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Analysis of diagnostic metabolites by capillary electrophoresis–mass spectrometry

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Abstract

We describe here a procedure by capillary electrophoresis–mass spectrometry (CE–MS) for the direct analysis of urine samples on diagnostic metabolites, which are present in patient urine with metabolic disorders. The method was demonstrated using urine samples spiked with diagnostic metabolites, including glutathione for gamma-glutamyl transpeptidase deficiency, pyroglutamate for generalized glutathione deficiency, adenylosuccinate for adenylosuccinase deficiency, ornithine for gyrate atrophy, histidine for histidinemia, and homogentisic acid for alcaptonuria, at concentrations similar to those found in patients' urine. A coaxial sheath liquid flow was used for coupling CE and MS in electrospray ionization mode. Identification of the metabolites is based on their molecular weights and fragmentation patterns. The CE–MS method is highly specific and sensitive comparing to the previously reported method using migration time and UV absorption for identification. It should find broad application in clinical and pharmaceutical research and development.

Keywords: Glutathione; Pyroglutamate; Adenylosuccinate; Ornithine; Histidine; Homogentisic acid

1. Introduction

Human metabolic disorders often cause the accumulation of specific metabolites in patient's serum and urine [1]. The detection of diagnostic metabolites will aid the identification of disease marker(s) for clinical studies and monitoring their changes will assist drug candidate selection processes for preclinical research and development in pharmaceutical research.

Commonly used techniques in the area of metabolite analysis, such as gas chromatography (GC)-mass spectrometry (MS), paper and thin layer chromatography, and high-performance liquid chromatography (HPLC), often require tedious sample derivatization treatment prior to the analysis [2–4]. Capillary electrophoresis (CE), a powerful analytical separation technique that permits the efficient separation of charged molecules in small sample volume, has been applied in clinical studies for multi-component analysis [5–7]. CE coupled with electrochemical detection has been shown to be able to profile clinically important metabolites in human urine samples, including carbohydrates and amino acids [8]. Recently, CE, when equipped with a diode-array detector, has been successfully applied to the analysis of diagnostic metabolites in urine samples from patients with metabolic disorders [9–11]. In this approach, the complete separation of co-eluting components was required since the identification of

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diagnostic metabolites was based on retention time and UV absorption.

Mass spectrometry has been an attractive detection method for CE analysis [12,13]. It provides the capability to determine the molecular weight as well as to obtain structure related fragmentation information on the analytes. The coupling of capillary electrophoresis with mass spectrometry combines the ease and power of electrophoretic separation with the sensitivity and selectivity of mass spectrometric compound detection [14].

In this study, we report the successful application of CE–MS to the direct analysis of diagnostic metabolites in urine samples. The mass spectrometric detection in CE–MS significantly enhances the selectivity comparing to UV detection in CE analysis. The CE–MS method is simple, rapid, selective and sensitive.

2. Experimental

2.1. Chemicals and urine sample preparation

Glutathione, pyroglutamate, adenylosuccinate, ornithine, histidine and homogentisic acid were purchased from Sigma (St. Louis, MO, USA) and used without further purification. Buffers were freshly prepared using HPLC grade water from Aldrich (Milwaukee, WI, USA) and filtered through a 0.45 μm filter. Human urine samples were collected from a normal donor and spiked with the above diagnostic metabolites. The concentrations of the spiked metabolites were according to well-documented clinical data (Table 1). All other reagents were of the highest purity commercially available, and were used without further purification.

2.2. CE–MS

A Beckman P/ACE 2050 CE system with a 111 cm×75 μm bare fused-silica capillary was used for all separations. The CE unit was upgraded to perform the simultaneous application of voltage and pressure. A new power supply from Beckman designed for CE–MS was installed to override the high voltage leakage error caused by the coupling with electrospray MS. The capillary was rinsed for 0.5 minute with 0.1 *M* sodium hydroxide and 5 minutes with the background electrolyte (BGE) prior to each run. The extensive wash of the bare fused-silica capillary was found necessary to maintain good elution reproducibility, which may not be as critical in CE–MS analysis. Urine samples were injected directly without any pre-treatment at a pressure of 0.5 psi for 3 s, corresponding to an injection volume of about 7.2 nl. For those samples to which positive mode ESI MS was applied, borate buffer (300 m*M*, pH 8.5 by ammonium hydroxide) was used for CE separation with a voltage of 30 kV; while for those analyzed by negative mode ESI MS, ammonium bicarbonate buffer (100 m*M*, pH 8.5 by ammonium hydroxide) was used for CE separation with a voltage of 20 kV.

