Determination of Amino Acids in Urine by Cyclodextrin-Modified Capillary Electrophoresis–Laser-Induced Fluorescence Detection

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Abstract

Capillary electrophoresis (CE) combined with laser-induced fluorescence detection is applied to the determination of amino acids in urine samples. The urine samples are first ultrafiltered, to remove proteins and large peptides, and the filtrates are then directly labeled by reaction with fluorescein isothiocyanate (FITC). Cyclodextrin-modified CE using α-cyclodextrin is employed for the separation of the FITC-labeled amino acids. Seven amino acids are clearly separated from side reaction products produced during the labeling reaction, when an 80mM borate buffer containing 45mM α-cyclodextrin is used as the running buffer. For quantitative analysis, rhodamine B is added to the labeled urine samples as an internal standard. The calibration curves for phenylalanine, glutamine, proline, glycine, serine, alanine, and valine are linear in the range of 10μM to 100μM. The concentration limits of detection for all of the amino acids are estimated to be 160~330nM. Conversely, the limit of quantitation (LOQ) was ~10μM and the limitations are due to the labeling efficiency rather than the sensitivity of the detector. Three amino acids in urine samples, glutamine, glycine, and alanine, are readily quantitated, while the concentrations of the others are below the LOQ. The present method would permit the determination of seven amino acids in urine successfully.

Introduction

Capillary electrophoresis (CE) is a powerful separation technique and is extensively used in the field of biochemical research including genomic, proteomic, and metabolomic analyses. High resolution of CE permits the determination of a wide variety of compounds found in cells and biological fluids. Metabolomics has recently become an important issue in post-genomic research (1,2). Biological fluids such as serum and urine are complex mixtures of many metabolites that are produced in the body. CE is a promising technique for analyses of biological fluids and cells because of its high resolving power. However, one of the problems associated with CE is the low sensitivity related to the use of the narrow capillary for the separations. To overcome this problem, a variety of sensitive detectors and on-column preconcentration techniques have been developed over the past 20 years. laser-induced fluorescence (LIF) detection has been extensively employed in the determination of amino acids and amines in serum (3), urine (3–5), plasma (4,6), cells and cell extracts (7–9), and biological tissues (10–13). Terabe and coworkers applied on-column concentration techniques to improve the sensitivity of CE for the determination of metabolites in cell extracts, human plasma, and urine (14–16). Lin et al. also reported on the determination of organic compounds in urine, plasma, and tissues using the sweeping technique (17,18). However, on-column preconcentration techniques require that the separation conditions be optimized because preconcentration and separation must be achieved simultaneously.

In this study, we describe the analysis of trace amounts of amino acids in urine samples by cyclodextrin-modified CE based on LIF detection. For the complete separation of the amino acids employed in this study, α-cyclodextrin was added to the running buffer. Seven amino acids were selected as analytes to be determined because these amino acids could be successfully separated from side reaction products of a fluorescent labeling reagent, fluorescein isothiocyanate (FITC). Some of the amino acids are importantly related to amino aciduria (e.g., phenylketonuria leads to an excessive excretion of phenylalanine, the hartnup syndrome shows the high value for serine, and ostemalacia with excessive glycine excretion is reported) (19). Therefore, the method was applied to the determination of amino acids in urine samples.

Experimental

Apparatus

The experimental setup for CE–LIF is essentially the same as that reported previously (20). A high-voltage power supply, a model HCZE-30PN0.25 (Matsusada Precision Devices, Shiga,

