SIMULTANEOUS DETERMINATION OF *TRANS,TRANS*-MUCONIC ACID AND *S*-PHENYLMERCAPTURIC ACID BY HIGH PRESSURE LIQUID CHROMATOGRAPHY AND ITS APPLICATION

Prapin Tharnpoophasiam[1], Pornpimol Kongtip[2], Waranya Wongwit[1], Wijitr Fungladda[1] and Dwip Kitayaporn[1]

[1]Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University; [2]Department of Occupational Health and Safety, Faculty of Public Health, Mahidol University, Bangkok, Thailand

Abstract. The simultaneous determination of urinary *trans,trans*-muconic acid (*t,t*-MA) and *S*-phenylmercapturic acid (*S*-PMA) was performed by liquid extraction with ethyl acetate and reversed-phase high performance liquid chromatography (RP-HPLC) on a Hypersil-ODS column using the gradient mobile phase of methanol and 0.0012 N perchloric acid and diode array detection at 205 and 264 nm for *S*-PMA and *t,t*-MA, respectively. The retention times for *t,t*-MA and *S*-PMA were 3.8 and 12.3 minutes, respectively. The recoveries of *t,t*-MA and *S*-PMA were > 97%; between-day precisions were all within 8% RSD (100x SD/mean). The method was applied to analyze the urinary *t,t*-MA and *S*-PMA of 59 service station attendants exposed to average benzene concentrations in the air of 0.20±0.18 ppm. Significant differences in pre-shift and post-shift urinary *t,t*-MA between smokers and non-smokers were found.

INTRODUCTION

Benzene is an important industrial chemical and is an intermediate in the synthesis of numerous chemicals. Since benzene is hematotoxic and has been classified as a group 1 carcinogen by the Internal Agency for Research on Cancer (IARC, 1989), monitoring and control of benzene exposure is important. Two minor urinary benzene metabolites, *trans,trans*-muconic acid (*t,t*-MA) (Inoue *et al*, 1989; Ghittori *et al*, 1996; Ruppert *et al*, 1997) and *S*-phenylmercapturic acid (*S*-PMA) (van Sittert *et al*, 1993; Ghittori *et al*, 1999; Melikian *et al*, 2002), were suggested as biomarkers of benzene exposure. *t,t*-MA analysis by gas chromatography with various detection methods has been used, including flame ionization detection (FID) (Bertczak *et al*, 1994; Yu and Weisel, 1996) and mass spectrometric (MS) detection (Ruppert *et al*, 1997; Rothman *et al*, 1998). Reversed-phase high performance liquid chromatography (RP-HPLC), with UV detection after solid phase extraction of the sample, is the method preferred for the determination of *t,t*-MA (Inoue *et al*, 1989; Ducos *et al*, 1990; Buratti *et al*, 1996; Ghittori *et al*, 1996), while the analytical methods employed for *S*-PMA are rather complex, *eg*, extraction through solid-phase extraction cartridges (Stommel *et al*, 1989; Ghittori *et al*, 1999; Buratti *et al*, 2001), requiring derivatization before analysis (Stommel *et al*, 1989; Einig and Dehnen, 1995; Ghittori *et al*, 1999; Buratti *et al*, 2001), using GC-MS (Stommel *et al*, 1989; Angerer *et al*, 1998) or LC-MS (Melikian *et al*, 1999; 2002). Most of the previously described procedures could detect either *t,t*-MA or *S*-PMA; only a few were carried out for simultaneous determination of both. Sabourin *et al* (1988) extracted urine and tissue from rats which had taken [^{3}H/^{14}C] benzene orally, and analyzed these two and other benzene metabolites by HPLC with a complicated mobile phase solvent system. Schad *et al* (1992) used a complex process of hydrolysis, separation with anion exchanger and extraction with diethyl ether before analyzing these two and other metabolites

Correspondence: Prapin Tharnpoophasiam, Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand.
Tel: +66 (0) 2354-9100 ext. 1562-3; Fax: +66 (0) 2354-9167

by HPLC. However, there was interference with the *S*-PMA signal. The studies of Melikian *et al* (1999; 2002) used radiolabeled *t,t*-MA and *S*-PMA as an internal standard, solid-phase extraction and analyzed them with electrospray-tandem mass spectrometry-selected reaction monitoring (LC-ES-MS/MS-SRM). These methods are complex and not appropriate for routine monitoring of *t,t*-MA and *S*-PMA in most laboratories.