Table 1
Diagnostic metabolites studied by capillary electrophoresis–mass spectrometry

Metabolic disease	Diagnostic metabolite	Concentration spiked into normal urine[a]	Secretion amount in urine per day [1]	Detection limit[b]
γ-Glutamyl transpeptidase deficiency	Glutathione	0.8 mg/ml	~850 mg	0.08 mg/ml
Generalized glutathione deficiency	Pyroglutamate	10 mg/ml	~30–40 g	0.1 mg/ml
Adenylosuccinase deficiency	Adenylosuccinate	0.4 mg/ml	~460 mg	0.04 mg/ml
Gyrate atrophy	Ornithine	0.4 mg/ml	~410 mg	0.04 mg/ml
Histidinemia	Histidine	0.6 mg/ml	~620 mg	0.06 mg/ml
Alcaptonuria	Homogentisic acid	1.4 mg/ml	~7 g	0.07 mg/ml

[a] Estimation based on the assumption of 1 l urine/day.
[b] Based on injection of 7.2 nl urine samples without any treatment.

The on-line mass spectrometric analysis was performed on a Finnigan LCQ ion trap mass spectrometer. The fast scan speed offered by the ion trap instrument makes it an excellent choice for coupling with CE where the analyte often elutes as a narrow peak. In our CE–MS setup, the normal MS interface provided by the manufacturer for LC-MS was used with a minor modification. The exit side of the nozzle was cut slightly to accommodate the wider bore CE capillary. The mass spectrometer was calibrated according to the manufacturer's standard protocol and tuned through infusion of the diagnostic metabolites. A sheath flow design with a coaxial arrangement was used for interfacing CE and MS. The sheath liquid, acetic acid–methanol–water (10:45:45, v/v/v) for the positive ion mode and methanol–water (50:50, v/v) for the negative ion mode, was delivered by a syringe pump at 5 μl/min. The on-line CE–MS–MS experiment for obtaining structural information was performed by programming the mass spectrometer to alternate between full scan and MS–MS scan when the most abundant ion in the full scan mode was detected over a pre-set threshold. Identification of the diagnostic metabolites was based on the molecular weights and the fragmentation patterns under the collision induced dissociation condition.

3. Results and discussion

3.1. Analysis of urine samples spiked with diagnostic metabolites by on-line CE–MS

The urine samples were directly analyzed by CE–MS. Borate buffer eliminated the problem of CE capillary blockage even with the untreated urine samples [9–11]. Therefore a sample pre-treatment step was removed. The selection of CE separation buffer was based on the separation efficiency and the effect on mass spectrometric detection. The formation of a moving ion boundary helps to achieve good mass spectrometric response even with the use of a nonvolatile buffer, such as borate buffer (300 m*M*, pH 8.5 by ammonium hydroxide) [15]. Sheath liquid has been found to have a significant effect on CE separation as well as on MS detection [16]. The acid in the sheath liquid helps to lower the pH of the BGE to improve MS detection in the positive ion mode analysis.

Diagnostic metabolites were spiked into freshly collected normal urine samples at concentrations similar to those found in the patients' urine. The LCQ mass spectrometer was set to scan m/z from 50 to 500 in the MS full scan experiment, and dependent ion scan mode in the MS–MS experiment, respectively. Total mass chromatograms displayed the different metabolic profiles in normal and ''diseased'' (i.e. spiked) urine samples while extracted ion chromatograms for selected diagnostic metabolites demonstrated the specificity and sensitivity of the assay.

Adenylosuccinate is a metabolite that accumulates in patients with adenylosuccinase deficiency and is excreted in the patients' urine [17]. The CE–MS analysis of normal urine was compared with urine spiked with adenylosuccinate. A prominent peak of the molecular ion of m/z 464.1 which could be attributed to adenylosuccinate was detected in the ''diseased urine'', but was absent in the normal urine (Fig. 1).

