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Simultaneous determination of the urinary metabolites of toluene, xylene and styrene using high-performance capillary electrophoresis Comparison with high-performance liquid chromatography

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Abstract

A simple and rapid method using high-performance capillary electrophoresis (HPCE) for the simultaneous determination of the urinary metabolites of toluene, xylene and styrene, plus creatinine and uric acid in human urine specimens and standard solutions is described. The compounds were well separated from each other on a fused-silica capillary utilizing a 20 m*M* sodium tetraborate buffer (pH 9.65) with 15 m*M* β-cyclodextrin and UV detection at 200 and 225 nm. The total analysis time was less than 6 min per sample. The capillary zone electrophoresis (CZE) method shows a good correlation with the high-performance liquid chromatography (HPLC) method with respect to urinary hippuric acid concentrations in the urine specimens of subjects exposed to the vapors of a solvent mixture of toluene and xylene. In comparing these two techniques, HPCE was found to be superior to HPLC because the analysis time is shorter, and the separation of *m*-MHA and *p*-MHA takes a long time with HPLC.

Keywords: Toluene; Xylene; Styrene; Creatinine; Uric acid; Hippuric acid; Methylhippuric acid; Mandelic acid

1. Introduction

Toluene and *o*-, *m*- and *p*-xylene have been widely used, separately and as mixtures, as organic solvents, ingredients of thinners, and in the synthesis of chemicals. In vivo, toluene is oxidized, then conjugated with glycine and excreted in urine as hippuric acid (HA). There are three isomers of xylene, *o*-, *m*- and *p*-xylene, and a mixture is commonly called 'xylol'. In vivo, these isomers are oxidized to *o*-, *m*- and *p*-toluic acid, then conjugated with glycine and excreted in urine as *o*-, *m*- and *p*-methylhippuric acid (MHA) [1]. HA and MHA are used as biological markers in studies of occupational exposure to these industrial solvents because they show a good correlation with the level of exposure [2–4]. Styrene monomer has been widely used in the synthetic resin industry. Styrene exposure can be quantitatively estimated by determining the amounts of its metabolites, phenyl glyoxylic acid (PGA), mandelic acid (MA) and HA, which are excreted in the urine [5].

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Determination of the concentration of urinary metabolites of organic solvents has been used in the biological monitoring of workers exposed to organic solvents. In urinary biological monitoring, expressing the amount of urinary metabolite per gram of creatinine has been used for spot samples of urine, as suggested by Elkins et al. [6] and Lauwerys [7]. This correction is thought to be particularly applicable for very concentrated and dilute samples. Therefore, the biological exposure indices (BEI) of urinary HA in toluene exposure, MHA in xylene exposure, and MA in styrene exposure were expressed as metabolite concentrations in urine corrected for creatinine [8]. For the simultaneous determination of metabolite concentrations in the urine of workers exposed to aromatic solvents, high-performance liquid chromatography (HPLC) has been widely used to obtain accurate and reliable results [2–4,9–14]. Also gas chromatography (GC) has been used for the determination of metabolite concentrations [15–20].

Recently, high-performance capillary electrophoresis (HPCE) has developed into an exciting, powerful analytical technique in diverse fields, and shows a high separation power with a short analysis time [21]. In the field of occupational health, there are not many HPCE methods for the determination of metabolites in the urine of workers exposed to aromatic solvents [22,23], although HPCE methods have been used for HA in whey [24], human urine [25], and human serum [26,27], and for creatinine [28–31] and uric acid [29,30,32–34].

The aim of this study was to assess the possibilities of using HPCE to determine urinary metabolites of toluene, xylene and styrene, simultaneously, while at the same time determining the endogenous urinary metabolites, creatinine. A second purpose was to do a comparative study of HPCE and HPLC.

