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Determination of organic acids in urine by capillary zone electrophoresis

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Abstract

The simultaneous measurement of organic acids was studied using capillary electrophoresis with direct measurement of UV absorption at 185 nm. The organic acids studied were oxalic, formic, malonic, fumaric, succinic, α-ketoglutaric, citric, acetic, pyruvic, lactic, isovaleric and hippuric acid. They were separated in a fused-silica capillary (100 cm × 75 μm I.D.) filled with 50 m*M* borax buffer (pH 10.0) containing cationic surfactant as the electroosmotic flow modifier. The method was successfully applied to the determination of organic acids in urine in comparison with an organic acid analyser.

1. Introduction

Organic acids in the living body are present as intermediate and ultimate metabolites. When metabolic disorders occur, accumulation in the body fluids or tissues and excretion in the urine and stool of particular organic acids are observed. Organic acids have been determined in urine and serum in order to diagnose numerous inborn errors of metabolism known as organic acidurias [1–3]. It is also necessary to measure the concentration of organic acids in foods with respect to quality control of foods and their storage. Organic acid analysers (OAA), a post-labelling HPLC system and gas chromatography–mass spectrometry have been used for the determination of organic acids [4–8]. However, these techniques are time consuming and need tedious operation.

Capillary electrophoresis is a modern analytical technique that permits rapid and efficient separations of charged components present in small sample volumes [6,7]. In conventional capillary electrophoresis, a specimen is injected on to the anodic electrode and detection is performed on the cathodic electrode, as electroosmotic flow (EOF) goes from the anode to the cathode because of the negative electric charge of the capillary wall. In the measurement of particular anions, such as inorganic acids and short-chain carboxylic acids, the reversal of the EOF with cationic surfactants together with sample injection from the anode is reported to give successful separations [9–14]. In those studies, indirect detection of anions using UV-absorbing electrolytes was employed. However,

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the indirect method was not applicable to the trace determination of organic acids in urine as described here. In this study, we developed analytical conditions for the simultaneous determination of organic acids with direct detection at 185 nm. In addition, the method was applied to the measurement of organic acids in urine and compared with an organic acid analyser.

2. Experimental

2.1. Reagents and samples

Oxalic acid, malonic acid, sodium isovalerate and hippuric acid were obtained from Kanto Chemical (Tokyo, Japan), α-ketoglutaric, pyruvic, DL-citric, malic, DL-tartaric and succinic acid and sodium tetraborate (borax) from Wako (Osaka, Japan) and L-lactic, fumaric and methylmalonic acid from Sigma (St. Louis, MO, USA). All solutions were prepared from distilled, deionized water. Stock solutions of the organic acids were prepared at a concentration of $1 \cdot 10^4$ ppm. 2-Nitrophenylhydrazine hydrochloride (ONPH) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were obtained from Tokyo Rikakikai (Tokyo, Japan). CIA OFM Anion-BT, the electroosmotic flow modifier, was purchased from Waters Division of Nihon Millipore (Tokyo, Japan).

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, then passed through a C_{18} solid-phase extraction cartridge (Waters) before injection.

2.2. Instrumentation and analytical conditions

Capillary electrophoresis was carried out with a Quanta 4000 system with a Model 820 data station (Waters). The separations were carried out using an uncoated fused-silica capillary (100 cm × 75 μm I.D.) obtained from Waters. The injection mode used was hydrostatic, urine samples being elevated to a height of 10 cm for 45 s. The injection volume was calculated as approximately 54 nl. Detection was carried out at 254 nm by the indirect method and at 185 nm by the direct method. The applied voltage was 20 kV using a negative power supply.

