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Rapid determination of six urinary benzene metabolites in occupationally exposed and unexposed subjects

Suramya Waidyanatha,[a] Nathaniel Rothman,[b] Guilan Li,[c] Martyn T. Smith,[d] Songnian Yin,[c] and Stephen M. Rappaport[a,*]

[a] *Department of Environmental Sciences and Engineering, School of Public Health, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7431, USA*
[b] *Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD 20892, USA*
[c] *Chinese Academy of Preventive Medicine, Institute of Occupational Medicine, Beijing, China*
[d] *School of Public Health, University of California, Berkeley, CA 94720, USA*

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Abstract

A gas chromatography-mass spectrometry method for measurement of the main urinary metabolites of benzene, namely, phenol, catechol, hydroquinone, 1,2,4-trihydroxybenzene (trihydroxybenzene), *t*,*t*-muconic acid (muconic acid), and *S*-phenylmercapturic acid (phenylmercapturic acid), is reported. The method is considerably simpler than existing assays. It was applied to urine from benzene-exposed subjects and controls from Shanghai, China. When subjects were divided into controls ($n = 44$), those exposed to $\leqslant$31 ppm benzene ($n = 21$), and those exposed to >31 ppm benzene ($n = 19$), Spearman correlations with exposure category were $\geqslant$0.728 ($p < 0.0001$) for all metabolites except trihydroxybenzene. When exposed subjects were compared on an individual basis, all metabolites, including trihydroxybenzene, were significantly correlated with benzene exposure (Pearson $r \geqslant 0.472$, $p \leqslant 0.002$) and with each other (Pearson $r \geqslant 0.708$, $p < 0.0001$). Ratios of individual metabolite levels to total metabolite levels provided evidence of competitive inhibition of CYP 2E1 enzymes leading to increased production of phenol, catechol, and phenylmercapturic acid at the expense of hydroquinone, trihydroxybenzene, and muconic acid. Since all metabolites were detected in all control subjects, the method can be applied to persons exposed to environmental levels of benzene.

Benzene is an important industrial chemical with a worldwide production of about 13.6×10^6 metric tons in 1992 [1]. As a prominent constituent of gasoline (currently regulated at 1% in the United States) [2], effluents of organic combustion [2], and tobacco smoke (about 87 μg/cigarette) [3], benzene is a ubiquitous contaminant in the environment. Although benzene has long been associated with hematotoxicity and leukemia in humans [4–6], its metabolism is complex and the particular mechanism(s) by which benzene exerts these effects is not well understood. It has been postulated that two or more benzene metabolites act in concert to produce toxic and carcinogenic effects [5,7,8].

Following exposure, about 20% of the absorbed benzene dose is eliminated passively in breath and urine [9] and the remaining 80% is metabolized via CYP 2E1 to benzene oxide–oxepin [10–13]. As shown in Fig. 1, benzene oxide–oxepin is further metabolized via both enzymatic and nonenzymatic pathways to numerous products, including phenol, hydroquinone, catechol, *t*,*t*-muconic acid (muconic acid), 1,2,4-trihydroxybenzene (trihydroxybenzene), and *S*-phenylmercapturic acid (phenylmercapturic acid), which are eliminated in urine either unchanged or as sulfate and glucuronide conjugates [5,13]. Formation of three of these products from benzene oxide–oxepin (hydroquinone, muconic acid, and trihydroxybenzene) requires a second CYP 2E1 oxidation.

Most of the urinary metabolites of benzene and unmetabolized benzene in urine have been extensively investigated as biological markers of exposure to benzene [14–25]. While most of these biomarkers are highly correlated with benzene, exposure above 5–10 ppm

* Corresponding author. Fax: 1-919-966-0521.
E-mail address: smr@unc.edu (S.M. Rappaport).

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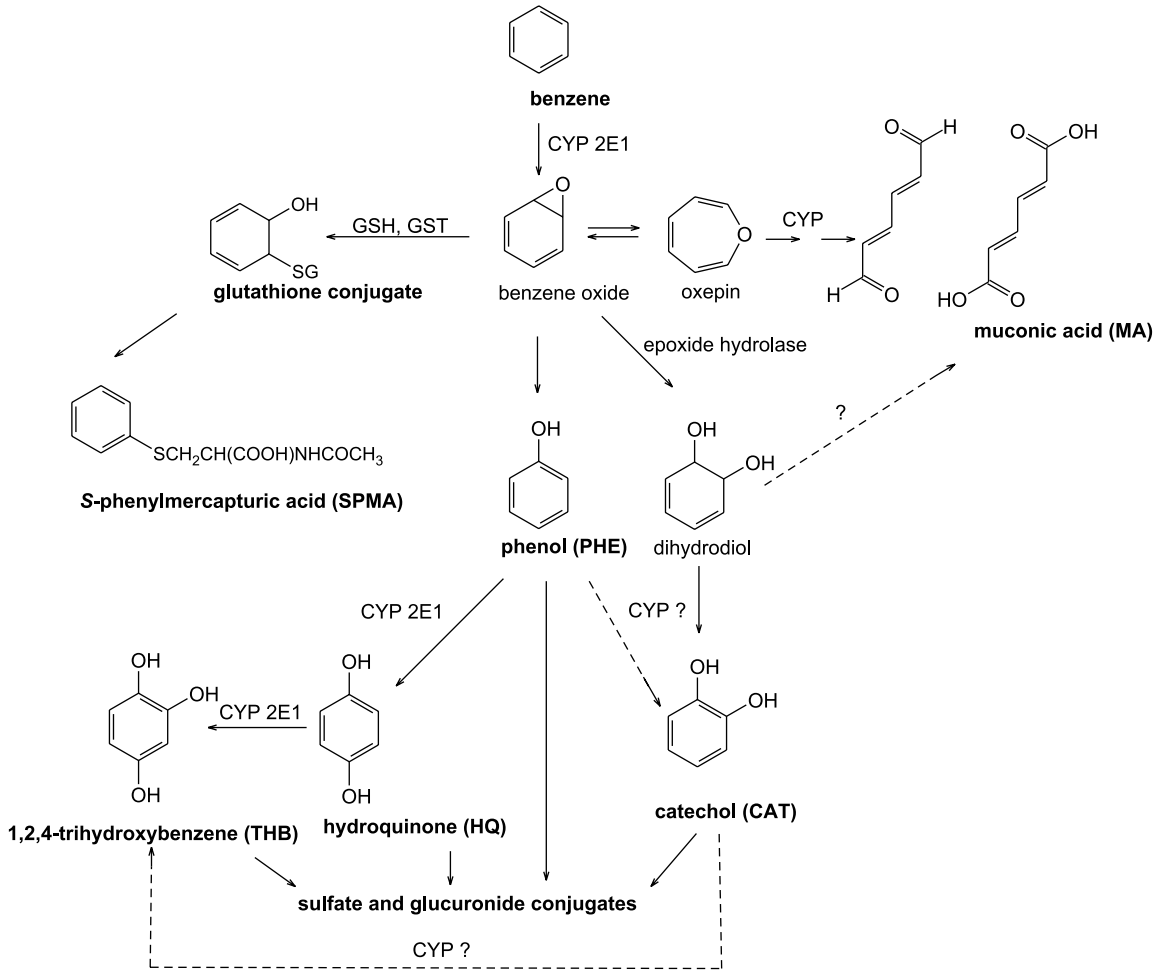

Fig. 1. Proposed metabolic pathways of benzene leading to the formation of urinary metabolites.

benzene in air, phenol, catechol, and hydroquinone have proven unreliable as biological markers at lower exposures, due to the presence of background levels of the same compounds arising from the diet, cigarette smoking, over-the-counter medicines, and endogenous sources [26–31]. Background levels of muconic acid have also been reported, being attributed to sorbic acid (a food additive), cosmetics, and pharmaceutical products [32,33]. Trihydroxybenzene has not received much attention as a biomarker, due either to the presence of high background levels or to the unavailability of suitable assays [15,34]. Hence, of all urinary biomarkers of benzene, phenylmercapturic acid and urinary benzene are regarded the most specific to benzene exposure [15,17,21,22,25,35].

