Rapid HPLC Method for the Simultaneous Determination of Eight Urinary Metabolites of Toluene, Xylene and Styrene

Cheol-Woo Lee,[a] Jeongmi Lee,[†,a] Jaehyun Lee, Han Young Eom, Min Kyung Kim, Joon Hyuk Suh, Hyesun Yeom, Unyong Kim, Jeong-Rok Youm, and Sang Beom Han[*]

Department of Pharmaceutical Analysis, College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea
[*]E-mail: hansb@cau.ac.kr
[†]Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9041, USA
Received May 15, 2009, Accepted July 27, 2009

Toluene, xylene and styrene are volatile organic solvents that are commonly used in mixtures in many industries. Because these solvents are metabolized and then excreted in urine, their urinary metabolites are thought to be biomarkers of occupational exposure to these solvents. Therefore, a simple, rapid, and yet reliable analytical method for determining the metabolites is required for accurate biological monitoring. In the present study, a simple and rapid HPLC-UV method was developed for the simultaneous determination of eight major metabolites of toluene, xylene and styrene: hippuric acid (HA), mandelic acid (MA), *o*-, *m*- and *p*-methylhippuric acids (*o*-, *m*- and *p*-MHAs), and *o*-, *m*- and *p*-cresols. A monolithic column was employed as the stationary phase and several conditions, including flow rate, composition of mobile phase and column temperature, were variables for the optimization of the chromatographic resolution. All eight metabolites were successfully resolved within 5 minutes in 10% aqueous ethanol containing 0.3% acetic acid and 1.6% β-cyclodextrin, using a flow rate gradient of 1.0 - 5.0 mL/min at 25 °C. The performance of this method was validated by linearity, intra- and inter-day accuracy, and precision. The linearity was observed with correlation coefficients of 0.9998 for HA, 0.9999 for MA, 0.9989 for *o*-MHA, 0.9998 for *m*-MHA, 0.9991 for *p*-MHA, 0.9997 for *o*-cresol, 0.9998 for *m*-cresol, and 0.9986 for *p*-cresol. The intra- and inter-day precision of the method were less than 5.89% (CV) and the accuracy ranged from 92.95 to 106.62%. The validity was further confirmed by analysis of reference samples that were prepared by the inter-laboratory quality assurance program of the Korea Occupational Safety and Health Agency (KOSHA, Seoul, Korea). All measured concentrations of the analytes agreed with the certified values.

Key Words: Urinary metabolites, HPLC-UV method, Monolithic column, β-Cyclodextrin, Flow rate gradient

Introduction

Toluene, xylene and styrene are commonly used separately or in mixtures in the paint, oil refining, and plastics industries.[1] Occupational exposure to these organic solvents is known to have adverse effects on workers' health, producing abnormalities in the central nervous system and development.[2] Furthermore, its association with cancer development has previously been suggested.[3] Therefore, periodic monitoring of the work environment together with biological monitoring is necessary to protect workers from the hazardous effects of these solvents.[4] The majority of these solvents are metabolized in the microsomes of the liver and excreted in urine after absorption; thus the metabolite levels in urine reflect the absorbed dose of the solvents in humans.[5] For this reason, the internal exposure levels to these solvents are often estimated by measuring the levels of their metabolites in urine.

Toluene is either metabolized via oxidization and conjugation to hippuric acid or metabolized to *o*-, *m*-, or *p*-cresol by hydroxylation (Figure 1). In the same fashion, xylene, which exists as three isomers, *o*-, *m*-, or *p*-, is excreted in the urine as the corresponding *o*-, *m*-, or *p*-methyl hippuric acid (*o*, *m*, *p*-MHA).

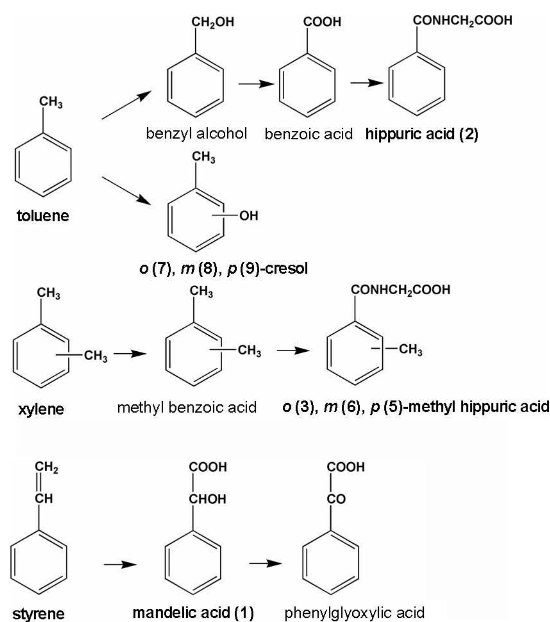

Figure 1. Urinary metabolites of toluene, xylene and styrene. The compounds that were analyzed in this study are in bold and numbered in parentheses and their numbers correspond with those found in the chromatograms.