A Model S300Z organic acid analyser (Tokyo Rikakikai) was used with an HC-5-500 anion-exchange column (50 cm × 5 mm I.D.) (Tokyo Rikakikai) [6]. The conditions for separation and postcolumn reaction for detection were as follows: mobile phase, 0.2 *M* HCl; reaction solution 1, 0.01 *M* ONPH in 0.1 *M* HCl; reaction solution 2, 0.15 *M* EDC in 4% pyridine solution in ethanol; reaction solution 3, 1.5 *M* NaOH; column temperature, 60°C; flow-rate of mobile phase, 0.25 ml/min; flow-rate of reaction solutions, 0.3 ml/min; injection volume, 100 μl; and detection wavelength, 530 nm.

3. Results and discussion

3.1. Assessment of detection wavelength

Kenney [14] and Wildman *et al.* [13] determined organic acids in foods and urine by the indirect method at 254 nm with potassium phthalate buffer and sodium chromate buffer. When we analysed urine samples for trace organic acids using similar methods the phosphate present interfered. The use of borax buffer with direct detection at 185 nm gave satisfactory results for organic acids without interference.

As the pK_a value of each organic acid expected in urine was lower than 7, the pH of the buffer was studied between 6 and 11 with 50 m*M* borax adjusted with sodium hydroxide or hydrochloric acid in the presence of 0.5 m*M* CIA OFM Anion-BT. Satisfactory results were obtained with borax buffer at pH 10 in respect of peak resolution, stability of the baseline and operation time. Similar migration times were obtained with buffers with pH < 8, but both the shape of the peaks and the separation of several acids were far inferior to those at pH 10, while the baseline became labile at pH 11.

Fig. 1 shows the effect of the concentration of borax buffer at pH 10.0 on the migration time of organic acids. While the resolution was improved

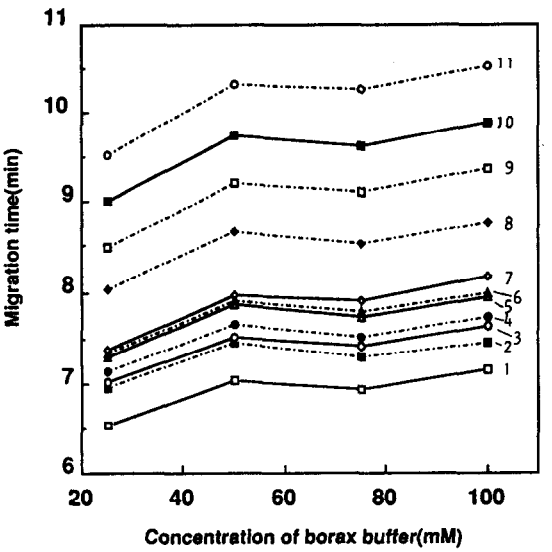

Fig. 1. Effect of concentration of borax buffer (pH 10.0) on migration time. Solutes: 1 = oxalic acid; 2 = formic acid; 3 = malonic acid; 4 = fumaric acid; 5 = succinic acid; 6 = α-ketoglutaric acid; 7 = citric acid; 8 = pyruvic acid; 9 = lactic acid; 10 = isovaleric acid; 11 = hippuric acid. For other conditions, see Experimental.

with increase in concentration, the operation time became longer and the electric current was elevated, as shown in Fig. 2 at higher concentration. As a high electric current should be avoided in order to suppress Joule heat generation [15], the concentration of the buffer adopted was 50 m*M* with an applied voltage of 20 kV.

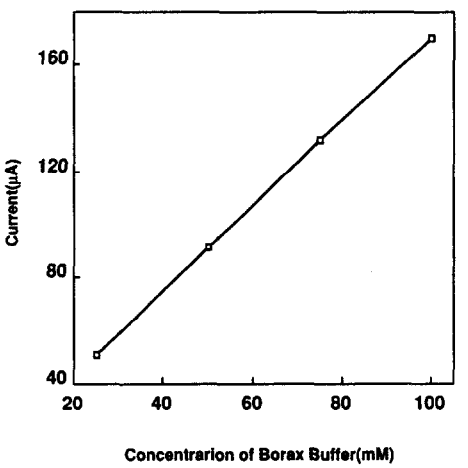

Fig. 2. Effect of concentration of borax buffer (pH 10.0) on electric current. Conditions as in Fig. 1.