Aside from applications involving biomonitoring, all urinary metabolites of benzene offer utility in understanding the metabolism of benzene at different levels of exposure. Thus, it is often desirable to measure most or all of these products in subjects exposed to a range of benzene levels. Numerous HPLC, GC-MS, and LC-MS-MS methods and immunoassays that measure either individual (phenol, phenylmercapturic acid, muconic acid, trihydroxybenzene) [15,16,32,34,36–40] or multiple (phenol, catechol, hydroquinone, and muconic acid or muconic acid and phenylmercapturic acid) [14,35,41] metabolites have been reported. However, no single assay is currently capable of quantitating all urinary metabolites of benzene. Furthermore, existing assays have limitations, namely, lack of specificity [39], undue complexity [41–43], and use of hazardous chemicals [40].

Here we report a simple GC-MS method to quantitate the six urinary metabolites of benzene in 0.5 ml of urine. We applied the assay to 42 benzene-exposed subjects (range 1.65–329 ppm) and 44 control subjects from a study of benzene biomarkers conducted in Shanghai, China [14,44–46].

Materials and methods

Chemicals

Phenol (99%+, redistilled), muconic acid (98%), trihydroxybenzene, and [$^2H_6$]phenol were obtained from

Aldrich Chemical (Milwaukee, WI). Catechol (99%) and hydroquinone (>99%) were obtained from Fluka Chemical (Switzerland). [$^2H_6$]catechol (98%) and [$^2H_6$]hydroquinone (98%) were from Cambridge Isotope Laboratories (Woburn, MA). Phenylmercapturic acid, Tri-Sil reagent, and conc. hydrochloric acid (Optima grade) were from TCI America (Portland, OR), Pierce (Rockford, IL), and Fisher Scientific (Pittsburgh, PA), respectively. Ethyl acetate (analytical reagent grade) and hexane (nanograde) were from Mallinckrodt Baker (Paris, KT). Anhydrous Na_2SO_4 was from J.T. Baker (Phillipsburg, NJ). 2,5-[$^{13}C_2$]Muconic acid and [$^2H_5$]phenylmercapturic acid were kindly provided by Drs. Avram Gold and Ramiah Sangaiah from The University of North Carolina at Chapel Hill.

Subjects, urine collection, and exposure assessment

Details of the study and subjects can be found elsewhere [25,44,45]. Briefly, 44 benzene-exposed workers were selected from three factories in Shanghai, China where benzene was used as a solvent to solubilize natural rubber, in the manufacture of adhesive tape, and to solubilize paint and varnish. Forty-four controls, frequency-matched to exposed subjects by age and gender, were selected from a sewing machine manufacturing plant and an administrative facility in the same geographic area.

Of 44 benzene-exposed subjects, 43 provided a spot urine sample at the end of the work shift. Control subjects provided a spot urine sample during the clinical phase of the study. Samples were aliquoted immediately after collection, transported on dry ice to the United States, and stored at −80 °C. After about 8 years of storage, benzene metabolites were measured in urine from 42 exposed workers and 44 controls.

Individual exposures were monitored using passive personal monitors worn by each worker for a full work shift on 5 consecutive workdays during the 1- to 2-week period prior to urine collection. The geometric mean (GM)[1] exposure of the five air measurements was used to estimate the overall exposure of each worker to benzene (note that 1 ppm = 3.2 mg benzene/m^3 air). Overall, the workers from this study were exposed to a median value of 31 ppm benzene [45]. Control subjects in the sewing machine factory were monitored for exposure to benzene with passive monitors on 1 day only; 3 of these subjects had exposures above the detection limit of 0.016 ppm (0.047, 0.052, and 0.110 ppm) and their GM exposures were set equal to these single measurements. The other sewing factory workers and all subjects from the administrative department, who had no exposure measurements, were assigned a GM exposure of 0.016 ppm (the detection limit) for statistical purposes. Individual exposures were also monitored in 37 of the 44 exposed workers on the same day that urine was collected.

Analysis of urinary benzene metabolites

The procedure used to quantitate urinary metabolites of benzene is outlined in Fig. 2. To 0.5 ml of urine in a 4-ml vial, was added 10 µl of a mixture containing 1.25 µg/µl [$^2H_6$]phenol, 0.625 µg/µl [$^2H_6$]catechol, 0.625 µg/µl [$^2H_6$]hydroquinone plus 10 µl of 0.05 µg/µl [$^2H_5$]phenylmercapturic acid, and 25 µl of 0.1 µg/µl [$^{13}C_2$]muconic acid (internal standards), all in ethyl acetate. After adding 50 µl of conc. HCl, the mixture was extracted with 1.5 ml of ethyl acetate to remove muconic acid, phenylmercapturic acid and free phenol, hydroquinone, trihydroxybenzene, and catechol and the organic layer was transferred to another 4-ml vial. Traces of ethyl acetate were removed from the aqueous layer under a gentle stream of N_2, and a second 10-µl portion of the phenolic internal standard mixture was added to standardize the conjugated phenolic metabolites. Then the aqueous layer was heated at 100 °C for 1 h to hydrolyze all phenolic conjugates for subsequent extraction. After cooling to room temperature, the aqueous layer was extracted with 1.5 ml of ethyl acetate and the two ethyl acetate layers were combined and dried with anhydrous Na_2SO_4. The volume of ethyl acetate was reduced under N_2, transferred to a 500-µl flat-bottomed insert, and brought to dryness under N_2. To the residue, 100 µl hexane was added and the mixture was derivatized with 100 µl Tri Sil reagent at 70 °C for 30 min.

Use of the Tri-Sil reagent is based on the procedure of Sweeley et al. [47] for the optimal conversion of organic hydroxylated and polyhydroxylated compounds and carboxylic acids to trimethylsilyl- (TMS-)ethers and esters. As shown in Fig. 3, this derivatization step converts all benzene metabolites and internal standards to the corresponding TMS-ethers and TMS-esters before GC-MS analysis. Due to the presence of two Si atoms, we observed an 8.1% contribution from TMS-muconic acid (*m/z* 271) to TMS-[$^{13}C_2$]muconic acid (*m/z* 273) (the theoretical contribution is 7.1% for M + 2 when two Si atoms are present). Thus, we subtracted 8.1% of the abundance of TMS-muconic acid from [$^{13}C_2$]TMS-muconic acid before final quantitation. No significant contributions to TMS derivatives of deuterated internal standards were observed from the corresponding ^{12}C analogues of phenol, catechol, hydroquinone, and phenylmercapturic acid. Phenylmercapturic acid levels were quantifiable only in 25 of the 44 controls due to

[1] *Abbreviations used:* EI, electron ionization; GC-MS, gas chromatography-mass spectrometry; GM, geometric mean; muconic acid, *t,t*-muconic acid; *r*, Pearson correlation coefficient; r_s, Spearman correlation coefficient; trihydroxybenzene, 1,2,4-trihydroxybenzene; phenylmercapturic acid, *S*-phenylmercapturic acid; TMS-, trimethylsilyl derivative.

