Analysis of some metabolites of organic solvents in urine by high-performance liquid chromatography with β-cyclodextrin

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Abstract

Chromatographic separation of the metabolites derived from toluene, ethylbenzene, styrene and xylene was carried out on untreated urine samples from factory workers. The elution sequence was as follows: phenylglyoxilic acid, 3-hydroxy-2-butanone, hippuric acid, o-methylhippuric acid, p-methylhippuric acid, m-methylhippuric acid, p-cresol, m-cresol and o-cresol. The stability constants (K_G) of cresol and methylhippuric acid derivatives were evaluated. The capacity factor (k'), selectivity factor (α) and resolution (R_s) are described with a variety of mobile phases containing β-cyclodextrin (β-CD). The optimum concentration ratio of ethanol–water–acetic acid–β-CD was determined to be 20:80:0.3:1.4%. Under these conditions, k' values of the five metabolites were $2 < k' < 6$, and all α values were greater than 1.5. Simultaneous determinations of the metabolites were carried out in real urine samples from factory workers using the standard addition method. Validation of the method and the detection limit are described under the optimum conditions attained in this experiment. © 1997 Elsevier Science B.V.

Keywords: Phenylglyoxylic acid; 3-Hydroxy-2-butanone; Hippuric acid; Methylhippuric acid; Cresol

1. Introduction

Since carbohydrate compounds, such as toluene, xylene, ethylbenzene and styrene are widely used not only for industrial raw materials but also as solvents, exposure of humans to these compounds is highly likely. Factory workers, in particular, are occupationally exposed to these solvents [1]. Thus, it is important to establish a method for diagnosing if factory workers have been exposed to organic solvents or not.

The main metabolites of toluene are the three isomers, o-, m- and p-cresol, and those of o-, m- and p-xylene are o-, m- and p-methylhippuric acid (MHA) [1]. Phenylglyoxalic acid (PGA) and mandelic acid (MA) are the regenerated products of ethylbenzene [2]. 3-Hydroxy-2-butanone (3-H-2-B) is from methylethylbenzene [3]. The metabolism of ethylbenzene is the same as for styrene. Therefore, these compounds are used for determining the exposure index in humans. Of these organic solvents, extensive studies have been performed to detect and estimate the metabolites of toluene and xylene separately, but only a few papers have been published for benzene, toluene and xylene. Most researchers have detected metabolites in urine after

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some pretreatment step, but only a few papers have dealt with direct determination. The simultaneous determination of these metabolites is important in order to evaluate the health of factory workers, however, there have only been a few reports of the simultaneous determination of the metabolites derived from toluene, xylene, styrene and ethylbenzene [2].

The determination of some metabolites in urine has been studied by Ikeda and Ohtsuji [4], Umberg and Fiorese [5], Ogata et al. [6] and Tomokuni and Ogata [7] using spectrometry. There have been a few reports regarding the separation of hippuric acid and methylhippuric acid using gas chromatography, in which the metabolites of toluene and xylene were found [8–10]. The weak points in these methods were in sensitivity, quickness, uniqueness and systematization. The separation of some metabolites by high-performance liquid chromatography (HPLC) has also been performed by Ogata et al. [11,12], Matsui et al. [13], Hansen and Dossing [14] and by Zukowski et al. [15]. In most cases, however, quantitative analysis was performed after pretreatment of the urine specimens. This pretreatment is inconvenient.

Thus, we have tried to detect the nine metabolites of toluene, xylene, ethylbenzene (or styrene) and methylethylketone, simultaneously, rapidly and easily. This highly reproducible method can be used not only for research data but to aid in the management of the health of workers who are exposed to these organic solvents. In this work, we applied this method to the direct analysis of urine samples from factory workers, without pretreatment, by adding β-cyclodextrin (β-CD) to the sample solution. β-CD was used as a mobile phase component because of it easily forms inclusion complexes with other molecules of an appropriate size [16,17]. The separation of the three toluene isomers was accomplished satisfactorily using a solvent containing β-CD [18,19]. We extensively considered the separation parameters for the metabolites by changing the percentage concentration of β-CD in various ratios of solvents, etc. Validation of the method and the detection limit were attained under the optimum experimental conditions obtained from this study, and stability constants are also described.