2. Experimental

2.1. Reagents

Hippuric acid (HA), *o*-, *m*- and *p*-methylhippuric acid (MHA), mandelic acid (MA), distilled deionized water, and sodium 1-decansulfonate were purchased from Tokyo Kasei (Tokyo, Japan). Creatinine, uric acid, and acetonitrile were purchased from Nacarai Tesque (Tokyo, Japan). Sodium tetraborate, β-cyclodextrin, and potassium phosphate were purchased from Wako Pure Chemical Industries (Osaka, Japan). All chemicals were of analytical-reagent or HPLC grade.

2.2. High-performance capillary electrophoresis

Capillary electrophoresis was carried out on a CAPI 3001 capillary electrophoresis system (Otsuka Electronics, Osaka, Japan) equipped with a multi-wavelength photodiode-array detector. The mode of HPCE is capillary zone electrophoresis (CZE) using β-cyclodextrin (β-CD), which is the most commonly used additive for the formation of inclusion compounds. A 20 m*M* sodium tetraborate buffer (pH 9.65) with 15 m*M* β-CD was used for the separations. All buffer solutions were filtered through a 0.2 μm membrane filter (Nihon Millipore, Tokyo, Japan) and then degassed in an ultrasonic bath for 30 min. Conditions were as follows: A fused-silica capillary [Otsuka Electronics, Osaka, Japan, 75 μm I.D.×50 cm length (37.5 cm to detector)] was used. The temperature was set at 25°C and a constant voltage of 15 kV with a current of 50–55 μA was applied. Injections were performed by raising the sample vial 20 mm above the level of the measuring vial for 30 s. The calculated injection volume was ca.12 nl. The wavelength of the UV detector was set at either 200 or 225 nm.

2.3. High-performance liquid chromatography

Simultaneous determination of the urinary metabolites of toluene, xylene and styrene was done by HPLC [4]. In this study, a Shimadzu Class LC-10 liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an SPD-M10 AVP diode array detector was used. A mixed solution of acetonitrile (15/85) was used as the mobile phase to separate urinary creatinine, uric acid, and the metabolites of toluene, xylene and styrene. Conditions were as follows: A stainless-steel column, ϕ 4.6 mm×150 mm, packed with octadecyl-silanized silica gel (TSK gel, ODS-80TM, 5 μm, Tosoh, Japan) was used. The flow-rate was 0.7 ml min^{-1}, producing a pressure of 56~60 kg cm^{-2} in the separation procedure. The column

temperature was set at 40°C. The injection volume was 20 μl.

2.4. Preparation of solutions

For HPCE, individual solutions of HA, three isomers of MHA, MA, creatinine and uric acid were prepared in a 50 m*M* sodium chloride solution at a concentration of 1 g l^{-1}. Each of these stock solution was then diluted with 50 m*M* sodium chloride to prepare solutions at concentrations from 6.25 to 50 mg l^{-1}.

For HPLC analysis, each stock solution by dissolving in water was mixed and diluted with 50% methanol to concentrations ranging from 10 to 30 mg l^{-1}. Each standard solution was filtered through a 0.2 μm membrane filter (Toyo Roshi Kaisha, Japan) and degassed in an ultrasonic bath for 30 min.

2.5. Urine samples

Urine specimens were obtained from healthy young female students (aged 21) who were not exposed to any organic solvents and thirty-six workers exposed to the vapors of a solvent mixture of toluene and xylene. For analytical tests, a standard solution was added to the urine of unexposed subjects at the final concentrations ranging from 6.25 to 100 mg l^{-1}.

The urine specimens from exposed subjects were used to compare the HPCE and HPLC methods in the simultaneous determination of the urinary metabolites of toluene and xylene. Each urine sample was diluted 10-fold with distilled water for HPCE, and 100-fold with 50% methanol for HPLC. Then the urine solutions were centrifuged at 1630 *g* for 10 min at 5°C, filtered through a 0.2 μm membrane filter, and degassed in an ultrasonic bath for 10 min before analysis.

3. Results and discussion

3.1. Capillary electropherograms

The electropherogram and individual absorption spectra of the seven standards at 200 nm are shown in Fig. 1. The migration times in minutes at a concentration of 12.5 mg l^{-1} were 3.07 for creatinine, 4.23 for *p*-MHA, 4.47 for *m*-MHA, 4.65 for *o*-MHA, 4.72 for HA, 5.17 for MA, and 5.57 for uric acid, and the coefficients of variation (C.V.) were 0.5, 0.5, 0.5, 0.7, 0.6, 0.6 and 0.7, respectively.