In the present study, the HPLC method was developed as a proposed practical method for the simultaneous determination of two minor urinary benzene metabolites, *t,t*-MA and *S*-PMA, using liquid-liquid extraction (LLE) instead of solid-phase extraction, which is commonly used for sample preparation with a short analysis time of 20 minutes. The application of the established method was used to analyze the urine of service station attendants for occupational benzene exposure.

MATERIALS AND METHODS

Chemicals

t,t-MA was purchased from Aldrich (Milwaukee, WI, USA); D,L-phenylmercapturic acid from Tokyo Kasei Chemicals (Tokyo, Japan); ethyl acetate (HPLC grade) from Labscan (Bangkok, Thailand); methanol (multisolvent grade) from Scharlau (Barcelona, Spain) and benzoic acid from Merck (Darmstadt, Germany). Distilled, deionized water was used to prepare all solutions.

Instrumentation

HPLC separation was carried out using an HPLC 1100 (Hewlett Packard, Waldbronn, Germany) equipped with a diode array detector, DAD G1315A (Hewlett Packard, Waldbronn, Germany) and data acquisition and elaboration were processed by means of LC-ChemStation software (Hewlett Packard, Waldbronn, Germany). The Hypersil ODS column (125 x 4 mm I.D., 5 μm particle size) and the Hypersil ODS guard column (4 x 4 mm I.D., 5 μm particle size) were obtained from Agilent Technologies (Avondale, PA, USA). The column temperature was controlled at 25°C. The eluent system consisted of methanol and 0.0012 N perchloric acid in water, pH 2.7. At the beginning of the chromatographic run, the initial mobile phase was 14.5:85.5 (methanol and 0.0012 N perchloric acid, v/v) at a flow rate of 1.0 ml/minute for 5 minutes. From the minutes 5.0 to 5.30, the mobile phases were gradually increased to 22.7:77.3 (methanol and 0.0012 N perchloric acid, v/v) and the flow rate was increased to 2.0 ml/minute and the system was maintained until reaching 20 minutes. After that, the system was allowed to re-equilibrate for 5 minutes at 1 ml/minute with 14.5:85.5 (methanol and 0.0012 N perchloric acid, v/v). The UV responses of *t,t*-MA and *S*-PMA were recorded at 264 and 205 nm, respectively.

GC separation was carried out using a Shimadzu GC-14B (Shimadzu, Kyoto, Japan) with a DB-1 capillary column (30 m x 0.53 mm I.D.), equipped with a flame ionization detector and an integrator (Shimadzu C-R7A, Shimadzu, Kyoto, Japan). The carrier gas was helium at a flow rate of 10 ml/minute. The GC condition was isothermal: column, 120°C; injector, 200°C; detector, 200°C.

A Shimadzu UV-160A spectrophotometer (UV-visible recording spectrophotometer) (Shimadzu, Kyoto, Japan) equipped with a Shimadzu TB-85 thermobath (Shimadzu, Kyoto, Japan) was used for creatinine determination.

Study subjects

The exposed subjects were service station attendants. Participation in the study was voluntary and subjects were included only after written informed consent was given. This study was reviewed and approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University. The subjects were 59 benzene-exposed workers, 47 men and 12 women. Both smokers and nonsmokers were included (23 smokers, 36 nonsmokers).

Analysis of benzene exposure

Benzene in the work environment of each individual was collected throughout the workshift (8-12 hours) using an activated charcoal tube (SKC-226-09, SKC, Eighty Four, PA, USA) connected to an SKC personal air sampling pump (SKC, Eighty Four, PA, USA) at a flow rate of 0.15 l/minute and was determined according to the NIOSH method 1501 (NIOSH, 1994).

Urine sample storage and processing

On the day of sampling, each participant was asked to provide a 20-ml urine sample in the morning before starting work and again near the end of the workshift. All urine samples were kept

in a dry ice box and shipped to the laboratory. All samples were frozen immediately and then stored at -35°C until analysis.