2. Experimental

2.1. Materials

Hippuric acid, PGA, o-, m- and p-MHA, 3-hydroxy-2-butanone and β-CD were purchased from Sigma (St. Louis, MO, USA). HPLC-grade ethanol, acetic acid and p-cresol were from Junsei Chemicals (Japan). o-Cresol and m-cresol were from Hanawa and Kokusan Chemicals (Japan), respectively. All other reagents were of the best commercial quality available. The real samples were urine samples from workers who work at the industrial fields that use various organic solvents in Pusan, South Korea.

2.2. Equipment and experimental conditions

An HPLC with a dual pump (CCPM, Toso), a UV–Vis detector (UV-8010, Toso), a data system (SC-8010, Toso) and a Beckman spectrophotometer was used. A Lichrosorb RP-18 (10 μm) column with octadecylsilane as the packing material was employed. The flow-rate of the eluent was 0.8 ml/min. The sample volume was 20 μl at a solvent ratio of EtOH–H_2O–CH_3COOH of 20:80:0.3. We recorded chromatograms to obtain the optimum conditions using various amounts of β-CD and different solvent ratios. A column heater from Waters (TCM-005822) maintained the temperature of the column at 40°C. The maximum wavelength of each of the nine metabolites was obtained separately using a UV–Vis spectrophotometer. The analytical wavelength of the mixture of the nine metabolites was 270 nm. In this experiment, p-chlorophenol was used as the internal standard.

To obtain a standard calibration plot, the concentrations of the standards used were as follows: PGA, 0.0028 g/l; 3-H-2-B, 2.8368 g/l; HA, 0.4255 g/l; o-MHA, 0.4255 g/l; p-MHA, 0.1064 g/l; m-MHA, 0.2128 g/l; p-cresol, 0.1418 g/l; m-cresol, 0.1418 g/l and o-cresol, 0.1418 g/l. To analysis the real urine of factory workers using the standard additions method, the above standard materials were mixed with distilled water at volume ratios of 1:1, 1:2, 1:3 and 1:4, respectively, then the mixing standard solutions were finally diluted into the real urine at a ratio of 1:1. A 20-μl volume of the sample

containing standards was loaded on the column and was eluted with solvents containing β-CD. The urine samples were frozen and stored after collection from the factory workers. Before being measured, a 5-ml volume of each sample was thawed without any pretreatment and filtered through an AP-type purifier (pore size, 0.45 μm; Millipore, USA). The samples containing the standard were then introduced to the injection port of the HPLC for determining the concentration of the nine metabolites by the standard addition method. Analysis of the actual sample was carried out using a zero blind method.

3. Results

The resolution of the metabolites was examined by changing the concentration of β-CD at a fixed ratio of the eluent (EtOH–H_2O–CH_3COOH, 20:80:0.3). p-MHA, m-MHA and o-, m- and p-cresol were not resolved without β-CD being present and the elution time of the last metabolite was 39.33 min (Fig. 1a). The chromatogram of p-MHA and m-MHA with 0.6% β-CD shows no resolution, and the elution time of the last metabolite, o-cresol, was 23.45 min. At 1.0% β-CD, almost the same phenomenon occurred. As the concentration approached 1.4%, all nine metabolites were resolved and the elution times of each of the compounds were as follows: PGA, 2.94 min; 3-H-2-B, 3.68 min; HA, 6.28 min; o-MHA, 8.17 min; p-MHA, 10.66 min; m-MHA, 11.70 min; p-cresol, 18.69 min; m-cresol, 21.25 min and o-cresol, 23.07 min. At 1.8% β-CD, all nine metabolites and the internal standard were resolved and the elution time of o-cresol (19.63 min) was shorter than at 1.4% β-CD (Fig. 1b). The mixture was composed of the nine metabolites and p-chlorophenol as an internal standard. The addition of the internal standard reduced the elution time: PGA, 2.88 min; 3-H-2-B, 10.14 min; HA, 5.81 min; o-MHA, 7.45 min; p-MHA, 9.13 min; m-MHA, 10.14 min; p-cresol, 15.34 min; m-cresol, 17.90 min; o-cresol, 19.63 min and internal standard, 21.60 min.