Creatinine (peak 1) in Fig. 1 had a broad shape when the standard solution was prepared with distilled water. In order to obtain a sharper peak, standard solutions were prepared in two different solvents, 100 m*M* and 50 m*M* sodium chloride. Sharper peaks were obtained with both sodium chloride solvents compared to the aqueous solution. However, the capillary could not be used repeatedly with the 100 m*M* sodium chloride solution. Therefore, the 50 m*M* sodium chloride solution was chosen for preparing the standard solutions. Photodiode-array detection (with UV spectra acquisition) has provided a powerful means of identification. The UV spectrum for each analyte is similar to the spectrum obtained by spectrometer. Uric acid, the most hydrophilic molecule among the seven analytes, migrated last. All electropherograms obtained from this CZE with β-CD showed distinct separation within 6 min after injection.

Fig. 2 shows the electropherograms of two human urine samples: (a) the urine specimen of a subject not exposed to any aromatic solvent vapors, (b) the urine specimen of a subject exposed to the vapors of a solvent mixture of toluene and xylene. In the urine specimen of the unexposed subject, only three major peaks, i.e. creatinine, HA and uric acid, were observed, while in the urine specimen of the exposed subject, seven peaks, i.e. creatinine (peak 1), *p*-MHA (peak 2), *m*-MHA (peak 3), *o*-MHA (peak 4), HA (peak 5), MA (peak 6) and uric acid (peak 7), were observed. Since commercial xylene mixtures contain some ethylbenzene as one of the minor components, a small MA peak was detected. Although creatinine is not separable from other neutral compounds in urine using this HPCE method, peak 1 shows a spectral similarity to the spectrum obtained by spectrometric analysis. Urinary creatinine has been recognized as a reference compound for correcting the excretion rates of HA, MHA and MA [7,8], and several procedures for the determination of urinary creatinine by HPCE have been developed using electrochemical detection [28], micellar electrokinetic chromatography [29,30], CZE with in-