[a]These authors contributed equally to this work

Styrene is mostly oxidized to mandelic acid (MA) and partially metabolized to phenylglyoxylic acid (PGA).[6] Since toluene, xylene and styrene are often used in combination in many industries, workers may be co-exposed to the solvents, and therefore comprehensive determination of the individual metabolites is desired for accurate estimation of the internal exposure to these solvents.

The urinary metabolites of toluene, xylene and styrene described above have previously been analyzed by various methods, including high-performance liquid chromatography (HPLC) with UV detection[7-12] and gas chromatography (GC) with flame ionization detection[13-15] and mass spectrometry (MS).[16-21] Although GC methods show good resolution for most of the metabolites, with a relatively short run time (usually less than 15 min), these methods involve time-consuming procedures for sample extraction and derivatization of the metabolites. HPLC-UV methods are more commonly utilized because of the simple sample preparation, which excludes extraction or derivatization. These methods, however, are not preferable for the analysis of a large number of samples due to the long analysis time (usually 20 ~ 50 min). Also, the chromatographic resolution of isomeric metabolites, such as *o, m, p*-MHAs and *o, m, p*-cresols, is usually not as good as that by GC. Therefore, it is of great importance to develop a simple and rapid method for the simultaneous determination of the prevalent metabolites that can be routinely used for efficient biological monitoring of a large number of exposed workers.

Previously, we developed a rapid HPLC-UV method using a monolithic column, by which HA, MA, PGA and *o*-MHA were simultaneously analyzed within 2 min; however, *m*- and *p*-MHAs were not completely resolved by this method.[22] In the present study, we further investigated the HPLC method using a monolithic column in order to develop a better method by which more metabolites could be analyzed with better resolution in a short analysis time. Eight major metabolites of toluene, xylene and styrene, *i.e.*, HA, *o, m, p*-cresols, *o, m, p*-MHAs and MA, were targeted for analysis in this study (Figure 1).

This is the first report on the simultaneous determination of eight metabolites within five minutes using HPLC-UV analysis. We also confirmed the validity of the developed method by assessing the metabolites in quality assurance urine samples[23] spiked with known amounts of metabolites.

Experimental

Materials and instruments. Hippuric acid (98%), mandelic acid (99%), *o*-methylhippuric acid (98%), *m*-methylhippuric acid (98%), *p*-methylhippuric acid (98%), *o*-cresol (99%), *m*-cresol (99%), *p*-cresol (99%) and β-cyclodextrin (98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *p*-Nitrophenol (99%) was obtained from Kanto Chemical (Tokyo, Japan) and acetonitrile, methanol, ethanol and acetic acid were from Merck (Darmstadt, Germany). All solvents were of analytical grade or HPLC grade. Urine specimens for quality assurance were provided by Industrial Safety and Health Research Institute, Korea Occupational Safety and Health Agency (KOSHA, Seoul, Korea), and stored at −80 °C until analysis.

For HPLC chromatography, a Hitachi HPLC system (Hitachi High-technologies Corp., Tokyo, Japan), comprising a L-2130 low-pressure pump, a L-2200 autosampler, a L-2300 column oven and a L-2400 UV detector, was used. Analytes were chromatographed on an Onyx monolithic RP-18 (4.6 × 100 mm, Phenomenex Inc., Torrance, CA, USA), a Luna phenyl hexyl (4.5 × 150 mm, 5 μm, Phenomenex Inc.) or a Hypersil GOLD (4.5 × 150 mm, 5 μm, Thermo Electron Corp., Waltham, MA, USA) column with a SecurityGuard column (Phenomenex Inc.). The data were obtained with the software, EZChrome Elite (Ver. 3.1.3).

Preparation of standard solutions and calibration curves. Eight stock solutions of the standards (HA, MA, *o*-, *m*-, *p*-MHA, *o*-, *m*-, *p*-cresol) and a stock solution of the internal standard (*p*-nitrophenol) were prepared by dissolving 5 mg of each compound in 5 mL of deionized water; these solutions were stored at −20 °C until further use. A portion of internal standard stock solution was diluted with deionized water to a concentration of 100 μg/mL to make the internal standard working solution. Each stock solutions of the standards were diluted in deionized water to produce a series of standard working solutions. The calibration standards were prepared by mixing 475 μL of each standard working solution with 25 μL of internal standard working solution to produce a series of standards of various concentrations ranging from 0.8 μg/mL to 12.8 μg/mL (0.8, 1.6, 3.2, 6.4, and 12.8 μg/mL) for MA and *o*-, *m*-, and *p*-cresol; 0.4 μg/mL ~ 6.4 μg/mL (0.4, 0.8, 1.6, 3.2, and 6.4 μg/mL) for *o*-, *m*-, and *p*-MHA; and 2.0 μg/mL ~ 32.0 μg/mL (2.0, 4.0, 8.0, 16.0 and 32.0 μg/mL) for HA. Plotting the peak area ratios of the standard compound to the internal standard *vs.* the analyte concentration generated calibration curves that were used for the analysis.