Capacity factors (k') were determined at various concentrations of β-CD (0, 0.6, 1.0, 1.4 and 1.8%). At 0% β-CD, none of the metabolites were resolved completely. The k' values at 1.8% β-CD were as follows: PGA, 0.58±0.01; 3-H-2B, 0.92±0.01; HA, 2.10±0.01; o-MHA, 3.00±0.01; p-MHA, 4.01±0.02; m-MHA, 5.01±0.01; p-cresol, 7.71±0.04; m-cresol, 9.01±0.03 and o-cresol, 9.91±0.05. From the above results, it could be determined that the k' values at 1.8% β-CD were higher than those at 1.4% β-CD and all k' values of the metabolites were less than ten.

The selectivity factor (α) at the different β-CD concentrations were determined. At 0.6% β-CD, the values of each were as follows: PGA/3-H-2-B, 1.17; 3-H-2-B/HA, 1.64; HA/o-MHA, 1.26; o-MHA/p-MHA, 1.34; p-MHA/m-MHA, 1.07; m-MHA/p-cresol, 1.89; p-cresol/m-cresol, 1.07 and m-cresol/o-cresol, 1.07. In this case, the standard deviation of the values was below ±0.01. The selectivity factors for a β-CD content of above 0.6% for the nine metabolites were similar to the values obtained at 1.8% β-CD. All of the selectivity factors at 1.8% β-CD were higher than one, so the selectivity was good, as seen in the following: PGA/3-H-2-B, 1.21; 3-H-2-B/HA, 1.64; HA/o-MHA, 1.28; o-MHA/p-MHA, 1.40; p-MHA/m-MHA, 1.10; m-MHA/p-cresol, 1.58; p-cresol/m-cresol, 1.15 and m-cresol/o-cresol, 1.09, and the standard deviation of the values was below ±0.01.

The resolution (R_s) at the different concentrations of β-CD were also determined for the nine metabolites. At 0% β-CD, p-MHA, m-MHA and o-, m- and p-cresol were not fully resolved. However, as the resolution of most of the compounds, with the exception of o-MHA/p-MHA and m-MHA/p-cresol were better at 1.8% β-CD than at 1.4% β-CD, the former was found to give the best resolution: PGA/3-H-2-B, 1.90±0.02; 3-H-2-B/HA, 6.01±0.04; HA/o-MHA, 3.27±0.02; o-MHA/p-MHA, 3.29±0.01; p-MHA/m-MHA, 1.34±0.01; m-MHA/p-cresol, 6.90±0.01; p-cresol/m-cresol, 2.24±0.01 and m-cresol/o-cresol, 1.45±0.01.

