HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR DETERMINATION OF URINARY METABOLITES OF TOLUENE, XYLENE AND STYRENE AND ITS APPLICATION

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Abstract. The purpose of this research was to determine mandelic, phenylglyoxylic, hippuric, o-, m- and p-methylhippuric acids, the six urinary metabolites of styrene, toluene and xylene by high performance liquid chromatography (HPLC). These metabolites were extracted in an acid medium, transferred into a basic solution and back extracted again using ethyl acetate, and the organic phase was evaporated to dryness under a compressed air flow at room temperature. The residue obtained was dissolved in 1 ml mobile phase solution of 0.01 M potassium orthophosphate in 0.3% acetic acid (adjusted to a pH of 2.5 with orthophosphoric acid):tetrahydrofuran:acetonitrile(87:5:8) and 100 μl was injected into a HPLC equipped with a 4.6 x 250 mm ODS3-C18 reversed phased column and ultraviolet (UV) detector at a wavelength of 254 nm. All metabolites were clearly separated within 21 minutes. The detection limits of the method were 1.1 ng/ml for PGA, 4.9 ng/ml for HA, 17.0 ng/ml for MA, 2.5 ng/ml for o-MHA, 1.7 ng/ml for p-MHA and 2.0 ng/ml for m-MHA. The percent recoveries of the six metabolites were 99.2-101.8% with percent coefficients of variation of less than 2%. The method was applied to the analysis of urine samples of twelve workers exposed to toluene, xylene and styrene in a paint factory. The 5-day post-shift urinary excretions of the six metabolites in these workers are presented. The metabolites were found at levels greater than the Biological Exposure Index (BEI) recommended by the American Conference of Governmental Industrial Hygienists (ACGIH).

INTRODUCTION

The organic solvents, toluene, xylene and styrene, are widely used in paint, varnishes, lacquers and polymer industrials. The main urinary metabolites of styrene are phenylglyoxylic acid (PGA) and mandelic acid (MA). The main urinary metabolites of o-, m-, p-xylenes are o-, m-, p-methylhippuric acids (o-, m-, p-MHAs), respectively, while for toluene the main metabolite is hippuric acid (HA). These metabolites have long been used for biological monitoring of these solvents (Yasuhiro *et al*, 2006). There are several methods used for analyzing urinary MA, PGA, HA and o-, m- and p-MHAs, including colorimetry (Ohtsuji and Ikeda, 1970), fluorometry (Chakrabarti, 1979), spectrophotometry (Pagnotto and Lieberman, 1967), gas chromatography (Flek and Sedivec, 1980; Kongtip *et al*, 2001; Lanchote *et al*, 2002) and high performance liquid chromatography (Masana and Reiko, 1978; Masana and Toyohiro, 1986; Deog *et al*, 1997; Laffon *et al*, 2001).

The gas chromatography (GC) method has been used for determination of MA, PGA, HA, o-, m- and p-MHAs, but this method requires

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a derivatization procedure using carcinogenic and toxic chemicals (Flek and Sedivec, 1980). However, separation of m-and p-MHAs could not be accomplished without the use of a DB-1 capillary column (Kongtip *et al*, 2001). Recently, a sensitive and selective GC-MS method reported complete separation of all of these metabolites (Yasuhiro *et al*, 2006).

High performance liquid chromatography (HPLC) has been widely used to determine some of these metabolites in urine without the derivatization process. Some have used solvent extraction for sample pretreatment (Masana and Reiko, 1978), others diluted urine samples and injected them directly (Masana and Toyohiro, 1986). The separation of m- and p-MHAs has been a difficult problem. The addition of β-cyclodextrin (Deog *et al*, 1997) and tetra-n-butylammonium bromide (Masana and Toyohiro, 1986) during the mobile phase helps separate the peaks of m- and p- MHAs.