Intra- and inter-day accuracy and precision and limit of quantification. The intra- and inter-day accuracies were investigated at the concentrations used to prepare calibration curves and were expressed as observed concentration relative to nominal concentration. The intra- and inter-day precision of the eight standards were examined at the concentrations within the linear range of the calibration curves and expressed in relative standard deviation (coefficient of variation, CV). The limits of quantification were the concentrations at the signal-to-noise ratio of 10 with precision of < 20% and accuracy between 80 ~ 120%.[24]

Quality assurance samples. The quality assurance urine samples provided by Industrial Safety and Health Research Institute, Korea Occupational Safety and Health Agency, were thawed at room temperature and a 475 μL sample spiked with 25 μL of internal standard working solution (100 μg/mL in deionized water) was used for analysis after vortexing. The sample was then diluted 20-fold in deionized water. After centrifugation at 4000 rpm for 5 min, the supernatant was filtered through a 0.45-μm membrane filter, and 20 μL of filtrate was injected for HPLC analysis.

Results

Optimization of liquid chromatographic separation of eight metabolites. The HPLC conditions were optimized for the rapid

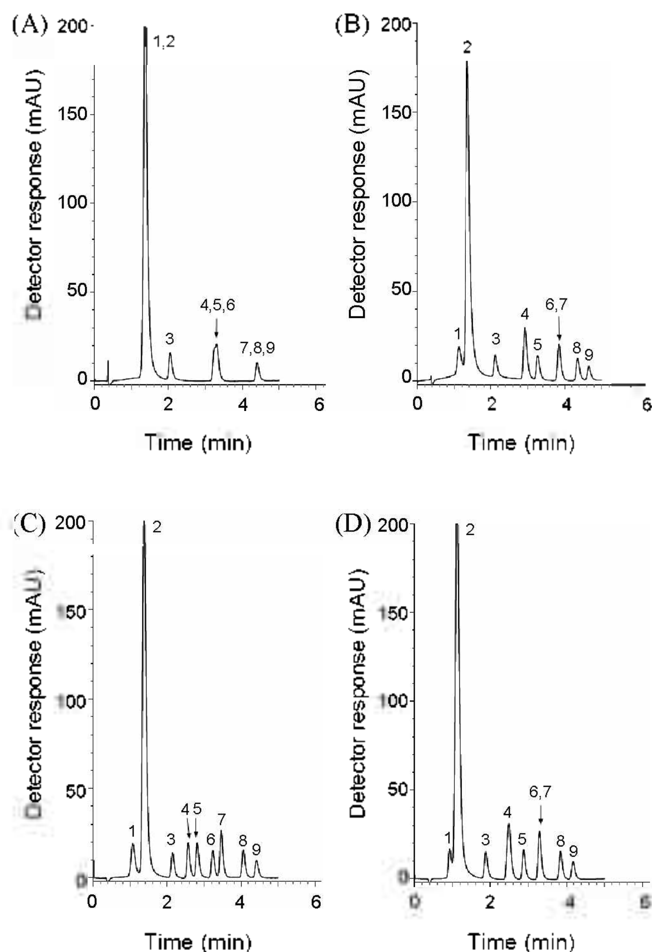

Figure 2. HPLC chromatograms of eight standard compounds. Chromatographic resolution depended on the concentration of β-cyclodextrin in the mobile phase. The composition of mobile phase was fixed at 10% ethanol, 0.3% acetic acid and 90% water while different concentrations β-cyclodextrin [0% in (A), 1.1% in (B), 1.6% in (C) and 2.0% in (D)] were added to the water. (Detection wavelength: 225 nm, column temperature: 25 °C, flow gradient: 5 mL/min for the initial 1 min, 5 → 1 mL/min over the next 4 min, compound numbering: 1. MA, 2. HA, 3. *o*-MHA, 4. *p*-Nitrophenol (I.S.), 5. *p*-MHA, 6. *m*-MHA, 7. *p*-Cresol, 8. *m*-Cresol, 9. *o*-Cresol).

and simultaneous analysis of eight metabolites (HA, *o*, *m*, *p*-cresols, *o*, *m*, *p*-MHAs and MA) in a monolithic silica column as follows: Analytes were detected at 225 nm in all chromatography procedures as is routine for analysis of urinary metabolites of toluene, xylene and styrene.[9,25,26] Several different mobile phases of varied compositions were examined for efficient isocratic elution. Ten percent ethanol produced the best chromatographic resolution and sensitivity among the tested solvents, which included acetonitrile, ethanol and methanol as the mobile phase in the presence of 0.3% acetic acid (data not shown). At each selected eluent condition, we varied the flow rate from 1 mL/min to 5 mL/min under a flow rate gradient in order to minimize the elution time while retaining the chromatographic resolution of the analytes. The chromatographic resolution was dependent on the concentration of β-cyclodextrin in the mobile phase (Figure 2). Complete separation of all eight compounds was observed when the

