Articles

Simultaneous Determination of Urinary Metabolites of Workers Exposed to Benzene, Toluene and Xylene Mixture with HPLC using β-Cyclodextrine

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The high-performance liquid chromatographic determination of eight metabolites was evaluated with a β-cyclodextrine (β-CD) added C_{18} column. The urinary metabolites were hippuric acid (HA), phenol, o-, m-, p-methylhippuric acid (MHA) and o-, m-, p-cresol in untreated urine samples of factory workers exposed to benzene, toluene and xylene mixture. Eluting sequence of eight metabolites was shown to be the order of HA, o-MHA, phenol, p-MHA, m-MHA, p-cresol, m-cresol, and o-cresol. The optimum ratio of the mobile phase was selected 20/80/0.3/1.4% to ethanol/water/acetic acid/β-CD, in which was shown $2 < k' < 6$ for five metabolites. Optimum concentration ranges and detection limits for the metabolites of factory worker's urine samples were determined.

Introduction

The simultaneous determination of the urinary metabolites is important to the biological monitoring of occupationally exposed people *i.e.* factory workers. However, few reports were available for the analysis of the metabolites from people exposed to the mixture of benzene, toluene, and xylene.[1] The separation of toluene metabolites had been satisfactorily accomplished with the advantage of β-CD inclusion complex.[2,3] The determination of metabolites using gas chromatography in a human urine sample had been reported for hippuric acid (HA) or methylhippuric acid (MHA) as person expose to toluene or xylene.[4-6] The liquid chromatographic determination of urinary metabolites has also been developed and practically applied.[7-10] Quantitative analysis with HPLC, in most cases, has been performed after the pretreatment of urine samples until recently. The aim of adding β-CD into an eluent in the present work is to improve the selective resolution of the metabolites without the pretreatment of a urine sample. The previous result investigated in our laboratory shows that the β-CD addition into a mobile phase has led to improve the selective separation of some metabolates.[11]

β-cyclodextrine ($C_{42}H_{70}O_{35}$, β-CD) is cycloheptaamylose and parallelogram shaped crystals. The non-polar central cavity of β-CD can selectively include various inorganic and/or organic species of neutral of ionic nature. Hydrophobicity and shapes of guest molecules might mainly affect formation of the inclusion complex with β-CD, *i.e.* by the fitness of the complexed molecule to the cavity of CD. β-CD has utilized the separation of steroidal drugs, benzene isomer or cispermethrinic acid isomers by GC or HPLC.[12-14]

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The present study is focus to the determination of eight metabolites simultaneously using the HPLC with β-CD. Worker's urine samples have been analyzed without pretreatment. β-CD, which forms easily inclusion complexes, was applied to a component of the mobile phase,[15-17] then practicability for the determination method were evaluated for the real urine samples.

Experimental

Reagents. Hippuric acid, o-methylhippuric acid, m-methylhippuric acid and p-methylhippuric acid (Tokyo Chemical, Tokyo, Japan), o-, m-, p-cresol (Kanto Chemical, Tokyo, Japan) were used as a standard solution mixture. p-chlorophenol (Tokyo Chemical, Tokyo, Japan) was used as an internal standard material. HPLC grade Ethanol as the eluent material was received from Fisher (New Jersey, USA) and β-cyclodextrine was from Sigma Co. (St. Louis, USA). All the following reagents were of extra pure grade or special grade for HPLC (up to 99.5% purity). Deionized water (18.2 MΩ/cm) was prepared by purification through Milli-Q system (Millipore).

Apparatus. HPLC was composed of a CCPM dual pump (Toso, Tokyo, Japan) and an uv/vis 8010 detector connected to a data system (Toso SC-8010). The columns (150 mm length, 4.6 mm internal diameter) were packed with a 10 μm particle size of the Lichrosorb resin (RP-18, E. Merk, Darmstadt, Germany). The flow rate of the eluent was 1.0 mL/min at a column temperature of 40(C and an urine specimen volume of 25 μL. For obtaining an optimum wavelength to detect the standard metabolites mixtures, the Beckman spectrophotometer (Fullerton, USA) was used. The maximum absorption wavelength (λ_{max}) of the standard solution of the metabolite mixture was adjusted to 270 nm.