The metabolic disorder gyrate atrophy results in hyperornithineuria. The disease is caused by a deficiency of ornithine-δ-amino-transferase and patients with gyrate atrophy excrete ornithine (0.4 g/day) in the urine [1]. The same experiment was carried out in the positive ion mode (Fig. 2). The molecular ion of ornithine was clearly observed in the ''diseased'' urine sample by CE–MS. No interference was found from the normal urine sample in the extracted ion chromatogram (m/z of 133.2, Fig. 2D).

Patients with inborn deficiency of γ-glutamyl transpeptidase excrete substantial amounts of glutathione in the urine [1]. The enzyme responsible for the disease is a member in the γ-glutamyl cycle. The extra peak in the ''diseased urine'' sample corresponds to the diagnostic metabolite glutathione (Fig. 3C and D), which is absent in the normal urine sample (Fig. 3A and B).

For those metabolites with no or low responses in the positive ion mode analysis, such as organic acids, the negative ion mode mass spectrometric analysis is the method of choice. Instead of an acidic solution, a methanol–water (50:50, v/v) solution was used as the sheath liquid to promote negative ion mode mass spectrometric detection. Homogentisic acid is an

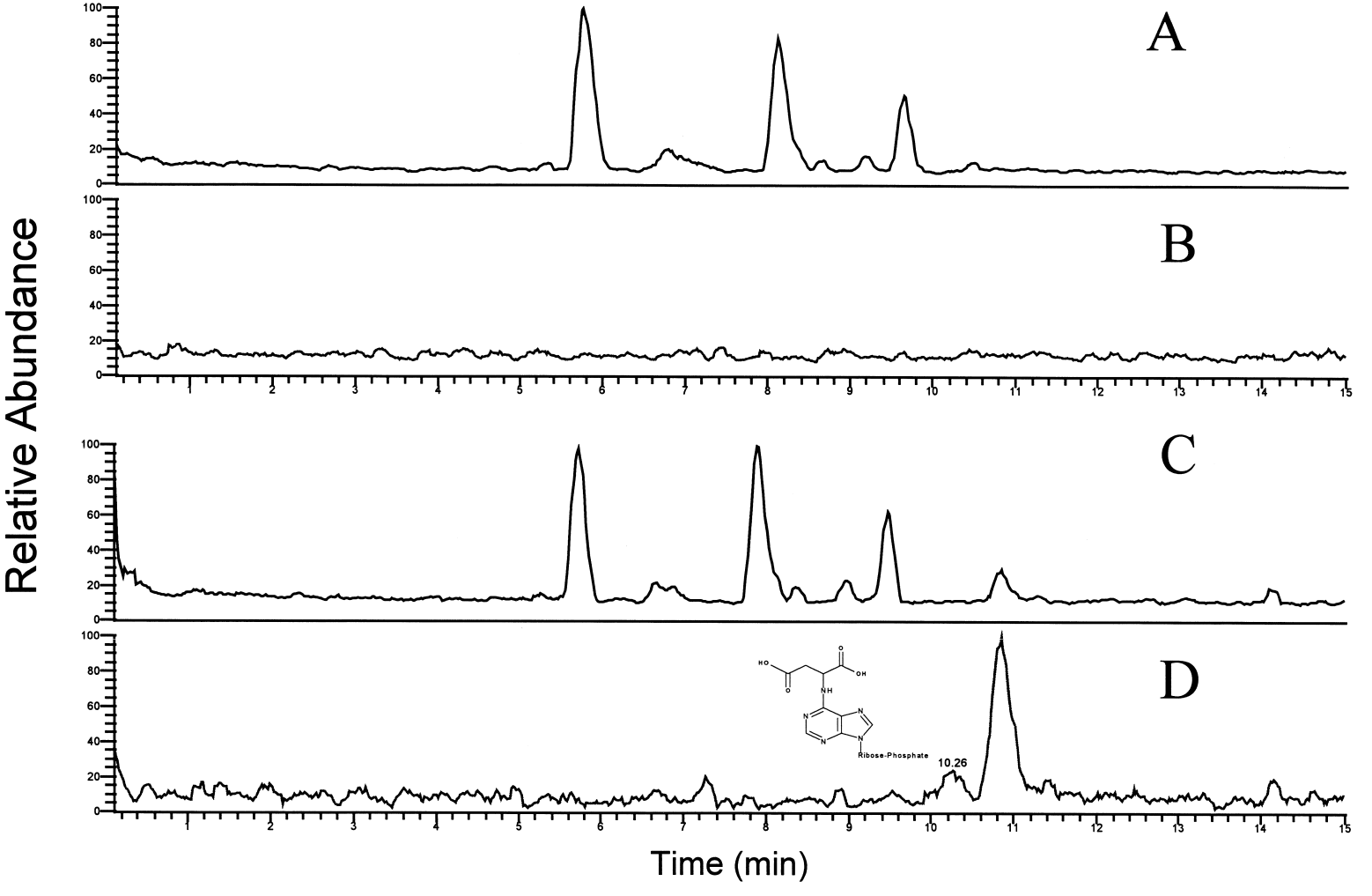

Fig. 1. CE–MS analysis of normal urine (A and B) and normal urine spiked with adenylosuccinate (C and D). Urine samples of 7.2 nl were injected without any treatment. Borate buffer (300 m*M*, pH 8.5 by ammonium hydroxide) was used and voltage for CE separation was 30 kV. ESI voltage was 5.5 kV in the positive ion mode. A and C: total mass chromatograms; B and D: extracted mass chromatograms of m/z 464.1 (MH^+ of adenylosuccinate).