The resolution of the nine metabolites were also determined at different solvent ratios using a fixed concentration of β-CD (1.8%). These solvent ratios were 30:70:0.3, 25:75:0.3, 20:80:0.3 and 15:85:0.3 (EtOH–H_2O–CH_3COOH). At a solvent ratio of EtOH–H_2O–CH_3COOH of 20:80:0.3, the elution times of each compound were as follows: PGA, 2.90 min; 3-H-2-B, 3.67 min; HA, 6.07 min; o-MHA,
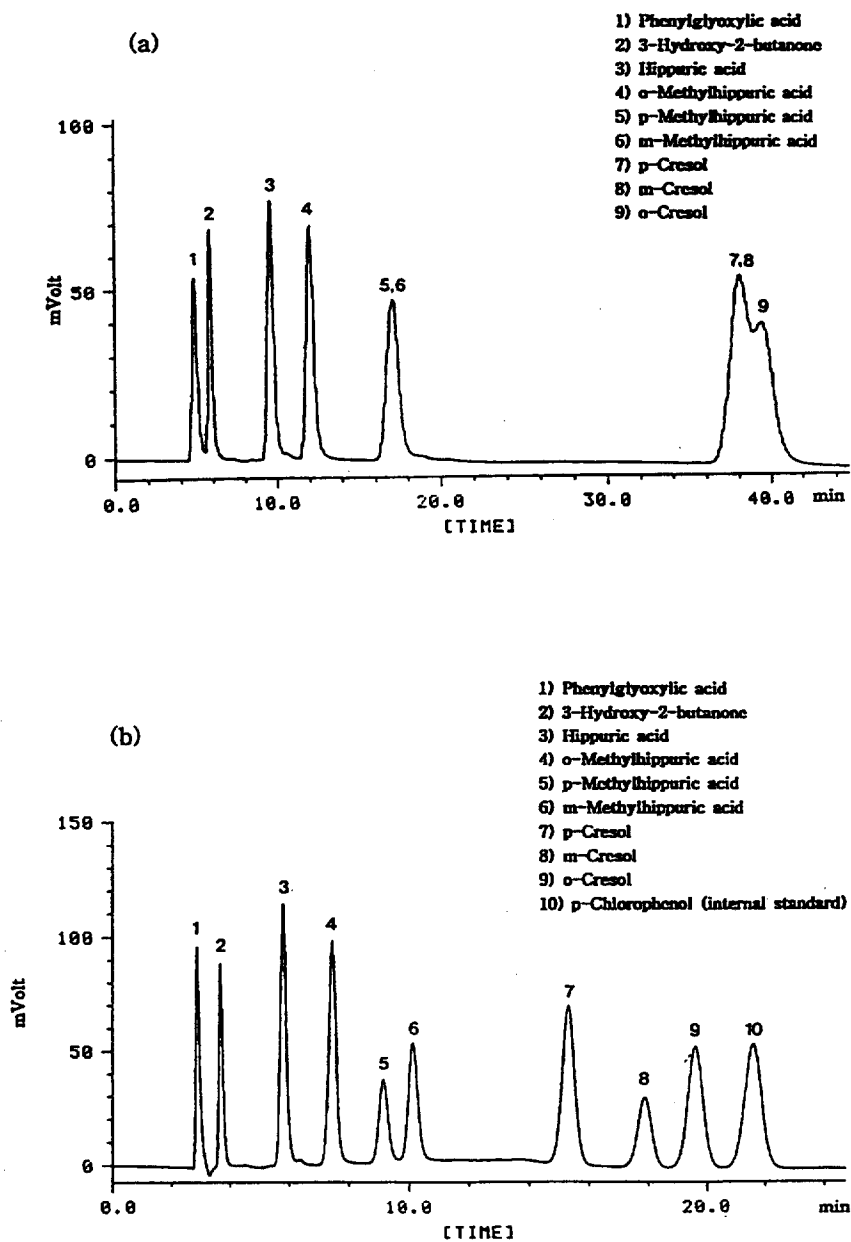
Fig. 1. Chromatograms of the nine metabolites (a) in EtOH–H$_2$O–CH$_3$COOH = 20:80:0.3 without β-CD and (b) in the eluent with β-CD and an internal standard.

7.87 min; p-MHA, 9.93 min; m-MHA, 11.01 min; p-cresol, 16.89 min; m-cresol, 19.59 min and o-cresol, 21.43 min. At ratios of 30:70:0.3 and 25:75:0.3 (EtOH–H$_2$O–CH$_3$COOH), the elution times were shorter, but p-MHA and m-MHA were

not resolved. At a ratio of 15:85:0.3 (EtOH–H$_2$O–CH$_3$COOH), not only did the elution time become longer, but PGA and 3-H-2-B were not resolved.

The capacity factors were determined using various solvent ratios. At a ratio of EtOH–H$_2$O–

CH_3COOH of 20:80:0.3, the capacity factor was larger than at other solvent ratios and only four species satisfied the $2<k'<6$ condition. The selectivity factor of the eluents at the different ratios were evaluated. At a $EtOH-H_2O-CH_3COOH$ ratio of 20:80:0.3, all of the values were larger than one and, therefore, good resolution was obtained. The selectivity factors (α) in this case were as follows; PGA/ 3-H-2-B, 1.21; 3-H-2-B/HA, 1.64; HA/o-MHA, 1.28; o-MHA/p-MHA, 1.26; p-MHA/m-MHA, 1.10; m-MHA/p-cresol, 1.59; p-cresol/m-cresol, 1.15 and m-cresol/o-cresol, 1.09. In this case, the standard deviation was below ±0.01. At a ratio of $EtOH-H_2O-CH_3COOH$ of 20:80:0.3, the resolution was also good, as previously described.

