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Personal exposure to different levels of benzene and its relationships to the urinary metabolites *S*-phenylmercapturic acid and *trans,trans*-muconic acid

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Abstract

This report is part of an extensive study to verify the validity, specificity, and sensitivity of biomarkers of benzene at low exposures and assess their relationships with personal exposure and genetic damage. The study population was selected from benzene-exposed workers in Tianjin, China, based on historical exposure data. The recruitment of 130 exposed workers from glue-making or shoe-making plants and 51 unexposed subjects from nearby food factories was based on personal exposure measurements conducted for 3–4 weeks prior to collection of biological samples. In this report we investigated correlation of urinary benzene metabolites, *S*-phenylmercapturic acid (*S*-PMA) and *trans,trans*-muconic acid (*t,t*-MA) with personal exposure levels on the day of urine collection and studied the effect of dose on the biotransformation of benzene to these key metabolites. Urinary *S*-PMA and *t,t*-MA were determined simultaneously by liquid chromatography–tandem mass spectrometry analyses. Both *S*-PMA and *t,t*-MA, but specifically the former, correlated well with personal benzene exposure over a broad range of exposure (0.06–122 ppm). There was good correlation in the subgroup that had been exposed to <1 ppm benzene with both metabolites (P-trend <0.0001 for *S*-PMA and 0.006 for *t,t*-MA). Furthermore, the levels of *S*-PMA were significantly higher in the subgroup exposed to <0.25 ppm than that in unexposed subjects ($n=17$; $P=0.001$). There is inter-individual variation in the rate of conversion of benzene into urinary metabolites. The percentage of biotransformation of benzene to urinary *S*-PMA ranged from 0.005 to 0.3% and that to urinary *t,t*-MA ranged from 0.6 to ~20%. The percentage of benzene biotransformed into *S*-PMA and *t,t*-MA decreased with increasing concentration of benzene, especially conversion of benzene into *t,t*-MA. It appears that women excreted more metabolites than men for the same levels of benzene exposures. Our data suggest that *S*-PMA is superior to *t,t*-MA as a biomarker for low levels of benzene exposure.

Keywords: Benzene; *S*-phenylmercapturic acid; *trans,trans*-Muconic acid

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1. Introduction

Benzene is a major industrial chemical. About 1.3 billion gallons are produced annually in the United States, this is about one-third of the worldwide production. Benzene is found in gasoline, below 1% in the United States, but above 5% in 'super unleaded' gas in other countries [1,2]. Benzene is present in the mainstream and sidestream of tobacco smoke (about 87 μg/cigarette) [3], as well as in many foodstuffs and consumer products. The average background level of benzene intake for non-smokers has been estimated at 0.5 mg/day [4]. Therefore, human exposure to benzene may be unavoidable.

Benzene is a known leukemia-inducing agent in humans and is a multisite carcinogen in rodents [5,6]. Chronic exposure to high levels of benzene in humans is associated with chromosomal damage, depression of the immune system, aplastic anemia, myelodysplastic syndromes, and leukemia, especially acute myeloid leukemia [7,8]. Recent studies in China also suggest that benzene exposure may be associated with non-Hodgkin's lymphoma, lymphatic leukemia, lung cancer, and nasopharyngeal cancer [9].

Though toxic effects of benzene at high exposures are well documented, the risk for adverse health effects at low levels of benzene exposure remains unknown. An international collaborative study was conducted in an occupational cohort in China to verify the validity, specificity, and sensitivity of biomarkers of benzene at low exposures (e.g. chromosomal damage, protein adducts, urinary metabolites) and assess their relationships with personal exposure and genetic damage. We report here a part of that study. Two minor urinary benzene metabolites namely *S*-Phenylmercapturic acid (*S*-PMA) [10–14] and *trans,trans*-muconic acid (*t,t*-MA) [15–22] were suggested as biomarkers of exposure. Individual personal exposure to different levels of benzene was monitored, the resulting correlation of exposure with urinary *S*-PMA and *t,t*-MA was established, and the effect of dose on the biotransformation of benzene to these key metabolites was examined.