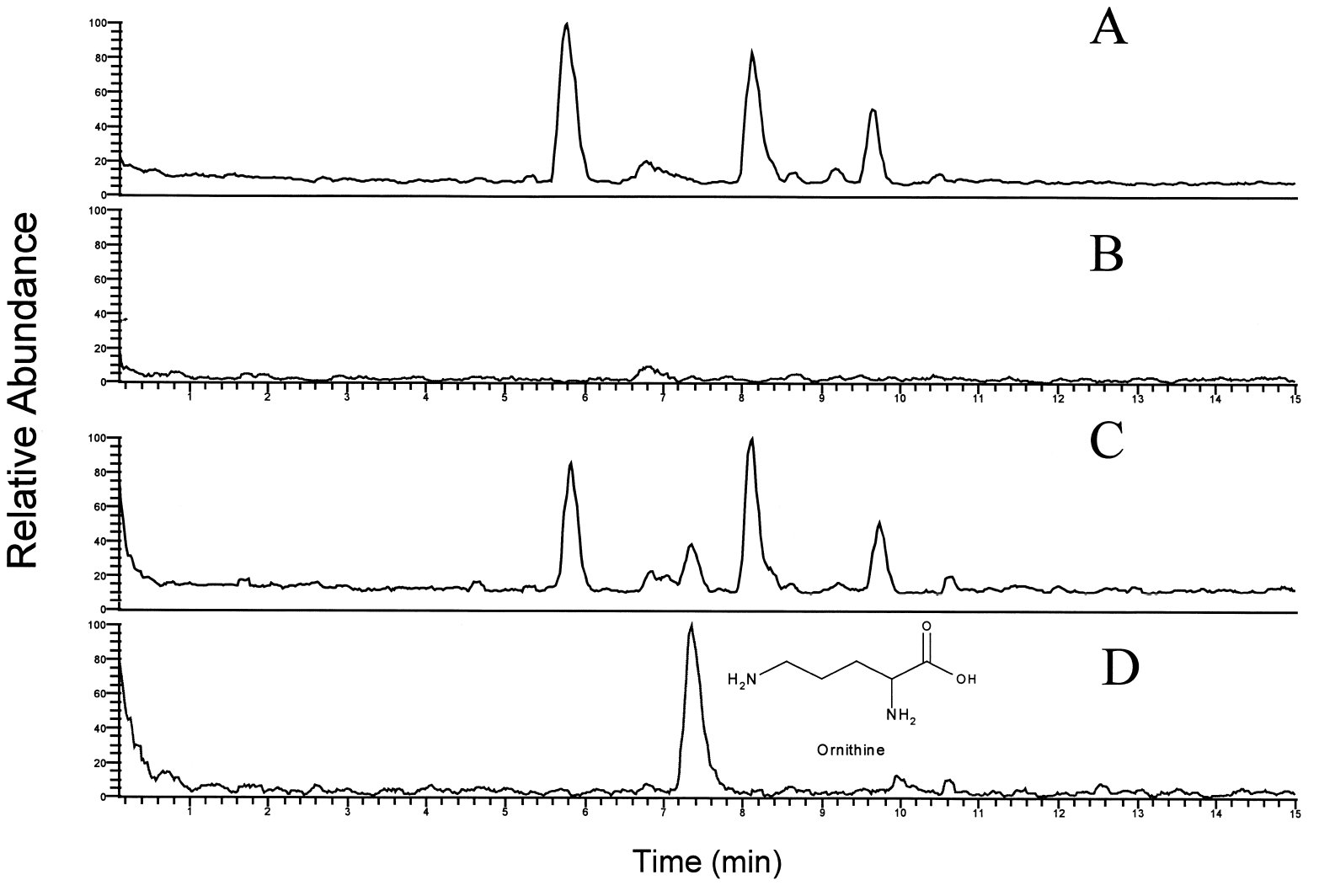

Fig. 2. CE–MS analysis of normal urine (A and B) and normal urine spiked with ornithine (C and D). Experimental conditions were the same as those in Fig. 1. A and C: total mass chromatogram; B and D: extracted Mass chromatogram of m/z 133.2 (MH^+ of ornithine).

