1 Introduction

Benzene, toluene, ethylbenzene, and o-, m-, and p-xylene (BTEX) are vital ingredients of commercial and industrial chemicals. They are widely used in fuels; for example, as primary components of motor vehicle gasoline, as well as solvents, and starting products in a variety of chemical syntheses. These applications have rendered BTEX ubiquitous in the environment. In addition, these volatile organic compounds are highly toxic. They are easily absorbed via the lungs and the skin may also be a significant absorption route [1].

In recent years, BTEX and corresponding metabolites have been widely studied, owing to the health risks associated with exposure to these organic compounds [2–10]. Minor metabolites of benzene, such as trans, trans-muconic acid (t-MA) and S-phenylmercapturic acid (PMA), conjugated with glutathione, are excreted in the urine. Primarily, alkylbenzenes are oxidized at the alkyl side chain, which in turn, give rise to aromatic carboxylic acids. Hippuric acid (HA) and o-, m-, and p-methylhippuric acid (MHA), respectively, are the chief metabolites of toluene and o-, m-, and p-xylene. Phenylglyoxylic acid (PGA) and mandelic acid (MA) are major metabolites of ethylbenzene [9]. MA, PGA, MHA, as well as a few minor metabolites are not endogenously produced on a large scale. Thus, they are diagnostically more specific for determining the extent of occupational or environmental exposure. The assessment of BTEX metabolite concentrations within biological materials permits the degree of exposure to these chemicals to be estimated. ACGIH (American Conference of Governmental Industrial Hygienists) proposed a maximum permissible level of 2.5 g of HA or 1.5 g of MHA per gram of creatinine in urine as a biological monitoring strategy for groups of workers [10].

Investigating BTEX metabolites requires a sufficient, rapid, sensitive, and potentially automated analytical method. To determine these metabolites in urine, high-performance liquid chromatography (HPLC) [11–14] and gas chromatography (GC) [15–16] have been applied. After the fluid samples were pretreated, quantitative analyses of BTEX metabolites by HPLC or GC were performed. MA and PGA were analyzed by both HPLC and GC methods, both of which involved complex extraction steps and, in the case of GC, a derivatization procedure prior to the analysis [15]. The detection limit was determined to be 0.020 mmol/L or less for PGA and 0.050 mol/L for MA with the HPLC method. MA and PGA in the urine of workers who are occupationally exposed to styrene were also analyzed by GC [16]. The urine sample, in an acidic medium, was extracted with chloroform and subsequently converted into the respective methyl esters. Recoveries of 66.7% and 95.4% were obtained for MA and PGA, respectively, with limits of quantitation of 0.03 g/L for MA and 0.02 g/L for PGA. Moon et al. reported
on an HPLC method that utilized a β-cyclodextrin mobile phase to analyze PGA, HA, and o, m, and p-MHA. The detection limits ranged from 0.6 to 2.1 mg/L with recoveries of over 99.8%[12]. The primary disadvantages of these methods are the required extraction step, the longer analysis time, and the additional derivatization procedure in the case of GC.

Since 1990, capillary electrophoresis (CE) has proven to be of considerable value in various fields [17–21]. The advantages of CE are high resolution, high separation efficiency, lower sample consumption, and rapid analysis. Capillary zone electrophoresis (CZE) [22–25] and micellar electrokinetic chromatography (MEKC) [26–27] have been employed to investigate one or a few BTEX metabolites, but a thorough and comprehensive investigation has not been reported.

A β-cyclodextrin (β-CD) modified capillary electrophoresis method is presented herein for determining eight BTEX metabolites. In addition, the selected reference compounds were the endogenous urinary metabolites creatinine and uric acid. The effects of pH and β-CD concentration on the separation of the analytes were examined. Finally, through a simple sample pretreatment, urine samples were analyzed using the optimized method developed here.

2 Experimental

2.1 Apparatus

The experiments were performed on a Beckman P/ACE system MDQ Capillary Electrophoresis System (Beckman Instruments, Fullerton, CA, USA) equipped with an UV-Vis diode-array detector. A personal computer controlled by P/ACE System MDQ software was used for data acquisition. Data analyses were also performed using this software. A 50 μm ID fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, USA) with a total length of 60.2 cm (50 cm to the detector) was used for the separations. The capillary column was assembled in the cartridge format. The temperature of the capillary tube during the electrophoresis was maintained at 25°C by means of a thermostating system. The electrophoretic separation was performed with an electric potential of 25 kV. Samples were pressure injected at 0.034 bar (0.5 psi). The detection wavelength was set at 200 nm except for trans, trans-muconic acid, which was at 265 nm.