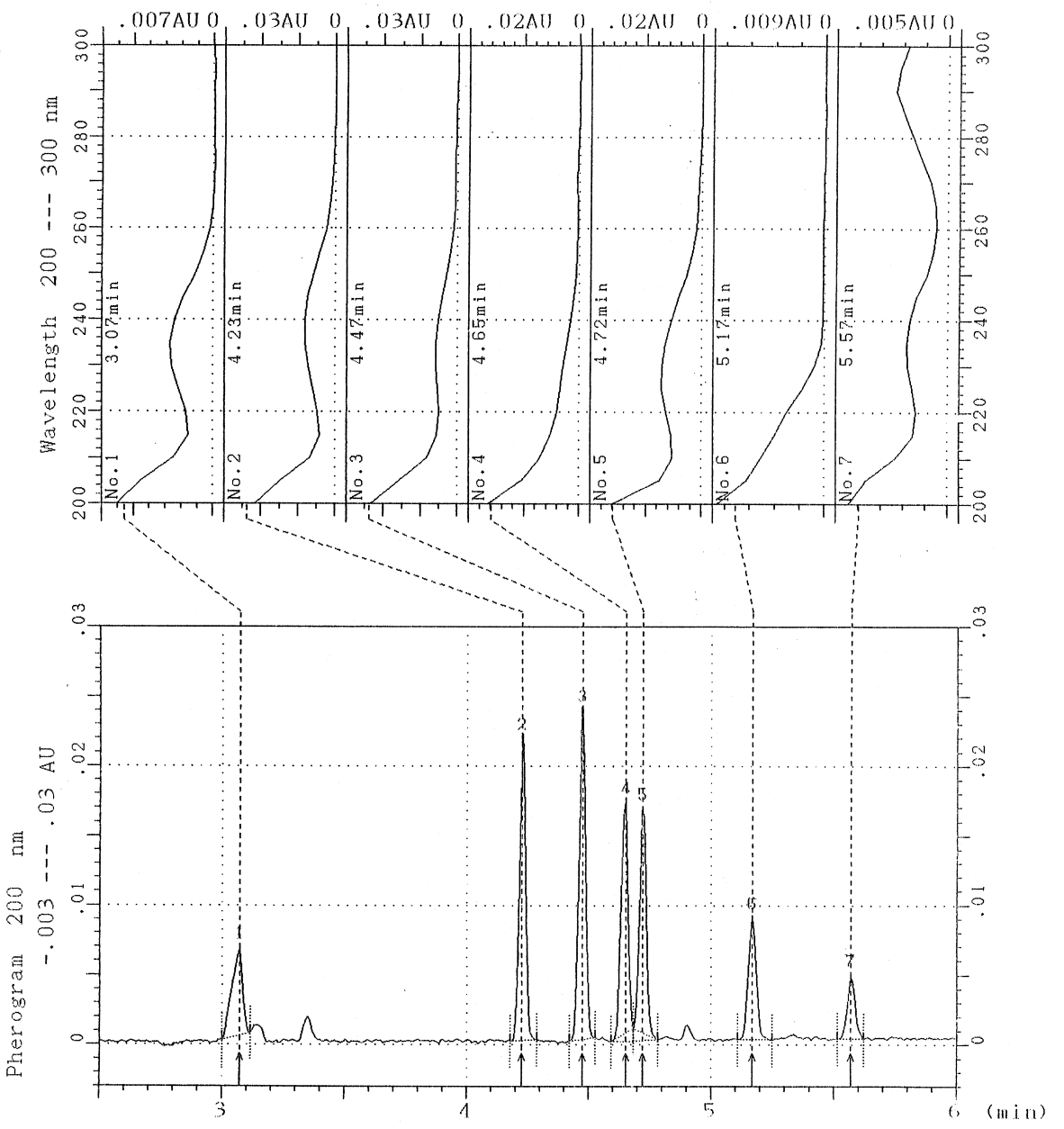

Fig. 1. Electropherogram and absorbance spectra of a 50 m*M* sodium chloride solution of HA, MHA, MA, creatinine and uric acid at a concentration of 12.5 mg l^{-1}. 1, Creatinine; 2, *p*-MHA; 3, *m*-MHA; 4, *o*-MHA; 5, HA; 6, MA; and 7, uric acid. Concentration of each solute is 12.5 mg l^{-1}.

direct chromatography [31]. Since creatinine is uncharged over a wide pH range, determination remains difficult with free zone capillary electrophoresis. Therefore, other procedures to separate and identify the neutral components in urine are currently being investigated.

Fig. 3 shows HPLC chromatograms of: (a) standard solutions of the five acids, creatinine and uric

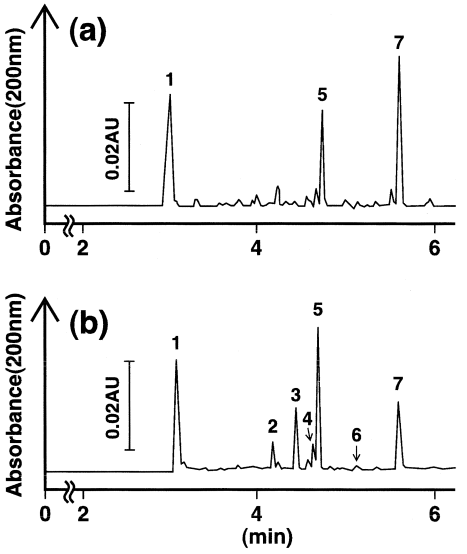

Fig. 2. Electropherograms of: (a) unexposed urine sample diluted 10-fold with water; (b) HA, MHA, MA, creatinine and uric acid in the urine specimen from a subject exposed to the vapors of a solvent mixture of toluene and xylene, diluted 10-fold with water. 1, Creatinine; 2, *p*-MHA; 3, *m*-MHA; 4, *o*-MHA; 5, HA; 6, MA; and 7, uric acid.

