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Short communication

Analysis of creatinine, vanilmandelic acid, homovanillic acid and uric acid in urine by micellar electrokinetic chromatography

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Abstract

A simultaneous determination of vanilmandelic acid, homovanillic acid, creatinine and uric acid using capillary electrophoresis was investigated. The optimum conditions of buffer concentration, pH and surfactant concentration were studied, and high resolution was obtained using a 30 m*M* phosphate buffer (pH 7.0) containing 150 m*M* sodium dodecyl sulfate. The detection was by UV absorbance at 245 nm and the column was a fused-silica capillary of 67 cm×75 μm I.D.. The determination of these metabolites in human urine was completed within 15 min without any interferences.

Keywords: Catecholamines; Creatinine; Vanilmandelic acid; Homavanillic acid; Uric acid

1. Introduction

Vanilmandelic acid (VMA) and homovanillic acid (HVA) are the end-products of catecholamine metabolism from hormonal biosynthesis in adrenal medulla, sympathetic and central nervous systems. The excretion of VMA and HVA in urine abnormally increases in patients with pheochromocytoma and neuroblastoma [1]. The latter is the second most frequent disease, leukemia being the first, seen in children with malignant tumors. Diagnosis of these diseases before the age of 1 year old is recommended because of highly effective therapy in the early stages. Thus, since 1985, diagnostic detection of VMA and HVA in urine has been adopted for the routine mass screening of 6-month-old infants in Japan [2].

While qualitative methods most commonly used were spot-test and thin-layer chromatography [3,4], introduction of quantitative determination with high-performance liquid chromatography (HPLC) [5] and enzyme-linked immunosorbent assay (ELISA) [6] methods have been designed and applied to mass screening. The HPLC methods with ultraviolet absorbance [7], electrochemical [8,9] and fluorimetric detection [10] have been described for the analysis of VMA and HVA. However, HPLC and ELISA methods require creatinine (Cr) measurement to correct VMA and HVA concentration in urine. Creatinine measurement has been carried out by Folin-Wu method based on the Jaffé reaction [11], and the ratio of VMA or HVA to Cr has been utilized as a

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diagnostic index of the diseases. A column and solvent switching technique combined with UV absorbance and fluorimetric or electrochemical detections was developed for the simultaneous determination of VMA, HVA and Cr [12–15]. This technique, however, required troublesome instrumental maintenance for routine analysis.

Recently, capillary electrophoresis (CE) has been developed as a rapid method with high resolution, and the micellar electrokinetic chromatography (MEKC) introduced by Terabe et al. [16] is one of the most widely used CE modes. Issaq et al. [17] separated VMA and HVA using capillary zone electrophoresis (CZE) with acetate buffer at pH 4 and UV absorbance detection at 214 nm. They did not examine Cr with VMA and HVA, and the peak of uric acid eluted near neutral pH and co-eluted with interfering solutes. This paper describes a simple and rapid separation of Cr, VMA, HVA and uric acid, a main end product of urine metabolism in kidney using the MEKC technique.

2. Experimental

2.1. Chemicals and samples

Cr, HVA, VMA and uric acid were obtained from Sigma (St. Louis, MO, USA), SDS from Nacalai Tesque (Kyoto, Japan). All solutions were prepared in distilled water. Standard solutions for analysis by CE were prepared at concentration of 50 μg/ml for Cr, VMA, HVA and uric acid in distilled water.

Human urine was collected from healthy volunteers and stored at −20°C until analysis. The samples were clarified by centrifugation at 800 *g* for 20 min to remove cells and other particulate matter. A 10 μl volume of clarified urine were diluted with 30 μl of distilled water and spiked with final concentrations of 40 μg/ml each for VMA and HVA.

2.2. Apparatus and analytical conditions

All CE experiments were carried out using a Beckman P/ACE 5500 instrument equipped with a photodiode-array detector (Beckman, Tokyo, Japan). The system was interfaced to a personal computer, and Beckman system Gold software (version 5.0, Beckman) was used for data collection and manipulation. The separations were carried out in a fused-silica capillary tube (67 cm×75 μm I.D.). UV absorbance detection was monitored at 245 nm. The temperature of the coolant liquid in the P/ACE instrument was controlled from 25±0.1°C. Injections were made using the pressure mode for 5 s at 3447.4 Pa.

A buffer consisting of phosphate was prepared by mixing equimolar amounts of sodium phosphate monobasic and sodium phosphate dibasic in distilled water.

Calibration curves were calculated from duplicate injections of six different levels (10–250 μg/ml) of standards. Peak height and time-corrected area were used for quantitation and a linear calibration curve were drawn.

3. Results and discussion

Since VMA and HVA are anionic compounds with a carboxylic group in their molecules, they have been generally determined by anion mode of CZE. The concentration of these two metabolites and uric acid varies with urine concentration, whereas Cr is excreted in the urine at a virtually constant rate. The measurement of Cr is required to correct the concentration of VMA, HVA and uric acid. MEKC, a useful technique that simultaneously determines both cationic and anionic ions, was assayed to examine these four metabolites.