2.2 Chemicals

Borax, β-cyclodextrin, creatinine, uric acid, DL-mandelic acid, and hippuric acid were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide and sodium chloride were obtained from Fluka (Buchs, Switzerland). o-, m-, and p-Methylhippuric acids, trans,trans-muconic acid, and phenylglyoxylic acid were purchased from Aldrich (Milwaukee, WI, USA). S-Phenylmercapturic acid was obtained from TCI (Tokyo, Japan). All other chemicals were of analytical grade. Water was purified with a Milli-Q water system (Millipore, Bedford, MA, USA) and filtered through a 0.22-μm filter.

2.3 Capillary electrophoresis procedures

The procedure used to condition a new capillary involved sequential treatment with 1.0 M NaOH, H₂O, 0.1 M NaOH, and H₂O for 30 min each. The capillary was washed between runs with 0.1 N NaOH and then water (3 min of high pressure rinsing at 138 kPa), followed by reconditioning with the running buffer, typically for 3 min. The pH 7.2 and pH 8.1 borate running buffers were prepared by mixing appropriate amounts of 0.2 M boric acid and 0.1 M borax. 0.1 M NaOH was used to adjust the solutions to pH 9.0, pH 10.0, and pH 10.5 borate buffers, which contained appropriate amounts of 0.1 M borax.

2.4 Preparation of standard solutions and urine samples

Stock solutions of the four analytes (p-methylhippuric acid, uric acid, S-phenylmercapturic acid and trans,trans-muconic acid) were prepared in a 50 mM NaOH solution at a concentration of 3 mg/mL. The other six standards were prepared in a 50 mM NaCl solution at the same concentration. Working standards over the range 1 to 300 μg/mL with the exception of t-MA, which was 1 to 75 μg/mL, were prepared by dilution of a standard stock solution with a 50-mM NaCl solution. Urine samples were obtained from a gas station worker who had worked there continuously for over one year and a non-exposed graduate student. The urine samples were collected in the afternoon of three consecutive days. These samples were centrifuged at 4000 g for 10 min at room temperature, diluted 6-fold with 50 mM NaCl solution, and degassed in an ultrasonic bath for 1 min before CE analysis. All stock and sample solutions were stored at −20°C prior to use. The recovery study followed the same procedure as described above by spiking 50 μg/mL of p-MHA, m-MHA, HA, and PGA, and the others were 10 μg/mL in gas station worker’s urine samples.

3 Results and discussion

Figure 1 illustrates the molecular structures of eight BTEX metabolites as well as two reference compounds, creatinine and uric acid. This figure also shows that each
metabolite possesses one or two carboxyl acid groups that would be negatively charged in a basic buffer solution. Moreover, there are three MHA isomers that differ relative to the positions of the substituted methyl group on the benzene ring. Except for \( t\)-MA, which absorbed at 265 nm, the maximum absorbance of the analytes was approximately 200 nm. Consequently, to determine optimal separation conditions, the detection wavelengths were set at 200 nm and 265 nm.

### 3.1 Effects of buffer pH value and \( \beta\)-CD concentration on the separation

Figure 2 summarizes the effects of buffer pH level on the migration behavior of the analytes, which ranged from 7.2 to 10.5. The experimental results indicate that these analytes, except for creatinine, carry a negative charge within the pH range used. Notably, \( t\)-MA displayed the lowest mobility among the analytes. \( t\)-MA carried the highest negative charge density, since it possessed two carboxyl acidic groups with a relatively small molecular weight. Uric acid contains no dissociated functional groups. However, owing to its molecular structure and hydrophilic nature, it does have a negative charge to mass ratio, which is slightly less than that of \( t\)-MA. The experimental findings revealed that with increasing pH of the running buffer, the effective mobility of MA and PGA decreased, which can be attributed to increasing proton dissociation. MA and PGA were not resolved at a buffer pH value of less than 8.1; however, they were gradually separated by increasing the pH of the buffer. The migration order of PGA, HA, MHA, and PMA was consistent with their increasing molecular size, while bearing the same charge. Notably, three MHA isomers migrated with the same mobility in the pH range employed, but failed to separate. Over a broad pH range, creatinine remained uncharged and coeluted with EOF. For the purpose of high separation efficiency, a pH 10.0 buffer was selected for further investigation. Since the three isomers of MHA failed to separate in the prescribed buffer, further improvements were required to separate all analytes.