Creatinine determination

Urinary creatinine determination was performed by the kinetic Jaffé method (Smith, 1985). The results were expressed as ratio of *t,t*-MA and *S*-PMA (μg *t,t*-MA or *S*-PMA /g creatinine).

Preparation of standard solution

Stock standard solutions of *t,t*-MA and *S*-PMA (1.0 mg/ml) were prepared by dissolving 10 mg of each compound in 10 ml of methanol; concentrations were adjusted to 200 μg/ml with water and stored at -20°C. Working standards for calibration were prepared daily by diluting stock standard solution with water to obtain concentrations of 0.25, 0.5, 1.0, 2.5 and 5 μg/ml, respectively. Benzoic acid, used as an internal standard, was prepared by dissolving 10 mg of compound in 10 ml of methanol and storing at -20°C. The concentration was diluted to 250 μg/ml with water at the time of analysis.

Analytical procedure

t,t-MA and *S*-PMA were separated from the urine matrix by LLE. An aliquot of urine (4.0 ml) was taken into a 15-ml screw-capped test tube, and mixed with 0.5 ml water, 0.1 ml internal standard solution, and 0.2 ml 17.8 N sulfuric acid. The purpose of adding sulfuric acid was to convert pre-PMA to *S*-PMA (Inoue *et al*, 2000). Within 10 minutes of adding the sulfuric acid, the strong acid mixture was made weakly acidic by adding 0.2 ml 9.8 N potassium hydroxide. The final pH of the mixture was approximately 1.0. The mixture was extracted twice with 3.0 ml of ethyl acetate by a 3-minute vortex mix and then centrifuged at 4,000 rpm to improve phase separation. The organic upper layers were transferred to another test tube and evaporated to dryness under a nitrogen stream at room temperature. The residue was dissolved with 500 μl of 14.5:85.5 (methanol and 0.0012 N perchloric acid, v/v). Fifty microliter of the solution was injected into the HPLC system.

Statistical analysis

Intercooled Stata Version 7.0 (College Station, TX, USA) was used to determine the statistically significant differences in means either by paired or unpaired *t*-tests. A Pearson's or Spearman's correlation was used when appropriate.

RESULTS

Chromatographic separation

Chromatographic profiles obtained from pooled urine, which was used as blank urine, without and with spiked standards, and a urine sample from a benzene exposed worker are shown in Figs 1, 2 and 3, respectively. *t,t*-MA and *S*-PMA were well separated from other urinary components within 13 minute (Fig 2) and the pattern of wavelength-spectrums of *t,t*-MA and *S*-PMA standards is shown in Fig 4. The *t,t*-MA and *S*-PMA found in the urine samples can be confirmed by comparison with this pattern. The retention times of *t,t*-MA, benzoic acid (internal standard), and *S*-PMA were 3.8, 10.3 and 12.3 minutes, respectively. The analysis was completed in 20 minutes and a 5-minute gap was scheduled to restore the initial conditions.

Calibration, recovery, and reproducibility

The calibration curve was obtained by analyzing three replications of *t,t*-MA and *S*-PMA at 0.25 to 5.0 μg/ml. Linear correlations were found between the acid concentrations and the relative peak area ratios. The parameters of the linear regressions for *t,t*-MA and *S*-PMA are shown in Table 1.

A pooled urine sample containing added *t,t*-MA and *S*-PMA, 0.5, 2.0 and 4.0 μg/ml, was used in determining the within-day accuracy and between-day precision of the method. The RSDs (100 x SD/mean) were calculated for 10 days for between-day precision. As Table 2 indicates, the accuracy of the overall method ranged from 97.2 to 99.8% and the calculated precision was within 8% RSD, ranging from 4.8 to 7.3% RSD. The detection limit of the method [based on a signal-to-noise ratio (S/N) of 10] was 16.7 μg/l for *t,t*-MA and 92 μg/l for *S*-PMA.

Table 1
Typical calibration parameters of *t,t*-MA and *S*-PMA in urine (relative peak area ratio *vs* concentration in μg/ml).