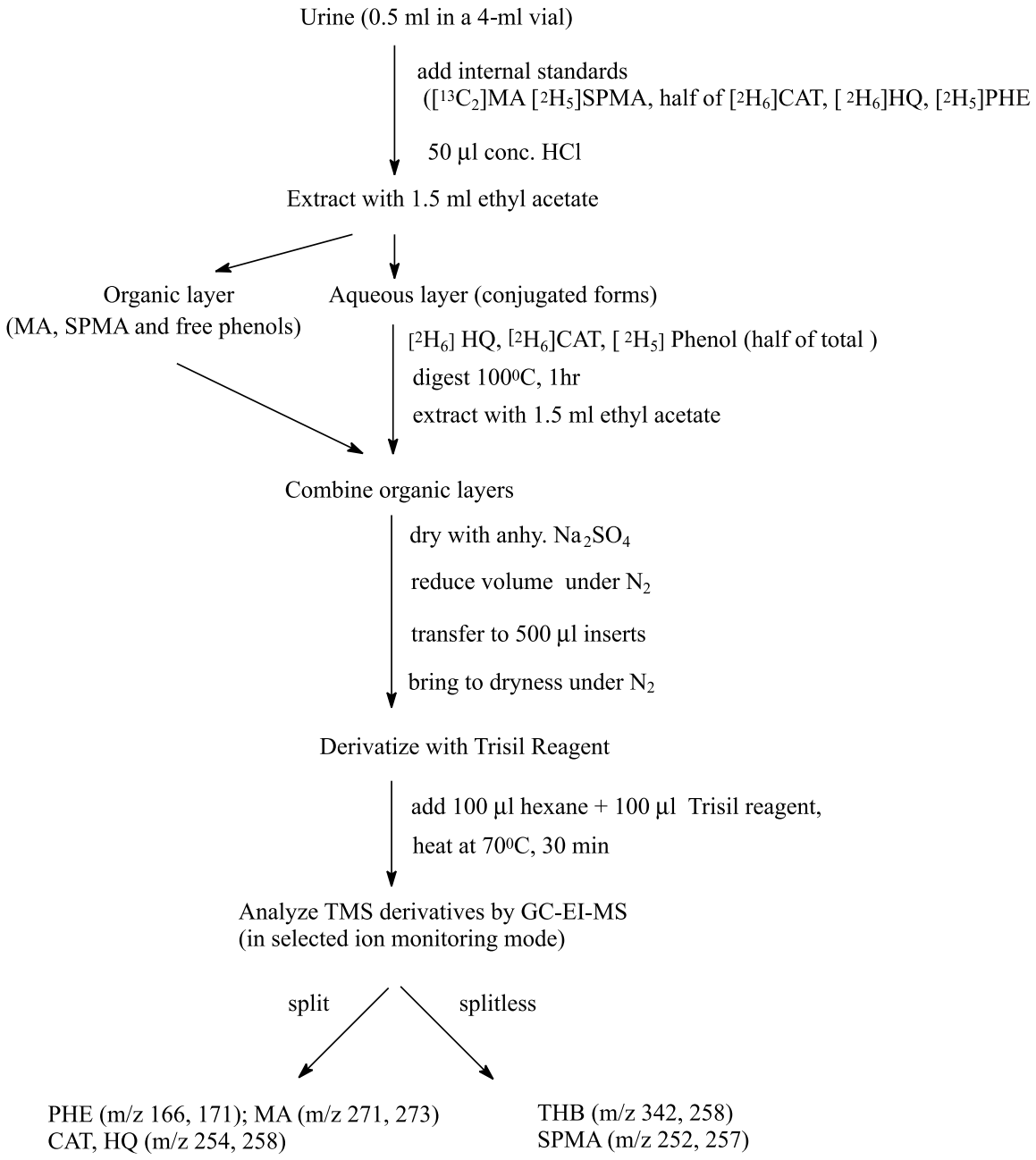

Fig. 2. Scheme for quantitation of six urinary metabolites of benzene. PHE, phenol; CAT, catechol; HQ, hydroquinone; SPMA, *S*-phenylmercapturic acid; THB, 1,2,4-trihydroxybenzene; MA, *t*,*t*-muconic acid; TMS-, trimethylsilyl derivative.

coelution of TMS-[$^2H_5$]phenylmercapturic acid with the TMS-derivative of 6-hydroxychlorzoxazone, a metabolite of chlorzoxazone which had been administered to 19 control subjects (to estimate CYP 2E1 activity) during the study [44].

For quality control purposes, human urine from unexposed volunteers was pooled and spiked with analytes to give final concentrations of phenol, catechol, hydroquinone, trihydroxybenzene, and muconic acid at 5 mg/L and phenylmercapturic acid at 1 mg/L (2- to 3-ml aliquots were stored in 4-ml vials at −80 °C). These were analyzed with each batch of samples to serve as controls.

GC-MS analysis

Samples were analyzed by GC-EI-MS using a HP 5980 Series II gas chromatograph coupled to a HP 5971-A mass selective detector. The injector, MS transfer-line, and ion source temperatures were 270, 280, and 186 °C, respectively, and the EI electron energy was 70 eV. A DB-5 fused silica capillary column (60 m, 0.25 mm i.d., 0.25 µm film thickness) was used with He as the carrier gas at a flow rate of 1.5 ml/min.

Since levels of phenol, catechol, hydroquinone, and muconic acid were high, even in control subjects, 1-µl

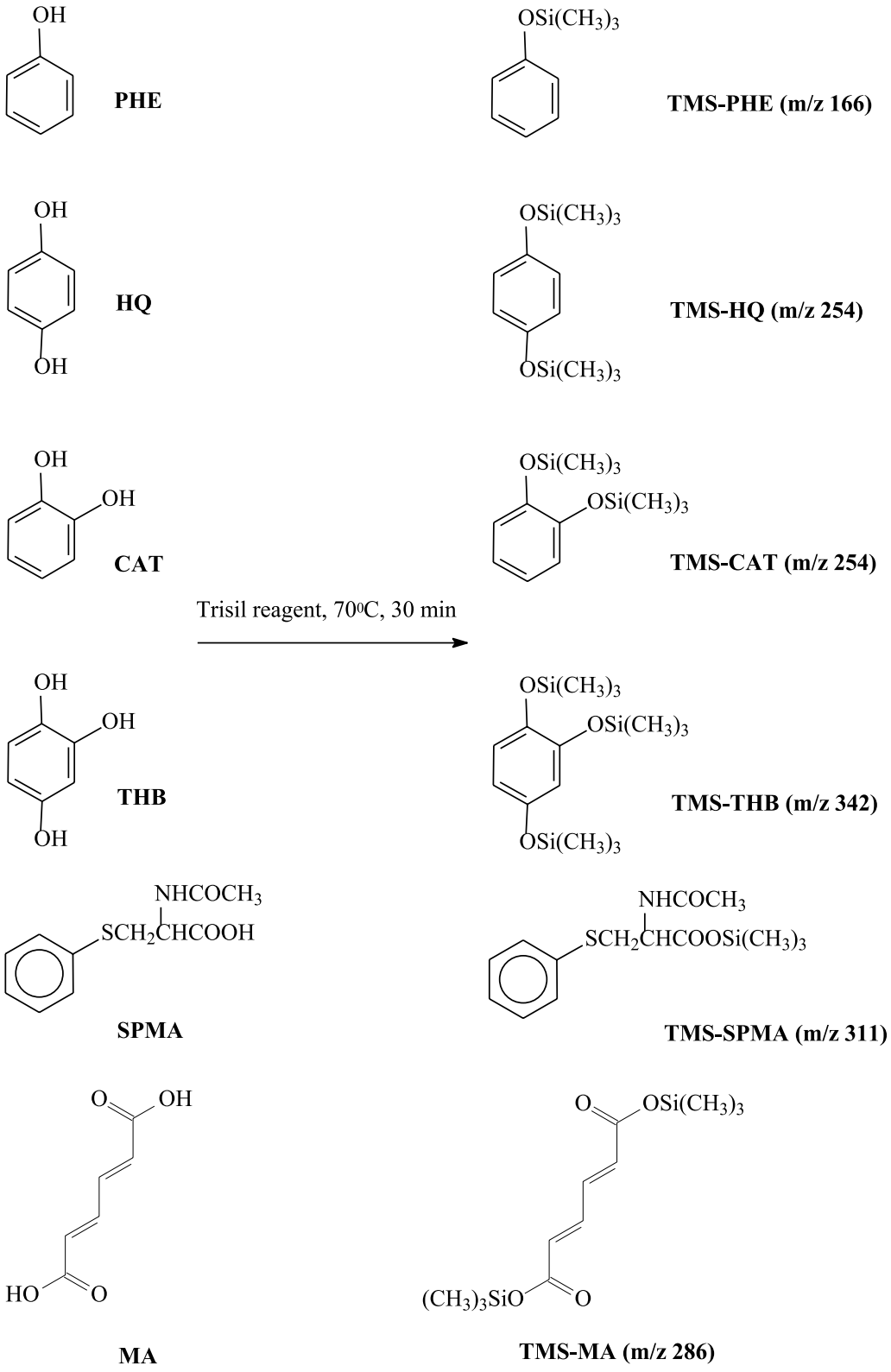

Fig. 3. Structures of benzene metabolites and their corresponding trimethylsilylated derivatives. TMS-, trimethylsilyl derivative; PHE, phenol; CAT, catechol; HQ, hydroquinone; SPMA, *S*-phenylmercapturic acid; THB, trihydroxybenzene; MA, *t,t*-muconic acid.

injections were made in split mode with a split ratio of 1:20 for quantitation of these analytes. The GC oven was held at 75 °C for 4 min and then ramped at 10 °C/min to 230 °C where it was held for 5 min. Since levels of trihydroxybenzene and phenylmercapturic acid were low, particularly in control subjects, 2-μl injections were

made in splitless mode for these analytes, with the GC oven held at 75 °C for 4 min. The temperature was then ramped at 10 °C/min to 245 °C where it was held for 10 min. In both cases, late-eluting compounds were removed by raising the oven temperature to 270 °C and holding there for 12 min.