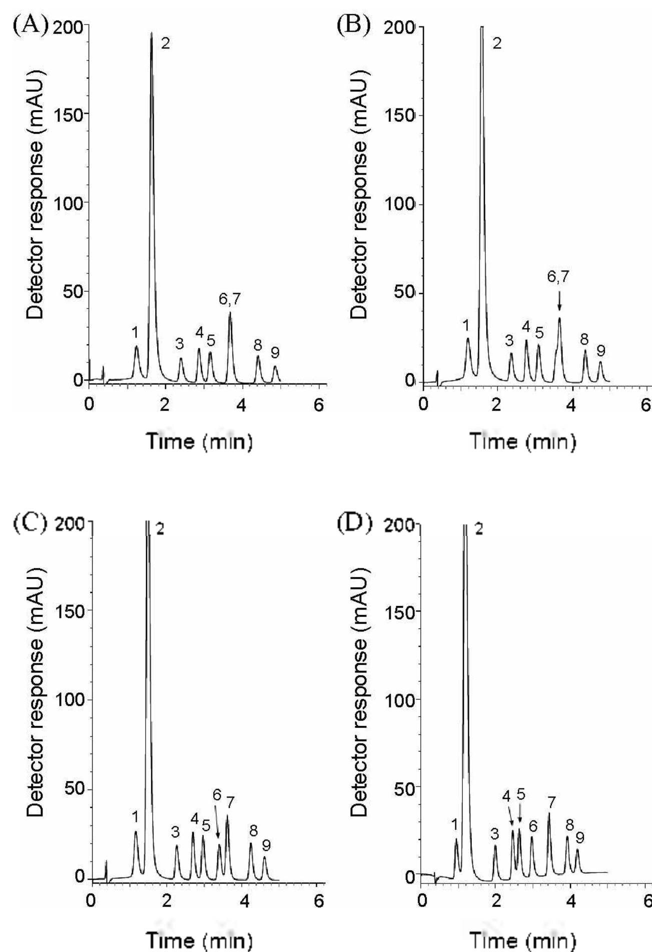

Figure 3. Temperature-dependence of chromatographic resolution on an Onyx monolithic column. Eight analytes were separated using a mobile phase composed of ethanol (10%), acetic acid (0.3%) and 1.6% β-cyclodextrin in water (90%) at 15 °C (A), 20 °C (B), 25 °C (C) and 30 °C (D). [1. MA, 2. HA, 3. *o*-MHA, 4. *p*-Nitrophenol (I.S.), 5. *p*-MHA, 6. *m*-MHA, 7. *p*-Cresol, 8. *m*-Cresol, 9. *o*-Cresol]. For the flow gradient, see Table 1.

Table 1. Optimized conditions for the HPLC-UV analysis of eight metabolites

Column	Onyx monolithic RP-18 (4.6 × 100 mm)
Column temperature	25 °C
UV detection wavelength	225 nm
Mobile phase	Ethanol/Acetic acid/1.6% β-cyclodextrin in water = 10/0.3/90
Flow rate gradient	0 min → 1 min: 5 mL/min 1 min → 5 min: 5 → 1 mL/min

analytes were eluted in 10% ethanol, 0.3% acetic acid and 90% water containing 1.6% β-cyclodextrin, using a flow rate gradient of 5 mL/min for the initial 1 min, which was decreased to 1 mL/min over the following 4 min (Table 1).

We also examined the effect of column temperature on the chromatographic performance. A column temperature of 25 °C yielded the best resolution among the tested temperatures from 15 °C to 30 °C (Figure 3). In the final optimized chro-

