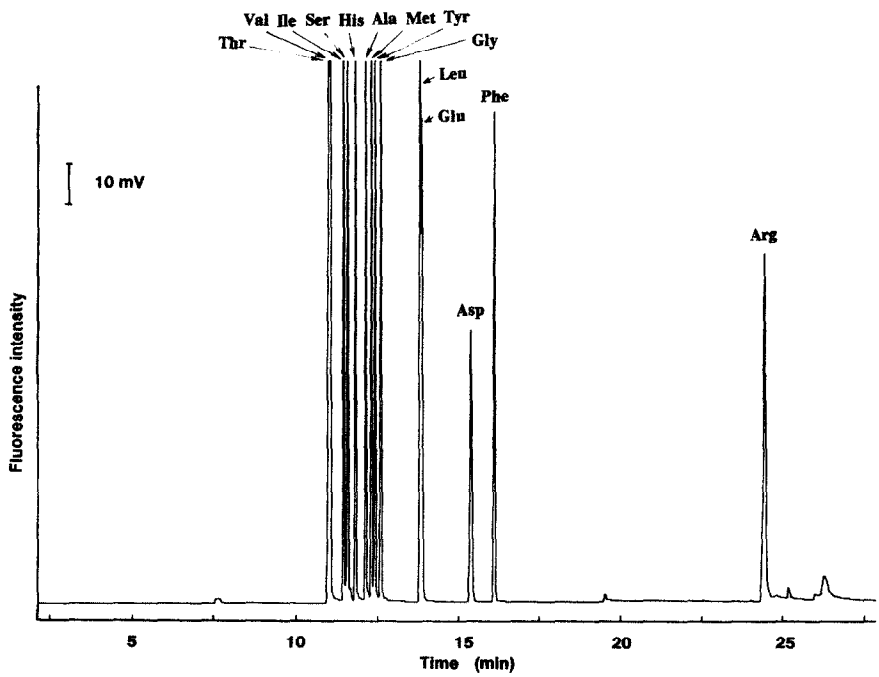

Fig. 5. Electropherogram of CBI-amino acids obtained by γ-CD-MEKC. Electrolyte, 10 m*M* γ-CD–50 m*M* SDS–100 m*M* borate buffer (pH 9.0), applied voltage, 15 kV; current, 36 μA; estimated injection volume, 2.5 nl; other conditions as in Fig. 2.

residues. Under these analytical conditions, CBI-Ala and CBI-Glu co-eluted, and the separation of CBI-Leu and CBI-Phe was incomplete by MEKC even at different pH values and/or higher concentrations of SDS. Manipulation of the migration time of CBI-Glu, which was possible by changing pH of the buffer solution, led to an improved separation of CBI-Glu and CBI-Ala at pH 7.0. Unfortunately, the sensitivity towards lysine and cystine was still poor. In Fig. 3, the small peak just before the CBI-Arg corresponded to di-derivatized cystine, and the peak just after the CBI-Arg contained di-derivatized lysine together with by-products of the NDA reaction. Although a cationic surfactant is available for MEKC, CBI-amino acids did not separate satisfactorily in the presence of cetyltrimethylammonium bromide, presumably owing to the ion-pair formation between this cationic surfactant and the negatively charged CBI-amino acids.

A more successful approach was to add CDs to the MEKC system. This technique has already been reported by Terabe *et al.* [18] for the separation of highly hydrophobic compounds. In this study, addition of CDs to the electrolyte was expected to improve the separation of derivatized amino acids as it did in reversed-phase chromatography by introducing another partition mechanism [19]. CD itself will migrate at a velocity identical with the electroosmotic flow. When a solute interacts with a CD through the formation of an inclusion complex, its migration time becomes shorter. The difference in migration time for a solute with CD is strongly dependent on the degree of complexation of the solute with CD. Therefore, addition of CD to the micellar solution can change the migration selectivity for the solutes in MEKC. This method, named CD-MEKC by Terabe *et al.* [18], was applied to the separation of CBI-amino acids in this study.

Fig. 4 shows the electropherogram of the CBI-amino acids obtained by β-CD-MEKC. Compared with Fig. 3, the separation of CBI-Leu and CBI-Phe was dramatically improved. CBI-Ala now migrated substantially faster than CBI-Glu and the migration times were all reduced. Other conditions being equal, the addition of β-CD reversed the elution order of CBI-Met–CBI-Ile and CBI-Asp–CBI-Val.

Fig. 5 shows the different elution profile obtained when β-CD was replaced with γ-CD. Table I lists the migration orders of the CBI-amino acids in MEKC, β-CD-MEKC, and γ-CD-MEKC. The reproducibility of migration times for CBI-amino acids was less than 2% relative standard deviation ($n = 5$) in these modes. It was obvious that even though this separation system contained the same concentration of SDS in the operating buffer solutions, addition of CDs produced different selectivities in terms of solute migration characteristics. It can be concluded that CDs are effective for separations where manipulation of selectivity is necessary in MEKC.