Compound	Slope	Intercept	Correlation coefficient (r)[a]
t,t-MA	0.3831[a]	0.0000[a]	0.9997[a]
S-PMA	0.0613[a]	-0.0135[a]	0.9988[a]

[a]$p < 0.01$

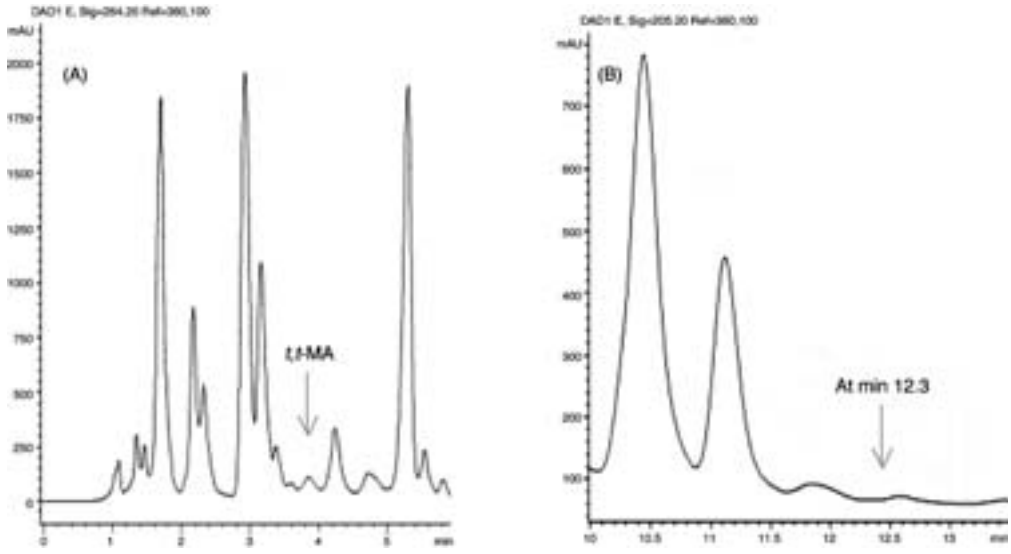

Fig 1–Chromatograms of blank urine sample without added standard; (A) detection of *t,t*-MA (0.09 μg/ml) at a wavelength of 264 nm and (B) at a wavelength of 205 nm.

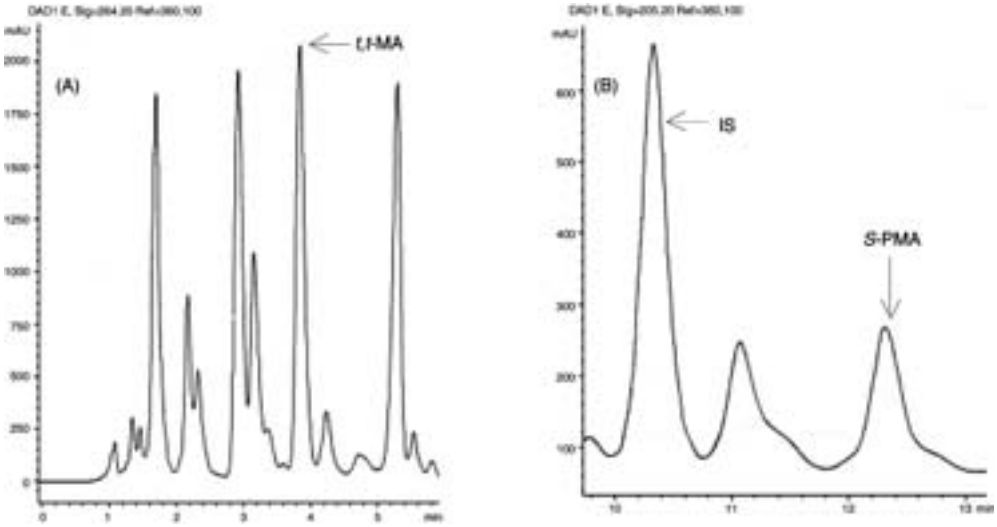

Fig 2–Chromatograms of blank urine sample with addition of 5.0 μg/ml of *t,t*-MA and *S*-PMA; (A) detection of *t,t*-MA at a wavelength of 264 nm and (B) detection of *S*-PMA at a wavelength of 205 nm.