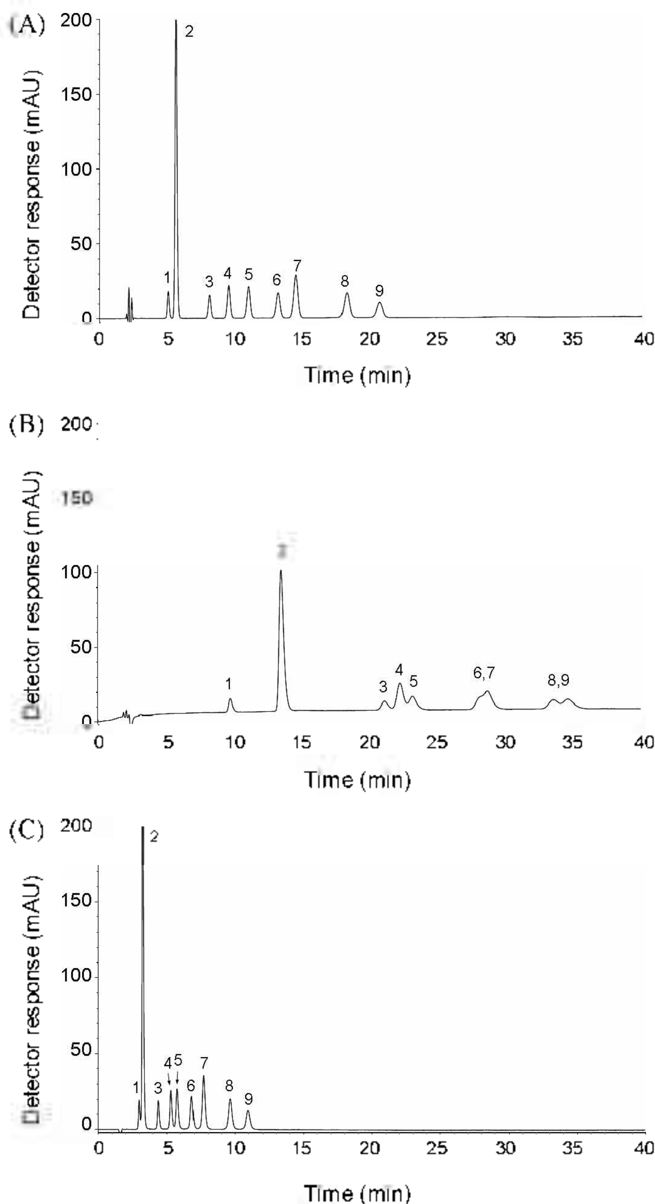

Figure 4. HPLC chromatograms of eight standards chromatographically resolved on a Hypersil GOLD column (A), a Luna phenyl hexyl column (B) and an Onyx monolithic column (C) in ethanol/acetic acid/1.6% β-cyclodextrin in water (10/0.3/90) at 25 °C column temperature at a flow rate of 1 mL/min. Analytes were detected at 225 nm. [1. MA, 2. HA, 3. *o*-MHA, 4. *p*-Nitrophenol (I.S.), 5. *p*-MHA, 6. *m*-MHA, 7. *p*-Cresol, 8. *m*-Cresol, 9. *o*-Cresol].

matographic conditions, displayed in Table 1, all eight metabolites were completely resolved within less than 5 min (Figure 5A).

We compared the chromatographic performance of the Onyx monolithic column with that of two conventional HPLC columns that were packed with particles (Hypersil GOLD, Luna phenyl hexyl) (Figure 4). The flow rate gradient that was used for the monolithic column was not applicable to the two particulate columns because of the high back-pressure involved. Therefore the analysis was performed using the optimized conditions described above except that the flow rate was fixed at 1 mL/min for all three columns. The Hypersil column

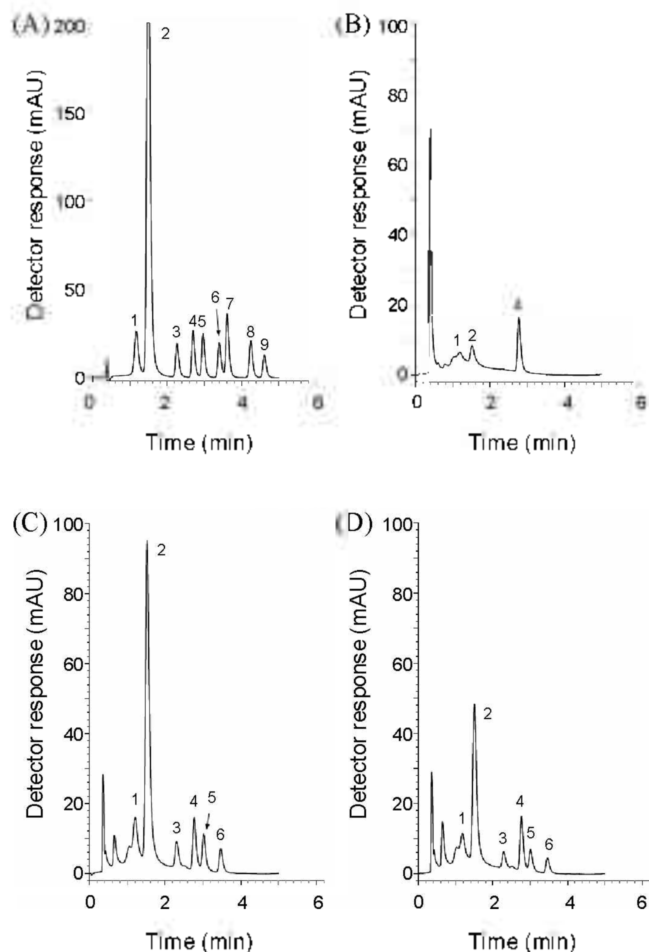

Figure 5. Simultaneous analysis of eight metabolites in urine specimens with known concentrations of metabolites prepared for quality assurance. HPLC chromatograms of the standards (A), a urine sample from an unexposed healthy individual (B), urine samples containing high (C) or low (D) concentrations of metabolites of toluene, xylene and styrene. For chromatographic conditions, see Table 1. [1. MA, 2. HA, 3. *o*-MHA, 4. *p*-Nitrophenol (I.S.), 5. *p*-MHA, 6. *m*-MHA, 7. *p*-Cresol, 8. *m*-Cresol, 9. *o*-Cresol].

successfully resolved all eight compounds but required about 22 min (Figure 4A). The phenyl hexyl column required almost 40 min for the analysis with incomplete separation of the compounds (Figure 4B). In the monolithic column, however, total analysis with complete resolution of the eight compounds was accomplished in less than 12 min (Figure 4C).