The purpose of this study was to determine the six urinary metabolites of toluene, xylene and styrene by HPLC. Tetrahydrofuran was added to improve the separation of m- and p-MHAs. A back extraction was used for sample pretreatment and an Inertsil ODS3-C18 column provided complete separation of MA, PGA, HA, o-, m- and p-MHAs. The method was applied to analyze these six metabolites in 5-days post-shift urine samples from twelve workers in a paint factory.

MATERIALS AND METHODS

Chemicals

Phenylglyoxylic acid (98%), hippuric acid (99%) and mandelic acid (99%) were obtained from Fluka (Switzerland). O-, m- and p-methylhippuric acids (98%) and p-hydroxybenzoic acid (99%) were purchased from Sigma-Aldich (Germany) and p-HBA was used as an internal standard (IS). Potassium dihydrogen orthophosphate (KH_2PO_4), orthophosphoric acid, acetic acid, methanol, acetonitile and tetrahydrofuran (THF) were obtained from Merck (Germany). Ethyl acetate, sodium hydroxide (NaOH) and sulfuric acid (H_2SO_4) were obtained from BDH (England). Milli-Q deionized water was used for all aqueous solutions. All chemicals were of analytical reagent or HPLC grade.

Instrumentation

A Hitachi 655A HPLC with an Inertsil ODS-3 C18 column (250 x 4.6 mm ID) equipped with a UV detector at a wavelength of 254 nm and an integrator (Hitachi 655-71 Processor B, Japan) were used. The HPLC condition was performed in the isocratic mode at a flow-rate of 1.2 ml/minute in an Inertsil ODS-3 C18 column (250 x 4.6 mm). The mobile phase was a mixture of 0.01 M KH_2PO_4 in 0.3% acetic acid adjusted to a pH of 2.5 with 85% orthophosphoric acid, tetrahydrofuran and acetonitrile at ratios of 87:5:8.

Stock standard mixture preparations

One hundred milligrams per liter of PGA, 400 mg/l of HA, 1,000 mg/l of MA, 350 mg/l of o-MHA, 85 mg/l of p-MHA and 180 mg/l of m-MHA were mixed in methanol and water (1:1,v/v) and stored at -20ºC. Working standards for calibration were prepared daily by diluting stock standard solution with the methanol/water mixture (1:1, v/v).

Sample preparations

A 2-ml urine sample was pipetted into a centrifuge tube, 50 μl p-HBA (IS) was added, and the solution was acidified with 5% sulfuric acid to a pH of 1.8 - 1.9. The solution was thoroughly mixed and 3 ml ethyl acetate, an extraction solvent, was added to the tube. The samples were mixed for 2 minutes, and separation of organic phases was achieved by centrifugation at 3,100*g* for 20 minutes. The organic upper phase was transferred and shaken with 1 ml of 0.5 M NaOH for 2 minutes, and the solution was centrifuged at 3,100*g* for 10 minutes. The basic phase solution was transferred and mixed with 0.5 ml 5% sulfuric acid then back extracted using 2 ml of ethyl

acetate. The organic phase was evaporated to dryness under compressed air flow at room temperature. This residue was dissolved in a 1-ml mobile phase solution and 100 μl was injected into the HPLC system.

The calibration curves for urinary MA, PGA, HA, o-, m- and p-MHAs

The calibration curves for the mixture were obtained from a 1 ml urine sample from persons not exposed to solvents; a 1 ml of mixture of PGA, HA, MA, o-, m- and p- MHAs standard solution and 50 μl p-HBA (IS) were added for reducing the extraction and analytical errors. It was extracted according to the method described above. The relative peak area ratios for PGA, HA, MA, o-, m- and p- MHAs to p-HBA (IS) were used for plotting a calibration curve in the range of 15-1,000 mg/l. All standard samples were analyzed in five replications.