Chromatograms of urine from a laborer who was exposed to organic solvents at a factory and from a normal person who was not exposed to solvents were recorded, without any pretreatment, and the results are shown in Fig. 2. Fig. 2a is a chromatogram of the urine sample from an exposed person and include the standard materials, Fig. 2b shows a chromatogram of the sample without the addition of the standard materials and Fig. 2c is of a sample from a person who was not exposed to the solvents. In Fig. 2a, all nine metabolites were determined by the standard addition method with a 1:1 ratio of standard material added to the urine sample, which was diluted by 50%. While we could detect PGA, 3-H-2-B, HA, p-MHA, p-cresol and m-cresol, o-MHA, p-MHA, m-MHA and o-cresol were not detected in the real sample because of their very low concentrations (see Fig. 2b). However, as shown in Fig. 2c, the chromatogram of the urine sample of the unexposed person had small peaks at the positions of PGA, 3-H-2-B and HA. This may have arisen due to interferences from some of the ~200 different species in the urine sample. Unfortunately, our experiment could not assign the species.

Calibration plots of the nine metabolites were detained at a ratio of the peak area with respect to one of the internal standards vs. concentration (not shown). These were linear for all of the metabolites. The equations of the plots are as follows: PGA, $y=3819.895x-0.008$; 3H-2-B, $y=3.730x+0.012$; HA, $y=43.067x-0.032$; o-MHA, $y=41.769x-0.016$; o-cresol, $y=155.713x-0.012$; m-cresol, $y=71.400x-0.106$; p-cresol, $y=153.661x+0.015$; m-

MHA, $y=61.646x-0.004$ and p-MHA, $y=55.018x+0.025$.

Detection limits and recoveries were also determined. The limits were found to be as follows: PGA, 1.4 μg/l; 3-H-2-B, 1.4 μg/l; HA, 0.6 mg/l; o-MHA, 0.6 mg/l; p-MHA, 1.0 mg/l; m-MHA, 2.1 mg/l; p-cresol, 4.4 mg/l; m-cresol, 7.0 mg/l and o-cresol, 4.4 mg/l. In this case, the relative standard deviations were ±6%. The recoveries were found to be: PGA, 103.5±1.9%; 3-H-2-B, 82.3±5.6%; HA, 99.8±1.4%; o-MHA, 104.1±2.0%; m-MHA, 106.3±2.5%; p-MHA, 101.5±1.8%; p-cresol, 95.7±2.8%; m-cresol, 93.3±2.1% and o-cresol, 96.4±3.2%.

Table 1 shows the concentration of the nine metabolites in the urine samples from 30 laborers who were exposed to the complex organic solvents. It was possible to detect seven of the nine compounds. Both m-MHA and o-cresol were not detected due to their low concentrations. The columns in this experiment have a limited life span, due to clogging by the re-precipitated β-CD and to sparingly soluble species in the urine samples at low temperature. However, the column thermostated at 40°C prevented re-precipitation of β-CD and allowed the column to be used for more than two months.