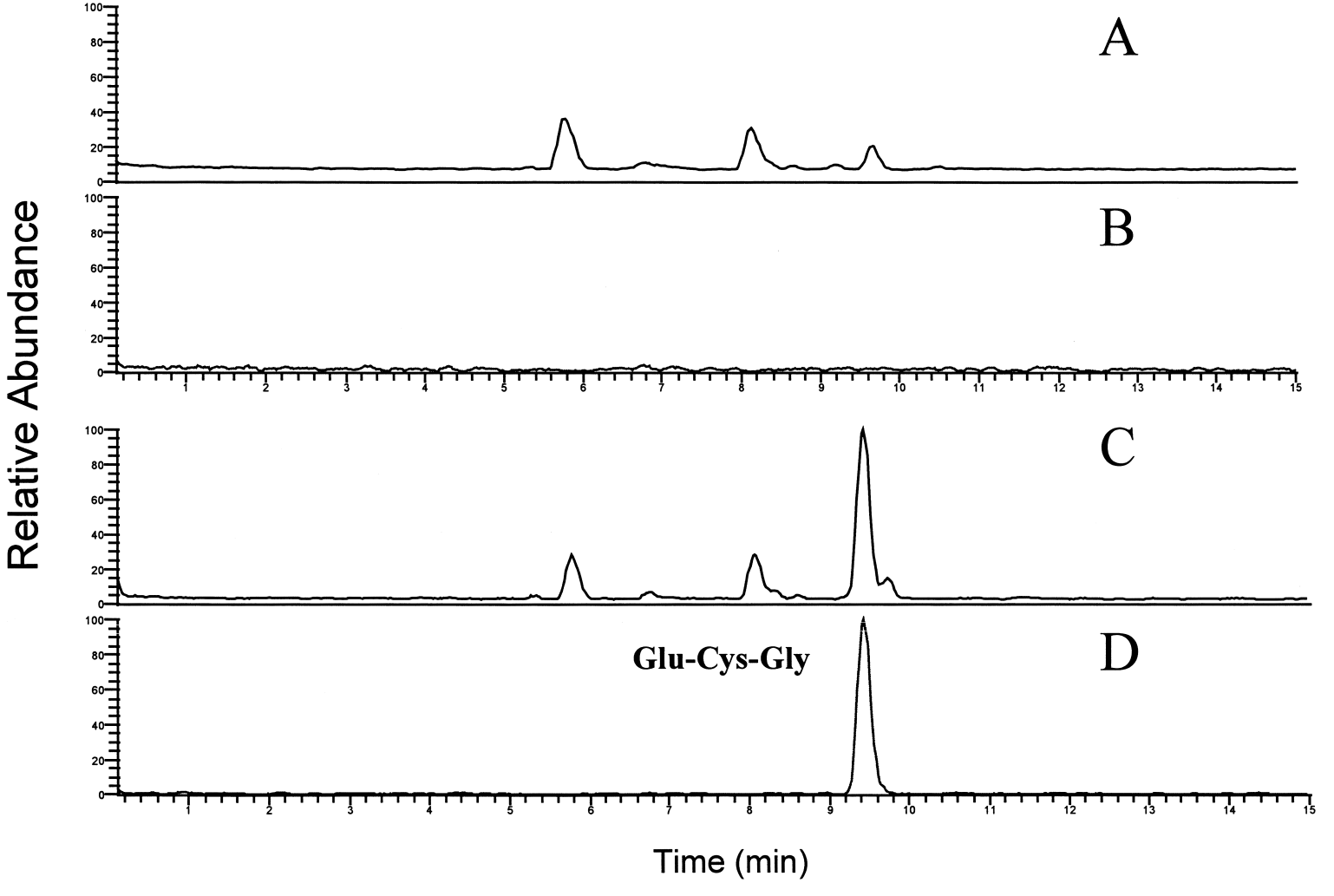

Fig. 3. CE–MS and CE–MS-MS analysis of normal urine (A and B) and normal urine spiked with glutathione (C and D). Experimental conditions for CE–MS analysis were the same as those in Fig. 1. The threshold for switching from MS full scan to MS-MS full scan in the CE–MS-MS analysis was 10^5 ion count, and the fragmentation energy was set to 15%. A and C: total mass chromatogram; B and D: extracted mass chromatogram of m/z 307.9 (MH^+ of glutathione). E: on-line CE–MS-MS spectrum of glutathione.