The detection limit

Evaluation of the detection limit was performed following the National Institute for Occupational Safety and Health (NIOSH) method (NIOSH, 1994). The ten-point concentrations of mixed MA, PGA, HA, o-, m-, p-MHAs standards ranged from 0.008 to 0.52 μg/ml for MA, 0.0008 to 0.05 μg/ml for PGA, 0.003 to 0.18 μg/ml for HA, 0.003 to 0.18 μg/ml for o-MHA, 0.001 to 0.08 μg/ml for p-MHA and 0.0007 to 0.04 μg/ml for m-MHA. The low level calibration standards were analyzed in three replicates to obtain linear regression equations and the standard error of the regressions. The limit of detection was calculated as three times the standard error of the regression divided by the slope of the regression.

The accuracy and precision of the method

A 1 ml volume of known concentration of PGA, HA, MA, o-, m- and p-MHAs standard mixture was added to 1 ml pooled unexposed urine then analyzed, as described above in five replication. The within and between day precision and accuracy were calculated as relative standard deviations (RSDs) and the percent recovery of the concentration was found.

The quality control of the method

To prepare quality control samples, the pooled unexposed urines were spiked with three different concentrations of PGA, HA, MA, o-, m- and p-MHAs, then analyzed in five replicates and analysis over 3 days. The means and standard deviations of the quality control samples were used for setting up the control chart to examine the accuracy of the sample analysis.

Field application

Paint factory. The paint factory was composed of an office area, a paint grinding section, a

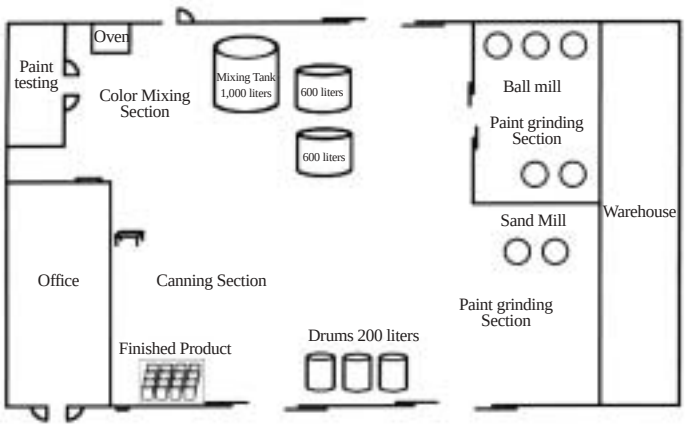

Fig 1–Diagram of paint factory layout.

color mixing section, and a canning section, as shown in Fig 1. The office was air conditioned. The factory had natural ventilation through wide slide gates, no mechanical ventilation was installed in the area. Several grinding tanks were situated in a closed room to reduce the noise level. The paint testing room had a small water curtain to collect paint pigments and organic solvents.

Study subjects. Twelve subjects were working in the small paint factory. They were included in this study after giving written informed consent. This study was reviewed and approved by the Ethics Committee of Mahidol University. The four types of jobs, namely office clerks, paint grinders, color mixers and canners, were as follows:

The office workers were responsible for checking the raw materials and paint products in the warehouse. They were exposed to organic solvents during the task. The office was an air conditioned room connected to the color mixing section with a small slide window which could be opened to communicate with workers in the factory.

The paint grinding workers were responsible for bringing paint pigment and organic solvents including styrene from a warehouse depending on the orders for that day. They cleaned the 600 liter mixing tank with 10-20 liters of xylene, mixed the pigment with organic solvents and then transferred the paint into 200 liter drums. They also put the used xylene and toluene into the 600 liter tank and left it over night and cleaned the tank the next morning. The paint grinding workers also mixed several types of organic solvents for other production sections.

The color mixing workers were responsible for taking the 200 liter drums from the paint grinding section and adding pigments or organic solvents to produce paint products in a 600 liter tank as required by customers. They also tested paint products by spraying them onto thin metal sheets and putting them in an oven to dry. They cleaned the metal sheets with used xylene and toluene before reusing the sheets.