To aid in the confirmation of each analyte, two characteristic ions were monitored based upon their abundance. (For muconic acid only one abundant ion was available.) The following characteristic ions were monitored: TMS-phenol [*m/z* 166, M^+; *m/z* 151, $(M\text{-}CH_3)^+$], TMS-[$^2H_5$]phenol [*m/z* 171, M^+; *m/z* 156, $(M\text{-}CH_3)^+$], TMS-catechol and TMS-hydroquinone [*m/z* 254, M^+; *m/z* 239, $(M\text{-}CH_3)^+$], TMS-[$^2H_4$]catechol and TMS-[$^2H_4$]hydroquinone [*m/z* 258, M^+; *m/z* 243, $(M\text{-}CH_3)^+$], TMS-muconic acid [*m/z* 271, $(M\text{-}CH_3)^+$], TMS-[$^{13}C_2$] muconic acid [*m/z* 273, $(M\text{-}CH_3)^+$], TMS-trihydroxybenzene [*m/z* 342, M^+; *m/z* 239, $(M\text{-}HOSi(CH_3)_3\text{-}CH_3)^+$], TMS-phenylmercapturic acid [*m/z* 311, M^+; *m/z* 252, $(M\text{-}NH_2COCH_3)^+$], and TMS-[$^2H_5$]phenylmercapturic acid [*m/z* 316 M^+; *m/z* 257, $(M\text{-}NH_2COCH_3)^+$]. The following ions were used for quantitation: *m/z* 166, TMS-phenol; *m/z* 171, TMS-[$^2H_5$]phenol; *m/z* 254, TMS-catechol and -hydroquinone; *m/z* 258, TMS-[$^2H_4$]hydroquinone and TMS-[$^2H_4$]catechol; *m/z* 271, TMS-muconic acid and TMS-[$^{13}C_2$]muconic acid; *m/z* 252, TMS-phenylmercapturic acid; *m/z* 257, [$^2H_5$]phenylmercapturic acid; and *m/z* 343, TMS-trihydroxybenzene (Fig. 3). The retention times were, respectively, 12.99, 12.95, 18.01, 17.98, 19.20, 19.18, 22.32, and 22.32 min for TMS-phenol, -[$^2H_5$]phenol, -catechol, -[$^2H_4$]catechol, -hydroquinone, -[$^2H_4$]hydroquinone, -muconic acid, and -[$^{13}C_2$]muconic acid following split injection and 18.01, 22.14, 29.95, and 29.91 for TMS-[$^2H_4$]catechol, -trihydroxybenzene, -phenylmercapturic acid, and -[$^2H_5$]phenylmercapturic acid following splitless injection.

Based on the mass spectrometric analysis of final TMS-derivatives, no proton exchange of [$^2H_5$]phenol, [$^2H_6$]catechol, and [$^2H_6$]hydroquinone was observed to give the corresponding protonated analogues of phenol, catechol, and hydroquinone, under the acidic conditions used in the assay.

Standard calibration curves

Stock solutions of standards and internal standards were prepared in ethyl acetate. Standards were prepared over the ranges of 0–100 mg/L for phenol, catechol, and hydroquinone, 0–50 mg/L for muconic acid, and 0–5 mg/L for trihydroxybenzene and phenylmercapturic acid. Standard calibration curves were prepared by spiking 0.5-ml portions of human urine from an unexposed volunteer with internal standards and standards and then carrying them through the assay as described for samples.

Final concentrations of internal standards in urine were 50, 25, 25, 5, and 1 mg/L for [$^2H_5$]phenol, [$^2H_6$]catechol, [$^2H_6$]hydroquinone, [$^{13}C_2$]muconic acid, and [$^2H_5$]phenylmercapturic acid, respectively. Quantitation was based on peak areas relative to the corresponding isotopically labeled internal standards except for trihydroxybenzene where the quantitation was based on peak areas relative to [$^2H_6$]catechol, monitored during the same injection.

Linearity, precision, and limits of detection

The linearity of the assay was evaluated with 0.5-ml aliquots of urine spiked with phenol, catechol, and hydroquinone over the range 0–100 mg/L, muconic acid over the range 0–50 mg/L, and trihydroxybenzene and phenylmercapturic acid over the range 0–5 mg/L. The precision of the assay was estimated by analysis of duplicate samples from 17 exposed and control subjects. The limits of detection for trihydroxybenzene, muconic acid, and phenylmercapturic acid were estimated by spiking 0.5 ml of urine from an unexposed person with analytes to give final concentrations of 0.005–0.05 mg/L and carrying the samples through the assay. Limits of detection were not estimated for phenol, catechol, and hydroquinone since there were high background levels of these analytes in control subjects.

Adjustment for urinary creatinine

Urinary metabolite levels are generally adjusted for urinary creatinine in an effort to control for urine volume at the time of collection. However, recent reports have questioned such adjustments because urinary creatinine is influenced by age, gender, diet, and other physiological factors and because measurement of creatinine introduces additional random error to analyte levels [48–52]. Thus, for most applications, we regard creatinine-adjusted metabolite levels as less reliable than unadjusted levels and report only unadjusted metabolite levels (mg/L of urine) in this study. For comparison of our data with other published reports, we assumed an average creatinine value of 1.53 g creatinine/L of urine, based on the averages from our laboratory [51,52] and from the literature [20,53].

Measures of cigarette consumption

The average number of cigarettes smoked per day during the past month was obtained from subjects via questionnaire (median = 10, range = 4–10). Measurements of urinary cotinine, a metabolite of nicotine, were found to be highly correlated with self-reported cigarette consumption in our subjects (Spearman $r_s = 0.86$, $p < 0.0001$) [45].

Statistical analysis

Subjects were divided into three exposure categories, namely controls, those with GM exposures less than or