Field application of the analytical method

With the purpose of an initial trial to test the developed method for monitoring benzene exposure, urinary *t,t*-MA and *S*-PMA were assessed in service station attendants who were occupationally exposed to benzene. Fifty-nine service station attendants from 12 petrol stations in the Bangkok area were included in this study (47 men and 12 women). Of these subjects, 23 were smokers and 36 were nonsmokers. All the smokers were men.

The average urinary *t,t*-MA and *S*-PMA for both smokers and nonsmokers who were occupationally exposed to benzene are presented in Table 3. Among smokers and non smokers, the pre-shift and post-shift urinary *t,t*-MA were significantly different, at $p < 0.01$. The average levels of benzene exposure in air and post-shift urinary *t,t*-MA in smokers were significantly greater than those in nonsmokers (Table 3). Detectable amounts of *S*-PMA were found in 3 post-shift

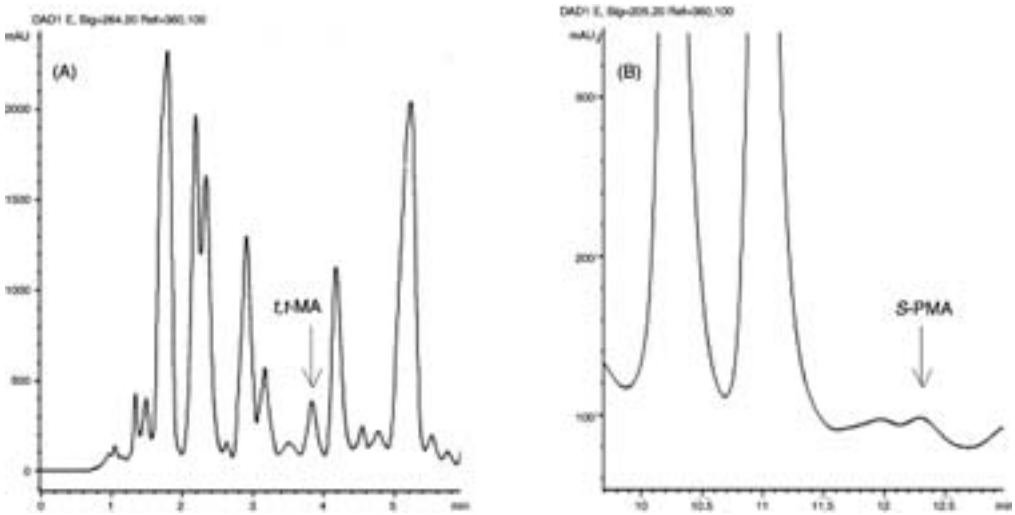

Fig 3–Chromatograms of urine sample from an exposed worker; (A) *t,t*-MA = 0.56 μg/ml (261.68 μg/g Cr) at a wavelength of 264 nm and (B) *S*-PMA = 0.35 μg/ml (194.44 μg/g Cr) at a wavelength of 205 nm.

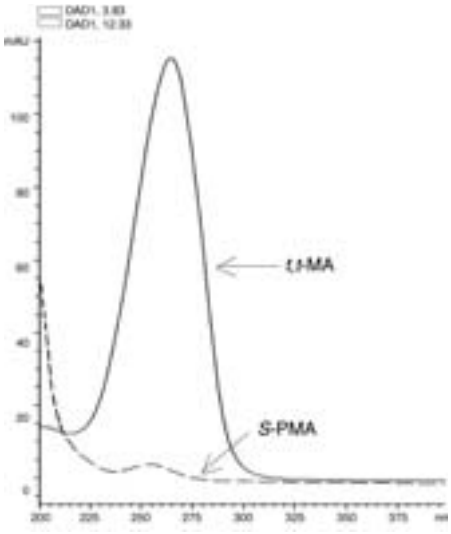

Fig 4–The pattern of wavelength-spectrums of *t,t*-MA and *S*-PMA standards.

urine samples and 1 pre-shift urine sample. All the detected *S*-PMA were from the urine samples of subjects who smoked approximately 10 cigarettes/day.

DISCUSSION

The purpose of the present study was to develop a practical HPLC method for the determination of urinary *t,t*-MA and *S*-PMA in a single chromatographic run. Sample extraction by ethyl acetate was eventually preferred because of its sufficient recovery. The established protocol enables the analysis to complete one assay within 20 minutes.