2. Experimental

2.1. Chemicals

[$^{13}C_6$]Benzene ($^{13}C_6$, 99%) and [$^{13}C_6$]aniline ($^{13}C_6$, 99%) were purchased from Cambridge Isotope Laboratory (Andover, MA). Sodium nitrite,

Table 1
Summary of the study to monitor benzene exposure and urinary metabolites

	Exposed group $n=130$ Mean±SD or % (Range)	Unexposed group $n=51$ Mean±SD or % (Range)
Sex (%Female)	52	53
Age (Years)	36.3±7.6	33.3±7.4
Smoking (self-reported) (%)	30	20
Smoking (based on urinary cotinine)[a] (%)	38	31
Urinary cotinine (μg/g creatinine)	628±1177	560±1125
Benzene exposure (ppm)[b]	9.7±16.6 (0.06–122)	–
Median	3.2	–
Mean of cumulative exposure (ppm-year)[c]	81.3±89.3	–
Median of cumulative exposure (ppm-year)[c]	51.2	–
Urinary *S*-PMA (μg/g creatinine), post-work	285.4±644.1 (0.6–2700)	1.9±1.9 (0.06–6.6)
Urinary *t,t*-MA (mg/g creatinine), post-work	6.2±6.48 (0.09–29.6)	0.26±0.27 (0.04–1.2)

[a] Urinary cotinine>100 μg/g creatinine were considered as smokers.
[b] Benzene exposure on the day of urine collection.
[c] Lifetime cumulative benzene exposure was estimated from individual work history and related historical exposure recorded at the factory.

N-acetyl-L-cysteine, copper, thiophenol, acetamidoacrylic acid, and *t,t*-MA were bought from Aldrich (Milwaukee, WI) and Whatman's strong anionic exchange (SAX) cartridges (1000 mg) were from Fisher Scientific Company (Fair Lawn, NJ). Mono- and di-sodium phosphates were purchased from Sigma (St. Louis, MO). Unless specified, solvents used were of HPLC grade from J.T. Baker (Phillipsburg, NJ).

2.2. Animals

Male F344/N rats were purchased from the Charles River Breeding Laboratories (North Wilmington, MA). The rats were 12-weeks-old at the onset of experiments.

2.3. Subject recruitment and urine sample collections

The exposed occupational group we studied was identified in glue- and shoe-making factories in Tianjin, China on the basis of their historical exposure records. The participating subjects were recruited by a three-step procedure. (1) Our research staff used a pre-designed questionnaire to interview all exposed workers in both factories. The information provided by the workers was then given to plant personnel staff for review and confirmation. (2) Workers with at least a 3-year exposure history, who have had no known disease and were not currently on medication, were then given a physical examination and laboratory tests for liver function and urinary protein levels. Subjects with any diagnosed disease were excluded from the study. (3) Those workers who passed the physical examination and laboratory tests were then monitored with 3 *M* organic vapor monitors for their personal benzene exposures at least three times within 1 month. This preliminary monitoring allowed the selection of an appropriate number of workers exposed to benzene in particular ranges of airborne concentrations. Based on the individual multiple exposure level, 130 workers were finally recruited. The control subjects were 51 workers from nearby food processing factories who had no occupational benzene exposure. Demographic characteristics among exposed and unexposed subjects were similar, as shown in Table 1. During the sampling days, each participant was first asked to provide 50-ml of urine sample in the morning before starting to work and was then monitored for exposure with duplicate 3 *M* monitors attached to the center of the upper chest for the whole workshift. Participants were asked to donate urine samples again at the end of the workshift. All urine samples were stored at −20°C until packed in dry ice and shipped to the United States. All samples were frozen immediately and stored at −20°C until analysis.

2.4. Preparation of unlabeled S-PMA standard and [^{13}C]S-PMA and [^{13}C]t,t-MA internal standards

Unlabeled *S*-PMA was prepared as previously described [23]. In brief, thiophenol (1.8 g; 16.4 mmol) was suspended in 20 ml of dioxane along with acetamidoacrylic acid (1.94 g; 15 mmol) and 0.4 ml piperidine, and refluxed under N_2 for 3 h. After removal of the solvent, the residue was partitioned between ether and sodium bicarbonate solution. The aqueous layer was neutralized, extracted with ether, and acidified to precipitate crude *S*-PMA. The precipitate was crystallized from aqueous methanol, characterized by NMR and mass spectra, and further purified by HPLC.

[$^{13}C_6$]*S*-PMA was prepared from [$^{13}C_6$]aniline by the Gattermann reaction as described previously [24]. In brief, the phenyldiazonium salt obtained from the reaction of [$^{13}C_6$]aniline (0.5 g; 5.3 mmol) and $NaNO_2$ (1.4 ml; 6 mmol) in the presence of HCl at <5°C was added to an *N*-acetyl-L-cysteine solution (7.5 ml; 4.8 mmol), and the resulting orange precipitate was centrifuged. The wet solid of *N*-acetyl-*S*-phenyldiazol-L-cysteine was dissolved in EtOH (6 ml), and after adding freshly prepared copper (0.64 g) and H_2O (14 ml), the suspension was refluxed at 80°C for 1.5 h. The residue was filtered, washed with hot H_2O, acidified, and extracted with $CHCl_3$. After removing the solvent, the crude [$^{13}C_6$]*S*-PMA residue was crystallized from aqueous EtOH and characterized by NMR and MS [24]. The purity of synthesized [$^{13}C_6$]*S*-PMA was >98% according to HPLC analysis.

