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Using urinary biomarkers to elucidate dose-related patterns of human benzene metabolism

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Although the toxicity of benzene has been linked to its metabolism, the dose-related production of metabolites is not well understood in humans, particularly at low levels of exposure. We investigated unmetabolized benzene in urine (UBz) and all major urinary metabolites [phenol (PH), *E*,*E*-muconic acid (MA), hydroquinone (HQ) and catechol (CA)] as well as the minor metabolite, *S*-phenylmercapturic acid (SPMA), in 250 benzene-exposed workers and 139 control workers in Tianjin, China. Median levels of benzene exposure were ~1.2 p.p.m. for exposed workers (interquartile range: 0.53–3.34 p.p.m.) and 0.004 p.p.m. for control workers (interquartile range: 0.002–0.007 p.p.m.). (Exposures of control workers to benzene were predicted from levels of benzene in their urine.) Metabolite production was investigated among groups of 30 workers aggregated by their benzene exposures. We found that the urine concentration of each metabolite was consistently elevated when the group's median benzene exposure was at or above the following air concentrations: 0.2 p.p.m. for MA and SPMA, 0.5 p.p.m. for PH and HQ, and 2 p.p.m. for CA. Dose-related production of the four major metabolites and total metabolites (μmol/l/p.p.m. benzene) declined between 2.5 and 26-fold as group median benzene exposures increased between 0.027 and 15.4 p.p.m. Reductions in metabolite production were most pronounced for CA and PH <1 p.p.m., indicating that metabolism favored production of the toxic metabolites, HQ and MA, at low exposures.

Introduction

Benzene is an important industrial chemical that is also emitted into the air from gasoline, engine exhausts and combustion of organic materials (including cigarette smoke) (1,2). Occupational exposures to benzene at air levels greater than ~10 p.p.m., have long been linked to hematotoxicity and to acute myelogenous leukemia (3–5). A recent report of hematotoxic effects in workers exposed to benzene <1 p.p.m. (6) has raised additional concerns regarding the health consequences of low exposures to this contaminant.

While it is generally accepted that benzene causes toxic effects via metabolism, the particular toxic metabolite(s) remains elusive (7). The major metabolic pathways are shown in Figure 1. Benzene is metabolized by CYP enzymes (primarily CYP2E1) to benzene oxide (BO, which is in equilibrium with its tautomer, oxepin), an electrophile that binds to macromolecules (8–11) and is the source of all other metabolites. Spontaneous rearrangement of BO produces phenol (PH), which can undergo another CYP oxidation to give hydroquinone (HQ). Hydrolysis of BO via epoxide hydrolase produces benzene dihydrodiol which can be converted to catechol (CA), via dihydrodiol dehydrogenases, or to benzene diolepoxides via CYP oxidation. HQ and CA can be oxidized to 1,4-benzoquinone and 1,2-benzoquinone, respectively, which also bind to macromolecules (11–13). A second CYP oxidation of oxepin, followed by ring opening, produces the muconaldehydes. These reactive species are also capable of binding to macromolecules (14–17) and are ultimately converted to *E*,*E*-muconic acid (MA) (18). The major phenolic metabolites, i.e. PH, HQ and CA, are easily conjugated and excreted in urine (19–21). Minor pathways of benzene metabolism include *S*-phenylmercapturic acid (SPMA) following reaction of BO with glutathione (16), and 1,2,4-trihydroxybenzene via CYP oxidation of HQ. Of all the benzene metabolites, 1,4-benzoquinone (derived from HQ) has most often been linked to the spectrum of toxic effects observed in humans and animals (7,22–24).

Since risks of hematotoxicity and leukemia are considered to arise from benzene metabolism, it is important that the dose-related production of benzene metabolites be understood. Two recent studies have reported levels of urinary metabolites in both benzene-exposed workers and control workers in China (25–28). Metabolite profiles from those studies suggested shifts among the competing metabolic pathways at increasing levels of benzene exposure. The Chinese studies also pointed to relatively high levels of phenolic metabolites, PH, CA and HQ, in control workers, presumably from dietary and endogenous sources of these compounds. These background levels of metabolites tend to obscure the amounts of PH, CA and HQ derived from ambient benzene exposure and from cigarette consumption. Also, because benzene air concentrations were not reported for control subjects in previous Chinese studies, they could not be used to determine the utility of the various metabolites as biomarkers of exposure to benzene at environmental levels.

In the present study we report levels of benzene in air and urine, as well as urinary levels of PH, CA, HQ, MA and

Abbreviations: BO, benzene oxide; CA, catechol; CV, coefficient of variation; EI, electron ionization; GC-MS, gas chromatography-mass spectrometry; GM, geometric mean; HQ, hydroquinone; LOD, limit of detection; MA, *E*,*E*-muconic acid; PH, phenol; SPMA, *S*-phenylmercapturic acid; TMS, trimethylsilyl derivative; UBz, urinary benzene.