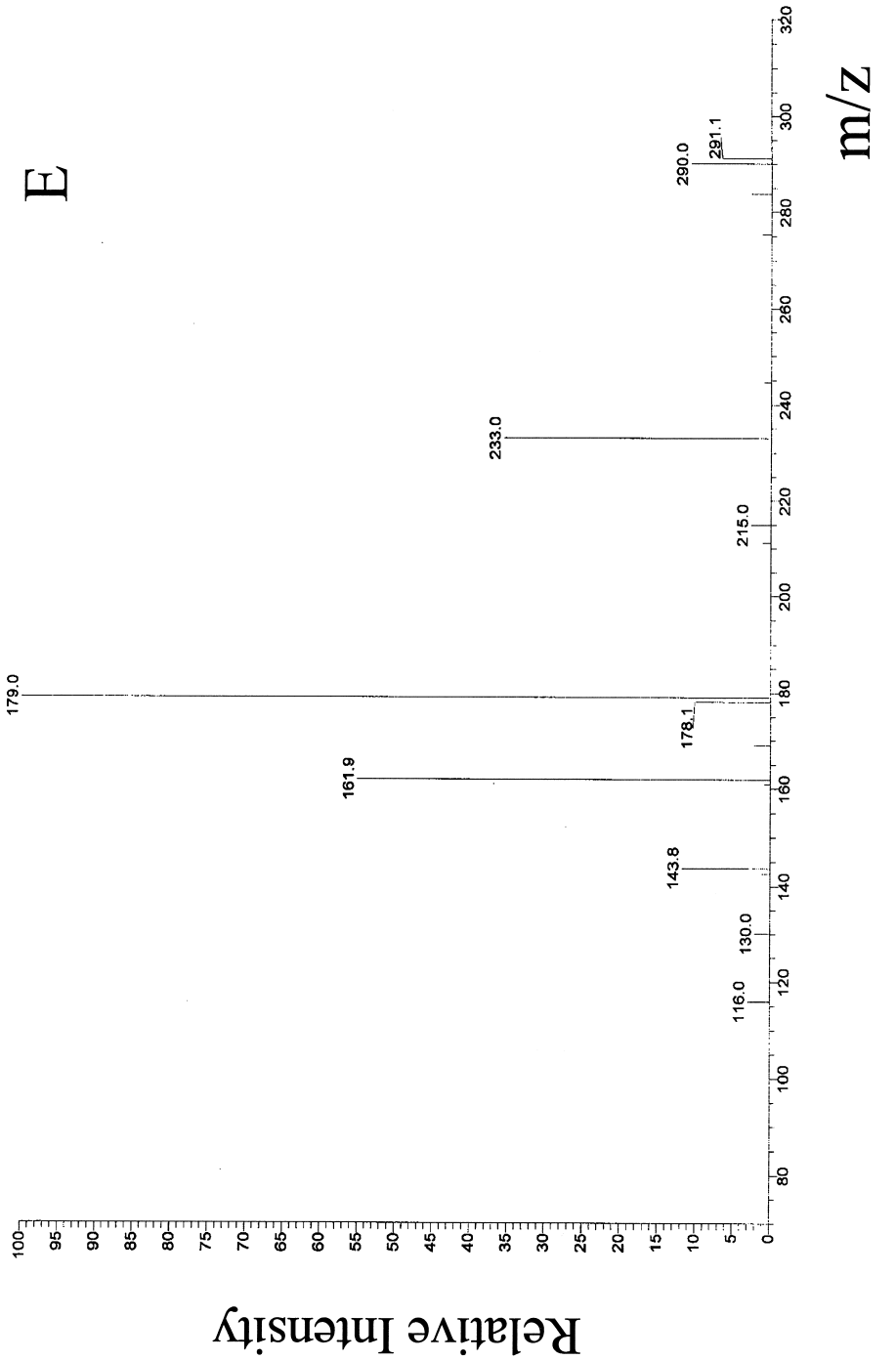

Fig. 3. (*continued*)