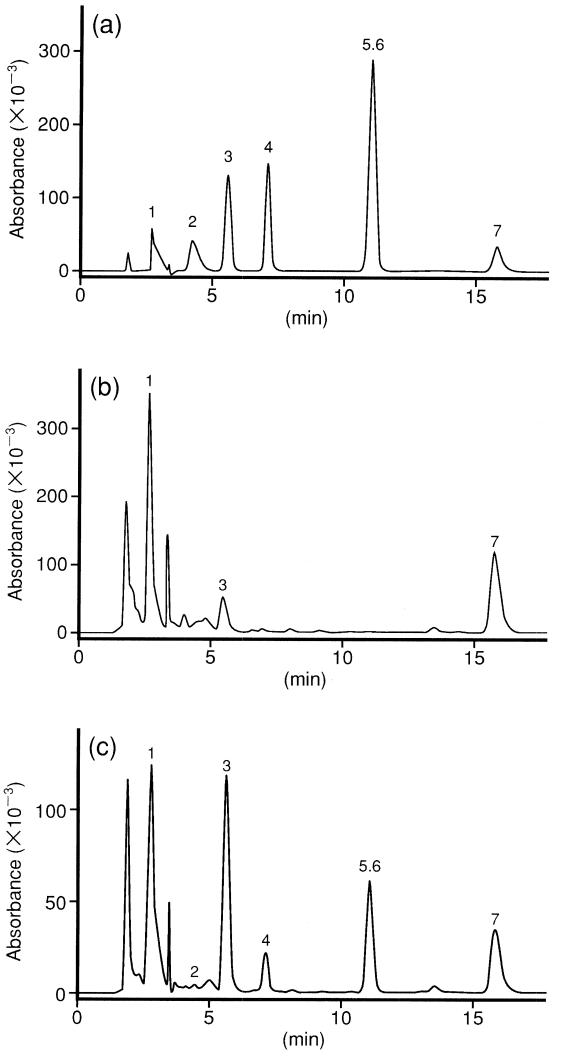

Fig. 3. Chromatograms of: (a) standard containing 10 mg l^{-1} of HA, MHA, MA and creatinine; (b) unexposed urine sample diluted 100-fold with 50% methanol; (c) urine specimen from a subject exposed to the vapors of a solvent mixture of toluene and xylene diluted 100-fold with 50% methanol. 1, Uric acid; 2, MA; 3, HA; 4, *o*-MHA; 5, 6, *m*- and *p*-MHA; and 7, creatinine.

acid, (b) the urine specimen of a subject not exposed to any aromatic solvent vapors, and (c) the urine specimen of a subject exposed to the vapors of a solvent mixture of toluene and xylene. HA, MA, *o*-MHA, creatinine, and uric acid were well separated from one another within 18 min after injection. However, *m*-MHA and *p*-MHA were not separated from each other because they have the same retention time. In comparing these two techniques, HPCE was found to be superior because analysis time is about one third that for HPLC. Also, the separation of *m*-MHA and *p*-MHA by HPLC is four times [3], eight times [10], or two times longer [14].

3.2. Calibration

Linearity of peak areas for the HPCE method was checked by measuring various concentrations, 6.25–50 mg l^{-1} range, of the five acids and creatinine. Linear relationships were found between the peak areas of the analytes and concentrations. The average slopes and y-intercepts of the regression line, and correlation coefficients (r) are shown in Table 1. Calibration plots and regression data for all analytes were linear ($r>0.9978$) at concentrations between 6.25 and 50 mg l^{-1}. The standard addition method (standard plus urine) was used in determining chemical interference of different acids. The slopes found for the calibration and standard addition graphs were

Table 1
Calibration graphs and standard addition method graphs for HPCE [a]