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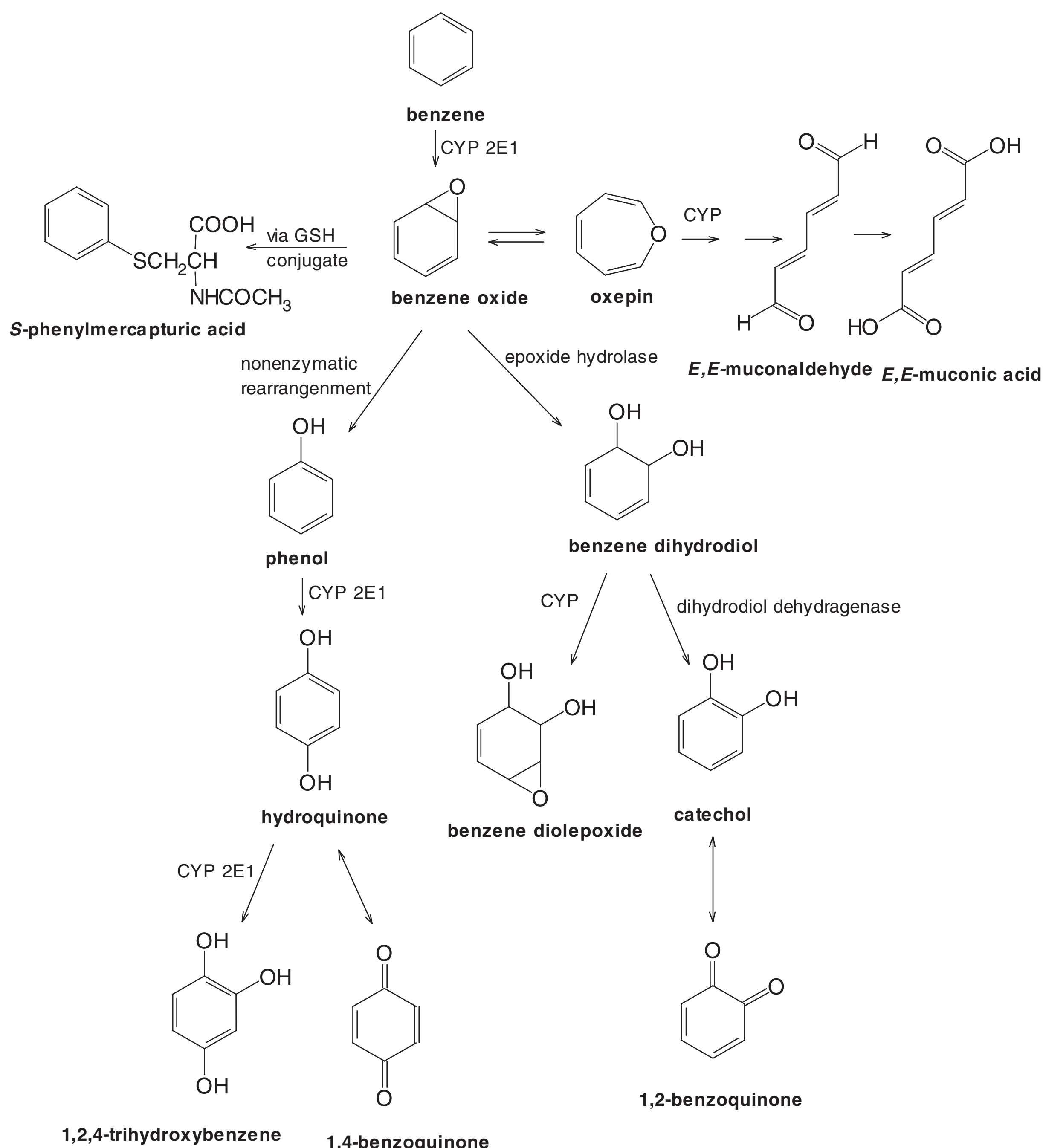

Fig. 1. Simplified metabolic scheme for benzene showing major pathways.

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SPMA, among 389 subjects (250 exposed and 139 controls) in a Chinese population that has shown evidence of hematotoxic effects (6). Because some personal measurements of airborne benzene were below the limits of detection (LODs) or were missing, we predict benzene exposures in these (mostly control) subjects from the levels of unmetabolized benzene in urine (UBz). Then, we use the data to characterize relationships between metabolite levels and benzene exposures in groups of subjects stratified by exposure level. This analysis identifies useful ranges of the various metabolites as biomarkers of benzene exposure. Finally, we estimate the dose-related production of each metabolite (μmol/l urine/p.p.m. benzene) after adjusting for background levels.

Materials and methods

Chemicals

Benzene, CA (99%) and HQ (>99%) were obtained from Fluka Chemical (Switzerland). NaCl was purchased from Fisher Scientific (Pittsburg, PA) and was heated at 120°C overnight prior to use. PH (99%+, redistilled), MA (98%) and $[^2H_6]$benzene (99.86%) were obtained from Aldrich Chemical (Milwaukee, WI). $[^{13}C_6]$PH (99%) and $[^2H_6]$CA (98%) were purchased from Cambridge Isotope Laboratories (Woburn, MA). Phenylmercapturic acid, Tri-Sil reagent and concentrated hydrochloric acid (Optima grade) were purchased from TCI America (Portland, OR), Pierce (Rockford, IL) and Fisher Scientific, respectively. Ethyl acetate (analytical reagent grade) and hexane (nanograde) were obtained from Mallinckrodt Baker (Paris, KT). Anhydrous Na_2SO_4 was purchased from J.T. Baker (Phillipsburg, NJ). 2,5-$[^{13}C_2]$MA, $[^2H_5]$SPMA and $[^{13}C_6]$HQ were kindly provided by Drs Avram Gold, Ramiah

Sangaiah and Alistair Henderson from the University of North Carolina at Chapel Hill. All headspace solid phase microextraction (HS-SPME) supplies were obtained from Supelco (Bellefonte, PA).

Subject recruitment and sample collection

Subjects ($n = 390$) were recruited with informed consent from five factories in Tianjin, China. Exposed subjects ($n = 250$) worked in two shoe-making factories, and control subjects ($n = 140$) worked in three clothes-manufacturing factories as described previously (6,29). The eligibility and exclusion criteria for participation were the same as in previous reports, and one control subject who did not provide a urine sample was removed. Exposed and control subjects were frequency matched by region and gender. As shown in Table I, demographic characteristics were similar among exposed and control workers in terms of gender, age and smoking status. Smoking status was highly correlated with gender. Personal protective equipment was not used.

Full-shift exposures to benzene were monitored with passive samplers (Organic Vapor Monitors, 3M Corporation, St Paul, MN), and urine was collected from each participant at the end of each working shift for which air had been monitored. Air samples were analyzed for benzene by gas chromatography with flame-ionization detection as described previously (29). The nominal LOD was 0.20 p.p.m. of benzene in air.

Urine samples were collected repeatedly (up to four times) in 139 subjects at intervals of ~42 days (median value) from June 2000 to June 2001. The other 250 subjects provided a single urine specimen. Urine samples were aliquoted immediately after collection, and stored at −80°C for 1.6–4.0 years (for UBz) and 2.2–3.8 years (for urinary metabolites) prior to analysis. Samples were identified by randomly assigned numbers. Information about exposure levels and demographic factors were released after all assays had been completed and results reported to collaborators. A total of 620 urine specimens were analyzed from 389 subjects. Urinary creatinine was quantified with a commercial kit (Monarch Instrument, Amherst, NH) as described previously (25).

For quality control purposes, positive controls were assayed with each batch of urine samples. They were prepared from a single urine specimen obtained from an unexposed volunteer to which had been added sufficient quantities of standard analytes to achieve the following concentrations: UBz, 24.5 nmol/l; MA, 32.3 μmol/l; SPMA, 2.65 μmol/l; and PH, 92.8 μmol/l; CA, 94.1 μmol/l and HQ, 69.7 μmol/l. Batches of positive controls were aliquoted into 2 ml vials and stored at −80°C prior to analysis.