Table 1. Capacity factors (k') and resolutions (R_s) according to β-CD addition in mobile phase

Metabolites (Resolutions)	β-Cd					
	none	0.7%	1.0%	1.2%	1.4%	2.0%
HA	0.96	1.16	1.26	1.34	1.34	1.17
o-MHA (HA/o-MHA)	1.45 (2.50)	1.75 (2.66)	1.92 (2.90)	2.00 (2.94)	2.14 (2.11)	1.85 (3.12)
phenol (o-MHA/phenol)	2.84 (4.77)	3.01 (4.30)	2.73 (3.12)	2.77 (3.03)	2.84 (3.04)	2.27 (1.62)
p-MHA (phenol/p-MHA)	3.21 (1.10)	3.25 (-)	3.13 (1.31)	3.28 (1.65)	3.30 (1.69)	2.77 (1.68)
m-MHA (p-MHA/m-MHA)	3.21 (-)	3.25 (0.43)	3.71 (1.59)	3.88 (1.63)	3.89 (1.87)	3.42 (1.99)
p-cresol (m-MHA/p-cresol)	8.38 (7.45)	7.38 (4.57)	5.72 (4.68)	5.81 (4.55)	6.05 (5.80)	4.58 (3.07)
m-cresol (p-cresol/m-cresol)	8.38 (-)	8.22 (1.47)	7.25 (2.99)	7.36 (3.06)	7.52 (3.32)	6.08 (3.46)
o-cresol (m-cresol/o-cresol)	8.38 (-)	8.95 (1.20)	8.23 (1.66)	8.34 (1.72)	8.51 (1.96)	7.07 (1.95)

Percent concentration ratios of all mobile phases were 20/80/0.3 in ethanol/deionized water/acetic acid. The abbreviations of hippuric acid and o-, m-, p-methylhippuric acid were expressed to HA and o-, m-, p-MHA, respectively. The numbers of parentheses are resolution (R_s) values. [(-) means no separation.]

Eluent Composition. An eluent was composed of ethanol (EtOH), deionized water (DW), acetic acid (HAc), and β-CD. The optimum composition of the eluent that composed of EtOH/DW/HAc/β-CD: 20/80/0.3/1.4 in the percent concentration. 196.7 mL of ethanol, 786.6 mL of distilled water and 2.95 mL of acetic acid were mixed in a Pyrex beaker and then 13.8 g of β-CD was dissolved well in the pre-mixed solution. Finally, the mixed solution was filtered with the AP type filter, then utilized as an eluent for HPLC.

Analysis of Urinary Metabolites. Separations of urinary eight metabolites were conducted employing 2 kinds of the eluent compositions, which are varied with a concentration of ethanol and water, or varied with a concentration of β-CD. The one of the component of the eluent constituted ethanol, deionized water, and acetic acid (20/80/0.3%) varied with the β-CD concentration from 0.7% to 2.0%. The other of an eluent constituted to acetic acid and β-CD (0.3/1.4, vol./vol.) varied with the concentration of ethanol and distilled water. Actual urine samples were filtered with an AP type purifier (0.45 μm of pore size, Millipore Co., Bedford, USA) before using. The separation was carried out by the internal standard method using p-chlorophenol as an internal standard material. The Calibration plot was detained to analyze the urinary samples. The 5 μL samples were injected.

Quality Control. For a quality control, the analysis of eight metabolites in worker's urine was carried out using the standard addition and the internal standard methods. The relative standard deviation in 20 urine samples in each metabolite is less than 5% for both of the standard addition method and internal standard method. Recoveries were 90-95% for all of the metabolites.