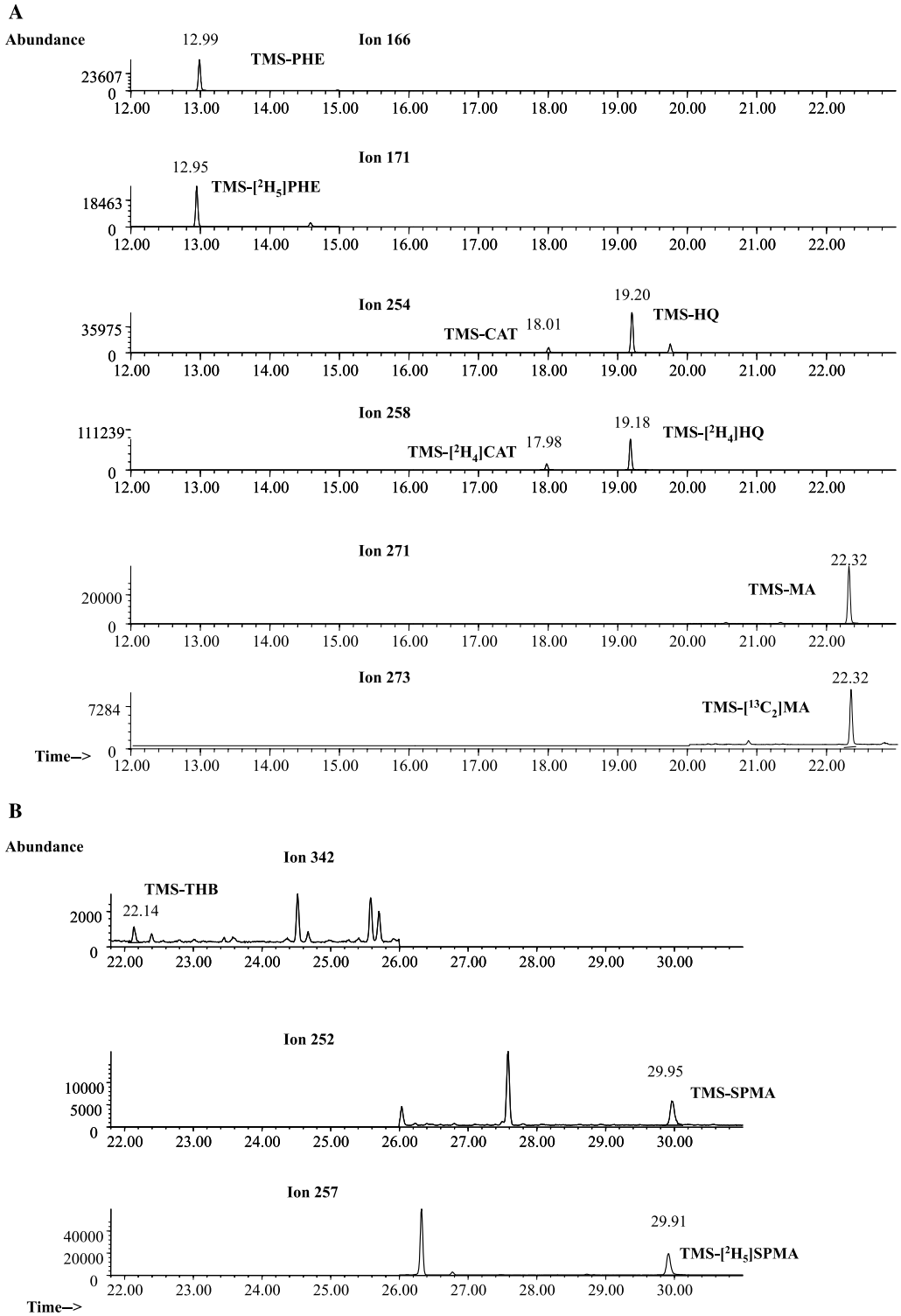

Fig. 4. GC-EI-MS selected-ion-monitoring chromatogram for 0.5 ml urine from a worker exposed to 14.04 ppm benzene. (A) Split injection; (B) Splitless injection. The following ions were monitored for trimethylsilyl (TMS) derivatives of phenol (PHE) (*m/z* 166): [$^2H_5$]phenol ([$^2H_5$]PHE) (*m/z* 171), catechol (CAT) and hydroquinone (HQ) (*m/z* 254), [$^2H_6$]catechol ([$^2H_6$]CAT) and hydroquinone ([$^2H_6$]HQ) (*m/z* 258), 1,2,4-trihydroxybenzene (THB) (*m/z* 342), muconic acid (MA) (*m/z* 271), [$^{13}C_2$]*t*,*t*-muconic acid ([$^{13}C_2$]MA) (*m/z* 273), *S*-phenylmercapturic acid (SPMA) (*m/z* 252), and [$^2H_5$]*S*-phenylmercapturic acid ([$^2H_5$]SPMA). Final concentrations of internal standards in urine were (μg/L) 50, 25, 25, 5, and 1 for [$^2H_5$]phenol, [$^2H_6$]catechol, [$^2H_6$]hydroquinone, [$^{13}C_2$]*t*,*t*-muconic acid, and [$^2H_5$]*S*-phenylmercapturic acid, respectively.

equal to the median exposure of 31 ppm, and those with GM exposures greater than 31 ppm. The correlations among these exposure categories and metabolite levels were tested using Spearman coefficients (r_s).

In light of the highly skewed distributions, all other statistical analyses were carried out using (natural) logarithmic transformation. Student's t test was used to test for differences in (logged) metabolite levels of exposed and control subjects and of smokers and non smokers in the control group. Linear relationships between (logged) individual metabolite levels, previously reported (logged) individual levels of urinary benzene [25], (logged) individual levels of benzene exposure (both the GM and the same-day exposure, available for 37 of the 42 exposed subjects), and cotinine levels were investigated using Pearson correlation coefficients (r) and least squares regression. Control subjects were excluded from the analysis of metabolite levels and same-day exposure because benzene exposures were not measured on the day of urine collection. Multiple linear regression was conducted to evaluate the impact of cigarette smoking on levels of urinary metabolites after adjusting for exposure.

The precision of the assay for different metabolites was estimated by performing a one-way ANOVA, using the log-transformed levels of metabolites. From this, the within-subject variance component (from the error term) was used to estimate the coefficient of variation as $CV = \sqrt{e^{s_w^2} - 1}$ where s_w^2 represents the estimated within-subject variance component [54].

All statistical analyses were performed using SAS system software (V. 8.1, SAS Institute, Cary, NC) using a significance level of 0.05 (two-tailed). Two subjects with reportedly high levels of benzene exposure, but with very low levels of all urinary products (benzene and all metabolites), were excluded from analysis.

Results

Linearity, precision, and limits of detection

For each analyte, there was no evidence of nonlinearity of standard calibration curves by visual inspection; $R^2 > 0.98$ in each case. The CVs for the analytes were as follows: 7.75% for phenol, 10.5% for catechol, 15.6% for hydroquinone, 31.9% for trihydroxybenzene, 14.7% for muconic acid, and 10.5% for phenylmercapturic acid. Based on a signal to noise ratio of 3, an injection volume of either 1 μl (muconic acid) or 2 μl (phenylmercapturic acid, trihydroxybenzene), and a final sample volume of 200 μl, the limits of detection corresponded to 3 μg/L for trihydroxybenzene and 2 μg/L for phenylmercapturic acid (splitless injection) and about 10 μg/L for muconic

Table 1
Summary statistics for benzene exposures[a] and urinary metabolite levels in workers exposed to benzene and control workers in Shanghai, China

	Parameter	Control	Lower exposure (≤31 ppm)	Higher exposure (>31 ppm)
Benzene exposure (ppm)	Mean (SD)	0.019 (0.016)	14.5 (8.95)	109 (73.0)
	Median (range)	0.016 (0.016–0.110)	13.6 (1.65–30.6)	92.0 (31.5–329)
	No. workers	44	22	22
	No. smokers	21	12	9
Phenol (mg/L)	Mean (SD)	4.71 (3.99)	39.9 (47.3)	192 (120)
	Median (range)	3.84 (0.311–21.0)	18.2 (3.87–175)	196 (27.1–374)
	No. workers	44	22	18
	No. smokers	21	10	6
Catechol (mg/L)	Mean (SD)	1.86 (2.14)	5.56 (6.05)	38.1 (26.5)
	Median (range)	1.27 (0.297–11.9)	3.09 (0.673–23.8)	40.3 (3.79–85.1)
	No. workers	44	22	18
	No. smokers	21	10	6
Hydroquinone (mg/L)	Mean (SD)	0.445 (0.369)	8.19 (10.1)	22.2 (13.8)
	Median (range)	0.346 (0.066–2.08)	3.97 (0.524–36.2)	22.1 (3.30–50.6)
	No. workers	44	22	18
	No. smokers	21	10	6
1,2,4-Trihydroxybenzene (mg/L)	Mean (SD)	0.192 (0.247)	0.072 (0.068)	0.114 (0.068)
	Median (range)	0.108 (0.017–1.06)	0.048 (0.028–0.345)	0.084 (0.043–0.262)
	No. workers	44	22	18
	No. smokers	21	10	6
t,t-Muconic acid (mg/L)	Mean (SD)	0.108 (0.074)	16.2 (22.2)	51.5 (34.9)
	Median (range)	0.093 (0.020–0.338)	7.14 (1.14–77.8)	41.2 (7.25–133)
	No. workers	44	22	18
	No. smokers	21	10	6
S-Phenylmercapturic acid (mg/L)	Mean (SD)	0.021 (0.018)	0.712 (1.33)	9.42 (7.76)
	Median (range)	0.018 (0.002–0.079)	0.175 (0.050–5.89)	7.69 (0.123–27.5)
	No. workers	25	22	18
	No. smokers	10	10	6

[a] Geometric mean levels for five personal air measurements per subject.

acid (1:20 split injection). (Limits of detection for phenol, hydroquinone, and catechol could not be estimated because of high background levels of these analytes.) Typical GC-EI-MS chromatograms (in selected-ion-monitoring mode) are shown in Figs. 4A and B, following analysis of urine from a worker exposed to 14.0 ppm benzene (same-day exposure) via split and splitless injections, respectively.

Urinary benzene metabolites in exposed and control workers

Median levels of phenol, catechol, hydroquinone, muconic acid, and phenylmercapturic acid in exposed workers (52.7, 7.63, 8.42, 17.0, and 1.15 mg/L, respectively) were significantly higher than those in controls (3.84, 1.27, 0.346, 0.093, and 0.021 mg/L, respectively) ($p < 0.0001$); this was not the case for trihydroxybenzene where levels for exposed and controls workers were 0.059 and 0.108 mg/L, respectively. Summary statistics of benzene exposure and urinary levels of all metabolites are given in Table 1 for workers categorized as controls, exposed to ⩽31 ppm benzene, and exposed to >31 ppm. All metabolite levels were highly correlated with exposure category ($0.728 \leqslant r_s \leqslant 0.933$, $p < 0.0001$) except those of trihydroxybenzene ($r_s = -0.090$, $p = 0.414$).