[$^{13}C_6$]*t,t*-MA as an internal standard was prepared biosynthetically as described previously [25]. A group of three rats were each given i.p. injections of 2.6 mmol [$^{13}C_6$]benzene/kg body weight in 0.2 ml corn oil once a day for 3 days. The urine voids were collected at 0°C during and after exposure on day four; they were then stored at −20°C until analysis. The [$^{13}C_6$]-labeled standard was isolated from the urine by a method described previously [13].

2.5. Liquid chromatography–electrospray–tandem mass spectrometry (LC–ES–MS/MS) analysis of urinary S-PMA and t,t-MA

The simultaneous measurement of *S*-PMA and *t,t*-MA by LC–ES–MS/MS was performed by a method described previously [13]. One-milliliter urine samples, spiked with 15 ng of [$^{13}C_6$]*S*-PMA and 30 ng of [$^{13}C_6$]*t,t*-MA as internal standards, then were subjected to SAX cartridge clean-up, followed by EtOAc extraction of eluted analytes from the cartridge [13]. After removal of the solvent, the residue was dissolved in 150 μl of MeOH:1% aqueous acetic acid (20:80 v/v), and a 30-μl sample of this solution was analyzed by LC–ES–MS/MS-selected reaction monitoring (SRM). A blank sample was included with each batch of urine cleanup.

The HPLC features included a Waters Model 600 pump, a Rheodyne model 7120 injector, and a Phenomenex Ultramex 5-μ C-18 narrow-bore column (250×2.0 mm). A pre-injector splitter was utilized to reduce the flow-rate from 0.9 ml/min (HPLC pump) to 160 μl/min. A linear gradient from 80% solvent A (0.5% aqueous acetic acid), 20% solvent B (MeOH) to 100% solvent B over 5 min was employed in the elution program for analysis of *S*-PMA and *t,t*-MA. The HPLC was interfaced with a Finnigan TSQ 700 triple-stage quadrupole mass spectrometer (San Jose, CA) via an electrospray source. The mass spectrometer was operated in the negative ion mode. The spray voltage was 4.1 kV, the capillary temperature 220°C. For selected reaction monitoring (SRM), the argon gas pressure in the collision cell (Q2) was adjusted so that the precursor beam suppression was approximately 75%. Between LC–MS urine sample analyses a solvent was injected to purge the instrument from possible carry over.

2.6. Creatinine and cotinine determination

Creatinine was determined with a Kodak Ektachem 500 Computer-Directed Analyzer and urinary cotinine was quantified by radioimmunoassay at the American Health Foundation's Clinical Biochemistry Facility according to previously described methods [25].

2.7. Analysis of benzene, toluene, and xylene in 3 M monitors

Duplicate organic vapor monitor samples were collected from each subject; one was analyzed on site in Beijing, China, and another was brought back to the United States for analysis. An extensive quality control program was instituted because of the required international shipping of samples and time between collection and analysis. Duplicate personal monitor samples were collected for 290 exposures. Excellent correlation was obtained between samples analyzed for benzene at the two sites [14]. The samples were desorbed in 1.5 ml CS_2 and analyzed for benzene, toluene, and xylene by gas chromatography at both sites [26].

2.8. Statistical methods and data analysis

All analyses were conducted using SPSS [27] statistical software and SigmaPlot software was used for Figs. 1 and 2 [28]. The urinary metabolites were measured as a continuous variable but proved to be right skewed. A logarithmic transformation was therefore applied to these variables for analysis. One-way analysis of variance, including contrast to evaluate trends, was applied to compare multiple ordered exposure groups. Multiple regression was employed to analyze benzene exposure as a continuous variable. The benzene exposure variable was the personal measurement on the day the urine samples were collected, while the main outcome variables were the urine metabolite levels at the end of the workday and the after-work measurement minus the before-work measurement.