This study was approved by the Institutional Review Boards of the University of North Carolina, the University of California, Berkeley, the US National Cancer Institute and the Chinese Academy of Preventive Medicine.

Measurement of urinary benzene

Levels of UBz were determined according to the method of Waidyanatha *et al.* (26), with minor modifications. Briefly, to 0.5 ml of urine in a 2 ml vial containing ca. 0.5 g of NaCl was added 1 μl of 59.4 μmol/l [$^{2}H_{6}$]benzene in methanol (internal standard). The vial was immediately sealed with a PTFE/silicone septum and benzene was extracted from the headspace with a 30 μm polydimethylsiloxane fiber (Supelco, PA) at 40°C for 15 min. Samples were analyzed by gas chromatography–mass spectrometry (GC–MS) in electron ionization (EI) mode using a HP 5980 Series II gas chromatograph coupled to a HP 5971-A mass selective detector. Sample injections were performed with a Varian Model 8200 autosampler (Walnut Creek, CA) equipped with a carousel (Strumenti Scientific, Padova, Italy). The injector, MS-transfer line and ion source temperatures were 200, 280 and 168°C, respectively. A DB-1 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 ml/min. The GC oven was held at 45°C for 9 min, and was then increased at 10°C/min to 100°C. Late eluting compounds were removed by rapidly raising the oven temperature to 250°C. The mass spectrometer was operated with an electron energy of 70 eV, and ions were monitored at m/z 78 and 84 for benzene and [$^{2}H_{6}$]benzene, respectively. The retention times were 8.56 and 8.50 min for benzene and [$^{2}H_{6}$]benzene, respectively. Quantification was based on peak areas relative to the isotopically labeled internal standard.

Table I. Demographic characteristics of the study population

Exposure status	Gender	*n* (%)	Age median (range)	Current smokers *n* (%)	Smoking intensity[a] median (range)
Control	Male	52 (37.4)	28 (18–51)	36 (69.2)	10 (1–40)
	Female	87 (62.5)	28 (18–51)	3 (3.45)	NR[b]
	All	139 (100)	28 (18–51)	39 (28.1)	10 (1–40)
Exposed	Male	86 (34.4)	23 (18–44)	47 (54.7)	10 (1–30)
	Female	164 (65.6)	33 (18–52)	5 (3.05)	4.5 (2–10)
	All	250 (100)	29 (18–52)	52 (20.8)	7 (1–30)

[a]Average number of cigarettes/day.
[b]Not reported.

Measurement of benzene metabolites

Levels of CA, HQ, MA, PH and SPMA were determined according to the method of Waidyanatha *et al.*, with minor modifications (30). Briefly, to 0.5 ml of urine in a 4 ml vial was added 10 μl of a mixture (in methanol) of 1.73 mmol/l [$^{13}C_{2}$]MA, 0.205 mmol/l [$^{2}H_{5}$]SPMA, 6.24 mmol/l [$^{13}C_{6}$]PH, 2.19 mmol/l [$^{2}H_{4}$]CA and 2.15 mmol/l [$^{13}C_{6}$]HQ (internal standards). After adding 50 μl of concentrated HCl, the mixture was extracted with 1.5 ml of ethyl acetate and the organic phase, containing MA, SPMA and free PH, CA and HQ, was transferred to another 4 ml vial. Then, a 10 μl aliquot of a mixture containing the above quantities of internal standards of PH, CA and HQ was added to the aqueous phase, which was heated at 100°C for 1 h to hydrolyze the conjugates. After cooling to room temperature, the aqueous phase was extracted with an additional 1.5 ml of ethyl acetate. The two organic extracts were combined, dried with anhydrous Na_2SO_4, reduced under N_2, transferred to a 500 μl flat-bottomed insert and brought to dryness under N_2. The residue was dissolved in 100 μl hexane and derivatized with 100 μl Tri-Sil reagent at 70°C for 1 h.

Samples were analyzed by GC–MS in EI mode with the same instrumentation described above using automated liquid injection. A 2 μl injection was analyzed in splitless mode for SPMA; here, the GC oven was maintained at 75°C for 4 min, and was then increased at 8°C/min to 245°C, where it was held for 10 min. A 1 μl portion was analyzed in (1:20) split mode for PH, CA, HQ and MA; here, the GC oven was maintained at 75°C for 4 min, and was then increased at 10°C/min to 230°C and held for 4 min. In both cases, late eluting compounds were removed by rapidly raising the oven temperature to 270°C and holding this temperature for 15 min. The following ions were monitored for trimethylsilyl (TMS)-derivatives of the analytes: TMS-PH [m/z 166, M^+], TMS-[$^{13}C_{6}$]PH [m/z 172, M^+], TMS-CA [m/z 254, M^+], [$^{2}H_{4}$]CA [m/z 258, M^+], TMS-HQ [m/z 254, M^+], TMS-[$^{13}C_{6}$]HQ [m/z 260, M^+], TMS-MA [m/z 271, $(M-15)^+$], TMS-[$^{13}C_{2}$]MA [m/z 273, $(M-15)^+$], TMS-SPMA [m/z 252, $(M-NH_2COCH_3)^+$] and TMS-[$^{2}H_{5}$]SPMA [m/z 257, $(M-NH_2COCH_3)^+$]. The respective retention times were 12.66, 12.66, 17.65, 17.62, 18.83, 18.83, 21.91 and 21.91 min for TMS-PH, TMS-[$^{13}C_{6}$]PH, TMS-CA, TMS-[$^{2}H_{4}$]CA, TMS-HQ, TMS-[$^{13}C_{6}$]HQ, TMS-MA and TMS-[$^{13}C_{2}$]MA in split mode, and 32.65 and 32.59 for TMS-SPMA and TMS-[$^{2}H_{5}$]SPMA in splitless mode, respectively. Quantification was based on peak areas relative to the corresponding isotopically labeled internal standards.

Creatinine adjustment for benzene metabolites

It is customary to adjust urinary metabolite levels for urinary creatinine to control for urine volume at the time of collection. However, such adjustments can introduce biases and reduce precision, due to variation of creatinine levels with gender, age, body mass index (BMI) and other physiological factors (31–33). In preliminary regression models, we observed lower adjusted R^2 values using creatinine-adjusted metabolite levels compared to unadjusted values. We also found significant effects of age, gender and BMI upon creatinine levels in our samples of workers. Thus, for all statistical analyses we used metabolite levels without adjustment for urinary creatinine. The following median levels of urinary creatinine were observed in our samples of workers: all subjects, 11.6 mmol/l; female controls, 10.4 mmol/l; male controls, 11.5 mmol/l; female exposed, 11.4 mmol/l; and male exposed, 13.9 mmol/l.