3.1. Influence of pH

In CE, the pH of the buffer solution greatly affects the velocity of the electro-osmotic flow (EOF) and the pH of phosphate buffer was studied to obtain appropriate separation (Fig. 1). The four solutes eluted closely at pH 6 and are completely separated at pH 7. As the pH of phosphate buffer was increased to 9, the two peaks of VMA and HVA eluted closely again. The migration order of the two acids was reversed beyond pH 9. At pH 10, VMA and uric acid co-eluted, and it was decided to carry out separation of VMA and HVA in this MEKC at pH 7 considering the pK_a and peak resolution of these acids.

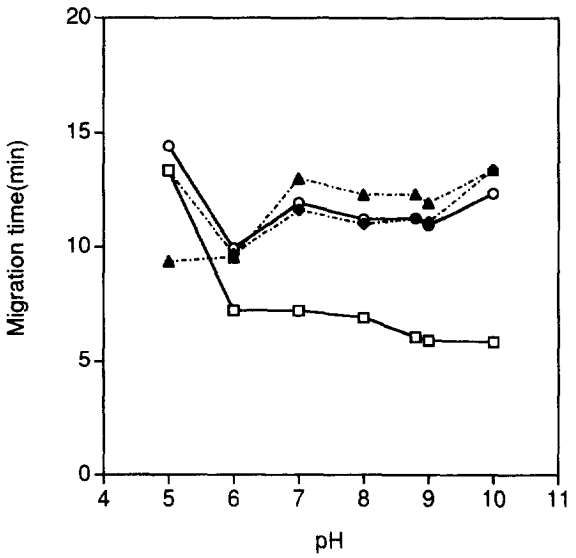

Fig. 1. Effect of the pH of phosphate buffer on migration time. Buffer: 30 m*M* phosphate buffer containing 150 m*M* SDS. Solutes: (□) Cr; (♦) VMA; (○) HVA; (▲) uric acid. Analytical conditions as in Section 2.2.

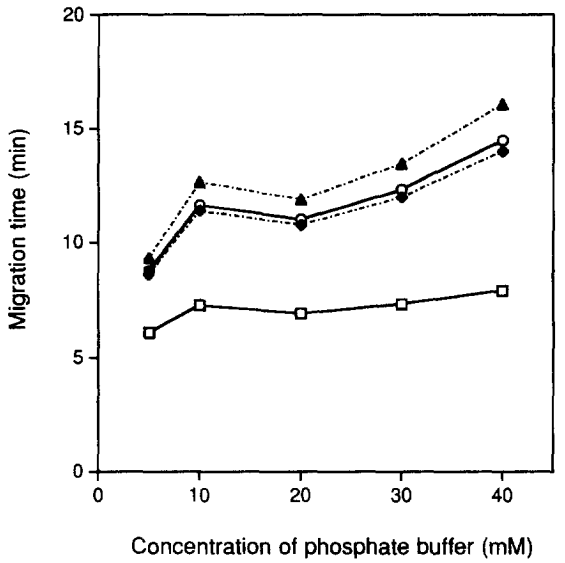

Fig. 2. Effect of phosphate buffer concentration on migration time. Buffer: phosphate buffer (pH 7.0) containing 150 m*M* SDS. Analytical conditions as in Section 2.2.

3.2. Influence of concentration of phosphate buffer

The buffer concentration is important in CE. Fig. 2 shows the influence of phosphate buffer concentration on MEKC. With increase in phosphate buffer concentration from 10 m*M* to 40 m*M*, the velocity of EOF decreased. As for the peak resolution of the analytes, improved separation was observed with increasing ionic strength of buffer concentration. On the other hand, migration time was delayed and the electric current was elevated. As high electric current should be avoided in order to suppress Joule heat generation [18], 30 m*M* phosphate buffer concentration was chosen for further investigation.

3.3. Influence of concentration of SDS

Surfactants in the running buffer at concentrations above the critical micelle concentration form micelles. Hydrophilic solutes that do not interact with the micelles elute with the EOF, and those that are totally retained elute with the micelles. Thus neutral solutes and substances that are hardly separated by CZE are separable by MEKC [19]. The separation of analytes is also affected by micelle concentration.

Fig. 3 shows the effect of SDS concentration on the separation of four urine metabolites. When the four solutes were analyzed without SDS, the two peaks of HVA and VMA eluted closely. Increasing SDS