Among the cationic surfactants tested, including cetyltrimethylammonium chloride and tetrabutylammonium bromide, CIA-Pak OFM Anion-BT gave the best separation and good peak shapes for simultaneous analysis. Fig. 3 shows the electropherogram of twelve organic acids employing 50 m*M* borax buffer at pH 10.0 with 0.5 m*M* CIA OFM Anion-BT.

It is known that the temperature in the capillary greatly affects electrophoresis [15]. As the instrument used in this study had no temperature control system except the forced cooling of the capillary by a fan, the influence of the temperature of the laboratory on the reproducibility of analysis was investigated. From the results with a standard solution in a laboratory without air conditioning, the migration time was greatly affected, with R.S.D. 9.50% ($n = 8$). In contrast, results obtained in an air-conditioned room (25 ± 1°C) showed good reproducibility, with R.S.D. 0.92% ($n = 8$). Therefore, all subsequent experiments were carried out in an air-conditioned room.

When we analysed urine samples, the delay in the migration times of organic acids became greater; the many impurities in the samples and

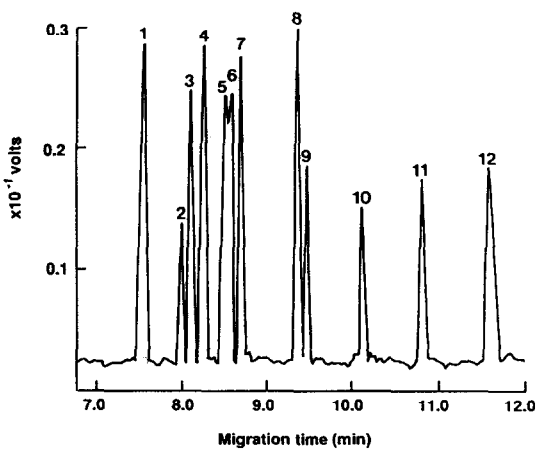

Fig. 3. Electropherogram of standard organic acids. Peaks: 1 = oxalic acid; 2 = formic acid; 3 = malonic acid; 4 = fumaric acid; 5 = succinic acid; 6 = α-ketoglutaric acid; 7 = citric acid; 8 = acetic acid; 9 = pyruvic acid; 10 = lactic acid; 11 = isovaleric acid; 12 = hippuric acid. For conditions, see Experimental.

the possibility of the occurrence of precipitates were thought to be responsible, and the effect of washing the capillary was investigated. Among the solutions studied, successive washing of the capillary with methanolic KOH solution (3 min), distilled water (3 min) and electrophoresis buffer (5 min) was suitable for precise analysis.

3.2. Calibration

The linearity of the method was evaluated between 10 and 250 ppm with respect to both peak-area and peak-height response. As the correlation coefficients for peak height (0.901–0.999) were better than those for peak area (0.878–0.999), calculations were carried out using peak height. The detection limits of oxalic, formic, malonic, fumaric, succinic, α-ketoglutaric, citric, acetic, pyruvic, lactic and isovaleric acid were 5 ppm and that of hippuric acid was 100 ppb.

3.3. Determination of organic acids in urine

Fig. 4 shows the electropherogram of organic acids in human urine using the developed method. The peaks were identified by adding a standard solution to urine according to the procedure of Wildman *et al.* [13]. The amounts of organic acids were different for each urine sample. Oxalic, formic, succinic, citric, pyruvic, lactic and hippuric acid were identified as shown in Fig. 4. The arrows numbered 3, 4 and 7 indicate the migration times of malonic, fumaric and acetic acid. Although the peaks of these acids in this sample did not appear larger than the background noise, they were reproducible and they appeared as peaks in other samples.