Analyte	Regression line	r	Concentration ($mg\,l^{-1}$)
Calibration			
Creatinine	$y=0.2940x-0.1621$	0.9992	6.25–50
HA	$y=0.4558x-0.0875$	0.9982	6.25–50
o-MHA	$y=0.4996x-0.2270$	0.9997	6.25–50
m-MHA	$y=0.6320x-0.0543$	0.9997	6.25–50
p-MHA	$y=0.5523x-0.3337$	0.9997	6.25–50
MA	$y=0.2855x-0.1321$	0.9978	6.25–50
Standard plus urine			
Creatinine	$y=0.2804x+92.0320$	0.9722	25–100
HA	$y=0.4638x+39.6220$	0.9950	25–100
o-MHA	$y=0.5087x+1.5478$	0.9976	25–100
m-MHA	$y=0.6431x-0.1740$	0.9990	25–100
p-MHA	$y=0.5774x+2.3895$	0.9956	25–100
MA	$y=0.2757x+0.4653$	0.9987	25–100

[a] y, Area$\times 10^{-4}$; x, concentration ($mg\,l^{-1}$); r, correlation coefficient.

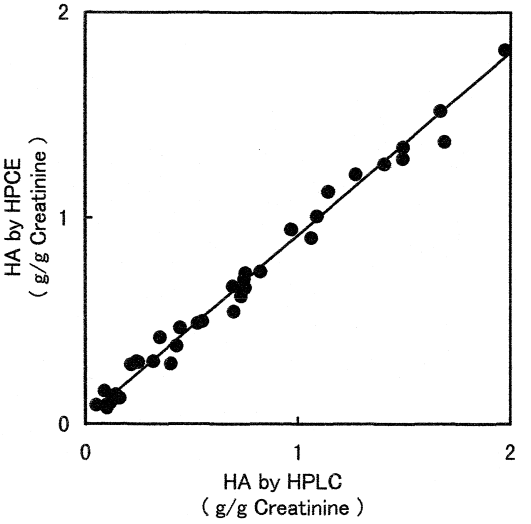

Fig. 4. Comparison of the HPLC and HPCE methods with respect to HA concentrations, corrected for creatinine, in the urine specimens of 36 subjects exposed to the vapors of a solvent mixture of toluene and xylene. Regression equation: $y=0.0239+0.891x$; $r=0.9936$; ●, individual value.

similar for each of the five acids when the error range of ±5% was taken into account.

Detection limits (LODs) were set at a signal-to-noise ratio of 3. The LODs of creatinine, *p*-MHA, *m*-MHA, *o*-MHA, HA and MA in the standard solutions were 0.44, 0.70, 0.17, 0.56, 0.31 and 0.65 $mg\,l^{-1}$, respectively. The LOD of HA was similar to one found in the literature [27].

3.3. Recovery

The analytical recovery by HPCE was evaluated by assaying six unexposed urine samples spiked with several concentrations of the standard solution. Analyses were done in triplicate for three concentrations, 25, 50 and 100 $mg\,l^{-1}$. As peaks of HA in unexposed urine samples were observed, each value for the untreated samples was subtracted from that of the spiked samples.

The mean recovery values with standard deviations (%) for spiked HA, *o*-, *m*- and *p*-MHA and MA of concentrations ranging from 25 to 100 $mg\,l^{-1}$ were 94.4±1.0, 98.0±1.9, 95.3±1.0, 99.5±0.7 and 96.9±1.0, respectively.

3.4. Comparison of the HPLC and HPCE methods with respect to HA concentrations in urine samples

Fig. 4 shows the correlation ($r=0.9936$) between the HPLC and HPCE methods for HA concentrations, corrected for creatinine, in the urine specimens of 36 subjects exposed to the vapors of a solvent mixture of toluene and xylene. A linear relationship was found between the HPLC and the HPCE methods. However, the HPLC values were slightly higher than HPCE, probably due to differences in methodology.

4. Conclusion

The CZE method with β-CD has been shown to be a good method for determining urinary HA, *o*-, *m*- and *p*-MHA, and MA, plus creatinine, simultaneously. Its advantages are short analysis time, and good separation of *m*-MHA and *p*-MHA compared to HPLC. The method requires minimal quantities of sample and thus can be useful for routine biological monitoring for occupational risk assessment.

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