TABLE I

ELUTION ORDER OF CBI-AMINO ACIDS IN MEKC AND CD-MEKC

Analytical conditions are given in Fig. 3 for MEKC, in Fig. 4 for β-CD-MEKC and in Fig. 5 for γ-CD-MEKC.

Elution order	MEKC	β-CD-MEKC	γ-CD-MEKC
1	Ser	Ser	Thr
2	Thr	Thr	Val
3	His	His	Ile
4	Gly	Gly	Ser
5	Ala, Glu	Ala	His
6	–	Glu	Ala
7	Asp	Tyr	Met
8	Tyr	Val	Tyr
9	Val	Asp	Gly
10	Met	Ile	Leu
11	Ile	Met	Glu
12	Leu	Leu	Asp
13	Phe	Phe	Phe
14	Arg	Arg	Arg

*Chiral separation of CBI-*DL*-amino acids*

During the course of this study, we found that the chiral separation of some CBI-DL-amino acids could be performed by CD-MEKC. This is not surprising, because CD or CD derivatives have been successfully applied to the enantiomeric separation of dansylated DL-amino acids in both HPLC [20,21] and other modes of HPCE [22,23]. The electropherogram shown in Fig. 6 illustrates the chiral separation of CBI-DL-amino acids obtained by β-CD-MEKC. The electrolyte contained 10 m*M* β-CD, 50 m*M* SDS, and 100 m*M* borate buffer (pH 9.0). Even though the difference in migration times for the D- and L-forms were very small, the inherently high efficiency of HPCE allowed the separation of these

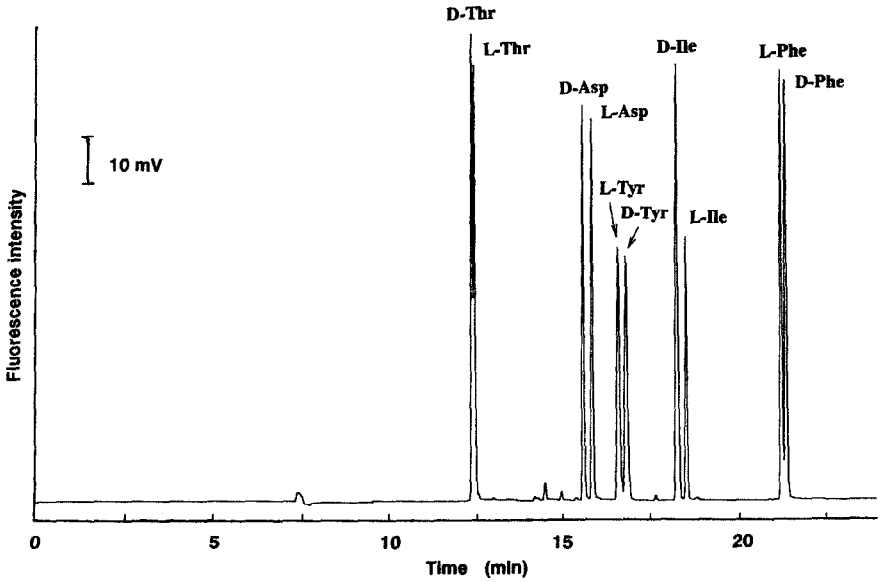

Fig. 6. Electropherogram of a mixture of five CBI-DL-amino acids obtained by β-CD-MEKC. Concentration of each pair of CBI-DL-amino acids, $4.0 \cdot 10^{-7}$ *M*; other conditions as in Fig. 4.

enantiomers, provided that the electrolyte contained SDS. The elution order of D- and L-enantiomers seemed to be dependent on the structure of the amino acids. As shown in Fig. 6, the D-enantiomers migrated faster than the L-enantiomers for derivatized aliphatic amino acids (Thr, Asp and Ile), whereas the reverse occurred for aromatic amino acids (Tyr and Phe). Even though all CBI-amino acids contain naphthalene rings in their structures, the functional group on each amino acid residue