The benzene exposure data showed one individual with an exposure level more than twice as high as that of anyone else. This carried the potential to be highly influential in regression analysis; this influ-

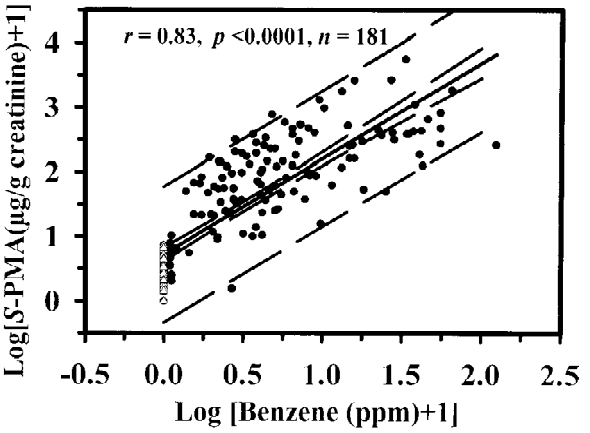

Fig. 1. Relation between urinary concentration of *S*-PMA and same day personal benzene exposure after logarithmic transformation (base 10) in 130 workers at the end of the shift (circles) and 51 unexposed workers (triangles). The solid line represent the average regression line and dashed lines the 95% CIs (inner lines on a group basis and outer lines on an individual basis).

ence was lessened by arbitrarily reassigning this high exposure value to be 20% greater than the next highest value. In addition, because the exposure data were right skewed, a semi-parametric analysis based on exposure ranks and outcome-variable ranks was conducted to see if it corroborated the parametric analyses.

The multiple regression analyses evaluated several covariates as potential confounders, including sex, age, cotinine levels and toluene exposure. These were routinely incorporated in the parametric analyses. Because of the right skewed nature of the cotinine and toluene values, logarithmic transformations of these variables were used in the analyses. In addition, sub-analyses were performed to evaluate the impact of potential confounding variables on the results.

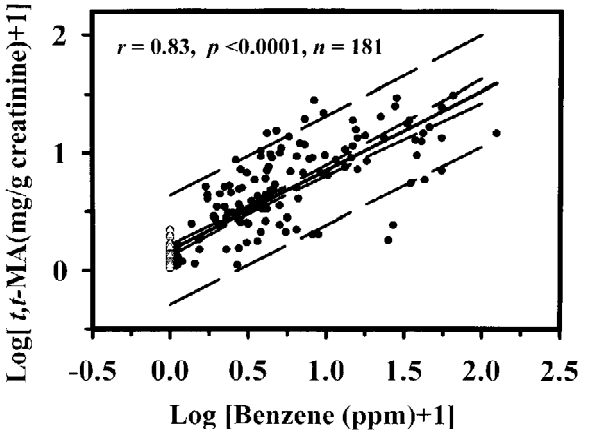

Fig. 2. Relation between urinary concentration of *t,t*-MA and same day personal benzene exposure after logarithmic transformation (base 10) in 130 workers at the end of the shift (circles) and 51 unexposed workers (triangles). The solid line represent the average regression line and dashed lines the 95% CIs (inner lines on a group basis and outer lines on an individual basis).

3. Results

3.1. Demographics of the study population

The characteristics of the benzene-exposed and unexposed study populations are summarized in Table 1. The study included 130 workers who were occupationally exposed to different levels of benzene and 51 unexposed control subjects from neighboring food-manufacturing industries. The percentage of women in the benzene-exposed group was 52% and that in unexposed group was 53%. The mean age (±standard deviation) of subjects in the exposed group was 36.3±7.6 years and in the unexposed group it was 33.3±7.4 years. Urinary cotinine was used as the indicator for smoking. Levels of urinary cotinine in several self-reported nonsmokers were relatively high. Therefore, any urine sample containing >100 μg cotinine/g creatinine (mean of two pre- and post-work urine sample analyses) was attributed to a smoker. The mean of urinary cotinine in the exposed group was 628±1177 μg/g creatinine, that in the unexposed group was 560±1125 μg/g creatinine. As shown in Tables 1 and 2 subjects in this study were exposed to a wide spectrum of benzene concentrations. The exposure distributions tended to be skewed, as shown by the large standard deviation (9.7 ppm±16.6) and discrepancies between the mean and median (9.7 and 3.2 ppm, Table 1). The mean for lifetime cumulative benzene exposure was 81.3±89.3 ppm-years, with a median of 51.2.