Validation of the developed analytical method: linearity, intra- and inter-day accuracy and precision and limit of quantification. The HPLC method in the present study was validated by assessing the linearity, intra- and inter-day accuracy, precision and limit of quantification. Linearity was observed for all eight standard compounds within the tested concentration ranges, as represented by their high correlation coefficients (r^2) of 0.9998 for HA, 0.9999 for MA, 0.9989 for *o*-MHA, 0.9998 for *m*-MHA, 0.9991 for *p*-MHA, 0.9997 for *o*-cresol, 0.9998 for *m*-cresol, and 0.9986 for *p*-cresol (Table 2).

The intra- and inter-day accuracy and intra- and inter-day precision were assessed from repeated experiments (n = 5) at five different concentrations of the eight standards. These are

Table 2. Calibration curve parameters for the analysis of eight metabolites

Compounds	Linear Range (μg/mL)	Regression Equation	Correlation Coefficient (r^2)
HA	2.0 ~ 32.0	y = 0.3798x + 0.0834	0.9998
MA	0.8 ~ 12.8	y = 0.0870x + 0.0151	0.9999
o-MHA	0.4 ~ 6.4	y = 0.1285x − 0.0183	0.9989
m-MHA	0.4 ~ 6.4	y = 0.1207x − 0.0115	0.9998
p-MHA	0.4 ~ 6.4	y = 0.1640x − 0.0179	0.9991
o-Cresol	0.8 ~ 12.8	y = 0.0458x − 0.0070	0.9997
m-Cresol	0.8 ~ 12.8	y = 0.0739x − 0.0093	0.9998
p-Cresol	0.8 ~ 12.8	y = 0.1333x − 0.0319	0.9986

Table 3. Intra- and inter-day precision and accuracy of the developed HPLC-UV method (n = 5)

Compounds	Concentrations (μg/mL)	Precision (CV, %)		Accuracy (%)	
		Intra-day	Inter-day	Intra-day	Inter-day
HA	2	0.96	1.57	103.59	103.50
	4	0.67	1.44	101.22	101.77
	8	0.42	0.88	100.79	100.64
	16	0.75	0.22	100.02	100.36
	32	1.06	0.41	100.51	100.48
MA	0.8	3.27	1.24	101.65	101.68
	1.6	0.90	0.42	99.99	100.90
	3.2	1.23	0.74	101.12	100.54
	6.4	0.93	1.09	99.98	100.76
	12.8	0.55	0.32	99.95	99.85
o-MHA	0.4	1.61	3.49	106.48	106.13
	0.8	3.85	4.05	103.39	102.25
	1.6	2.76	1.72	101.81	100.74
	3.2	0.85	1.54	100.34	101.29
	6.4	0.36	0.95	100.44	99.69
m-MHA	0.4	2.75	3.50	94.91	102.75
	0.8	4.91	3.76	97.68	100.55
	1.6	4.29	0.99	99.25	101.24
	3.2	2.89	2.07	98.61	99.93
	6.4	2.42	1.60	98.82	100.58
p-MHA	0.4	5.70	5.89	98.22	93.94
	0.8	4.00	2.71	95.15	92.95
	1.6	2.63	3.97	95.51	99.09
	3.2	0.92	1.87	98.42	99.72
	6.4	0.83	1.20	100.51	100.79
o-Cresol	0.8	3.75	3.16	100.03	101.49
	1.6	1.59	1.67	100.53	101.66
	3.2	1.01	0.26	100.55	100.42
	6.4	1.18	1.13	99.58	100.68
	12.8	0.90	0.37	99.74	99.89
m-Cresol	0.8	0.65	1.26	101.53	101.23
	1.6	1.62	3.38	102.52	101.55
	3.2	0.92	1.18	100.43	100.69
	6.4	0.70	0.68	100.14	100.57
	12.8	0.36	0.37	100.18	100.17
p-Cresol	0.8	2.24	3.97	106.62	103.05
	1.6	1.93	1.96	105.68	103.20
	3.2	1.83	1.84	101.91	100.86
	6.4	3.57	1.45	102.26	99.86
	12.8	3.51	0.76	104.12	100.32

Table 4. Determination of the metabolites in a urine sample from a healthy unexposed individual (A) and in urine samples spiked with high (B) or low (C) concentrations of metabolites. Total MHA indicates the combined amount of *o*-, *m*- and *p*-MHAs