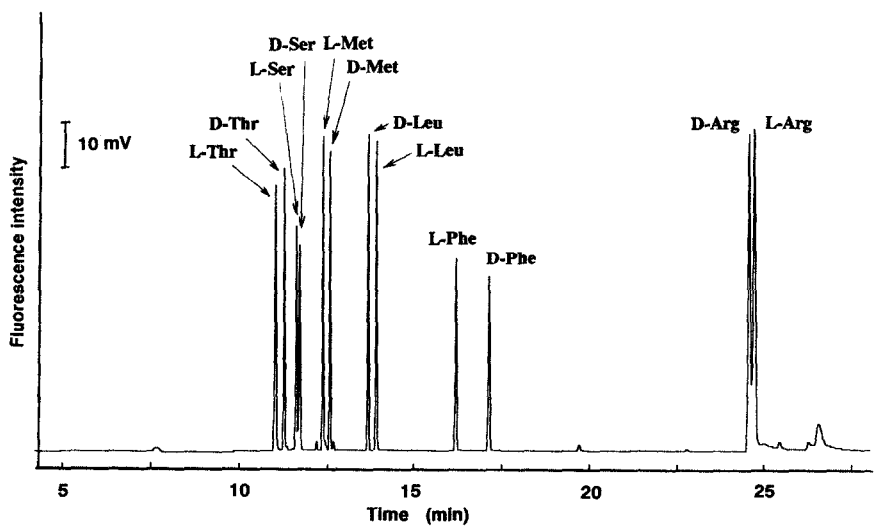

Fig. 7. Electropherogram of a mixture of six CBI-DL-amino acids obtained by γ-CD-MEKC. Concentration of each pair of CBI-DL-amino acids, $2.0 \cdot 10^{-7}$ *M*; other conditions as in Fig. 5.

might be important for their chiral recognition as these functional groups govern the degree of the formation of an inclusion complex.

To investigate further the nature of the chiral separation in CD-MEKC, γ-CD was employed with the same micellar solution. Fig. 7 shows the chiral separation of the CBI-DL-amino acids by γ-CD-MEKC. Here, the L-form of CBI-Phe still migrated faster than the D-form, whereas the elution order of the enantiomers of CBI-DL-Thr was reversed: the resolution of both pairs was enhanced. Indeed, all the D/L-forms shown in Fig. 7 were better separated than by β-CD-MEKC, with the exception of D- and L-Asp, which could not be separated (not shown). In the future, optimization of analytical conditions such as pH, concentration of SDS and CD, addition of organic solvent and buffer species will be necessary to understand the mechanism of the chiral separation of amino acids in CD-MEKC.

Detection limits

To assess the limits of detection (LOD) for the CBI-amino acids in HPCE, mixtures of the derivatives were injected at a concentration of $2.5 \cdot 10^{-7}$ *M* for each amino acid (corresponding to $2.5 \cdot 10^{-6}$ *M* before derivatization). The estimated injection volumes were 2.9 nl for CZE and 2.5 nl for MEKC. Table II lists the mass detection limits for the amino acids obtained by CZE, MEKC and β-CD-MEKC. The sensitivity varied little, the estimated detection limits ranging from 0.7 amol ($2.4 \cdot 10^{-10}$ *M*) to 2.3 amol ($9.2 \cdot 10^{-10}$ *M*). The detector produced a linear response over the range of 10^{-9}–10^{-6} *M* using the derivatized sample solutions serially diluted with the electrolytes.

TABLE II

LIMITS OF DETECTION FOR CBI-AMINO ACIDS IN HPCE–LIF

Limits of detection were calculated based on a signal-to-noise ratio of 2.

Amino acid	LOD (amol)		
	CZE	MEKC	β-CD-MEKC
Ser	0.7	1.0	0.9
Thr	–	1.1	1.1
His	–	1.1	1.0
Gly	0.7	1.0	0.9
Ala	0.7	–	1.0
Glu	1.1	–	1.0
Tyr	–	1.1	1.0
Val	–	1.1	0.9
Asp	1.5	2.3	1.6
Ile	–	1.1	1.0
Met	–	0.9	0.9
Leu	–	–	0.8
Phe	–	–	0.9
Arg	–	0.9	0.9

As the spot diameter of the laser beam is *ca.* 25 μm on the capillary surface, a "detection cell" volume of *ca.* 35 pl can be estimated. As a medium detection limit of $5.0 \cdot 10^{-10}$ *M* of a CBI-amino acid in 35 pl corresponds to 0.018 amol (10 500 molecules) of the analyte, and the collection efficiency of the microscope objective used in this study was considered to be 4%, the observed fluorescence is emitted from about 420 molecules of the analyte. This calculation was made to assess the theoretical limits of detection in the present system and the results indicate that there is still room for improvement. Further increases in sensitivity could be achieved by increasing the cell volume, so far as this can be done without losing resolution, increasing the collection efficiency of the emitted fluorescence and increasing the laser power. A new tagging reagent, having a high molar absorptivity at the excitation wavelength of the laser and high quantum efficiency, would also improve the detection limits. Investigations involving both these aspects of LIF detection are being continued.

CONCLUSIONS

The results of this work demonstrate (1) the improvement of the selectivities for CBI-amino acids by MEKC and CD-MEKC compared with CZE, (2) the chiral separation of CBI-DL-amino acids by CD-MEKC and (3) the high sensitivity obtained for these derivatives by using an LIF detector. Overall, the HPCE–LIF system can provide a powerful microanalytical method for biological molecules such as amino acids and peptides.

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