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Japan), was used as the high voltage source. A capillary (50 µm i.d., 375 µm o.d., total length 60 cm or 50 cm, effective length 50 cm or 40 cm, GL Sciences Inc., Tokyo, Japan) was positioned perpendicular to the laser beam axis using a homemade holder constructed on a three-dimensional (XYZ) translation stage (Σ707-60PC, Sigma Koki, Saitama, Japan). An argon ion laser emitting at 488 nm (Stabilite2017, Spectra-Physics Laser Inc., Mountain View, CA) was focused on the capillary by an objective lens (Nikon, Tokyo, Japan, magnification ×5). The power of the laser was adjusted to 20 mW. Fluorescence was collected by an objective lens (Olympus, Tokyo, Japan, magnification ×50) and passed through a notch (488 nm; bandwidth, < 10 nm, Kaiser Optical Systems, Ann Arbor, MI) and an aperture (homemade, 0.5 mm i.d.). The signal from a photomultiplier (R3896, Hamamatsu Photonics, Shizuoka, Japan) was interfaced with a personal computer (GV1700GGI, Applied, Fukuoka, Japan).

Chemicals

Boric acid, sodium hydroxide, pyridine, acetone, α-cyclodextrin, rhodamine B, glycine, L-alanine, L-glutamine, L-valine, L-phenylalanine, L-proline, and L-histidine were purchased from Wako Pure Chemical Industries (Osaka, Japan). L-Serine was obtained from Kishida Chemical Company (Osaka, Japan). Fluorescein isothiocyanate isomer I was obtained from Dojindo (Kumamoto, Japan). Deionized water was supplied by a water purification system (Elix, Millipore Co. Ltd., Molsheim, France). A borate buffer solution was prepared by dissolving boric acid in deionized water and adjusting the pH using sodium hydroxide. The urine samples were obtained from a volunteer of a healthy boy of six years. No careful attention was paid in handling of the urine samples.

Derivatization procedure

Standard solutions of each amino acid were prepared by dissolving 50 µmol (final concentration, 1mM) of amino acid in 50 mL of 150mM borate buffer (pH 9.0). For the identification of amino acids, each standard solution was independently labeled with FITC in the presence of an excess amount of the amino acid; 1 mL, containing 17mM of FITC dissolved in acetone, was added to 3 mL of a standard solution containing 10mM of amino acid. The derivatization reaction was catalyzed by the addition of 0.1% of pyridine. The reaction was carried out overnight in the dark at 20°C, and the resulting solutions of FITC-labeled amino acids were stored at 4°C.

For constructing calibration curves, amino acid mixtures were prepared by diluting standard solutions containing 1mM of amino acids at concentrations ranging from 10µM to 100µM with 150mM borate buffer (pH 9.0). Each mixture was labeled with FITC as follows; a 250 µL aliquot of the mixture was placed in a reaction vial, and 750 µL of 150mM borate buffer (pH 9.0), 1 µL of pyridine, and 500 µL of 17mM FITC were then added to the mixture. The reaction was carried out overnight in the dark at 20°C, and the resulting solutions of FITC-labeled amino acids were stored at 4°C.

Within 6 h after collection, urine samples were reacted with FITC. The urine samples were filtered by ultrafiltration (~10000 *g*, 30 min) to remove proteins and large peptides with an ultrafree-MC (Millipore: cut off molecular weight, 3000), before the derivatization reaction. In the labeling reaction, a 250 µL aliquot of urine was placed in a reaction vial, and 750 µL of 150mM borate buffer (pH 9.0), 1 µL of pyridine, and 500 µL of 17mM FITC solution were then added to the solution. The reaction was carried out overnight in the dark at 20°C.

For a recovery test, 5 nmol of each amino acid was added to 20 µL of filtered urine. The solution was diluted with 150mM borate buffer to 1 mL, and then labeled with FITC using the same procedure as described earlier.

Determination of amino acids

In the determination of amino acids, 100 µL of the labeled sample and 10 µL of 500µM rhodamine B, employed as an internal standard, were placed into a vial, and then diluted to 1 mL with water. Dilution of the sample improved the separation of the amino acids from side reaction products produced during the labeling with FITC. The samples were injected into the capillary by gravity. After injecting the sample, a voltage of –15 kV was applied for electrophoretic separation. Calibration curves were constructed by measuring ratio of the peak area for each amino acid to that for rhodamine B.