Sample	Analyte	Measured Conc. (g/L)	Reference Value (g/L)	Tolerance Range (g/L)
A	HA	< LOQ	N.A.[a]	N.A.
	MA	< LOQ	N.A.	N.A.
	Total MHA	< LOQ	N.A.	N.A.
B	HA	1.77	1.75	1.57 - 1.93
	MA	0.87	0.94	0.85 - 1.03
	Total MHA	1.24	1.21	1.06 - 1.36
C	HA	0.87	0.85	0.67 - 1.03
	MA	0.53	0.55	0.46 - 0.64
	Total MHA	0.75	0.74	0.62 - 0.86

[a]: not applicable.

summarized in Table 3. The intra-day accuracy was 94.91 ~ 106.62% and the inter-day accuracy was 92.95 ~ 106.13%. The intra-day precision was 0.36 ~ 5.70% and the inter-day precision was 0.22 ~ 5.89%. The limit of quantification was 2 μg/mL for HA, 0.8 μg/mL for MA, 0.4 μg/mL for *o*-, *m*- and *p*-MHAs, and 0.8 μg/mL for *o*-, *m*- and *p*-cresols (Table 2).

Confirmation of the validity of the developed method. The method was applied to the determination of the metabolites of toluene, xylene and styrene in human urine samples in order to evaluate its validity. Three different specimens prepared by the inter-laboratory quality assurance program from Korea Occupational Safety and Health Agency (KOSHA, Seoul, Korea) were analyzed: one specimen was from a non-exposed healthy individual and the other two contained known amounts of HA, MA and MHAs at high or low concentrations. As shown by the chromatograms of the three samples in Figure 5, we successfully detected the individual metabolites in the two urine samples containing high or low concentrations of the metabolites, but detected no significant levels of any metabolites in the sample from a non-exposed healthy individual. Because the reference values for MHAs were given as a mixture of three structural isomers (*o*-, *m*- and *p*- MHAs), we combined the measured values of the individual isomers of MHA to compare the numbers to the reference. Compared to the reference values given by the specimen provider (KOSHA), all of our measured values for HA, MA and MHAs fell within the tolerance range (Table 4).

Discussion

Many industrial workers are likely to be co-exposed to toluene, xylene and styrene because these solvents are often used in combination in a variety of industries. For effective biological monitoring of their occupational exposure, an accurate and comprehensive evaluation of the individual metabolites of the solvents is preferable and a simultaneous determination of as many metabolites as possible in a single, short run is ideal for automated analysis of a large number of samples. For

this purpose, we developed a time- and cost-effective method based on the HPLC-UV system.

Monolithic columns, which are made of single piece of porous silica, are increasingly used in the analyses of drugs and metabolites because they allow higher flow rates and therefore faster separations than particulate columns.[27-30] In the present study, we compared the metabolite analysis using the Onyx monolithic column with two particulate columns under the same chromatographic conditions using a flow rate of 1 mL/min. The monolithic column was superior to the other two columns, providing a much faster elution pattern with a fair chromatographic resolution (Figure 4). When an eluent flow rate gradient[31,32], which cannot be performed in conventional packed columns because of high back-pressure, was applied to the monolithic column, the total analysis time was markedly reduced without compromising the resolution.

β-Cyclodextrin, a cyclic oligosaccharide composed of seven glucose molecules, is occasionally used as a mobile phase additive for separation of structural isomers.[33-35] We have shown, for the first time, that the combined use of the monolithic column, eluent flow rate gradient and β-cyclodextrin in the mobile phase allowed for the simultaneous determination of eight metabolites within less than 5 min. Optimized column temperature also further improved the chromatographic performance by increasing the sensitivity and chromatographic resolution.

The validity of the developed method was confirmed when we analyzed human urine samples that had been prepared by KOSHA for quality assurance purposes.

The relatively long analysis time that has been the biggest pitfall for HPLC methods was overcome through the use of a monolithic column, and the use of a flow rate gradient instead of an eluent gradient could save a huge amount of time when many samples are analyzed because the eluent will not need to be equilibrated after each run. On the whole, our newly-developed method offers a very efficient analysis of the metabolites of toluene, xylene and styrene in a time-, cost- and performance-effective manner.

Acknowledgments. This research was supported by the Chung-Ang University Research Scholarship Grant in 2007.