Predicting low-level exposures from urinary benzene

All air samples from control subjects were below the LOD ($n = 160$) and some measurements from exposed subjects were either below the LOD ($n = 70$) or were missing ($n = 23$). In order to predict the levels of benzene exposure in these samples, we used the log-scale linear regression of UBz on air benzene (228 data pairs) shown in Figure 2A. For subjects having multiple air and urine samples, the mean values of the natural logarithms of urine and air levels were used. The following calibration curve was derived from the parameters of the simple linear regression: air benzene $= \exp[(\ln(\mathrm{UBz}) - 5.42)/0.886]$. Note that these predicted values of benzene exposure (in p.p.m.) include contributions from smoking and UBz is measured in nmol/l. Uncertainties in this relationship were evaluated via a bootstrapping technique as described below.

Investigating exposure–metabolite relationships

The relationships between levels of metabolites and exposure were explored using median values and interquartile ranges. Based upon 13 groups of 30 subjects stratified by exposure the following median air concentrations (p.p.m.) were observed: 0.001, 0.002, 0.004, 0.007, 0.027, 0.273, 0.508,

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0.689, 1.02, 1.57, 2.41, 5.45 and 15.4. Multiple comparison tests were utilized to generate step functions showing statistically significant increases in geometric mean (GM) metabolite levels from group to group. These step functions were used to define levels of benzene exposure at which metabolite concentrations were statistically distinguishable from background values.

A similar grouping scheme (30 subjects stratified by exposure level) was used to investigate the dose-related production of each benzene metabolite, after adjustment for background metabolite levels. Background levels were estimated as median metabolite concentrations in 60 subjects with the lowest benzene exposures (median predicted benzene concentration = 0.002 p.p.m.). Adjustment involved subtracting background metabolite levels from the corresponding urine concentrations observed in each subject. After grouping by benzene exposure ($n = 30$ per group) the median metabolite level in each group was divided by the median air concentration of benzene to estimate dose-related production of each metabolite (μmol/l urine/p.p.m. benzene) for the nine groups with estimated median benzene exposures between 0.027 and 15.4 p.p.m. Uncertainties in estimated median values of benzene exposure and background-adjusted metabolite levels were evaluated via a bootstrapping technique as described below.

Statistical analyses

All statistical analyses were performed using SAS software for Windows v. 9.12 (SAS Institute, Cary, NC). Between 1 and 4 concurrent air and urine samples from each subject were available for statistical analysis (median = 2/subject). For subjects with multiple samples, estimated individual GM levels were used for all analyses. Non-detectable levels of urinary analytes were replaced by LOD/$\sqrt{2}$ [UBz: $n = 8$ (1.29%); SPMA: $n = 30$ (4.84%)] (34). The precision of the assay for each urinary analyte was estimated as the coefficient of variation (CV) from 84 pairs of duplicate urine samples (from exposed and control workers), which had been aliquoted and assigned random identification numbers prior to shipment to the laboratory. The CV was estimated as $CV = \sqrt{\exp(s_\varepsilon^2) - 1}$, where s_ε^2 is the estimated error variance obtained from a one-way analysis of variance of the log-transformed levels of each analyte (Proc NESTED of SAS).

To evaluate possible effects of sample storage at −80°C upon the levels of UBz and the benzene metabolites, the logged level of each urinary analyte was regressed upon the corresponding logged air concentration of benzene and the time of storage using a mixed-effects model, which included a random-subject effect to account for multiple urine specimens being collected from some persons (Proc MIXED of SAS).

Median levels of benzene exposure and those of the urinary metabolites were compared by exposure status, gender and smoking status using the Wilcoxon two-sample test (two-sided tests with a significance level of 0.05, Proc NPAR1WAY of SAS). Trends of (median) metabolite levels versus benzene exposure were tested with Spearman correlation coefficients, (Proc CORR of SAS). Differences in mean values of logged metabolite levels among groups of 30 subjects aggregated by benzene exposure were tested using Tukey's multiple comparisons test (Proc GLM of SAS). These tests were used to determine levels of benzene exposure where metabolite levels could be differentiated from background values.

We predicted air exposures of control subjects from their levels of urinary benzene and used these predicted exposures to group subjects for aggregated analyses. Thus, to estimate dose-related profiles of metabolite production, and the associated uncertainties, bootstrapping was performed based upon median values of background-adjusted metabolite levels and the corresponding levels of benzene exposure. First, resampling was conducted (with replacement) 1000 times to predict non-detected air exposures from urinary benzene levels, using the 228 data pairs shown in Figure 2. For each of the 1000 samples, predicted air concentrations were combined with observed air concentrations, median background levels from the lowest 60 exposed persons were subtracted from individual metabolite levels and data were aggregated for groups of 30 subjects. Then, 5th, 50th and 95th percentiles of the distributions of estimated median values of air and metabolite concentrations were obtained. Differences in dose-related production of each metabolite between adjacent exposure groups were evaluated with Kruskal–Wallis tests for overall group effects and trends (Proc FREQ of SAS).