4. Discussion

All nine metabolites were resolved at a concentration higher than 1.4% β-CD and it was not possible to resolve the nine metabolites without β-CD. Resolution was achieved through the formation of inclusion complexes between β-CD and analytes, in which a hydroxyl group of o-, m- or p-cresol was included in the cavity of β-CD. The stability constants were calculated from Eq. (1) using the least squares method. The values obtained were 0.12±0.08, 0.47±0.28, 1.36±0.49 for o-, m- and p-cresols, respectively. As the stability constants of the complexes became larger, the interaction between the mobile phase and the complexes became stronger, which may have caused the cresols to be eluted in order of their stability constants. This order coincides with the stability constants of Zukowski et al. [15]. The equation used to calculate the stability constant in the RP-18 column was:
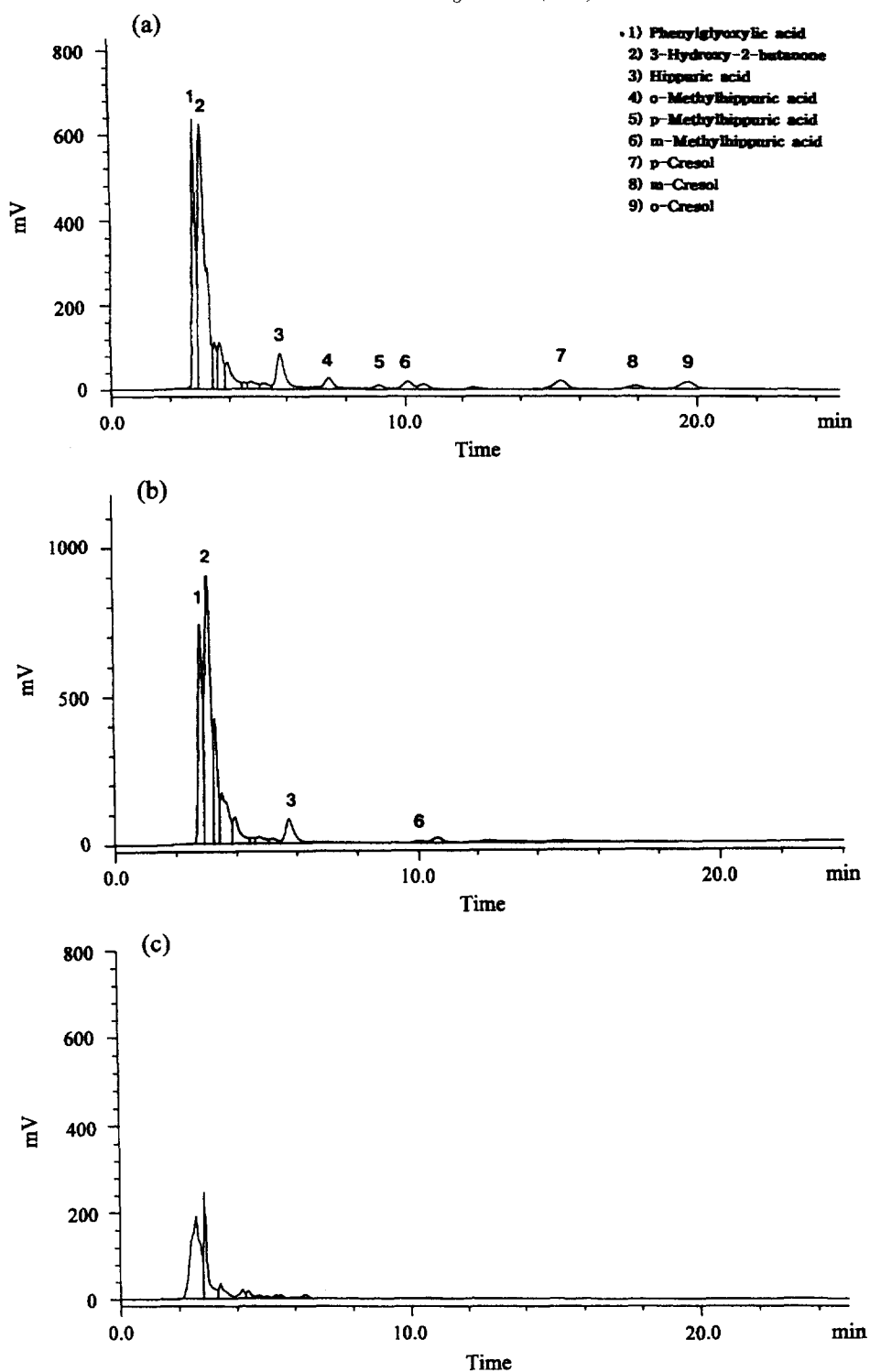
Fig. 2. Chromatograms of the metabolites in the urine samples of a factory worker exposed to the organic solvents (a) with addition of the standard materials, (b) without addition of the standard materials and (c) in the urine sample from an unexposed person.