Table 2
Levels of urinary *S*-PMA, *t,t*-MA in benzene-exposed and unexposed workers[a]

Variable[b]	Unexposed	≤1 ppm	>1–5 ppm	>5–15 ppm	>15–30 ppm	>30 ppm
Number of subjects	51	25	57	26	8	14
Female (%)	53	76	44	38	75	57
Smokers (%)	31	12	49	42	38	36
Mean age (years)	33	36	37	36	37	34
Mean benzene exposure (ppm±SD)	0	0.34±0.32	2.56±1.10	9.12±3.37	22.6±4.0	49.1±23.2
t,t-MA (mg/g creatinine), pre-work[c]	0.34±0.58	0.61±0.77	1.06±0.68	2.48±3.52	6.31±7.57	5.39±7.03
t,t-MA (mg/g creatinine), post-work[c]	0.26±0.27	0.96±1.16	4.1±3.39	9.3±6.29	13.5±10.13	13.7±7.03
t,t-MA (mg/g creatinine), post–pre[c]	−0.08±0.62	0.35±0.73	3.06±3.14	6.85±5.60	7.13±8.97	8.30±5.97
S-PMA (μg/g creatinine), pre-work[c]	1.80±4.3	31.5±62.3	52.6±63.4	146±255	196±222	109±118
S-PMA (μg/g creatinine), post-work[c]	1.87±1.86	23.9±37.9	117±143	464±608	580±872	938±1441
S-PMA (μg/g creatinine), post–pre[c,d]	0.07±4.45	−7.7±32	65±119	317±366	384±868	829±1360
Cotinine (μg/g creatinine)[e]	560±1124	115±270	786±1216	901±1597	384±658	527±1134

[a] The means±SD of urinary benzene metabolites are from urine samples collected on the day when benzene exposure was monitored.
[b] For the urinary metabolites, the means and standard deviations reported are the raw variables, but the statistical tests were performed on the log transformed data.
[c] $P \leq 0.001$, test for exposure-response trend.
[d] Four samples at very low exposures showed higher levels of *S*-PMA pre-work than post-work.
[e] Mean of pre- and post-work values.

3.2. Urinary t,t-MA, and S-PMA in benzene-exposed and unexposed workers

Urinary creatinine was determined and used for adjusting the variations in concentration of all urine samples due to differences in liquid uptake between subjects. The mean concentration of urinary *S*-PMA in exposed workers ($n=130$) at the end of shift was 285.4±644.1 μg/g creatinine, ranging from 0.6 to 2700 μg/g creatinine, that in the control group ($n=51$) was 1.9±1.9 μg/g creatinine, ranging from 0.06 to 6.6 μg/g creatinine. The mean concentration of *t,t*-MA in the exposed group was 6.2±6.48 mg/g creatinine, ranging from 0.09 to 29.6 mg/g creatinine; and in unexposed volunteers it was 0.26±0.27 mg/g creatinine, ranging from 0.04 to 1.2 mg/g creatinine (Table 1). Figs. 1 and 2 show relationships of urinary *S*-PMA and *t,t*-MA, respectively, at the end of the shift from exposed workers (circles) and unexposed controls (triangles) with the same day personal benzene exposure after logarithmic transformation. The exposure-response trend was not adjusted for any possible confounding variables in these graphs. However, over this broad spectrum of exposures, ranging from not detectable to 122 ppm benzene, a highly significant correlation between *S*-PMA and benzene exposure, $r=0.83$, $n=181$, $P<0.0001$, and between *t,t*-MA and exposure, $r=0.83$, $n=181$, $P<0.0001$, was established. There was also a significant correlation between urinary *t,t*-MA and *S*-PMA after logarithmic transformation ($r=0.83$, $n=181$, $P<0.0001$).

Table 2 shows the urinary metabolites broken down into five subgroups with different levels of exposure, where the relevant benzene exposure was taken on the day of the urine collection. We had anticipated seeing a substantial number of subjects with benzene levels between zero and one ppm. However, after assessment of individual exposures we found that many workers (27%) were exposed to levels between 1 and 3 ppm benzene, only 13% were exposed to 0–0.25 ppm, and 6% were exposed to levels between 0.5 and 1 ppm benzene. In all subjects a distinct elevation in both *t,t*-MA and *S*-PMA was found in the samples collected at the end of the shift compared with levels before exposure (Table 2); however, four workers showed levels of *S*-PMA in the morning to be greater than at the end of the shift. This is reflected in the negative values shown in Table 2 in the subgroup that had exposure to between 0 and 1 ppm benzene. Table 3 shows the results for low levels of exposure <1 and <0.25 ppm. In a few cases, where exposure was 0.1 ppm or less, relatively high concentrations of *S*-PMA (up to 44 μg/g creatinine) or *t,t*-MA (up to 1.1 mg/g creatinine) were detected in the urine.

Table 3
Comparison of urinary metabolites in unexposed and low levels of benzene-exposed workers[a]

Variable	Unexposed $n=51$ (53% F)	0.06–0.25 ppm $n=17$ (94% F)	>0.5–1 ppm $n=8$ (38% F)	*P* For trend over range 0–1 ppm	*P* Unexposed vs. <0.25 ppm
Benzene (ppm)		0.14±0.11	0.76±0.17		
t,t-MA (mg/g creatinine)	0.26±0.27	0.36±0.34	2.25±1.24	0.006	
S-PMA (μg/g creatinine)	1.87±1.86	6.49±11.30	60.80±48.40	<0.0001	0.001
Cotinine (μg/g creatinine)	560±1124	64±180	222±396	0.01	

[a] Data from post-shift.