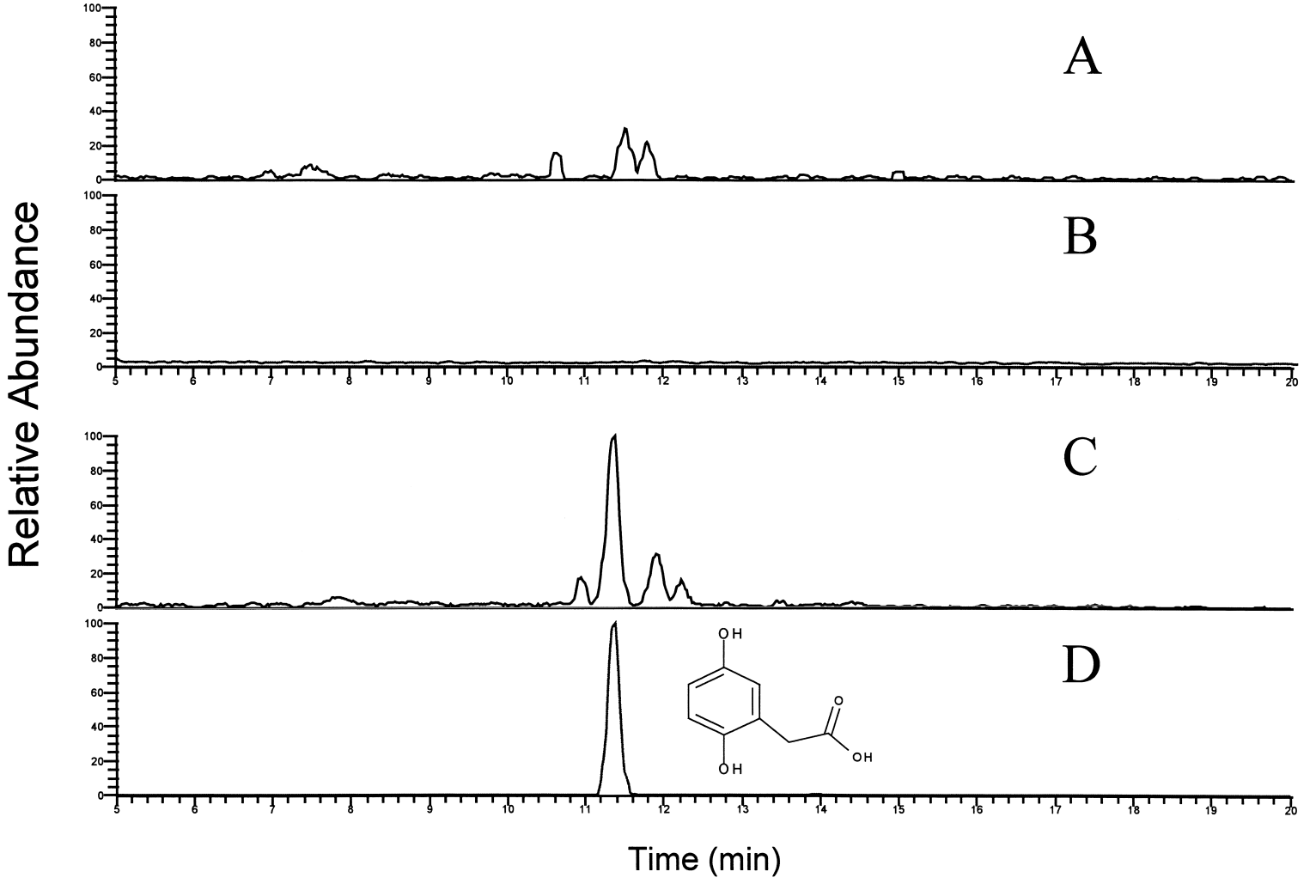

Fig. 4. CE–MS analysis of normal urine and normal urine spiked with homogentisic acid. Urine samples of 7.2 nl were injected without any treatment. Bicarbonate buffer (100 m*M*, pH 8.5 adjusted with ammonium hydroxide) was used and voltage for CE separation was 20 kV. ESI voltage was 4 kV in the negative ion mode. A and C: total mass chromatogram; B and D: extracted mass chromatogram of m/z 167.1 (MH^- of homogentisic acid).

intermediary product in the metabolism of phenylalanine and tyrosine, which accumulates in patients with alcaptonuria. The cause of the disease is homogentisic acid oxidase deficiency. The CE–MS analysis of normal and ''diseased urine'' samples indicated the presence of homogentisic acid in the ''diseased urine'', and absence in the normal urine (Fig. 4).

In addition, histidine and pyroglutamate for histidinemia and generalized glutathione deficiency, respectively, were also analyzed and the results are summarized in Table 1.

One advantage of CE–MS approach over the previously reported CE method based upon UV absorption detection [9–11] is higher selectivity and specificity, therefore the complete separation of the interfering components is not required. The method is rapid with a cycle time of less than 20 min. No decrease in signal intensity was observed during the continuous analysis of urine samples for a week (8 hours/day). The latest design of spray source, such as Z-spray from Micromass, when incorporated in the set-up, should further enhance the robustness of the method. At the same time, it should be noted that metabolites with low mass spectrometric responses may escape detection under the current scheme, although most of the compounds do respond to electrospray MS in either positive or negative ion mode.

3.2. Analysis of urine samples spiked with diagnostic metabolites by on-line CE–MS–MS

Collision induced dissociation MS provides another dimension of increased specificity when analyzing a specific metabolic disease marker or to obtain structural information for metabolic profiling. In this study, the instrument was set to automatically switch between MS full scan and MS–MS scan. The molecular ion and the fragment ion spectra of the metabolite