When analyzed on an individual basis, as shown in Table 2, the logged levels of all metabolites were significantly correlated with the individual GM exposure levels ($0.729 \leqslant r \leqslant 0.955$, $p < 0.0001$), except for trihydroxybenzene ($r = -0.113$, $p = 0.308$). When the corresponding comparisons were made with same-day exposure levels (exposed workers only), all metabolite levels (including trihydroxybenzene) were significantly correlated with exposure ($0.576 \leqslant r \leqslant 0.839$, $p < 0.0003$). The levels of urinary metabolites were also highly correlated with each other and with urinary benzene ($0.823 \leqslant r \leqslant 0.962$, $p < 0.0001$), except for comparisons with trihydroxybenzene ($r \leqslant 0.154$, $p > 0.163$) (Table 2).

Metabolite levels were not correlated with levels of urinary cotinine when analyzed on an individual basis (Table 2). However, multivariate analyses of metabolite levels, using exposure category and smoking status as independent variables, revealed levels of hydroquinone

Table 2
Correlation matrix for levels of exposure and urinary biomarkers for benzene- exposed and control subjects[a]

	Same-day exposure	UB	PHE	CAT	HQ	THB	MA	SPMA	Cotinine
Geometric mean exposure	0.862 <0.0001 36	0.925 <0.0001 81	0.834 <0.0001 84	0.729 <0.0001 84	0.885 <0.0001 84	−0.113 0.308 84	0.955 <0.0001 84	0.863 <0.0001 65	−0.010 0.651 86
Same-day exposure[b]		0.839 <0.0001 35	0.833 <0.0001 35	0.826 <0.0001 35	0.764 <0.0001 35	0.576 0.0003 35	0.809 <0.0001 35	0.812 <0.0001 35	−0.249 0.142 36
UB			0.876 <0.0001 81	0.823 <0.0001 81	0.920 <0.0001 81	0.001 0.991 81	0.954 <0.0001 81	0.912 <0.0001 65	−0.094 0.143 36
PHE				0.910 <0.0001 84	0.943 <0.0001 84	0.082 0.456 84	0.904 <0.0001 84	0.905 <0.0001 65	−0.033 0.765 84
CAT					0.899 <0.0001 84	0.154 0.163 84	0.837 <0.0001 84	0.837 <0.0001 65	−0.019 0.859 84
HQ						0.084 0.449 84	0.962 <0.0001 84	0.904 <0.0001 65	0.074 0.504 84
THB							−0.042 0.702 84	0.113 0.369 65	0.160 0.145 84
MA								0.914 <0.0001 65	0.013 0.904 84
SPMA									−0.0332 0.793 65

Pearson correlation coefficients, p values, and n are shown for each comparison, based on log-transformed data.

UB, urinary benzene; PHE, phenol; CAT, catechol; HQ, hydroquinone; THB, 1,2,4-trihydroxybenzene; MA, *t,t*-muconic acid; SPMA, *S*-phenylmercapturic acid.

[a] Urine from 42 exposed subjects and 44 control subjects was available for analysis of metabolites.

[b] Exposures on the day of urine collection were available for 37 of the 42 exposed subjects.

and muconic acid to be significantly greater for smokers than for nonsmokers ($p \leqslant 0.02$) while those of phenol, catechol, trihydroxybenzene, and phenylmercapturic acid were not significantly different between smokers and nonsmokers ($p \geqslant 0.073$).

Among control subjects, catechol, hydroquinone, and muconic acid were significantly correlated with urinary benzene ($r \leqslant 0.322$, $p < 0.040$) (Table 3). Also, levels of urinary metabolites were significantly correlated with each other ($0.378 \leqslant r \leqslant 0.707$, $p < 0.011$), except for trihydroxybenzene and phenylmercapturic acid.

Least squares regressions of log-transformed metabolite levels on exposure are given in Table 4 for all subjects (based upon individual GM exposures, $n = 84$) and for exposed workers only (based upon same-day exposure, $n = 35$). Scatter plots of metabolite levels versus same-day exposure are shown in Fig. 5. For each metabolite, clear trends toward higher levels with increasing exposure are seen among exposed subjects.

Discussion

Since the 1980s, benzene exposures have been extensively evaluated via measurement of urinary metabolites. Although HPLC, GC-MS, and LC-MS-MS methods have been developed to quantitate benzene metabolites, none are currently capable of measuring all major

Table 3
Correlation matrix for urinary biomarkers in control subjects[a]

	UB	PHE	CAT	HQ	THB	MA	SPMA[b]
Geometric	0.061	0.201	−0.075	0.058	0.209	−0.045	0.170
mean exposure	0.703	0.191	0.628	0.706	0.172	0.773	0.415
	41	44	44	44	44	44	25
UB		0.151	0.372	0.344	0.084	0.322	0.087
		0.345	0.016	0.028	0.601	0.040	0.686
		41	41	41	41	41	24
PHE			0.562	0.640	0.083	0.378	−0.031
			<0.0001	<0.0001	0.594	0.011	0.882
			44	44	44	44	25
CAT				0.719	0.095	0.573	−0.067
				<0.0001	0.539	<0.0001	<0.0001
				44	44	84	25
HQ					0.247	0.707	0.150
					0.106	<0.0001	0.475
					44	44	25
THB						0.037	0.118
						0.813	0.574
						44	25
MA							−0.003
							0.988
							25

Pearson correlation coefficients, p values, and n are shown for each comparison, based on log-transformed data.

UB, urinary benzene; PHE, phenol; CAT, catechol; HQ, hydroquinone; THB, 1,2,4- trihydroxybenzene; MA, *t*,*t*-muconic acid; SPMA, *S*-phenylmercapturic acid.

[a] Urine from 44 control subjects was available for analysis of metabolites.

[b] SPMA levels were quantified in 25 of 44 control subjects.

Table 4
Results of least squares regression of urinary metabolite levels (mg/L)[a] on exposure levels (ppm) among benzene-exposed and control workers in Shanghai, China

Metabolite	Exposed workers[b]	Exposed and control workers[c]
Phenol (PHE)	ln(PHE) = 1.42 + 0.832[ln(Benzene)]	ln(PHE) = 2.71 + 0.394[ln(Benzene)]
Catechol (CAT)	ln(CAT) = −0.440 + 0.859[ln(Benzene)]	ln(CAT) = 1.31 + 0.285[ln(Benzene)]
Hydroquinone (HQ)	ln(HQ) = −0.034 + 0.684[ln(Benzene)]	ln(HQ) = 0.643 + 0.437[ln(Benzene)]
1,2,4-Trihydroxybenzene (THB)	ln(THB) = −3.45 + 0.269[ln(Benzene)]	ln(THB) = −2.46 − 0.028[ln(Benzene)]
t,*t*-Muconic acid (MA)	ln(MA) = 0.337 + 0.792[ln(Benzene)]	ln(MA) = 0.417 + 0.700[ln(Benzene)]
S-Phenylmercapturic acid (SPMA)[d]	ln(SPMA) = −3.34 + 1.15[ln(Benzene)]	ln(SPMA) = −1.85 + 0.604[ln(Benzene)]

Analyses were performed with natural logarithms of metabolite and exposure levels.

[a] Metabolite levels were measured in 42 exposed and 44 control subjects.

[b] Relationships are given using same-day exposures for 35 exposed subjects.

[c] Relationships are given based on geometric mean exposures for 40 exposed and 44 control subjects.

[d] *S*-Phenylmercapturic acid levels were quantified in 25 of the 44 control subjects.