Fig. 3 displays mean levels of biotransformation of each ppm benzene to urinary *S*-PMA (black bars) and *t,t*-MA (white bars) in the five subgroups of Table 2 that are exposed to different levels of benzene. Fig. 3 indicates that metabolism of benzene to urinary *S*-PMA and especially that of *t,t*-MA decreases in subgroups with ascending level of benzene exposure. Figs. 4 and 5 show the inter-individual variation in transformation of each ppm benzene exposure to urinary *S*-PMA and *t,t*-MA, respectively, in three subgroups exposed to different levels of benzene. *X*-axes show the excretion of metabolites per ppm benzene exposure and *Y*-axes represent number of individuals with the same rate of transformation. The concentration of 12 μg/g creatinine *S*-PMA per ppm benzene exposure was frequent in all three subgroups of Fig. 4A–C. Whereas, Fig. 5A–C demonstrate that transformation of 1 ppm benzene to 1.3 mg *t,t*-MA/g creatinine is frequent in individuals in the subgroup with relatively low exposure (0.06–5 ppm benzene), and this value decreases to 0.4 mg *t,t*-MA/g creatinine in the high exposure subgroup (15–122 ppm).

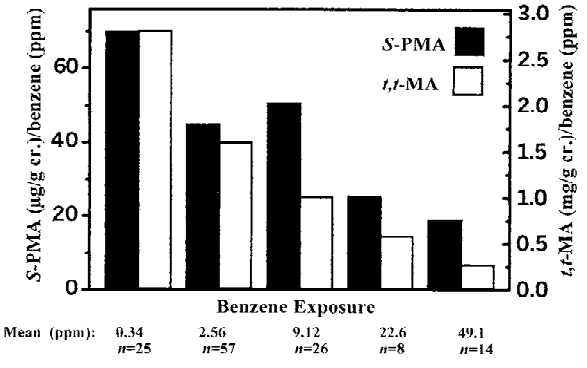

Fig. 3. Levels of bio-transformation of each ppm personal benzene exposure to urinary *S*-PMA (black bars) and *t,t*-MA (white bars) in exposed workers broken-down into the five subgroups based on the levels of exposure. The mean are of benzene exposure and number of workers in each subgroup are shown in *X*-axes.

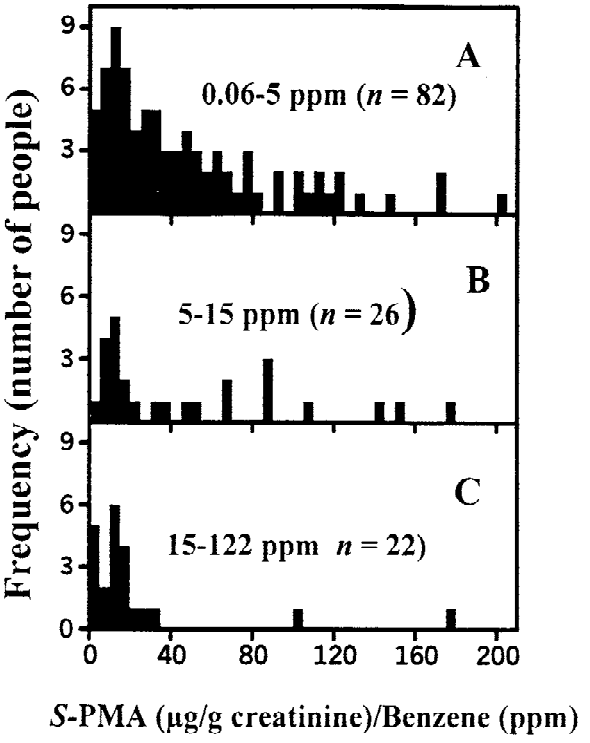

Fig. 4. Inter-individual variation in conversion of 1 ppm personal benzene exposure to urinary *S*-PMA at the end of the shift in subgroups of 82 workers exposed to 0.06–5 ppm (A); 26 workers exposed between 5 and 15 ppm (B); and 22 workers exposed between 15 and 122 ppm benzene.