could be obtained from a single CE–MS run. In the example of glutathione, the fragmentation pattern from on-line CE–MS–MS analysis correlates with the structure of glutathione (Fig. 3E). This approach thus significantly increases the selectivity and specificity of the CE–MS analysis over the CE method based on UV absorption for diagnostic metabolites in a complicated biological matrix. Co-migration may not be a problem even if the compounds possess the same molecular weight provided they generate unique fragments under the collision induced dissociation condition.

3.3. Sensitivity study

Even though sensitivity did not become an issue in this study, our CE–MS detection sensitivity was much higher than that required for the detection of the metabolites from the patients' urine. Good signal was obtained with one tenth or less of the concentration clinically found for diagnostic metabolites by full scan analysis (Table 1). Selective reaction monitoring, an MS scanning mode relying on the detection of a specific parent and a daughter ion upon fragmentation, provides another ten-fold increase in detection sensitivity (data not shown). Thus this method provides the capability of early detection of the diseases and ability to monitor the disease progression. Further improvement on sensitivity may be obtained by using on-line sample preconcentration [18,19].

4. Conclusion

CE–MS has been shown to be a powerful method for the direct analysis of diagnostic metabolites in urine samples. For the metabolic diseases we investigated in this study, selected ion monitoring is selective, sensitive and may provide rapid diagnostic and possible early detection of the diseases. The fragmentation information obtained from on-line CE–MS–MS experiment further improves the selectivity and specificity of the analysis when needed. The method is robust and rapid. Complete separation of the overlapping components and absolute reproducible elution is not necessary since the identification is not based on migration time. We believe that CE–MS should find broad applications for diagnostic metabolite analysis in clinical diagnostics and hold great potential in preclinical research and development.

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