Table 1
Actual concentration ranges of the nine metabolites in the urine samples of various factory workers, which were analyzed by the standard addition method

Metabolite	Number of samples	Concentration
PGA	30	7.44±7.90 mg/l
3-H-2-B	30	470±304 μg/l
HA	30	1430±1350 mg/l
o-MHA	13	30±29 mg/l
p-MHA	30	71±48 mg/l
m-MHA	–	–
p-Cresol	18	5.89±3.21 mg/l
m-Cresol	18	505±423 mg/l
o-Cresol	–	–

PGA = Phenylglyoxylic acid; 3-H-2-B = 3-hydroxy-2-butanone; HA = hippuric acid and o-, m- and p-MHA = o-, m- and p-methylhippuric acid.

$$k' = \frac{k'_G - k'}{K_G[CD]} + k'_{G \cdot CD} \qquad (1)$$

where K_G is the stability constant. k'_G and $k_{G \cdot CD}'$ are the capacity factor of the free guest molecule (G) and the CD complex (G·CD).

The benzene rings in o-, m- and p-MHA molecules were included in the cavity of β-CD because the -CONHCH$_2$COOH group attached to the benzene ring was too big. The stability constants of the o-, m- and p-MHA complexes obtained from Eq. (1) were: o-MHA, 3.4±1.0; m-MHA, 8.4±1.2 and p-MHA, 10.3±3.2. The order of elution in relation to the stability constants was p-, m- and o-MHA. However, o-MHA was eluted first, followed by p- and m-MHA in this study. This unexpected order probably arose from the weak interaction of o-MHA with the stationary phase, due to steric effects, although its stability constant is the smallest of the MHAs. For the other metabolites, HA, PGA, 3-H-2-B, it was considered that the elution time was altered because the stability constants of the complexes were different, as well as one in the degree of interaction of the β-CD complex to the stationary phase.

From the capacity factor, the selectivity factor and the resolution obtained at various concentrations of β-CD, the concentration of 1.8% β-CD was found to be the optimum quantity. The best eluent ratio with 1.8% β-CD was EtOH–H$_2$O–CH$_3$COOH = 20:80:0.3. Thus, the optimum ratio was EtOH–H$_2$O–CH$_3$COOH–β-CD = 20:80:0.3:1.8. The detection

limits of the nine metabolites were better than those obtained by Otaga and Tageuchi [19], which were 5, 2, 6, 8 and 8 mg/l for PGA, HA, o-, m- and p-MHA, respectively, and the elution time was within 25 min, therefore, this method was found to be a better and more economical method. The recoveries of the metabolites using this method were found to be better than those obtained using Ogata's method.

In this study, the average concentration of PGA in the workers' urine samples was 7.44±7.90 mg/l, which is lower than the limit deemed to be an occupational hazard (40 mg/l) in South Korea. However, since this value is obtained with the sulfuric acid formalin method, we could not make a direct comparison. The determination of such metabolites was performed using other methods [16,20], but, due to the limited detection limits, PGA could not be detected. However, in this study, it was possible to determine the concentration of PGA by the standard addition method. The average concentration of 3-H-2-B in urine was 470±304 μg/l, which was lower than obtained by Perbellini et al. [21] of 645±354 μg/l. The average concentration of HA was 1430±1350 mg/l. This value was similar to that of Lee et al. [22] (1560±950 mg/l), but was higher than that of Lee et al. [23] and was much lower than the value of 3423±2229 mg/l obtained by Cho [24], who used a spectrometric method. To ascertain the validity of this method, consideration was taken of the fact that the analysis method and the target of investigation were different from those of other methods. Augusta et al. [25] suggested the quantitation method only but could not make the detection because it was lower than the detection limit. From the above results, the analysis method from this study was superior to previous methods, and it is also more applicable for the determination of the nine metabolites in urine.