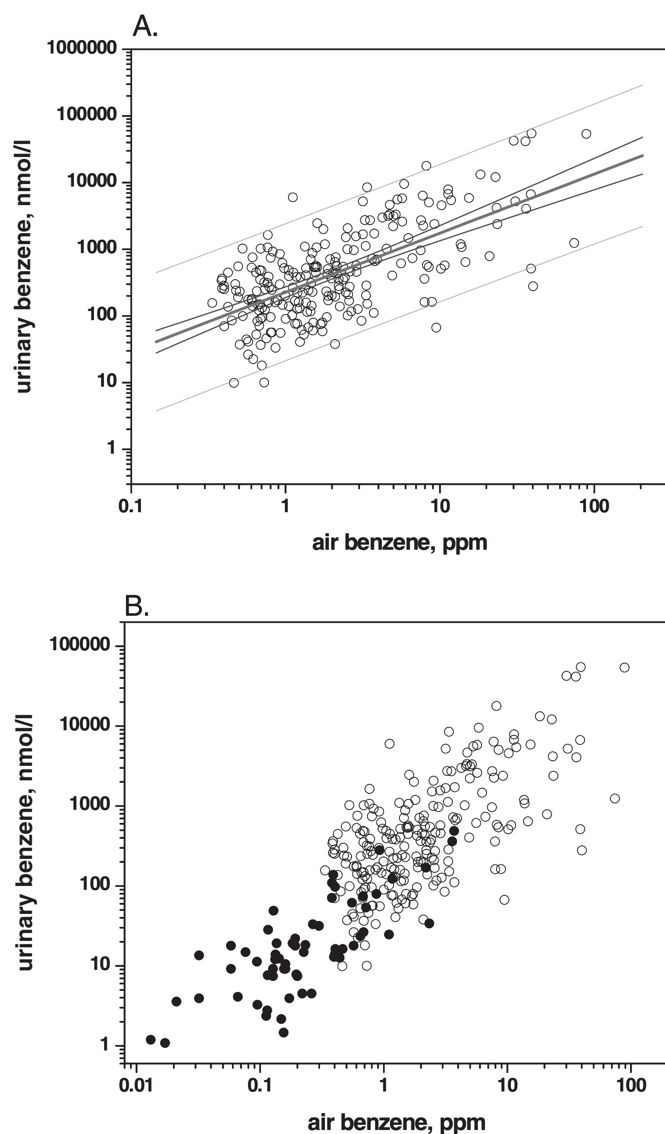

Fig. 2. Calibration curve for predicting air concentrations of benzene from the corresponding urinary levels. (**A**) Points represent observed air and urine concentrations and the line represents the least-squares regression equation: ln(urinary benzene, nmol/l) = 5.42 + 0.886 × ln(air benzene, p.p.m.) ($n = 228$, adjusted $R^2 = 0.428$; 95 confidence of intercept, 5.24–5.61; 95% confidence interval of slope, 0.752–1.02). The curves represent 95% confidence intervals and 95% prediction intervals. (B) Scatter plot of urinary benzene versus air benzene from the current study (open circles) and data from Ghittori *et al.* (39) obtained from measurements from 63 non-smoking subjects exposed to benzene (closed circles).

Results

Precision, sensitivity and storage stability

Estimates of CVs for the urinary analytes were as follows: UBz, 21.1%; MA, 6.15%; SPMA, 46.2%; PH, 4.97%; CA, 6.50% and HQ, 4.81%. Based on a volume of 0.5 ml urine and a signal-to-noise ratio of 3:1, the following LODs were estimated: UBz, 0.128 nmol/l, MA, 5.66 nmol/l (1 μl injected with 1:20 split) and SPMA, 0.836 nmol/l (2 μl with splitless injection). Values of LODs were not estimated for PH, CA and HQ due to the presence of background levels of these compounds in all urine specimens. The LOD for measurements of airborne benzene had previously been assigned a nominal value of 0.2 p.p.m. (29). When the period of storage was included as a fixed effect in mixed-effects models of the logged levels of UBz and the benzene metabolites on the logged benzene air concentrations, the observed regression coefficients were very small relative to the intercepts and showed no evidence of statistical significance ($P > 0.2$). This indicates that all analytes were stable during long periods of storage at −80°C in this study.

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Table II. Summary statistics for exposure to benzene and urinary analytes

Variable	Control				Exposed			
	Female (n = 87)	Male (n = 52)	Non-smokers (n = 100)	Smokers (n = 39)	Female (n = 164)	Male (n = 86)	Non-smokers (n = 198)	Smokers (n = 52)
Air benzene[a]								
Median	3.40 p.p.b.	3.71 p.p.b.	3.09 p.p.b.	6.07 p.p.b.	1.28 p.p.m.	1.05 p.p.m.	1.18 p.p.m.	1.18 p.p.m.
Range	0.146–21.2 p.p.b.	0.146–533 p.p.b.	0.146–21.2 p.p.b.	0.146–533 p.p.b.	0.017–88.9 p.p.m.	0.122–50.2 p.p.m.	0.017–88.9 p.p.m.	0.122–40.1 p.p.m.
IQ range	1.70–6.80 p.p.b.	2.13–9.71 p.p.b.	1.70–5.91 p.p.b.	2.56–17.5 p.p.b.	0.523–4.21 p.p.m.	0.572–2.36 p.p.m.	0.520–3.34 p.p.m.	0.588–3.02 p.p.m.
P-value	0.273		0.003		0.588		0.889	
UBz (nmol/l)								
Median	1.48	1.59	1.36	2.47	283	216	267	197
Range	0.091–7.47	0.091–130	0.091–7.47	0.091–130	6.21–53900	19.4–42600	6.21–53900	19.4–41600
IQ range	0.799–2.73	0.973–3.74	0.797–2.41	1.15–6.32	87.3–1020	96.3–643	96.3 – 810	85.3–654
P-value	0.273		0.003		0.635		0.702	
MA (μmol/l)								
Median	1.06	1.09	1.06	1.11	13.5	10.3	12.3	12.4
Range	0.152–6.17	0.132–5.78	0.152–6.17	0.132–4.96	0.644–426	1.50–370	0.644–426	2.15–347
IQ range	0.549–1.75	0.774–1.72	0.543–1.70	0.803–1.74	6.33–34.5	5.39–22.4	6.13–28.5	6.22–28.4
P-value	0.565		0.384		0.272		0.473	
SPMA (nmol/l)								
Median	1.94	3.24	1.96	3.17	262	137	228	171
Range	0.591–86.4	0.591–68.1	0.591–86.4	0.591–68.1	1.50–29400	3.68–33000	1.50–29400	4.06–33000
IQ range	1.17–3.62	1.04–6.93	1.13–4.25	1.09–7.37	50.6–788	48.9–353	50.1–631	47.9–412
P-value	0.479		0.493		0.048		0.638	
pH (μmol/l)								
Median	61.4	53.2	61.3	56.3	171	134	158	135
Range	9.48–208	7.58–336	9.48–208	7.58–336	15.9–3740	41.8–4140	15.9–4140	41.8–3380
IQ range	45.0–103	28.8–82.0	43.7–89.6	29.0–98.5	102–310	86.6–190	100–261	84.6–313
P-value	0.117		0.415		0.038		0.391	
CA (μmol/l)								
Median	13.3	12.7	12.1	15.7	21.8	21.0	20.5	23.7
Range	2.30–61.1	2.45–46.6	2.30–61.1	2.45–46.6	3.50–420	4.20–438	3.50–438	7.08–348
IQ range	8.06–17.0	7.07–18.9	7.50–15.8	9.90–19.8	13.3–33.0	12.6–29.4	12.6–32.1	13.8–35.5
P-value	0.786		0.034		0.411		0.153	
HQ (μmol/l)								
Median	5.95	7.35	5.93	8.52	17.7	18.5	17.1	19.6
Range	1.54–39.1	1.32–41.7	1.40–39.1	1.32–41.7	2.29–427	3.20–341	2.29–427	5.76–375
IQ range	4.44–8.88	4.48–11.7	4.11–8.86	5.65–13.9	11.2–36.8	11.5–27.8	10.4–29.1	14.9–35.9
P-value	0.106		0.001		0.925		0.084	

IQ range, interquartile range; benzene, benzene in air.
[a]Exposure to benzene for control subjects was predicted from the relationship between UBz and air exposure.