Distinct CDs are effective modifiers, which separate the molecular structure of isomers within CE. According to the molecular structure including size, hydrophobic property, and relative position of substituents of an analyte, stable inclusion complexes can be formed with CDs. Figure 3 presents the effects of \( \beta\)-CD concentration on the migration behaviors of the analytes. Altering the \( \beta\)-CD concentration in the buffer influenced the effective mobility of most of the analytes. Furthermore, PMA was affected to the greatest degree. When \( \beta\)-CD was added to the running buffer, the three MHA isomers were resolved. In addition, an increased \( \beta\)-CD concentration enhanced the resolution. \( \beta\)-CD influenced the effective mobility of \( p\)-MHA to a greater
extent than \( m \)- and \( \alpha \)-MHA. This suggests that \( \rho \)-MHA and \( \beta \)-CD form a relatively stable inclusion complex. It is noteworthy that an increasing \( \beta \)-CD concentration increased the resolution between PGA and MA. The planar structure of the substituent on PGA undergoes greater interactions with \( \beta \)-CD than MA. Owing to its relatively linear structure and hydrophilic property, \( t \)-MA was largely unaffected. Moreover, within the \( \beta \)-CD concentration range used, the migration sequence of the analytes remained unchanged. The addition of 7 mM \( \beta \)-CD to the pH 10.0 borax/NaOH buffer yielded the optimized approach to separate the ten analytes, according to the peak resolution calculated for different \( \beta \)-CD concentrations.

**Table 1.** Average migration times, repeatabilities, slopes, intercepts, correlation coefficients, and theoretical detection limits of the ten analytes.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Migration time(^a) (min)</th>
<th>R.S.D.(^a) (%)</th>
<th>Slope(^b) (( \times 10^4 ))</th>
<th>Intercept(^b) (( \times 10^4 ))</th>
<th>Correlation coefficient(^b) (( r ))</th>
<th>Detection limit (( \mu )g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>3.20</td>
<td>0.38</td>
<td>0.0156</td>
<td>0.0380</td>
<td>0.999</td>
<td>0.20</td>
</tr>
<tr>
<td>S-Phenylmercapturic acid</td>
<td>4.09</td>
<td>0.60</td>
<td>0.0120</td>
<td>0.0220</td>
<td>0.999</td>
<td>0.30</td>
</tr>
<tr>
<td>4-Methylhippuric acid</td>
<td>4.45</td>
<td>0.66</td>
<td>0.0279</td>
<td>0.0416</td>
<td>0.999</td>
<td>0.11</td>
</tr>
<tr>
<td>3-Methylhippuric acid</td>
<td>4.63</td>
<td>0.71</td>
<td>0.0332</td>
<td>-0.0118</td>
<td>0.999</td>
<td>0.10</td>
</tr>
<tr>
<td>2-Methylhippuric acid</td>
<td>4.74</td>
<td>0.73</td>
<td>0.0272</td>
<td>-0.0118</td>
<td>0.999</td>
<td>0.12</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>4.85</td>
<td>0.71</td>
<td>0.0286</td>
<td>-0.0136</td>
<td>0.999</td>
<td>0.13</td>
</tr>
<tr>
<td>Phenylglyoxylic acid</td>
<td>4.98</td>
<td>0.75</td>
<td>0.0228</td>
<td>0.0938</td>
<td>0.998</td>
<td>0.13</td>
</tr>
<tr>
<td>Mandelic acid</td>
<td>5.23</td>
<td>0.80</td>
<td>0.0151</td>
<td>0.0608</td>
<td>0.998</td>
<td>0.25</td>
</tr>
<tr>
<td>Uric acid</td>
<td>5.83</td>
<td>0.42</td>
<td>0.0237</td>
<td>0.1196</td>
<td>0.998</td>
<td>0.22</td>
</tr>
<tr>
<td>( t,t )-Muconic acid</td>
<td>9.46</td>
<td>0.93</td>
<td>0.0495</td>
<td>0.0705</td>
<td>0.999</td>
<td>0.10</td>
</tr>
</tbody>
</table>

\(^a\) \( n = 5 \), analyte concentration: 15 \( \mu \)g/mL.

\(^b\) Concentration range of analyte: 1 to 300 \( \mu \)g/mL for the analytes except \( t \)-MA with 1 to 75 \( \mu \)g/mL.
Table 1. Content and recovery of eight metabolites of BTEX in the urine sample of a gas station worker.