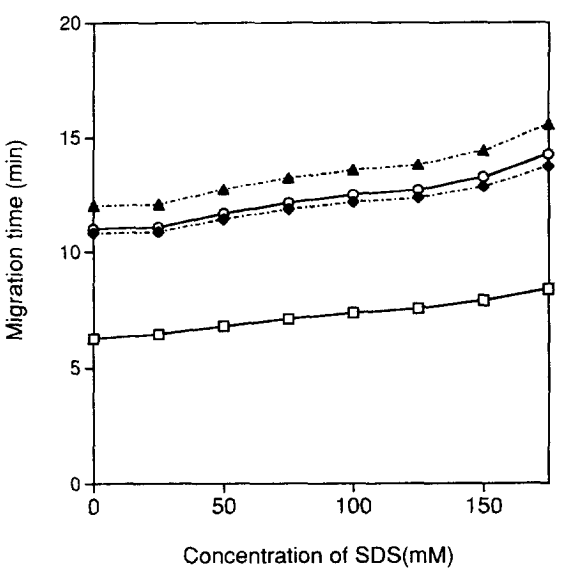

Fig. 3. Effect of SDS concentration on migration time. Buffer: 30 m*M* phosphate buffer (pH 7.0). Analytical conditions as in Section 2.2.

concentration resulted in excellent resolution of the analytes, and 150 m*M* SDS concentration provided the most favorable peak shapes and separation.

3.4. Reproducibility

Factors such as temperature and capillary conditioning by a washing procedure greatly affect reproducibility of migration time [20]. The temperature was controlled at 25±0.1°C and capillary tube was washed with 70% methanol, followed by distilled water and then buffer.

The relative standard deviations (R.S.D.) of migration time for Cr, VMA, HVA and uric acid were 1.89%, 5.57%, 6.00% and 6.30% (n=8), respectively. Migration time reproducibility in CE has been often discussed and researchers tried to improve the R.S.D. by such means as the use of internal standard or calculated mobility relative to the EOF [21,22]. The migration times of each compound were calculated by the migration time relative to the EOF. The R.S.D.s of Cr, VMA, HVA and uric acid were 0.44%, 0.29%, 0.01% and 0.34% (n=8), respectively.

The R.S.D.s of peak height and peak area were 7.29–10.50% and 9.57–11.70%, respectively (n=8).

3.5. Calibration

The linearity of the four compounds was assessed from 10 to 250 μg/ml and the correlation coefficients of these analytes were from 0.9800 to 0.999 for peak height and from 0.885 to 0.998 for peak area. The detection limits for Cr, VMA, HVA and uric acid in standard solution were 5, 10, 10 and 1 μg/ml, respectively (S/N=3).

3.6. Determination of Cr, VMA, HVA and uric acid

The electropherogram of the four standards is shown in Fig. 4A. Uric acid, the most hydrophilic molecule among the four analytes, eluted last; this may be explained by its strong interaction with the micelles.

Fig. 4B shows the electropherogram of a human urine spiked with 40 μg/ml each of VMA and HVA. A photodiode-array detector with wavelengths of both 200 and 245 nm was employed. Detection at shorter wavelengths caused interferences, and the Cr peak overlapped with other solutes in urine. Improved separation from other possible interfering metabolites was accomplished at 245 nm.

It is hoped that the method presented here will meet the laboratory requirements for routine mass screening of pheochromocytoma and neuroblastoma.

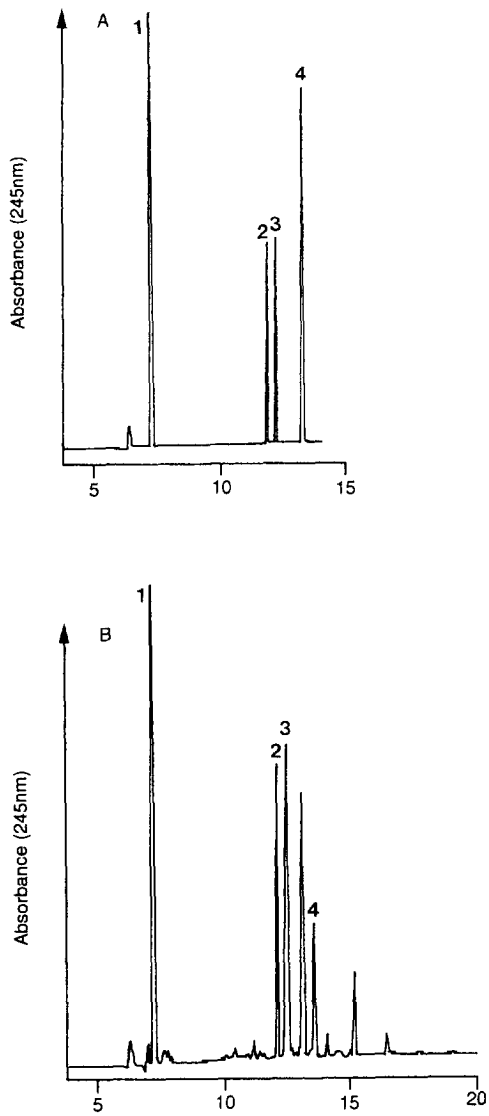

Fig. 4. (A) Electropherogram of a standard mixture of Cr, VMA, HVA and uric acid. (B) Electropherogram of normal urine spiked with VMA and HVA. Analytical conditions as in Section 2.2. Buffer: 30 m*M* phosphate buffer (pH 7.0) containing 150 m*M* SDS. Peak identification: 1=Cr; 2=VMA, 3=HVA, 4=uric acid.

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