Results and Discussion

Optimization of the separation condition

Urine contains variable concentrations of several amino acids and metabolites. Therefore, it is essential that the amino acids be completely separated. Unfortunately, it is difficult to separate twenty essential amino acids labeled with FITC by conventional CE (21–23). In addition, labeling with FITC produces several side reaction products that can overlap with the contents in urine. Figure 1 shows the electropherogram of seven amino acids obtained by conventional CE. Only alanine and glycine were separated from an overlapped peak of phenylalanine, valine, glutamine, proline, histidine, and serine. Conversely, Nakamura

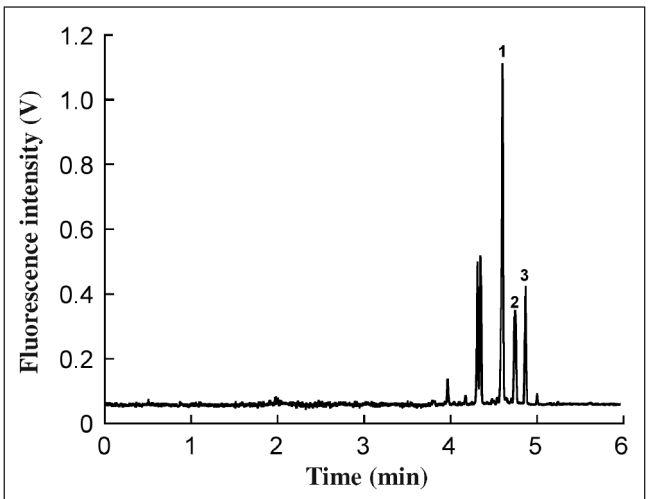

Figure 1. Electropherogram of FITC-labeled amino acids: 1, phenylalanine, valine, glutamine, proline, histidine, and serine; 2, alanine; 3, glycine. Conditions: migration buffer, 20mM borate (pH 9.0); capillary; total length, 50 cm, effective length, 40 cm; electric potential, –15 kV (5 µA).

et al. employed some organic solvents and cyclodextrin, including α-, β-, and γ-cyclodextrins, as additives for improving the separation of FITC-labeled amino acids (24). They concluded that the best separation of nineteen essential amino acids could be obtained, when a running buffer containing 45mM of α-cyclodextrin was used. Thus, we also attempted the separation of FITC-labeled amino acids under the same conditions. However, a practical separation of nineteen amino acids in which large amounts of FITC are required for the efficient labeling of analytes is nearly impossible because FITC would produce a large number of the side reaction products in the labeling reaction. Figure 2 shows the separation of the FITC-labeled amino acids employed in this study using the same conditions as reported by Nakamura et al. The sample contained the following eight amino acids: phenylalanine, valine, glutamine, proline, glycine, serine, alanine, and histidine. The inset shows the complete electropherogram of Figure 2. As seen in the inset of Figure 2, a number of the peaks related to side reaction products are present. The recommended conditions permit the separation of seven amino acids, phenylalanine, valine, glutamine, proline, glycine, serine, and alanine, although histidine, indicated by an asterisk, could not be separated from the peak for a side reaction product.

Derivatization condition for urine samples

In the determination of amino acids in urine samples, the amino acids must be efficiently labeled with FITC. In previous publications, it was reported that the preferable molar ratio of FITC to amino acid is 30:1 (11,12). However, the concentration of amino acids and amines in a urine sample is unknown. Therefore, to optimize the labeling conditions, the volume of the 17mM FITC solution was varied in the range from 10 µL to 500 µL in the derivatization of a urine sample. Figure 3 shows the effect of the volume of FITC on the ratio of the peak area for glycine in the urine sample to that for Rhodamine B, an internal standard. Glycine was identified by adding the standard solution of FITC-labeled glycine to the labeled urine sample. As seen in Figure 3, glycine in urine would be efficiently labeled when more than 200 µL of FITC was added. No difference in the relative intensities of the peaks was observed between the electropherograms for the urine samples added 400 µL and 500 µL of FITC. Therefore, 500 µL would be sufficient to label the amino acids contained in urine samples. Consequently, 500 µL of FITC was added to urine samples in the derivatization procedure.