<table>
<thead>
<tr>
<th>Analytes</th>
<th>Day 1 (µg/mL)</th>
<th>Day 2 (µg/mL)</th>
<th>Day 3 (µg/mL)</th>
<th>Similarity index (%)a)</th>
<th>Recovery (%)b)</th>
<th>R.S.D. (%)b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>1790</td>
<td>1770</td>
<td>1790</td>
<td>90.0</td>
<td>105</td>
<td>8.89</td>
</tr>
<tr>
<td>S-Phenylmercapturic acid</td>
<td>–c)</td>
<td></td>
<td>151</td>
<td>97.3</td>
<td>3.63</td>
<td></td>
</tr>
<tr>
<td>4-Methylhippuric acid</td>
<td>49.4</td>
<td>56.9</td>
<td>61.3</td>
<td>91.3</td>
<td>90.7</td>
<td>2.90</td>
</tr>
<tr>
<td>3-Methylhippuric acid</td>
<td>41.8</td>
<td>32.5</td>
<td>29.6</td>
<td>99.8</td>
<td>90.7</td>
<td>2.25</td>
</tr>
<tr>
<td>2-Methylhippuric acid</td>
<td>–c)</td>
<td></td>
<td>146</td>
<td>97.6</td>
<td>97.1</td>
<td>6.07</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>24.9</td>
<td>29.1</td>
<td>36.3</td>
<td>99.1</td>
<td>93.0</td>
<td>1.11</td>
</tr>
<tr>
<td>Phenylglyoxylic acid</td>
<td>151</td>
<td>144</td>
<td>146</td>
<td>96.7</td>
<td>97.1</td>
<td>6.07</td>
</tr>
<tr>
<td>Mandelic acid</td>
<td>–c)</td>
<td></td>
<td>100</td>
<td>100</td>
<td>98.7</td>
<td>2.34</td>
</tr>
<tr>
<td>Uric acid</td>
<td>357</td>
<td>350</td>
<td>372</td>
<td>90.3</td>
<td>95.0</td>
<td>2.83</td>
</tr>
<tr>
<td>t,t-Muconic acid</td>
<td>–c)</td>
<td></td>
<td>357</td>
<td>92.5</td>
<td>2.90</td>
<td></td>
</tr>
</tbody>
</table>

a) Peak purity data from photodiode array detector.
b) n = 3, spiked concentrations: 50 µg/mL of p-MHA, m-MHA, HA, and PGA, and the others were 10 µg/mL.
c) Not detected.

The optimized CE method was employed to determine the urinary BTEX metabolite concentrations for occupational exposure to these organic compounds. The migration times RSDs (n = 5) were lower than 0.93%. The metabolite concentrations were quantified from the peak area of the electropherogram. Good linearities of calibration graphs were observed with a minimum of two orders of magnitude from 1 to 300 µg/mL, with the exception of t-MA, which was 1 to 75 µg/mL. The correlation coefficients all exceeded 0.998. At a signal-to-noise ratio of 3, the theoretical detection limits for the analytes ranged from 0.10 to 0.30 µg/mL, which were lower than the data obtained previously using HPLC and GC methods [12, 15–16].

3.2 Determination of BTEX metabolites in urine sample

The optimized CE method was employed to determine the urinary BTEX metabolite concentrations for occupational exposure to these organic compounds. Figure 5 shows the electropherograms of urine samples from a gas station worker and a graduate student. More than ten peaks were detected in these urine samples. Four BTEX metabolites including p-MHA, m-MHA, HA, and PGA were detected in the urine sample from gas station worker. The migration times of four detected BTEX metabolites were 4.56, 4.73, 5.01, and 5.14 min respectively. Despite the complicated urinary matrix, those metabolites were adequately resolved from other unknown compounds. By comparing each peak migration time and the corresponding UV spectrum with those of the standard allowed those peaks to be identified. The peak purity data from the photodiode array detector of these peaks exceeded 91.3%. Moreover, to further confirm the identities of the analytes, a standard compound, spiked into the urine sample, was employed.

p-MHA, m-MHA, HA, and PGA are metabolites of p-xylene, m-xylene, toluene, and ethylbenzene, respectively, which are the most volatile components of unleaded gasoline. The experimental findings indicate that, due to the occupational exposure to unleaded gasoline in the workplace, the worker was exposed to BTEX compounds. Table 2 summarizes the BTEX metabolite content in the gas station worker’s urine sample, as well as its average recovery and relative standard deviation. The urine samples were collected for three consecutive days at approximately the same time each day. The mean
recovery values of analytes in urine samples exceeded 90.7%. Their RSDs were less than 6.07%. This result also confirms that the β-CD modified CE method is adequate for analyzing these metabolites in urine samples. As the data in Table 2 show, none of the urinary metabolites exceeded the values recommended by ACGIH.

4 Concluding remarks

Eight BTEX metabolites were separated by applying the β-CD modified CE method with a total analysis time of 10 minutes. The advantages of analyzing BTEX metabolites by this method are high resolution, high separation efficiency, short analysis time, and adequate reproducibility. Also, the sample needs no complicated extraction step or additional derivatization procedure. In addition, the method effectively determined the BTEX metabolites in the urine sample of a gas station worker. Therefore, the β-CD modified CE technique potentially offers a simple and rapid method for monitoring routine urine samples of persons who are occupationally exposed to such volatile organic compounds.

Acknowledgment

This research was supported by Grant NSC 89-2113-M-009-029 from the National Science Council of the Republic of China.

References


[JSS 1170]