Results

Resolution. The resolution of the metabolites was examined by changing the concentration of β-CD at a fixed ratio of the eluent. m-MAH, m- and o-cresol were not resolved without β-CD being present in the eluent. (Table 1) The capacity factors (k') and resolutions (R_s) calculated in various concentration of β-CD are shown in Table 1. R_s value obtained by the β-CD addition were better compared to another. k' value for 1.0% β-CD addition showed good selectivity coefficients in all of the separated metabolites. Especially, the R_s value of phenol/p-MHA for 1.0% β-CD addition in the mobile phase was determined to be 1.31, indicating no complete separation. R_s values for 1.4% β-CD addition are rather better than others. The k' values decreased when β-CD exceeds 1.4% in mobile phase. Table 2 shows k' values obtained in the various aqueous eluents containing 1.4% β-CD. The k' values among o-, p-, m-Cresol and m-, p-MHA were well satisfied with the range of $2 < k' < 6$.[23] Thereafter the optimum condition with the mobile phase composed of ethanol/water/acetic acid/β-CD were given as 20/80/0.3/1.4%.

Chromatographic Separation of Eight Metabolites. The chromatogram in Figure 1 shows a variation of the resolution among, (a) without β-CD, (b) 1.0% β-CD, and (c) 1.4% β-CD containing the eluent of ethanol : distilled water : acetic acid (20 : 80 : 0.3, v/v/v). While Figure 1(a) shows incomplete resolution of the peaks, both of them using β-CD had not overlapped peaks but a good resolution. The peaks in Figure 1(c) obtained with an addition of 1.4% β-CD had more clear resolution than that of 1.0% β-CD. Assay of eight metabolites were undertaken during about 30 min in case of a 1.4% β-CD addition that had shown a longer time than that of 1.0% β-CD. The retention times in each metabolite with 1.4% β-CD addition were shown 6.75 for

Table 2. Capacity factors (k') and resolutions (R_s) of metabolites according to the composition of eluents containing 1.4% β-CD

Metabolites (Resolutions)	EtOH : DW[a]		
	15 : 85	20 : 80	25 : 75
HA	1.28	1.34	0.61
o-MHA (HA/o-MHA)	1.92 (3.06)	2.14 (2.11)	0.96 (2.00)
phenol (o-MHA/phenol)	2.58 (2.80)	2.84 (3.04)	1.59 (2.99)
p-MHA (phenol/p-MHA)	3.20 (2.13)	3.30 (1.69)	1.86 (1.12)
m-MHA (p-MHA/m-MHA)	3.78 (1.66)	3.89 (1.87)	1.86 (-)
p-cresol (m-MHA/p-cresol)	5.58 (4.44)	6.05 (5.80)	4.00 (7.27)
m-cresol (p-cresol/m-cresol)	6.93 (2.93)	7.52 (3.32)	4.63 (1.67)
o-cresol (m-cresol/o-cresol)	7.77 (1.57)	8.51 (1.96)	5.13 (1.21)

The abbreviations of ethanol, deionized water, hippuric acid and o-, m-, p-methylhippuric acid were expressed to EtOH, DW, HA and MHA, respectively. [a] Percent concentration ratios were used. All 3 kinds of eluents were added to 0.3% with acetic acid and 1.4% with β-CD respectively.