Determination of amino acids in urine

Calibration curves were constructed for seven amino acids: phenylalanine, valine, glutamine, proline, glycine, serine, and alanine, by labeling mixtures of the amino acids with FITC. For all amino acids, the correlation coefficients of the calibration curves were greater than 0.992 (r^2) in the range from 10 to 100µM. The limit of quantitation (LOQ) was estimated to be ~ 10µM. In the electropherogram of the standard solution containing each amino acid at the concentration of 10µM, the signal-to-noise ratios (S/Ns) of amino acids were calculated to be 90~190. Assuming that the peak height is proportional to the concentration of the analyte, the concentration limits of detection (S/N = 3) were estimated to be 160~330nM. As a result, the LOQ is governed by the labeling efficiency rather than the sensitivity of the detector. A higher concentration of FITC is preferable for improving the labeling efficiency. However, side reaction products of FITC degrade the separation of the amino acids. A typical electropherogram of a urine sample is shown in Figure 4. When the concentration of FITC was increased, the peak denoted by an asterisk was wide and tailed, resulting in overlap with the valine and glutamine peaks. Thus, the concentration of FITC was not increased.

Table I shows the results of recovery for the seven amino acids. Three amino acids: glutamine, glycine, and alanine, were identified in the determination of amino acids in a urine sample. The concentrations of the amino acids were several hundred µM.

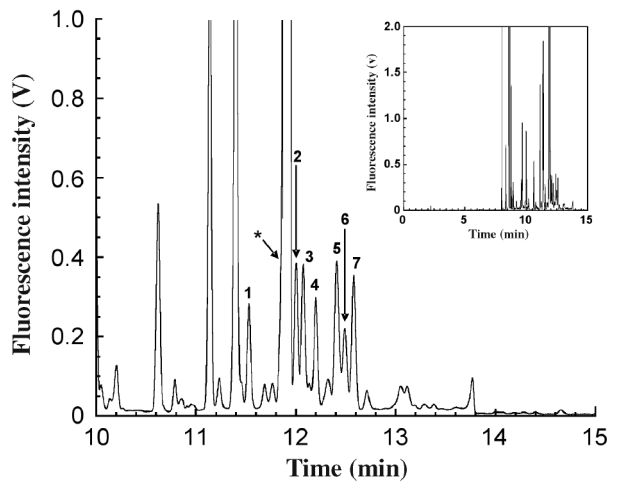

Figure 2. Electropherogram of FITC-labeled amino acids. 1, phenylalanine; 2. valine; 3. glutamine; 4, proline; 5, glycine; 6, serine; 7, alanine. An asterisk indicates histidine overlapped with FITC. The inset shows the complete electropherogram. Conditions: migration buffer, 80 mM borate (pH 9.2) containing 45mM α-cyclodextrin; capillary; total length, 60 cm, effective length, 50 cm; electric potential, –15 kV (5 µA).