The canning workers had to clean metal cans with xylene before packaging. They took the paint from the color mixing tanks and poured it into the smaller cans. They also mixed organic solvents to produce thinner.

At the end of each day, workers in each section usually cleaned their hands with used xylene. All workers were required to clean their working areas with used xylene and toluene at the end of each work week.

Urine samples. Approximately 20 ml of post-shift urine samples were collected for five consecutive days from each of the twelve workers who had been exposed to toluene, xylene and styrene in the paint factory and stored at -20ºC. Urinary creatinine concentrations were determined using the kinetic Jaffe' colorimetric method using picric acid (Smith, 1985). The urine samples were analyzed using HPLC following the method described above.

Statistical analysis

The general characteristics of the data were analyzed for the median, mean, standard deviation (SD) and relative standard deviation (RSD).

RESULTS

Calibration curves for urinary mandelic, phenylglyoxylic, hippuric, o-, m- and p-methylhippuric acids and p- hydroxybenzoic acid (internal standard)

The chromatograms for PGA, HA, MA, o-, m- and p- MHAs are shown in Fig 2. All metabolites were separated completely within 21 minutes. The retention times were 5.8 minutes for PGA, 8.9 minutes for HA, 10.6 minutes for MA, 12.4 minutes for o-MHA, 17.4 minutes for p-HBA (IS), 19.0 minutes for p-MHA and 20.1 minutes for m-MHA. An analysis of

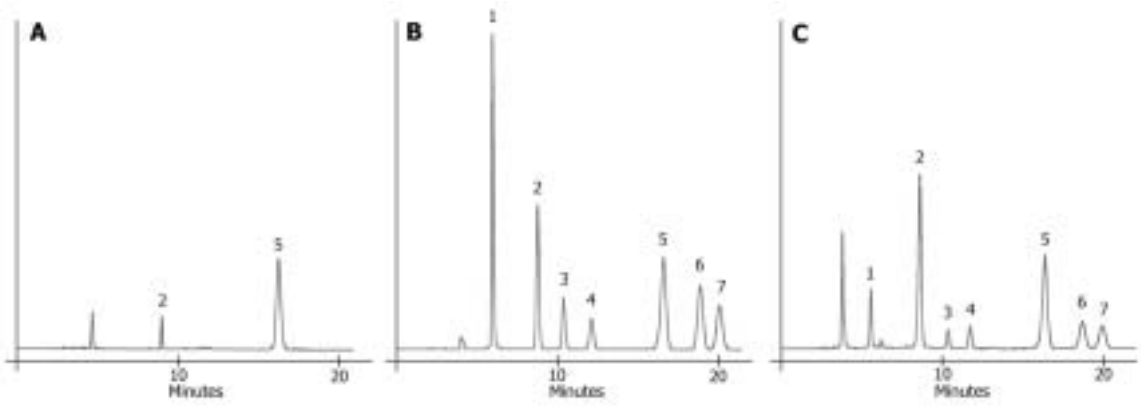

Fig 2–Chromatograms of the 6 metabolites (A) blank urine, (B) unexposed urine with 6 added metabolites, and (C) extracted urine from an exposed worker. The peaks and retention times are 1:PGA-5.8 minutes, 2:HA-8.9 minutes, 3:MA-10.6 minutes, 4:o-MHA-12.4 minutes, 5:p-HBA (IS)-17.4 minutes, 6:p-MHA-19.0 minutes and 7:m-MHA-20.1 minutes.

blank urine showed the concentrations of HA, IS and the normal components of human urine. The urine samples from the workers exposed to organic solvents had low levels of interference peaks due to the use of back extraction.