Summary statistics

Estimates of median levels, ranges and interquartile ranges of air exposures to benzene and all urinary analytes are summarized in Table II, after stratification by exposure status, gender and smoking status. Among occupationally exposed subjects, air levels of benzene ranged from 0.017 to 88.9 p.p.m. (interquartile range: 0.529–3.34 p.p.m.), with median values of ~1.2 p.p.m. in all categories of subjects. Among control subjects, median exposures (predicted from UBz levels) were between 3 and 6 p.p.b. depending upon gender and smoking status. Although air and urinary concentrations of benzene were comparable in all categories of exposed workers, females had significantly higher median levels of SPMA ($P = 0.048$) and PH ($P = 0.038$) than males. No significant differences were observed between smokers and non-smokers in the exposed group. Control subjects who were smokers had higher median levels of all urinary analytes than non-smokers, notably UBz ($P = 0.003$), CA ($P = 0.034$) and HQ ($P = 0.001$), indicating the likely presence of these analytes in cigarette smoke. Although there were no significant effects of gender on metabolite levels in control subjects, when non-smokers were further stratified by gender, females ($n = 84$) had significantly higher median levels of PH (63.3 versus 46.4 μmol/l, $P = 0.037$) and CA (13.2 versus 7.99 μmol/l, $P = 0.010$) than males ($n = 16$).

Distinguishing benzene metabolites from background sources

Figure 3 shows median levels and interquartile ranges for concentrations of each metabolite among groups of 30 subjects aggregated by benzene exposure. As exposure increased, the levels of metabolites also increased (estimated Spearman coefficients of median values for PH, 0.973; HQ, 0.945; CA, 0.934; MA, 0.973 and SPMA, 0.973; all with $P < 0.001$). The step functions signify nominal exposure concentrations at which statistically significant increases in metabolite levels were observed. As exposure increased, the contribution of benzene to each metabolite could consistently be discerned at some point (Tukey's multiple comparisons test), as indicated by an arrow in

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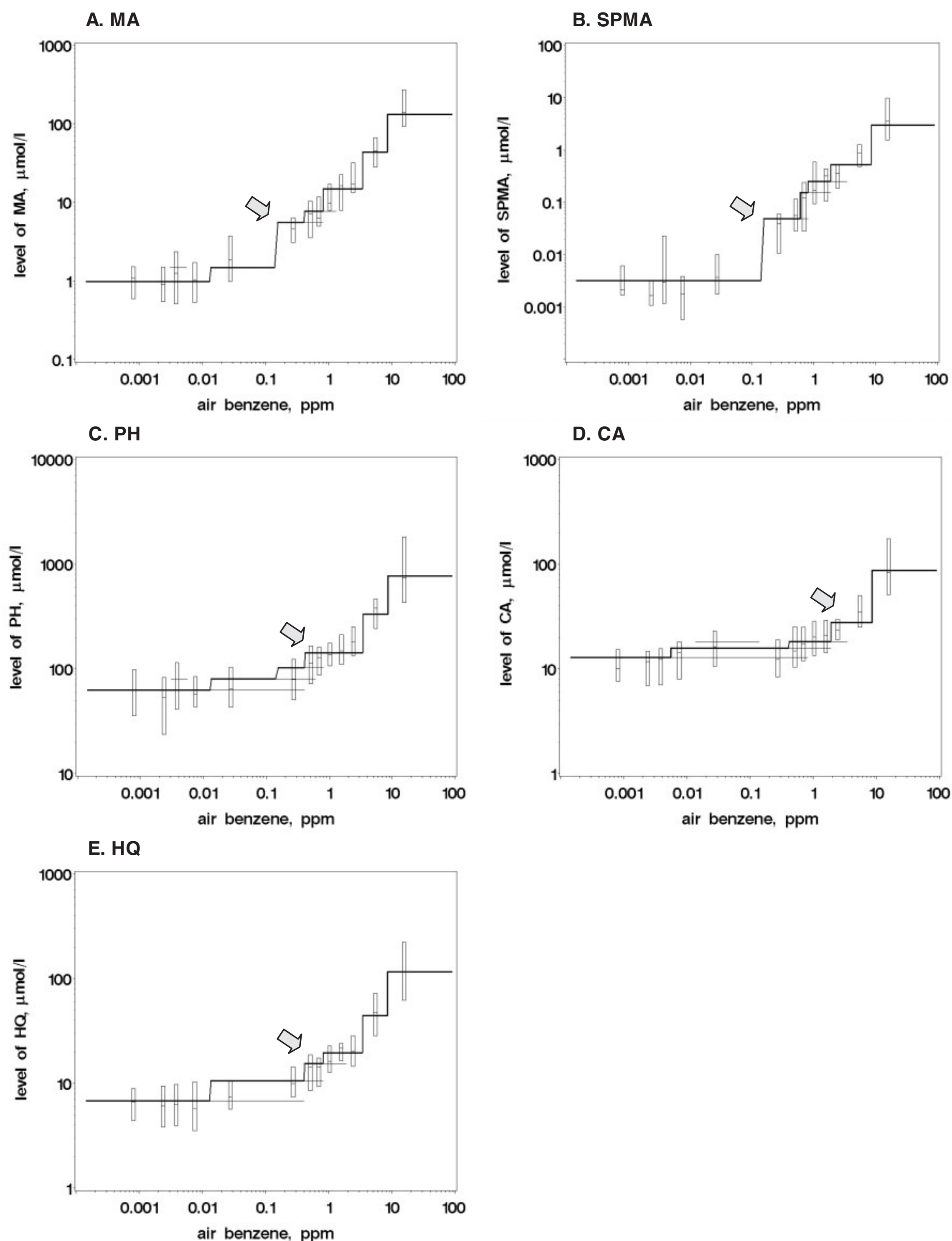

Fig. 3. Levels of urinary metabolites versus air exposures to benzene. Each vertical box depicts median, 25th and 75th percentile values of a given metabolite at the median level of benzene exposure for data grouped by exposure levels (30 subjects per group). Horizontal lines represent groups with equivalent metabolite levels (Tukey's multiple comparison tests). Arrows represent air exposure levels where it is possible to distinguish benzene metabolites from background sources.