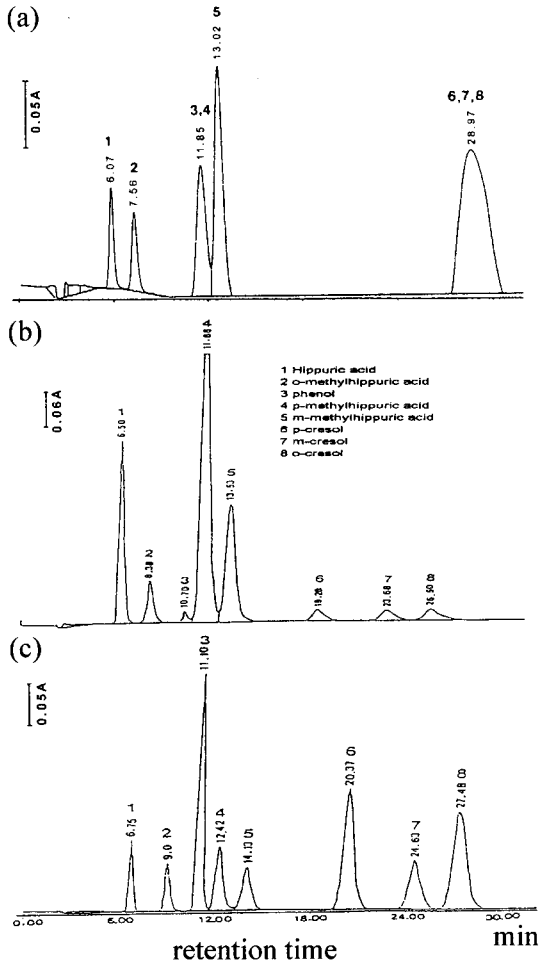

Figure 1. HPLC separation of urinary 8 metabolites (a) without β-CD, (b) with 1.0% of β-CD, and (c) with 1.4% of β-CD in ethanol/deionized water/acetic acid (20/80/0.3, v/v/v).

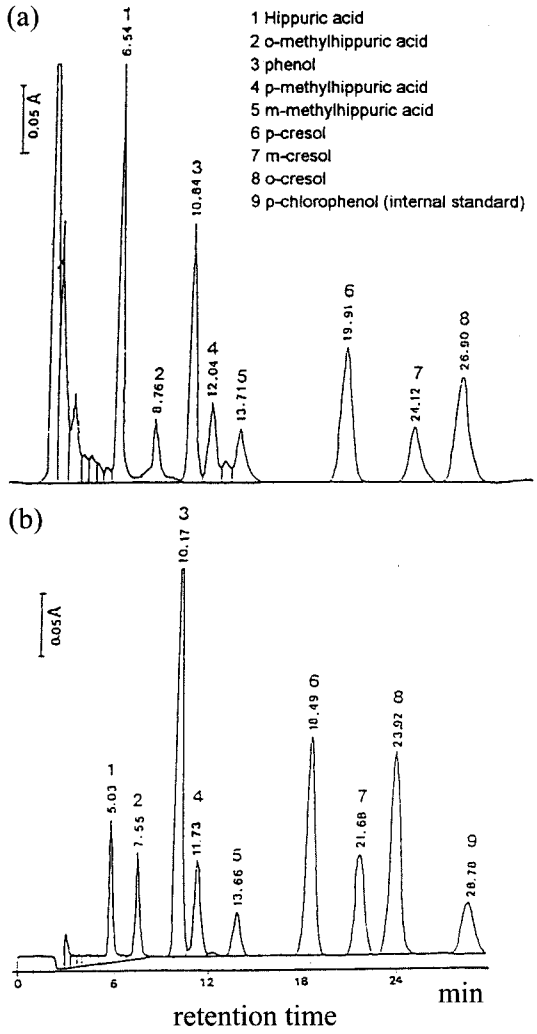

Figure 2. HPLC separation of untreated urine samples (a) added standard materials and (b) an internal standard as p-chlorophenol.

HA, 9.00 for o-MHA, 11.10 for phenol, 12.42 for p-MHA, 14.13 for m-MHA, 20.37 for p-cresol, 24.63 for m-cresol, 27.48 min for o-cresol.

Figure 2. shows the chromatograms recorded for the untreated urine samples by using (a) the standard materials and (b) p-chlorophenol as an internal standard. In case of using standard materials (Figure 2(a)), untreated urine sample was mixed with the mixture of standard materials in 1 : 4 ratio. Concentrations of the mixture of standard materials to detect eight metabolites were 0.19 for HA, 0.77 for o-MHA, 0.64 for phenol, 0.09 for p-MHA, 0.19 for m-MHA, 2.58 for m-cresol, and 1.29 mg/mL for o-cresol. The retention times were 6.54, 8.76, 10.84, 12.04, 13.71, 19.91, 24.12, and 26.90 min among the metabolites. Figure 2(b) illustrates a chromatogram for the mixture of eight metabolites of a urine sample and p-chlorophenol as an internal standard material. In this case, the retention time of the internal standard was 28.78 min.