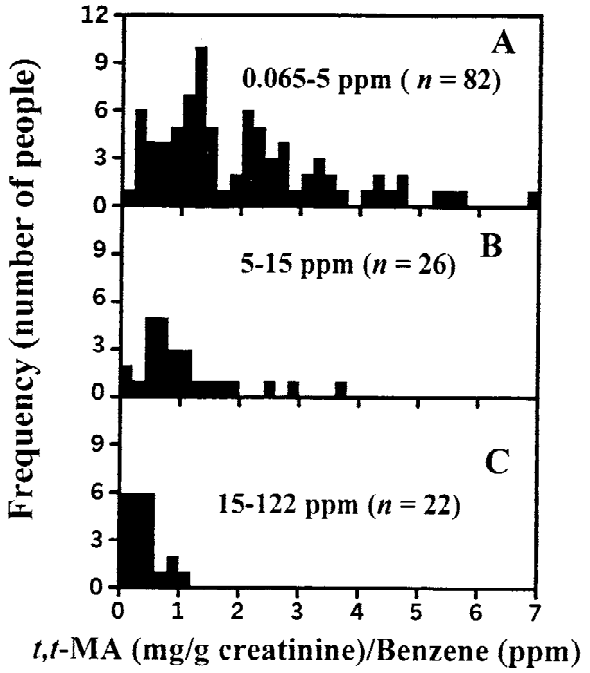

Fig. 5. Inter-individual variation in conversion of 1 ppm personal benzene exposure to urinary *t,t*-MA at the end of the shift in 82 workers exposed to between 0.06 and 5 ppm (A); 26 workers exposed to between 5 and 15 ppm (B); and 22 workers exposed to between 15 and 122 ppm benzene.

3.3. The effect of gender, smoking status and toluene exposure on activation of benzene to urinary metabolites

Women excreted more metabolites than did men for the same levels of benzene exposure. For the post-work urine sample, the *S*-PMA benzene regression coefficient±SE in males was 0.054±0.012, and in females it was 0.104±0.012 (interaction $P<0.0001$). The corresponding value for *t,t*-MA in males was 0.023±0.006 and that in females was 0.056±0.006 (interaction $P<0.0001$). The interactive effects of smoking were examined both across the entire exposure and among relatively low exposures <5 ppm. The results are difficult to interpret because the direction of interactions was about evenly divided between a greater effect among smokers and nonsmokers. Furthermore, there was little consistency between results across the exposure range and the corresponding results in the lower dose range only. For toluene interaction, only five out of 37 variables analyzed showed evidence of interaction at a conventional level of significance ($P<0.05$), and there did not seem to be any consistent pattern of the high- or low-toluene exposure group showing a greater effect.

4. Discussion

Previously, we had validated the use and predictive abilities of six urinary benzene metabolites including *t,t*-MA, *S*-PMA, 1,2,4-trihydroxybenzene, hydroquinone, catechol, and phenol as biomarkers of exposure to benzene in humans [14]. These biomarkers were evaluated for their sensitivity, replicability, and the elimination half-lives in 25 subjects exposed to relatively high concentrations of benzene, and in 25 controls. The result indicated that ring-hydroxylated urinary benzene metabolites are not reliable markers for benzene exposure at low levels due to their high background levels even though hydroquinone and catechol correlated well with exposures in the range of 6–122 ppm. On the other hand, our results indicate that among the observed biomarkers *S*-PMA and *t,t*-MA were the most specific and sensitive indicators of benzene exposure. Here we expand our investigation and report validation of sensitive biomarkers for low levels, the exposure–response relationships, and effects of the exposure dose on biotransformation to the urinary metabolites. In this study, urinary *t,t*-MA and *S*-PMA were determined by LC–tandem mass spectrometry using $^{13}C_6$-*t,t*-MA and $^{13}C_6$-*S*-PMA as internal standards. We have shown that from a laboratory standpoint this method is a practical, sensitive, and specific assay for the determination of these analytes in humans [13,24].

The results of this study indicate that both *S*-PMA and *t,t*-MA, but especially the former correlate well with personal exposures in a broad spectrum of exposures as shown in Figs. 1 and 2. There is also a good correlation with both metabolites (*P*-trend is <0.0001 for *S*-PMA and 0.006 for *t,t*-MA) in the subgroup that was exposed to <1 ppm benzene (Table 3). In fact, the correlation is again significant in the unexposed vs. the >0 to 0.25 ppm exposure subgroup for *S*-PMA ($P=0.001$) (Table 3).