The calibration curves for the six urinary metabolites were linear over the concentration ranges of 15 - 90 mg/l for PGA, 75 - 380 mg/l for HA, 200 - 1,000 mg/l for MA, 70 - 350 mg/ml for o-MHA, 15 - 85 mg/l for p-MHA and 35 - 180 mg/l for m-MHA, due to the expected concentration found in the urine samples. Linear correlations were found between the acid concentrations and the relative peak area ratio. The average slopes and y-intercepts of the regression lines, and the correlation coefficients are shown in Table 1; the correlation coefficients were all above 0.995. From the regression curve of the concentrations of PGA, HA, MA, o-, m- and p-MHAs in the urine, the detection limits for the method were 1.1 ng/ml for PGA, 4.9 ng/ml for HA, 17.0 ng/ml for MA, 2.5 ng/ml for o-MHA, 1.7 ng/ml for p-MHA and 2.0 ng/ml for m-MHA.

The accuracy and precision of the method

A pooled urine sample containing added PGA, HA, MA, o-, m- and p-MHAs was used in determining the within-day and between-day accuracy and precision of the method. The RSDs (100 X SD/mean) were calculated for three days for an overall average as shown in Table 2. The percent recoveries of these metabolites were 99.2-101.8%, with RSDs of less than 2%.

Application of the method in the paint factory

The method was applied to analyze the post-shift urine samples for five consecutive days for twelve workers who were exposed to organic solvents containing toluene, xylene and styrene in a paint factory. The ranges of urinary metabolite concentrations in the four groups of workers are presented in Table 3. The results show the canning workers had the highest urinary excretions of PGA, HA, and m-MHA and the paint grinding workers had the highest urinary excretions of MA and o-MHA. The results of the urinary metabolites for all job types were over the Biological Exposure Index (BEI) recommended by the American Conference of Governmental Industrial Hygienists (ACGIH). Of the 60 post-shift urine samples, 83.3% exceeded the BEI of 400 mg/g creatinine for PGA+MA, 71.7% exceeded the BEI

Table 1
Calibration parameters for urinary PGA, HA, MA, o-, m- and p-MHAs.

Compound	Slope	Intercept	Correlation coefficient (*r*)
PGA	65.590	-0.2151	0.9982
HA	7.0975	-0.4642	0.9994
MA	0.8903	-0.0366	0.9980
o-MHA	2.8196	-0.0412	0.9954
p-MHA	27.179	-0.0841	0.9992
m-MHA	10.884	-0.0767	0.9983

Table 2
Within and between day precision and accuracy for measuring urinary PGA, HA, MA, o-, m- and p- MHAs.

	Known conc (g/l)	Within-day RSDs	Recovery (%)	Between-day RSDs	Recovery (%)
PGA	0.01112	1.64	101.3	1.72	100.9
	0.05560	1.08	100.3	0.79	100.7
	0.10008	0.18	100.5	0.78	100.2
HA	0.03541	0.87	101.8	0.75	99.8
	0.17705	0.94	100.8	0.71	100.0
	0.31869	0.88	100.7	0.85	100.1
MA	0.10392	1.09	100.4	1.08	100.3
	0.51960	0.88	99.8	0.54	99.7
	0.93528	0.13	100.3	0.22	99.6
o-MHA	0.03550	1.31	100.6	1.26	100.3
	0.17750	0.90	100.4	0.57	99.7
	0.31950	0.25	100.0	0.50	100.1
p-MHA	0.00890	1.03	100.8	0.91	99.5
	0.04450	1.07	100.5	0.92	100.0
	0.08011	0.56	100.0	0.51	100.0
m-MHA	0.01659	0.41	99.8	0.50	99.7
	0.08295	0.86	99.2	0.61	99.8
	0.14931	0.47	99.6	0.42	99.6

of 1.6 g/g creatinine for HA and 41.7% exceeded the BEI of 1.6 g/g creatinine for o-, m-, p- MHAs.