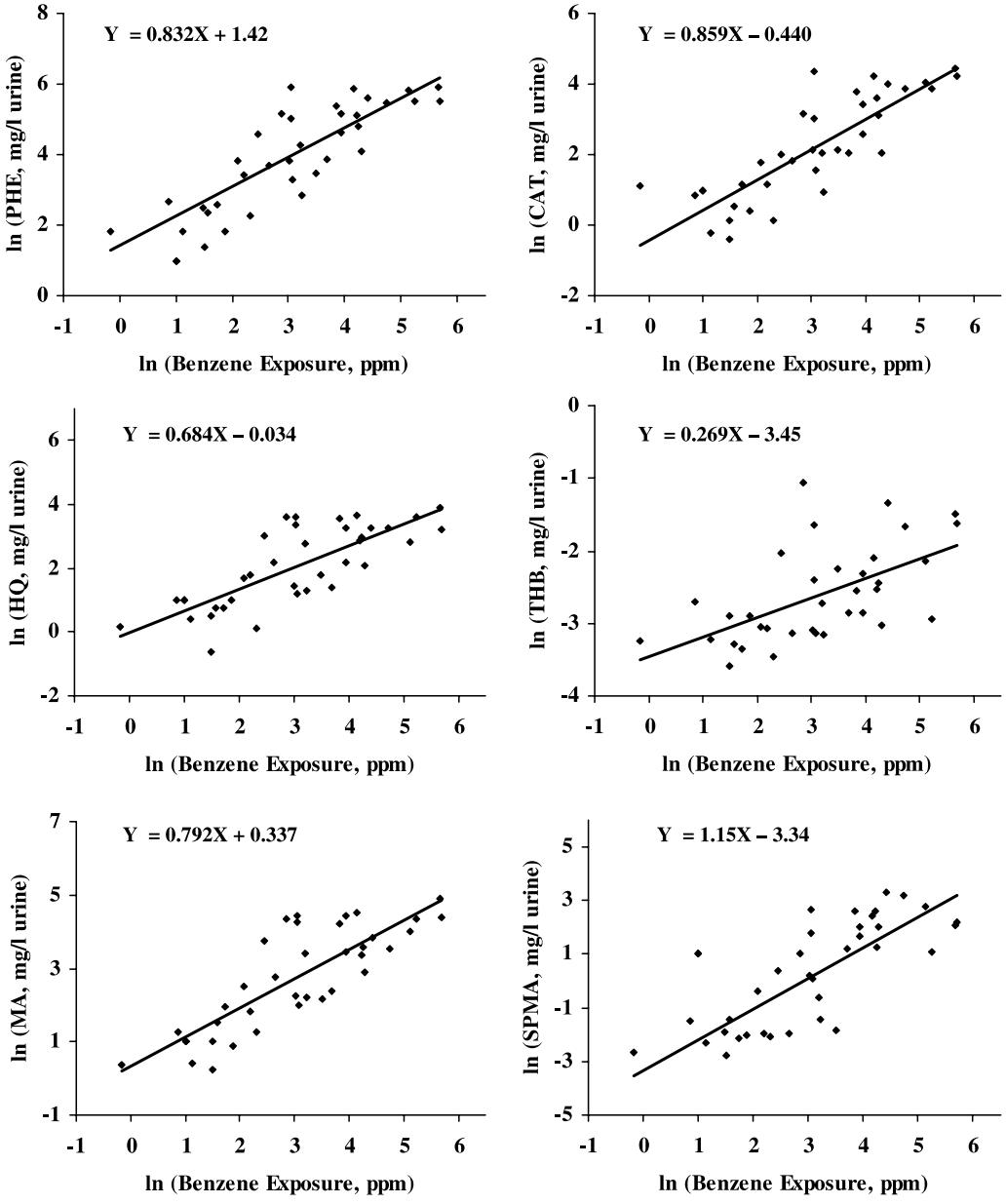

Fig. 5. Scatter plots of log-transformed same-day benzene exposure (ppm) vs log-transformed metabolite levels (mg/L) in exposed workers. PHE, phenol; CAT, catechol; HQ, hydroquinone; SPMA, *S*-phenylmercapturic acid; THB, trihydroxybenzene; MA, muconic acid.

metabolites in one assay. Here, we report a method based on solvent extraction of 0.5 ml of acidified urine, derivatization, and GC-MS analysis to measure the six most prominent urinary metabolites of benzene. The use of trimethylsilylation for derivatization of these polar metabolites avoids additional cleanup of samples prior to GC-MS. The method is simple, specific, reasonably precise (median $CV = 12.6\%$), and sufficiently sensitive for application among persons without occupational exposures to benzene. It is also much less cumbersome than existing HPLC-UV and LC-MS-MS methods. The higher $CV = 31.9\%$ for trihydroxybenzene probably reflects the less-than-optimal quantitation of this metabolite (using [$^2H_6$]catechol as internal standard).

The instrumental limit of detection for phenylmercapturic acid, the analyte regarded as the most difficult to quantitate, was 0.01 ng, based on a signal to noise ratio of 3, which is comparable to the current LC-MS-MS method (0.02 ng). Given 0.5 ml urine, an injection volume of either 1 µl (muconic acid) or 2 µl (phenylmercapturic acid), and a final sample volume of 200 µl, the limits of detection for muconic acid and phenylmercapturic acid (the two most specific metabolites of benzene) correspond to about 10 and 2 µg/L, respectively, values comparable to those generated by use of existing methods [24,36,43,55,56].

To illustrate application of the assay, we measured benzene metabolites in a sample of 42 benzene-exposed

and 44 control workers from Shanghai, China [44,45]. All metabolites, except trihydroxybenzene, were highly correlated with individual GM exposures to benzene (measured during 5 days prior to urine sampling, $n = 84$), with individual same-day exposures to benzene ($n = 35$), and with previously measured individual levels of urinary benzene ($n = 81$). Furthermore, catechol, hydroquinone, and muconic acid were significantly correlated with urinary benzene among controls, suggesting that environmental benzene contributed to these metabolites in our subjects (Table 3).

The mean trihydroxybenzene level observed in exposed subjects (0.090 mg/L) was lower than that observed for the control group (0.192 mg/L), suggesting that control subjects were exposed to a source of trihydroxybenzene other than benzene. For exposed workers, significant correlations between urinary trihydroxybenzene and same-day exposure to benzene (Fig. 5) and other urinary products were observed ($r \geqslant 0.472$, $p < 0.0021$). To our knowledge, such associations have not been reported before for trihydroxybenzene. In their study, Qu et al. [15] attributed the lack of correlation between trihydroxybenzene with benzene exposure and with other urinary analytes to instability of trihydroxybenzene. The mean trihydroxybenzene level reported in their study for the exposed group (8.8 mg/g creatinine) was not different from that for the control group (9.7 mg/g creatinine) but was about 145-fold higher than that observed for the exposed group in the present study (0.058 mg/g creatinine, assuming an average creatinine level of 1.53 g/L). The high levels of trihydroxybenzene reported by Qu et al. [15] point to possible interference by a coeluting contaminant.

Based upon measurements of benzene oxide–albumin adducts in a population of benzene-exposed workers, Rappaport et al. [57,58] reported apparent supralinear metabolism of benzene at exposures above 1 ppm. Assuming that exposure to 1 ppm represents a reasonable upper limit for linear benzene metabolism in humans, we used regression parameters from Table 4 (same-day exposure) to predict the following metabolite levels (mg/L at 1 ppm benzene) in decreasing order: 4.14 for phenol, 1.40 for muconic acid, 0.966 for hydroquinone, 0.644 for catechol, 0.035 for phenylmercapturic acid, and 0.032 for trihydroxybenzene.

When the metabolite levels predicted at 1 ppm benzene were compared with the mean levels observed in controls (mg/L) (4.14 vs 4.75 for phenol, 1.40 vs 0.108 for muconic acid, 0.996 vs 0.445 for hydroquinone, 0.644 vs 1.86 for catechol, 0.035 vs 0.021 for phenylmercapturic acid, and 0.032 vs 0.192 for trihydroxybenzene), only hydroquinone, muconic acid, and phenylmercapturic acid levels were elevated. This finding is consistent with other investigations indicating that most of the phenolic metabolites cannot be used to monitor exposures to benzene below 5 ppm [23,24]. However, we cannot draw a conclusion with regard to trihydroxybenzene since control subjects in our population appeared to be exposed to a source of trihydroxybenzene other than benzene.

Table 5 summarizes predicted muconic acid and phenylmercapturic acid levels at 1 ppm benzene using the log–log relationships reported in selected published studies and the corresponding control levels. Predicted values at 1 ppm benzene were 2- to 32-fold greater than corresponding control values for muconic acid and 2- to 18-fold greater for phenylmercapturic acid. This indicates that muconic acid and phenylmercapturic acid are generally suitable for biomonitoring over the full range of benzene exposures. Based on all predicted values in Table 5, exposure to 1 ppm benzene should result in phenylmercapturic acid levels of 0.023–0.046 mg/g of creatinine and muconic acid levels of 0.500–1.88 mg/g creatinine. The corresponding ranges of control values from these studies were 0.0009–0.014 mg/g creatinine for

Table 5
Summary of urinary *S*-phenylmercapturic acid (mg/g creatinine) and *t,t*-muconic acid (mg/g creatinine) levels at 1 ppm benzene exposure[a] from biomonitoring studies

Study	Occupation	SPMA at 1 ppm	SPMA in controls	MA at 1 ppm	MA in controls
Ducos et al. (1992)[b]	Perfume industry			0.772	0.086
Ghittori et al. (1995)	Chemical plant	0.046	0.009, 0.002[c]	0.500	0.228, 0.062[c]
Ong et al. (1995)[d]	Car mechanics and shoe manufacturing			1.70	0.110[d]
Boogaard et al. (1996)	Petrochemical industry	0.044	0.004, 0.002	1.88	0.058, 0.036[c]
Ghittori et al. (1996)	Chemical plant			0.660	0.255, 0.114[c]
Qu et al. (2000)	Glue- and shoe-making	0.016[e]	0.0009	0.930[e]	0.310
Current study[f]	Benzene used as solvent	0.023	0.014	0.917	0.071

SPMA, *S*-phenylmercaptiric acid; MA, *t,t*-muconic acid.
[a] Exposure to 1 ppm was estimated using log–log relationships given for metabolite levels and air levels of benzene.
[b] Values were converted to mg/g creatinine using the average creatinine concentration of 1.508 mg/L from the same study.
[c] Values are for smokers and nonsmokers, respectively.
[d] Levels below 0.02 mg/L were not detected.
[e] Reported values are adjusted to include the background levels for comparison with other studies.
[f] Levels expressed as mg/g creatinine assuming a creatinine concentration of 1.53 g/L.