Figure 3. Although there were marginal increases in median levels of MA, PH, CA and HQ at median benzene exposures as low as 0.02 p.p.m., elevations were not unambiguous below air concentrations of 0.2 p.p.m. for MA and SPMA, 0.5 p.p.m. for PH and HQ and 2 p.p.m. for CA. From these results, it appears that none of the metabolites would be useful for biomonitoring of benzene at environmental levels (generally <0.01 p.p.m.).

Dose-related production of benzene metabolites

Median urine concentrations of all metabolites per p.p.m. of benzene exposure are presented in Figure 4 for the nine

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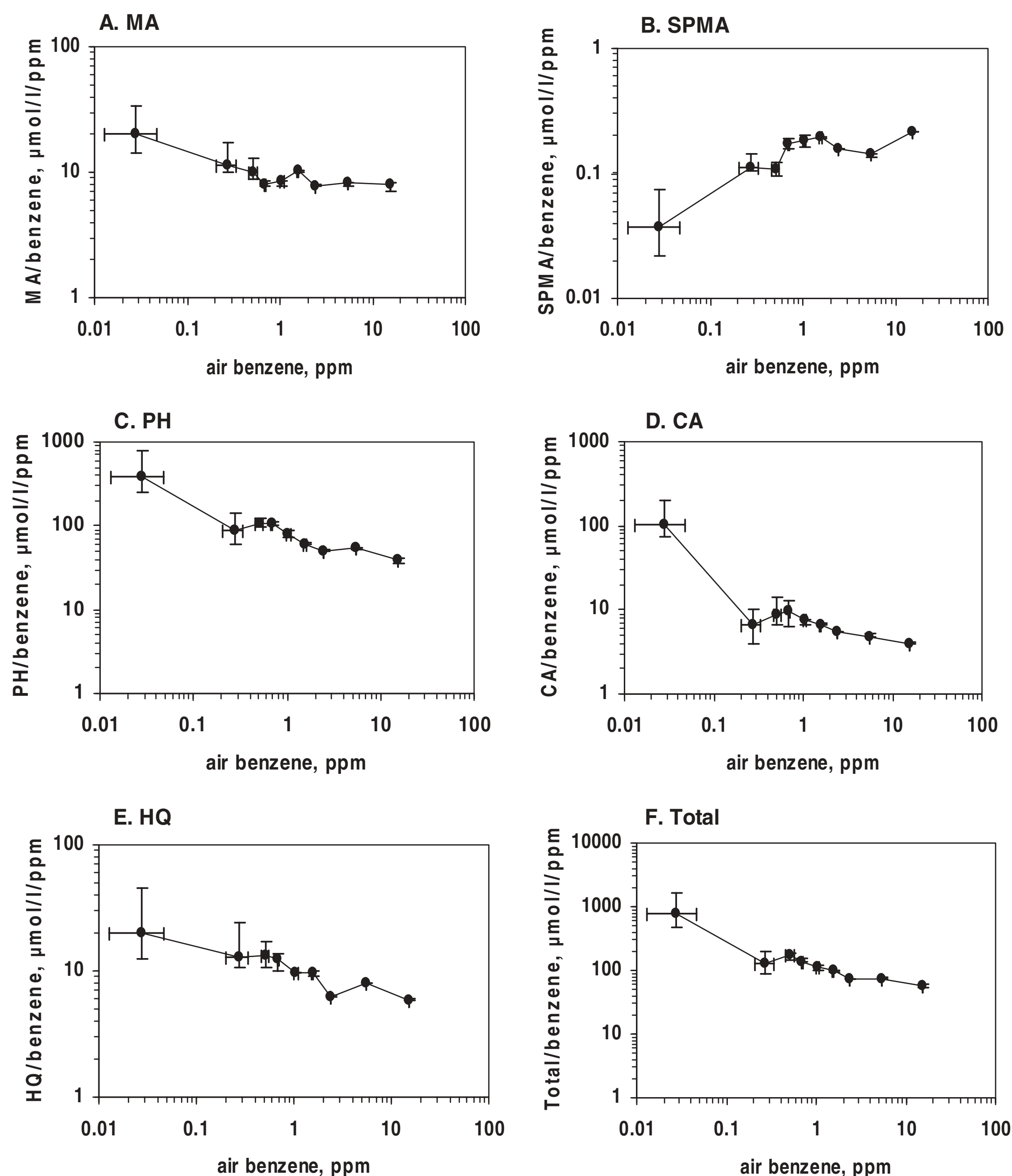

Fig. 4. Dose-related production of urinary metabolites versus air exposure to benzene. Each point and the corresponding error bars represent median values, and 5th and 95th percentiles of distributions of estimated median values of air benzene and dose-related metabolite levels (background-adjusted metabolite concentration/air benzene concentration) for groups of 30 subjects, determined by bootstrapping (1000 iterations). Total, total metabolites.

groups of workers having exposures >0.01 p.p.m. (after adjustment for background values). Each point in the figure and the corresponding error bars represent median values and 5th and 95th percentiles of distributions of median values, as determined by bootstrapping. These curves point to the dose-related production of benzene metabolites according to the pathways shown in Figure 1. Over the range of exposures investigated (median benzene concentrations between 0.027 and 15.4 p.p.m.) significant downward trends in metabolite production were observed ($P < 0.001$, Kruskal–Wallis tests) for all major metabolites (PH, CA, HQ and MA) and for total metabolites, while a significant upward trend was observed for SPMA ($P < 0.001$). The downward trend of total benzene metabolites is consistent with partial saturation of CYP metabolism of benzene between 0.027 and 15.4 p.p.m. The transitions were particularly apparent between the first and second exposure groups (median benzene concentrations between 0.027 and 0.274 p.p.m.) for all metabolites.