This method could also be applicable to urine samples without dilution. A chromatogram of organic acids in the same sample obtained with the organic acid analyser is shown in Fig. 5. The organic acids indicated were identified. Glucuronic acid was appeared in some samples. Under these conditions, it was difficult to determine fumaric and hippuric acid, because they eluted as broad peaks at *ca.* 4.5 and 6.5 h, respectively. The detection limits of other acids were *ca.* 500 ppb with the organic acid analyser.

When the concentrations of some acids that are abundant in urine, such as citric acid, were calculated, the values determined by capillary electrophoresis were similar to those obtained with the organic acid analyser.

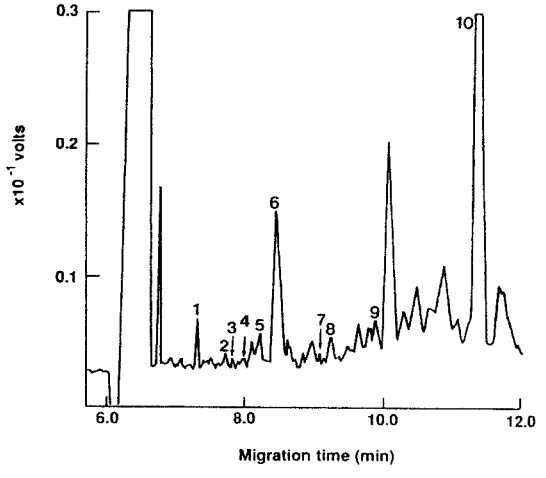

Fig. 4. Electropherogram of organic acids in urine. Peaks: 1 = oxalic acid; 2 = formic acid; 3 = malonic acid; 4 = fumaric acid; 5 = succinic acid; 6 = citric acid; 7 = acetic acid; 8 = pyruvic acid; 9 = lactic acid; 10 = hippuric acid. The arrows numbered 3, 4 and 7 indicate the migration time of malonic, fumaric and acetic acid, respectively. Conditions as in Fig. 4.

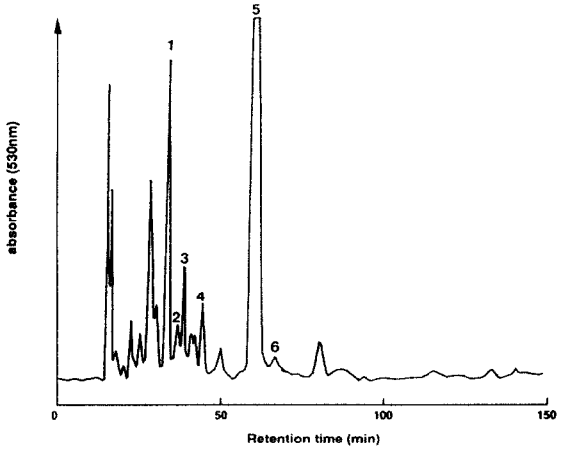

Fig. 5. Chromatogram of organic acids in urine obtained with the organic acid analyser. Peaks: 1 = lactic acid; 2 = acetic acid; 3 = pyruvic acid; 4 = formic acid; 5 = citric acid; 6 = succinic acid. For conditions, see Experimental.

4. Conclusions

We have discussed the fundamental conditions for the determination of organic acids in urine by capillary electrophoresis. Using detection at 185 nm and an applied voltage of 20 kV, favourable separation was achieved in a fused-silica capillary of 100 cm × 75 μm filled with 50 m*M* borax buffer (pH 10) without interference from phosphate present in urine. The capillary electrophoresis method was to an organic acid analyser especially with respect to operating time and the amount of the sample required, but the latter was more sensitive than the former.

The determination of organic acids is also important in food manufacturing and quality control of foods. From our preliminary results for the analysis of food samples, such as wines and soy sauce, the method described was applicable except for a few acids contained in these samples which overlapped others. Slight modification of analytical conditions is necessary and is under study for the determination of organic acids in foods and other samples.

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