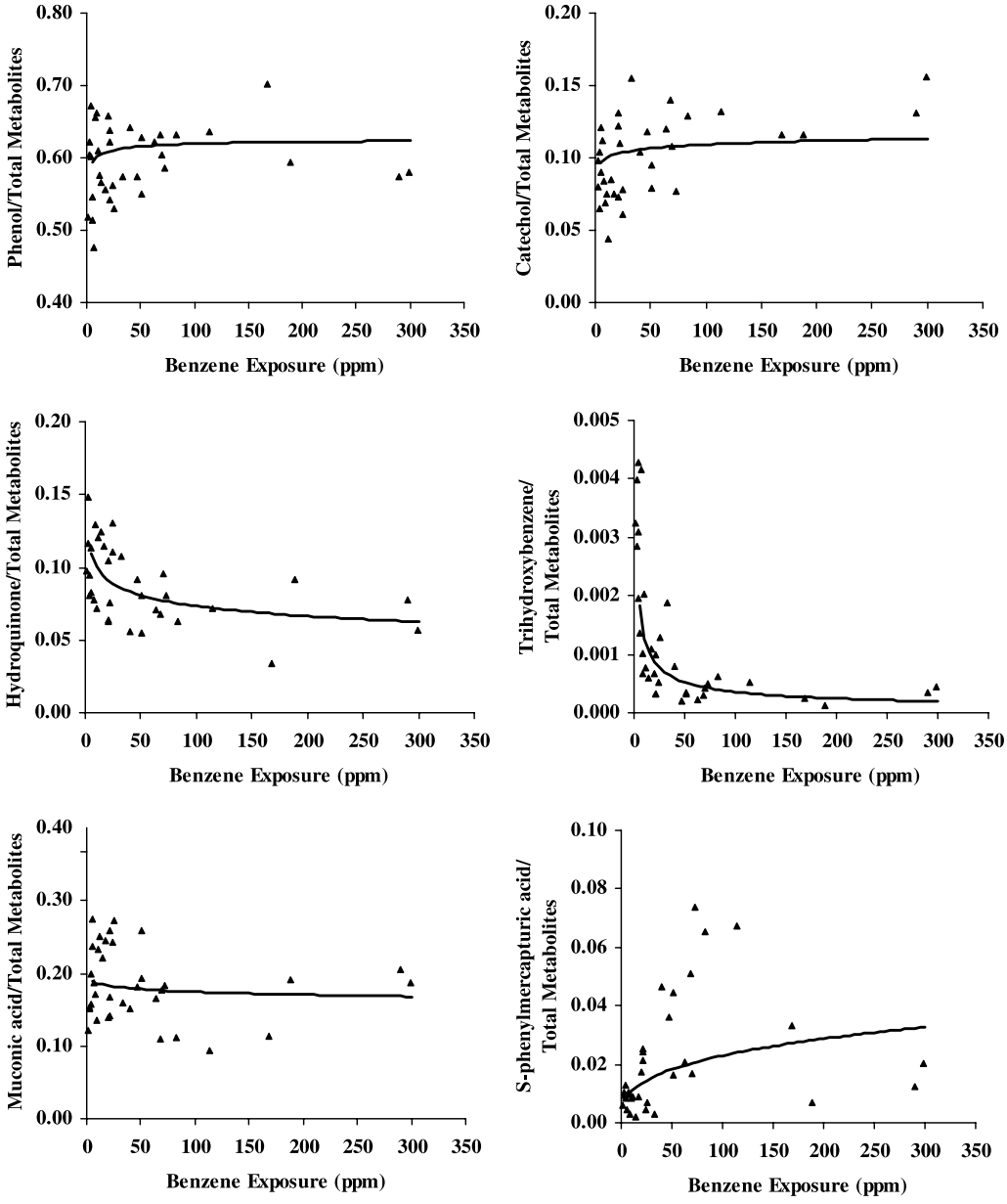

Fig. 6. Plots of ratios of individual metabolites to total metabolites vs same-day exposure in benzene-exposed subjects. Solid lines represent values predicted using the log–log relationships given in Table 4.

phenylmercapturic acid and 0.058–0.310 mg/g creatinine for muconic acid. These 2- to 15-fold ranges probably reflect both population variability and differences in the particular assays.

Of particular interest is the result that cigarette smoking significantly elevated the levels of hydroquinone ($p = 0.004$) and muconic acid ($p = 0.019$) but not of phenol, catechol, trihydroxybenzene, and phenylmercapturic acid ($p = 0.073$–0.533) in these workers. Since hydroquinone is the precursor of 1,4-benzoquinone, this finding is consistent with our previous observation that levels of albumin adducts of 1,4-benzoquinone were elevated in the same smoking subjects [59]. Among controls, mean values of all metabolites (mg/L) were higher in smokers than in nonsmokers (phenol, 5.12 vs 4.33; catechol, 2.07 vs 1.69; hydroquinone, 0.619 vs 0.286; trihydroxybenzene, 0.276 vs 0.141; muconic acid, 0.141 vs 0.078; phenylmercapturic acid, 0.029 vs 0.016); these differences were significant for hydroquinone, muconic acid, and phenylmercapturic acid ($p < 0.012$). Elevated levels of hydroquinone, catechol, muconic acid, and phenylmercapturic acid in smokers have also been reported in populations otherwise unexposed to benzene [17,21,38,40,56,60–65].

Since subjects in our study were exposed to a wide range of benzene levels on the day of urine collection (0.85–290 ppm), it is likely that substantial saturation of

CYP 2E1 occurred. To investigate this phenomenon, we plotted the ratio of individual metabolite levels to total benzene metabolites vs benzene exposure, as shown in Fig. 6. Consistent with previous investigations [14,35], our data point to greater than proportional production of phenol, catechol, and phenylmercapturic acid and to less than proportional production of hydroquinone, muconic acid, and trihydroxybenzene with increasing benzene exposure.

These results are consistent with competitive inhibition of benzene, phenol, benzene oxide–oxepin, and hydroquinone for the same CYP 2E1 enzymes (Fig. 1). Such competitive inhibition would reduce the proportions of metabolites requiring subsequent oxidation(s) (i.e., hydroquinone from phenol, muconic acid from benzene oxide–oxepin, and trihydroxybenzene from hydroquinone) while increasing the proportions of metabolites requiring only the initial oxidation of benzene (phenol, catechol, and phenylmercapturic acid). This supports the hypothesis that catechol is derived mainly from benzene oxide–oxepin via benzene dihydrodiol and that the formation of catechol from phenol via CYP 2E1 oxidation, observed in rats, probably represents a minor pathway in humans [66]. Inhibition of trihydroxybenzene is particularly dramatic (Fig. 6), suggesting the involvement of several CYP 2E1 oxidations in producing this metabolite.

Conclusion

We report a method for rapid quantitation of six metabolites of benzene (phenol, muconic acid, catechol, hydroquinone, phenylmercapturic acid, and trihydroxybenzene) in 0.5 ml of urine, based upon extraction, trimethylsilylation, and GC-MS. The method was applied to urine from 86 subjects, including 42 benzene-exposed workers and 44 control workers in Shanghai, China. Significant correlations were observed between all metabolites and benzene exposure and between pairs of metabolites. Since metabolites were detected in all control subjects, the method can be applied to persons exposed to ambient levels of benzene. By comparing the levels of all metabolites over a wide range of benzene exposures, our data point to competitive inhibition of pathways requiring two or more CYP 2E1 oxidation steps.

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