Most of service station attendants (51 of 59) in this study had higher post-shift urinary *t,t*-MA concentrations than pre-shift, and the means of pre-shift and post-shift urinary *t,t*-MA were sig-

Table 2
Within-day and between-day assays for the determination of *t,t*-MA and *S*-PMA in urine.

	Spiked concentration (μg/ml)	Average concentration (μg/ml) ± SD	Recovery (%) (n=5)	RSD (%) (n=10)
t,t-MA	0.5	0.50±0.03	99.2	4.8
	2.0	1.98±0.18	98.5	6.7
	4.0	3.90±0.34	97.5	7.2
S-PMA	0.5	0.49±0.04	98.6	7.3
	2.0	1.94±0.12	97.2	6.4
	4.0	3.91±0.29	99.8	6.9

Table 3
Average environmental benzene exposure, urinary *t,t*-MA and *S*-PMA of subjects.

	n	Mean ± SD (range) of benzene exposure (ppm)	Mean ± SD (range) of *t,t*-MA (μg/g cr)	Mean (range) of *S*-PMA (μg/g Cr)
Total workers	59	0.20±0.18 (0.01-0.94)		
Pre-shift			127.37±115.87 [b]	87.27 [d]
			(1.80-485.21)	-
Post-shift			276.80±180.71 [b]	148.25 [e]
			(32.89-1,087.63)	(123.60-194.44)
Non smokers	36	0.16±0.14 (0.01-0.63) [a]		
Pre-shift			106.63±109.44	ND
			(1.80-485.21)	-
Post-shift			229.93±130.61 [c]	ND
			(32.89-542.17)	-
Smokers	23	0.26±0.21 (0.04-0.94) [a]		
Pre-shift			159.83±120.57	87.27 [d]
			(23.14-458.96)	-
Post-shift			350.15±223.01	148.25 [e]
			(79.55-1,087.63) [c]	(123.60-194.44)

ND = non-detectable, Cr = creatinine; [a] $p = 0.045$ (unpaired t-test, equal variance); [b] $p < 0.01$ (paired *t*-test); [c] $p = 0.026$ (unpaired *t*-test, unequal variance); [d] Detectable amount of urinary *S*-PMA in 1 benzene exposed worker; [e] Detectable amount of urinary *S*-PMA in 3 benzene exposed workers.

nificantly different by paired t-test. This result indicates the urinary excretion of *t,t*-MA resulted from benzene exposure during the work-shift. There were significant differences in average levels of benzene exposure and post-shift urinary *t,t*-MA between smokers and non smokers (Table 3). These data indicate that smoking is likely to affect urinary excretion of *t,t*-MA. Since benzene is also a constituent of tobacco smoke (Wallace, 1989), the uptake of benzene by a smoker who smokes 32 cigarettes/day is 1.8 mg (55 μg/cigarette x 32 cigarettes). With a conversion rate of benzene to urinary *t,t*-MA of 2-25%, smoking should, on average, increase daily *t,t*-MA excretion by 0.026-0.62 mg (Scherer *et al*, 1998). The significant differences in urinary *t,t*-MA, between smokers and non smokers in this study, agreed with previous studies (Lee *et al*, 1993; Buratti *et al*, 1996; Ghittori *et al*, 1996; Ruppert *et al*, 1997). The average post-shift urinary *t,t*-MA in service station attendants in the present study, of 276.80±180.71 (ranging from 32.89-1087.63) μg/g creatinine, was similar to the study of Ong *et al* (1995), which found that among car mechanics and service station attendants exposed to <1 ppm, post-shift urinary *t,t*-MA was 360±220 (ranging from 90-1560) μg/g creatinine.

In conclusion, the proposed method is a quick, reliable, and practical method for determining both urinary *t,t*-MA and *S*-PMA. Results of field application show that the sensitivity for *t,t*-MA is high for routine biological monitoring of benzene exposure, but the sensitivity for *S*-PMA is less for low levels of benzene exposure in the air of 0.20 ppm. Therefore, the sensitivity of urinary *S*-PMA analysis should be further improved for low levels of benzene exposure in the air.

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