5. Conclusion

The new method to determine the nine metabolites in urine with HPLC was performed by changing the solvent ratios and the concentration of β-CD in the eluent. The results were as follows: (i) The optimum composition for the mobile phase was EtOH–H$_2$O–CH$_3$COOH–β-CD = 20:80:0.3:1.8. (ii) The detection

limits of the nine metabolites with relative standard deviations of 6% or less were: PGA, 1.4 μg/l; 3-H-2-B, 1.4 μg/l; HA, 0.6 mg/l; o-MHA, 0.6 mg/l; p-MHA, 1.0 mg/l; m-MHA, 2.1 mg/l; p-cresol, 4.4 mg/l; m-cresol, 7.0 mg/l and o-cresol, 4.4 mg/l. (iii) The recovery ratios were: PHA, 103.5±1.9%; 3-H-2-B, 82.3±5.6%; HA, 99.8±1.4%; o-MHA, 104.1±2.0%; m-MHA, 106.3±2.5%; p-MHA, 101.5±1.8%; p-cresol, 95.7±2.8%; m-cresol, 93.3±2.1% and o-cresol, 96.4±3.2%. (iv) The concentration of the nine metabolites in the urine of laborers who had been exposed to the complex organic solvents were: PHA, 7.44±7.90 mg/l; 3-H-2-B, 470±304 μg/l; HA, 1430±1350 mg/l; o-MHA, 31±29 mg/l; p-MHA, 7.0±48 mg/l; p-cresol, 6.0+3.21 mg/l and m-cresol, 505±423 mg/l. m-MHA and o-cresol were not detected in this case. (v) The column could be used for more than two months with our experimental conditions. Analysis of the nine metabolites could be achieved within about 30 min using our experimental conditions.

6. Acknowledgement

References

[1] T. Inoue, M. Ikeda and M. Ogata, Jpn. J. Ind. Health, 26 (1984) 518.

[2] T. Sakai, Y. Takeuchi, Y. Ikeya, T. Araki and K. Ushio, J. Ind. Health, 31 (1989) 9.

[3] S. Kezic and A.C. Monster, J. Chromatogr., 428 (1988) 275.

[4] M. Ikeda and H. Ohtsuji, Br. J. Ind. Med., 26 (1969) 26.

[5] C.J. Umberg and F.F. Fiorese, Clin. Chem., 9 (1963) 91.

[6] M. Ogata, K. Tomokuni and Y. Takazuka, Br. J. Ind. Med., 26 (1969) 330.

[7] K. Tomokuni and M. Ogata, Clin. Chem., 18 (1972) 349.

[8] P.B.V. Roosmalen and I. Drunandez, Br. J. Ind. Med., 35 (1978) 56.

[9] R. Careros and J.G. Fernandez, Br. J. Ind. Med., 34 (1977) 229.

[10] J.P. Buchet and R.R. Lauwerys, Br. J. Ind. Med., 30 (1973) 125.

[11] M. Ogata, R. Sugihara and S. Kira, Int. Arch. Occup. Environ. Health, 39 (1977) 199.

[12] M. Ogata, K. Tomokuni and Y. Takazuka, Br. J. Ind. Med., 27 (1970) 43.

[13] H. Matsui, M. Kasao and S. Imamura, Br. J. Ind. Med., 34 (1977) 310.

[14] S.H. Hansen and M. Dossing, J. Chromatogr., 229 (1982) 141.

[15] J. Zukowski, D. Sybilska and J. Jurezak, Anal. Chem., 57 (1985) 2217.

[16] K. Fujimura, T. Ueda, M. Kitagawa, H. Takayanagi and T. Ando, Anal. Chem., 58 (1986) 2668.

[17] A.O. Pulley and D. French, Biochem. Biophys. Res. Commun., 5 (1961) 11.

[18] F. Cramer, Rev. Pure Appl. Chem., 5 (1955) 143.

[19] M. Ogata and T. Tageuchi, Int. Arch. Occup. Environ. Health, 59 (1987) 263.

[20] G. Poggi, M. Giusiani and U. Palagi, Int. Arch. Occup. Environ. Health, 50 (1982) 25.

[21] L. Perbellini, F. Brugnone and P. Mozzo, Int. Arch. Occup. Environ. Health, 54 (1984) 73.

[22] C.U. Lee, H.L. Seen and B.M. Cho, J. Kor. Prev. Med., 27 (1988) 374.

[23] S.H. Lee, H.A. Kim and B.K. Lee, Kor. Ind. Health, 27 (1988) 4.

[24] B.M. Cho, J. Pusan Med. Coll., 29 (1989) 109.

[25] B. Augusta, P. Palo and A. Carlo, J. Chromatogr., 535 (1990) 311.