Application to Exposure Monitoring of Workers. Arithmetic means and detection limits of urine samples of workers exposed to benzene, toluene and xylene in factories were shown in Table 3. The detection limits of all metabolites were 0.5 mg/L to 2.1 mg/L. HA as a main toluene metabolite was 850 mg/L in arithmetic mean among worker's urine samples. o-, m-, p-methylhippuric acid as main xylene metabolites were 20 to 31 mg/L. Phenol as a main benzene metabolite was 497 mg/L. m-cresol and p-cresol as minor metabolites of benzene and toluene were 505 and 5.89 mg/L, respectively. o-Cresol was below the detection limit (0.5 mg/L) in the value of arithmetic mean.

Table 3. Metabolites concentrations and detection limits of 20 worker's urine samples

Metabolites	AM±ASD	Detection limit
HA	850±231	0.6
o-MHA	31±29	0.6
phenol	497±104	0.5
p-MHA	20±11	1.0
m-MHA	25±10	2.1
p-cresol	5.89±3.21	0.5
m-cresol	505±423	0.5
o-cresol	ND	0.5

units: mg/L. ND expressed no detection. Hippuric acid and o-, m-, p-methylhippuric acid were abbreviated to HA and o-, m-, p-MHA, respectively.

Discussion

β-CD was applied to assay the eight urinary metabolites in the present study. The percent concentration of β-CD in a mobile phase was set up at 1.4% and a good separation was shown. This result may be ascribed due to the difference in the magnitude of the interaction between β-CD and the test molecules in the mobile phase. When the chromatogram was recorded for the separation of eight metabolites without β-CD or less than 0.7% of β-CD, they had shown an incomplete separation. HPLC column (RP-18) was often clogged during the metabolite separation due to a low solubility of β-CD even though it was filtered with the AP type. Retention times were hardly related directly to the amounts of β-CD added into the eluent.

In this study, the hydroxy groups of o-, m-, and p-cresol could be complexed into the cavity of β-CD, and the stability constants (k_G) of the complex was evaluated by plotting the linear relationships between k' and $(k'_G-k')/[CD]$. These were given as 0.12 ± 0.48, 0.47 ± 0.28, 1.36 ± 0.49. Adding over 1.0% β-CD made a good separation. This is highly like to the formation of an inclusion complex. Increase of the stability constants decreased the capacity factor to some extent in the presence of β-CD. As a result showed in the presence of β-CD, the retention times would be shorter than that without β-CD. In the detection of cresol, the eluting sequence of p-cresol, m-cresol, and o-cresol were confirmed to be equal in the magnitude of the stability constant reported by Zukowski *et al.*.[12] This sequence was coincident with our result. In the separation of MHAs, the benzene ring can be more easily formed the complex in a β-CD cavity than the bulky $CONHCH_2COOH$ group. The stability constants (K_G), evaluated from the above equation for o-, m-, p-MHA, were given as 3.4 ± 1.0, 8.4 ± 1.2, 10.3 ± 3.2, and the eluting sequence was expected to be the order of p-MHA, m-MHA, and o-MHA. However, the detection sequence was shown as the order of o-MHA, p-MHA, and m-MHA in this study. This result may be due to the differences in the interaction of positional isomers of MHAs with β-CD on the stationary phase. The difference may also be owed to the methyl group of o-MHA rather than that of p- and m-MHA. A hydrogen bonding or hyper-conjugation easily forms the inclusion complex with $CONHCH_2COOH$ group. The retention times of phenol and HA was much influenced by the extent of the adsorption on the stationary phase. The formation of the inclusion complex with β-CD in the mobile phase might be more closely related to the difference of the stability constant and the extent of the adsorption on a RP-18 system.

Conclusion

Analysis of the eight metabolites in urine with HPLC was carried out with changing the solvent ratios and β-CD concentration in the eluent. β-CD applied to assaying urinary metabolites derived from benzene, toluene and xylene exposure. Optimum eluent composition for HPLC assaying the eight urinary metabolites was determined to be 20/80/0.3/1.4% (ethanol/water/acetic acid/β-CD).

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