References

1. Seedorff, L.; Olsen, E. *Ann. Occup. Hyg.* **1990**, *34*, 371.
2. Chen, Z.; Liu, S. J.; Cai, S. X.; Yao, Y. M.; Yin, H.; Ukai, H.; Uchida, Y.; Nakatsuka, H.; Watanabe, T.; Ikeda, M. *Occup. Environ. Med.* **1994**, *51*, 47.
3. Persson, B.; Fredriksson, M.; Olsen, K.; Boeryd, B.; Axelson, O. *Cancer* **1993**, *72*, 1773.
4. Heinrich-Ramm, R.; Jakubowski, M.; Heinzow, B.; Christensen, J. M.; Olsen, E.; Hertel, O. *Pure Appl. Chem.* **2000**, *72*, 385.
5. Imbriani, M.; Ghittori, S. *Int. Arch. Occup. Environ. Health* **2005**, *78*, 1.
6. Hee, S. Q. *Biological Monitoring; an Introduction*; Van Nostrand Reinhold: New York, U. S. A., 1993; p 471-478.
7. Inoue, O.; Seiji, K.; Suzuki, T.; Watanabe, T.; Nakatsuka, H.; Satoh, H.; Ikeda, M. *Bull. Environ. Contam. Toxicol.* **1991**, *47*, 204.
8. Astier, A. *J. Chromatogr.* **1993**, *643*, 389.
9. Fujii, T.; Kawabe, S.; Horike, T.; Taguchi, T.; Ogata, M. *J. Chromatogr. B* **1999**, *730*, 41.
10. Yang, Y. D. *Biomed. Chromatogr.* **1998**, *12*, 47.
11. Chua, S. C.; Lee, B. L.; Liau, L. S.; Ong, C. N. *J. Anal. Toxicol.* **1993**, *17*, 129.
12. Hansen, S. H.; Dossing, M. *J. Chromatogr.* **1982**, *229*, 141.
13. Chakroun, R.; Faidi, F.; Hedhili, A.; Charbaji, K.; Nouaigui, H.; Laiba, M. B. *J. Forensic. Sci.* **2008**, *53*, 232.
14. Kongtip, P.; Vararussami, J.; Pruktharathikul, V. *J. Chromatogr. B* **2001**, *751*, 199.
15. Kataoka, H.; Manabe, K.; Nakase, S.; Makita, M. *J. Pharm. Biomed. Anal.* **1991**, *9*, 699.
16. Pacenti, M.; Dugheri, S.; Villanelli, F.; Bartolucci, G.; Calamai, L.; Boccalon, P.; Arcangeli, G.; Vecchione, F.; Alessi, P.; Kikic, I.; Cupelli, V. *Biomed. Chromatogr.* **2008**, *22*, 1155.
17. Jiye, A.; Huang, Q.; Wang, G.; Zha, W.; Yan, B.; Ren, H.; Gu, S.; Zhang, Y.; Zhang, Q.; Shao, F.; Sheng, L. *J. Anal. Biochem.* **2008**, *379*, 20.
18. Marais, A. A.; Laurens, J. B. *J. Sep. Sci.* **2005**, *28*, 2526.
19. Szucs, S.; Tóth, L.; Legoza, J.; Sárváry, A.; Adány, R. *Arch. Toxicol.* **2002**, *76*, 560.
20. Saito, T.; Takeichi, S. *J. Pharm. Biomed. Anal.* **2002**, *30*, 365.
21. Laurens, J. B.; Mbianda, X. Y.; Spies, J. H.; Ubbink, J. B.; Vermaak, W. J. *J. Chromatogr. B* **2002**, *774*, 173.
22. Han, S. B.; Lee, S. J.; Lee, C. W.; Yoon, S. H.; Joung, S. K.; Youm, J. R. *Anal. Sci. & Tech.* **2006**, *19*, 433.
23. Yang, J. S.; Lee, M. Y.; Park, I. J.; Moon, Y. H.; Kang, S. K. *Int. Arch. Occup. Environ. Health* **1997**, *69*, 361.
24. International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use; Validation of analytical procedures; ICH-Q2A: Geneva, 1995.
25. Ogata, M.; Taguchi, T. *Int. Arch. Occup. Environ. Health* **1986**, *58*, 121.
26. Poggi, G.; Giusiani, M.; Palagi, U.; Paggiaro, P. L.; Loi, A. M.; Dazzi, F.; Siclari, C.; Baschieri, L. *Int. Arch. Occup. Environ. Health* **1982**, *50*, 25.
27. Arapitsas, P.; Turner, C. *Talanta* **2008**, *74*, 1218.
28. Liu, J.; Sun, J.; Zhang, W.; Gao, K.; He, Z. *J. Pharm. Biomed. Anal.* **2008**, *46*, 405.
29. Adcock, J. L.; Francis, P. S.; Agg, K. M.; Marshall, G. D.; Barnett, N. W. *Anal. Chim. Acta* **2007**, *600*, 136.
30. Lubda, D.; Cabrera, K.; Nakanishi, K.; Minakuchi, H. *J. Sol-Gel Sci. Tech.* **2002**, *23*, 185.
31. Tzanavaras, P. D.; Themelis, D. G. *J. Pharm. Biomed. Anal.* **2007**, *43*, 1526.
32. Deeb, S. E.; Schepers, U.; Wätzig, H. *J. Sep. Sci.* **2006**, *29*, 1571.
33. de Melo, N. F.; Grillo, R.; Rosa, A. H.; Fraceto, L. F. *J. Pharm. Biomed. Anal.* **2008**, *47*, 865.
34. Xiao, K. P.; Xiong, Y.; Rustum, A. M. *J. Chromatogr. Sci.* **2008**, *46*, 15.
35. Chen, D.; Jiang, S.; Chen, Y.; Hu, Y. *J. Pharm. Biomed. Anal.* **2004**, *34*, 239.