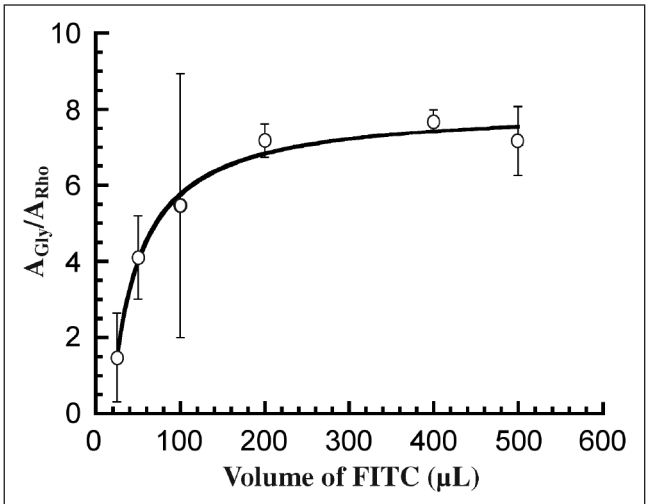

Figure 3. Effect of the volume of FITC added for labeling urine on the area of the glycine peak. A_{Gly} and A_{Rho} are the peak areas for glycine and rhodamine B, respectively. Capillary; total length, 50 cm, effective length, 40 cm; electric potential, –15 kV (6 µA). Other conditions are the same as in Figure 2.

All of the amino acids showed a more than 76% recovery. The recovery values for glutamine and proline were slightly lower than those for the other amino acids. This fact implies that labeling of these amino acids were insufficient under the derivatization conditions used for the urine sample. Phenylalanine, valine, proline, and serine were not found in the urine sample in this study. The recoveries for these amino acids were also greater than 77%, so that the method would also permit the determination of these amino acids.

There are a few reports on the simultaneous determination of amino acids contained in urine samples by CE. Boulat et al. have reported that the LOQ was 1µM for glycine and aspartic acid in human plasma, in which a fluorogenic labeling reagent, 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA), was employed in LIF detection (6). In the labeling reaction of CBQCA, it is necessary to add potassium cyanide, which is extremely toxic. Soga and coworkers demonstrated quantitative analysis of amino acids by CE-electrospray ionization-tandem mass spectrometry, in which the method permits the limit of detection at 0.5–14µM, although the LOQ is not shown (25). Electrospray ionization-tandem mass spectrometry has an advantage that no derivatization is needed, whereas it is expensive. The LOQ presented in this study is one order of magnitude higher than those obtained in the previous publication. However, the present method would be more conventional and inexpensive than those as described earlier.

Table I. Results for the Determination of Amino Acids in a Urine Sample

Amino acids	Found (µM)*	Recovery (%)
phenylalanine	nd	94
valine	nd	98
glutamine	200	76
proline	nd	77
glycine	100	82
serine	nd	93
alanine	430	117

* nd = not detected.

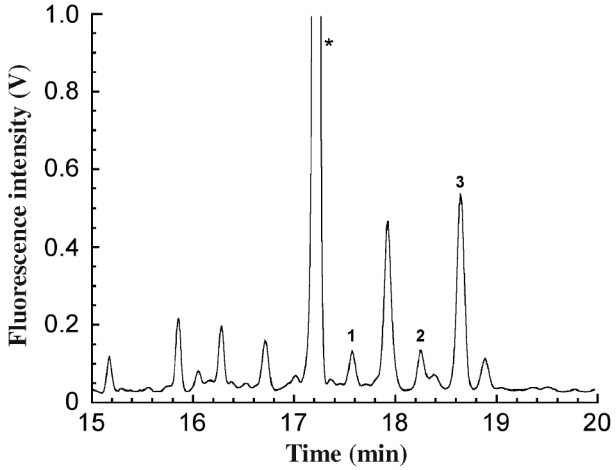

Figure 4. Electropherogram of a urine sample labeled with FITC. 1, glutamine; 2, glycine; 3, alanine. An asterisk indicates FITC. Other conditions are the same as in Figure 2.

Conclusion

Cyclodextrin-modified CE coupled with LIF detection was successfully applied to the determination of amino acids in urine. The method permits the analysis of seven amino acids without the need for complicated pretreatment procedures. Three types of amino acids were used in this study, with the recoveries in the range of 76~117%. The LOQ is estimated to be ~10µM for all of the amino acids measured in this study. The method permits the conventional analysis of seven amino acids. For analysis of the other amino acids and amines, which were not determined in this study, it will be necessary to optimize the separation conditions including buffer concentration, pH, and additives.

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