The mean concentrations of excreted urinary *S*-PMA and of *t,t*-MA at the end of the shift for each ppm benzene exposure obtained from the entire exposed group were 22.4 μg *S*-PMA/g creatinine and 0.62 mg *t,t*-MA/g creatinine (Figs. 1 and 2). However, these values varied when the exposure group was broken down into the five subgroups with different exposure levels. In subgroups of workers who had a mean exposure to 0.34-, 2.56-, 9.12-, 22.6- and 49.1-ppm benzene, levels of urinary *S*-PMA per ppm benzene exposure were 70.2-, 45.7-, 50.8-, 25.6-, and 19.1-μg *S*-PMA/g creatinine; levels of transformation to *t,t*-MA per ppm benzene exposure were 2.8- 1.6- 1.02-, 0.59-, and 0.28-mg *t,t*-MA/g creatinine at the end of the shift. The possible reasons why metabolism of benzene is lower at higher exposures are: there may be less uptake of benzene from inhaled air; it may also be that metabolic activation of benzene becomes saturated at higher doses. In a study where workers were exposed to less than 5 ppm benzene, Boogaard and van Sittert have reported that exposure to 1 ppm benzene (8 h TWA) will lead to an average excretion of 1.74 mg *t,t*-MA/g creatinine and 47 μg *S*-PMA/g creatinine [10]. Bechtold and coworkers also reported that in a group of 14 Chinese female workers who were exposed to 4.4 ppm benzene (8 h TWA), 1.4 mg *t,t*-MA/g creatinine for each ppm exposure were excreted at the end of the shift [16]. These values are in agreement with current data for the subgroup whose mean of personal benzene exposure was 2.56 ppm.

Benzene is metabolically activated by CYP 2E1 to benzene epoxide, and detoxification of benzene epoxide by GSH leads to the excretion of minor amounts of urinary *S*-PMA. As shown in Fig. 4A–C, there is inter-individual variation in the biotransformation of inhaled benzene to urinary *S*-PMA. From Fig. 4A–C we estimated that, at the end of shift, the fraction of benzene that is converted to *S*-PMA ranges between 0.005 and 0.3%. Gittori et al. recently reported that the biotransformation of benzene to *S*-PMA in smokers is significantly variable from 0.01 to 0.21% [12]. Boogaard and van Sittert also reported that in exposed workers the conversion of benzene to *S*-PMA ranged from 0.05 to 0.26% [10].

Similarly, there is inter-individual variation in the rate of conversion of benzene into *t,t*-MA at the end of the shift, ranging from 0.6 to ~20% in 8-h inhaled benzene (Fig. 5). Biotransformation of benzene into *t,t*-MA decreases remarkably with ascending concentration of benzene exposure (Figs. 3 and 5A–C). Fig. 5A indicates that in exposures <5 ppm the transformation rate of 1.3 mg/g creatinine per each ppm exposure or 4.1% benzene exposure was frequent. This value decreases to 0.96% in the subgroup exposed to >15 ppm benzene (Fig. 5C). This is in agreement with the finding that a greater percentage of benzene is metabolized to *t,t*-MA at lower doses of exposure in laboratory animal model studies [29–31]. Similar results were reported previously in studies of environmental tobacco smoke [20]. These observations suggest that the determination of *t,t*-MA alone may not accurately reflect the personal exposure in workplaces where people are exposed to high concentrations of benzene. In addition, *t,t*-MA may also not be a sufficiently specific biomarker of low exposures because it is known that sorbic acid, a food preservative and fungistatic agent, is metabolized to *t,t*-MA and excreted in urine [21,22,32,33]. The uptake of sorbic acid is about 25 mg/day in the United States [20] and 6–30 mg/day in Europe [22]. Biotransformation rates of sorbic acid into *t,t*-MA are estimated to be in the range of 0.05 to 0.51% [33,22]. The inter-individual variability of the conversion rates of sorbic acid into *t,t*-MA might constitute another source of uncertainty in the interpretation of urinary levels of *t,t*-MA.

There was gender interaction in metabolism of benzene in that women excreted more metabolites than men did. However, only one out of 23 variables showed significant interaction for chromosomal damage (data not shown). Toluene is a known competitive inhibitor of benzene metabolism, because it is metabolized by the same Cytochrome P450 2E1 and thus reduces benzene metabolism [34–36]. Toluene does not seem to play an appreciable role in the activation of benzene to urinary metabolites in the current study.

In conclusion, our data show that the determination of *t,t*-MA and *S*-PMA by LC–MS is sensitive and has the ability to reflect low levels of benzene exposures. The evaluation of these metabolites has a promising role in the monitoring of benzene exposure. However, decreasing levels of *t,t*-MA excretion

with increasing levels of benzene exposure, and the fact that sorbic acid uptake may be contributing to the excretion of *t,t*-MA, lower the degree of specificity of this metabolite as a marker of benzene exposure and makes *S*-PMA the superior biomarker at low benzene exposures.

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