Discussion

The connections between benzene metabolism and toxicity have been extensively debated (15,22,23,35,36). To gain a better understanding of human benzene metabolism, we measured the key metabolites (PH, CA, HQ, MA and SPMA) and UBz in 250 benzene-exposed workers and 139 control workers from a Chinese population. Of the studies devoted to urinary biomarkers of benzene in Asian populations, none compared levels of all key metabolites with air levels representing both environmental and occupational exposures (25,27,28,30, 37,38). Our goals were to determine the usefulness of the urinary metabolites as biomarkers of benzene exposure in

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exposed and control subjects and to elucidate the dose-related patterns of benzene metabolism along the prominent pathways shown in Figure 1.

Since control workers in our study were exposed to benzene in environmental air and tobacco smoke, we predicted their exposures from the corresponding UBz concentrations, using a calibration curve obtained from exposed subjects (Figure 2). (We attribute difference between the calibration curve shown in Figure 2A and one previously reported by Waidyanatha *et al.* (26) to differences in urine collection and storage). Such predictions should be reasonably unbiased, given previous findings of strong linear trends between levels of benzene in urine and environmental air (39–42). This conjecture is reinforced by Figure 2B which plots levels of UBz versus air benzene from the current study (open circles) as well as those reported by Ghittori *et al.* (39) for 63 non-smoking workers exposed to measured benzene concentrations between 0.01 and 4 p.p.m. (closed circles). The two sets of measurements point to a linear log-scale relationship down to the lowest measured air level of ~0.01 p.p.m. Also, the median benzene air concentration predicted from UBz levels for our control subjects (3.55 p.p.b., $n = 139$) is similar to air concentrations estimated from personal monitoring of adult populations in the U.K. (3.82 p.p.b. daytime mean value, $n = 50$) (43), in Germany (3.44 p.p.b. median value, $n = 113$) (44), in the U.S. (2.29–7.01 p.p.b. range of median values for five cities, $n = 421$) (45) and Italy (2.82 and 1.88 p.p.b. for control subjects in two cities, $n = 107$) (46).

Comparing levels of urinary analytes between smoking and non-smoking controls, the median values of UBz, CA, HQ, MA and SPMA were all higher in smokers, while the median level of PH was marginally lower in smokers (Table I). The apparent anomaly for PH is probably related to gender because male controls had lower PH levels than females and 92% of the smokers were male. [Among male controls, smokers had a higher median level of PH (58.7 μmol/l, $n = 36$) than male non-smokers (46.4 μmol/l, $n = 16$)]. Other investigators have reported that non-occupationally exposed smokers had significantly higher urinary levels of UBz (39–42), MA (30,40,47–50) and SPMA (30,40,51) than non-smokers.

To estimate the equivalent air level of benzene from cigarette consumption, we regressed the (logged) predicted air concentration of benzene on the number of cigarettes smoked per day among control subjects. This led to the following relationship:

ln(benzene, p.p.m.) = −5.81 + 0.050 (cigarettes/day) ($n = 134$, P-value for slope = 0.003), from which we predict that smoking 20 cigarettes/day would be equivalent to an occupational benzene exposure of 8.2 p.p.b. (26 $\mu g/m^3$). This estimated benzene exposure of 8.2 p.p.b. is the same as that predicted from British data (52) for an urban smoker consuming 20 cigarettes/day.

In order to distinguish benzene-derived metabolites from background levels of the same compounds, we investigated median metabolite levels for groups of 30 subjects, aggregated by benzene exposure (Figure 3). Using statistical significance to discern exposure-related increases in urinary levels, we detected marginal elevations at benzene exposures between 0.02 and 0.1 p.p.m. for all metabolites except SPMA. However, when we considered exposure levels which produced unambiguous increases in metabolite concentrations, MA and SPMA were the most sensitive biomarkers of exposure to benzene (~0.2 p.p.m.), followed by PH and HQ (~0.5 p.p.m.) and CA (~2 p.p.m.).

Using aggregated data, we also observed changes in dose-related metabolism of benzene (Figure 4) for median benzene exposures between 0.027 and 15.4 p.p.m. We anticipated that saturable metabolism would be detected in this range, given results from other Chinese studies (25–27,30), and because benzene, oxepin and PH are all thought to compete for the same enzymes (CYP2E1) (53–55). And, in fact, downward trends were apparent for dose-related production of PH (9.9-fold), CA (26-fold), HQ (3.4-fold), MA (2.5-fold) and total metabolites (14-fold) (Figure 4). The 2.5-fold decrease observed for MA is comparable to a 3.3-fold reduction that we estimated from grouped data reported by Melikian *et al.* (27) (using differences between post-work and pre-work measurements of MA) for Chinese workers exposed to benzene concentrations between 0.34 and 22.6 p.p.m. (estimated mean values).

Surprisingly, SPMA displayed an upward trend of production, with a 5.8-fold increase between 0.027 and 15.4 p.p.m. (Figure 4). This might reflect the action of glutathione-*S*-transferase or be the result of mechanisms (16) or sites of formation of SPMA which differ fundamentally from those of the other metabolites.

It is noteworthy that the most dramatic reductions in dose-related metabolism occurred between the first two groups in Figure 4, with benzene exposures of 0.027 and 0.274 p.p.m., respectively. In this range, production of CA dropped by 16-fold and that of PH by 4.4-fold. Yet, only marginal reductions were observed in this exposure range for production of MA (44% reduction) and HQ (36% reduction). Thus, it appears that metabolism shifted away from CA and PH at low doses in favor of MA and HQ, the only major benzene metabolites requiring two CYP oxidations (Figure 1). When exposures exceeded ~1 p.p.m., the curves in Figure 4, representing major metabolites (PH, MA, HQ and CA), became quasi-parallel, suggesting that metabolism of benzene to BO had become rate-limiting.

If our conjecture that exposures to benzene <1 p.p.m. favor production of HQ and MA is correct, there could be important implications for risk assessment. Certainly, HQ is the precursor of 1,4-benzoquinone, which is generally regarded as most hematotoxic of the benzene metabolites (18,22,24, 36,56), and MA is derived from the extremely reactive and toxic muconaldehydes (14,15,17). We are currently applying various non-linear models to these data to more fully examine the dose-related metabolism of benzene and to estimate effects of physiological and genetic factors upon benzene metabolites.

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Conflict of Interest Statement: M.T.S. has received consulting and expert testimony fees from law firms representing both plaintiffs and defendants in cases involving exposure to benzene. G.L. has received funds from the American Petroleum Institute for consulting on benzene-related health research.

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