DISCUSSION

The purpose of this study was to develop a simple and reliable HPLC method for the determination of MA, PGA, HA and o-, m-, p-MHAs in human urine and to investigate the optimal conditions for HPLC analysis in order to completely separate the six metabolites. The addition of 5% H_2SO_4 was used to adjust the pH of urine samples to 1.8-1.9 for increasing the recovery of metabolites. The urine samples from the workers exposed to organic

Table 3
The range and median post-shift urine concentrations of PGA, HA, MA, o-, p- and m-MHAs in paint factory workers.

Work description	No. of workers	No. of samples	Range and median concentrations (g/g creatinine)					
			PGA g/g creatinine	HA g/g creatinine	MA g/g creatinine	o-MHA g/g creatinine	p-MHA g/g creatinine	m-MHA g/g creatinine
			0.017-0.235	0.244-4.708	0.263-0.957	0.090-1.164	0.025-0.282	0.050-0.650
Office work	4	20	0.045	1.007	0.420	0.373	0.088	0.203
			0.045-0.189	1.934-11.444	0.526-1.669	0.253-4.458	0.134-0.521	0.181-2.002
Paint grinding	2	10	0.087	5.839	0.725	1.005	0.372	0.531
			0.040-0.331	0.788-9.658	0.257-1.245	0.096-1.829	0.074-1.163	0.160- 2.962
Color mixing	3	15	0.168	4.009	0.708	0.537	0.400	0.896
			0.050-0.412	1.040-20.622	0.225-1.570	0.350-1.187	0.093-0.711	0.097-3.107
Canning	3	15	0.091	7.037	0.820	0.643	0.372	0.672

solvent had low levels of interference peaks due to the use of back extraction.

In regard to the mobile phase (a mixture of 0.01 M KH_2PO_4 in 0.3% acetic acid adjusted to a pH of 2.5 with 85% orthophosphoric acid, tetrahydrofuran and acetonitrile at a ratio of 87:5:8), appropriate percentage of acetic acid caused sharp peaks of PGA and MA in the C18 column (Laffon *et al*, 2001). A pH of 2.5 was selected as best for separation of PGA and MA peaks. If the pH of the buffer is not suitable, the PGA peak will overlap the MA peak. The separation of m- and p-MHAs has been a persistent problem. Deog *et al* (1997) recommended the addition of β-cyclodextrin in a mobile phase to help separate the m- and p-MHAs peaks. The disadvantage of using β-cycloextrin in the mobile phase is that this will reduce the life span of the HPLC column due to clogging of re-precipitated β-cyclodextrin. In this research, only the addition of tetrahydrofuran improved the separation of m- and p-MHAs peaks.

The detection limits of the six metabolites using this method were 1.1 ng/ml for PGA, 4.9 ng/ml for HA, 17.0 ng/ml for MA, 2.5 ng/ml for o-MHA, 1.7 ng/ml for p-MHA and 2.0 ng/ml for m-MHA. When the detection limits of this study were compared with previous studies, this approach was found capable of detecting metabolite concentrations lower than the other methods. Previous studies by Laffon *et al* (2001), reported detection limits of 1.2, 1.5, 3.0 and 7.6 μg/ml for MHA, HA, PGA and MA, respectively. Deog *et al* (1997) reported detection limits of 5, 2, 6, 8 and 8 μg/ml for PGA, HA, o-, m- and p-MHAs, respectively.

When the results of this study are compared with a previous study in two paint factories (Kongtip *et al*, 2000), the earlier study found 0.02-4.6 g/g creatinine for HA and 0-0.7 g/g creatinine for MHA. This study found higher concentrations of HA and MHA than a previous study. This is probably because the

workers in this current study handled large amounts of organic chemicals each day and did not use respirators or gloves to protect themselves, and the working area did not have any mechanical ventilation.

In conclusion, a convenient method for the determination of PGA, HA, MA, o-, m and p-MHAs in urine samples was developed. This method is sensitive and can be performed without interference from other substances after back extraction. This methodology is suitable for evaluating exposure to low concentrations of toluene, xylene and styrene in the workplace.

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