Urinary excretion of phenol, catechol, hydroquinone, and muconic acid by workers occupationally exposed to benzene

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Abstract

Objectives—Animal inhalation studies and theoretical models suggest that the pattern of formation of benzene metabolites changes as exposure to benzene increases. To determine if this occurs in humans, benzene metabolites in urine samples collected as part of a cross sectional study of occupationally exposed workers in Shanghai, China were measured.

Methods—With organic vapour monitoring badges, 38 subjects were monitored during their full workshift for inhalation exposure to benzene. The benzene urinary metabolites phenol, catechol, hydroquinone, and muconic acid were measured with an isotope dilution gas chromatography mass spectroscopy assay and strongly correlated with concentrations of benzene air. For the subgroup of workers (n=27) with urinary phenol >50 ng/g creatinine (above which phenol is considered to be a specific indicator of exposure to benzene), concentrations of each of the four metabolites were calculated as a ratio of the sum of the concentrations of all four metabolites (total metabolites) and were compared in workers exposed to >25 ppm and ≤ 25 ppm.

Results—The median, 8 hour time weighted average exposure to benzene was 25 ppm. Relative to the lower exposed workers, the ratio of phenol and catechol to total metabolites increased by 6.0% (p=0.04) and 22.2% (p=0.007), respectively, in the more highly exposed workers. By contrast, the ratio of hydroquinone and muconic acid to total metabolites decreased by 18.8% (p=0.04) and 26.7% (p=0.006), respectively. Similar patterns were found when metabolite ratios were analysed as a function of internal benzene dose (defined as total urinary benzene metabolites), although catechol showed a more complex, quadratic relation with increasing dose.

Conclusions—These results, which are consistent with previous animal studies, show that the relative production of benzene metabolites is a function of exposure level. If the toxic benzene metabolites are assumed to be derived from hydroquinone, ring opened products, or both, these are assumed to be derived from hydroquinone metabolism of phenol, a more complex, quadratic relation with increasing dose.

Occupational exposure to benzene has been causally associated with increased risk of aplastic anaemia, myelodysplastic syndromes, and acute non-lymphocytic leukaemia. The evaluation of risk posed by exposure to relatively low concentrations of benzene from occupational and environmental sources is an active area of research, given the ubiquity of benzene in the general environment. Because human data are sparse on risk of leukaemia due to low concentrations of benzene, the risk assessment process has generally relied on the extrapolation of observations made in highly exposed populations. However, extensive evaluation of the effects of dose, dose rate, route of exposure, and species on benzene absorption, metabolism, and excretion in experimental animals has shown that the relative proportions of benzene metabolites are highly dependent on dose level, raising concerns about high to low dose extrapolations.

For example, a model developed by Schlosser et al. based on benzene and phenol metabolism in mouse and rat liver microsomes suggests that as benzene concentration increases, metabolism of phenol to hydroquinone, a more toxic benzene metabolite along with its triol and quinone derivatives, should decrease due to competition for P-450 activation.

By contrast with the extensive database on benzene metabolism in animals, relatively little is known about potential benzene metabolite shifts in humans over wide ranges of exposure to benzene. To gain insight into the early biological effects of exposure to benzene in currently exposed workers, we carried out a cross sectional study of workers exposed to benzene and unexposed controls in Shanghai, China. We have previously shown a dose-response relation between exposure to benzene and haematotoxic, aneuploidogenic, clastogenic, and genotoxic events in this population. Here, we report how the patterns of the four most important urinary metabolites of benzene—phenol, hydroquinone, catechol, and muconic acid vary over a broad range of exposure.

Keywords: benzene; phenol; catechol; hydroquinone; muconic acid; biomonitoring
Materials and methods

SUBJECT ENROLMENT
Details of the study design and demographic characteristics of study subjects have been previously reported. Briefly, three factories in China were selected in which workers were occupationally exposed to benzene with minimal coexposures to other aromatic solvents. Controls were selected from workplaces without exposure to benzene and were frequency matched to the exposed subjects on age and sex. Forty four workers exposed to benzene and 44 controls were enrolled into the study. Exclusion criteria for subjects were a history of cancer, therapeutic radiation, chemotherapy, or current pregnancy. Informed consent was obtained from all study participants with Institutional Review Board approved procedures. Data were collected by interview on demographics and on current tobacco and alcohol use, and the height and weight of each subject were measured.

EXPOSURE ASSESSMENT
Occupational exposure to benzene and other organic vapours was monitored with passive dosimeters (3M No 3500, St Paul, MN) worn by each worker for a full work shift on the day of the study. Badges were analysed by the badgemakers’ methods, which used gas chromatography (GC) with flame ionisation detection for benzene, toluene, and xylene. For quality control, two separate badges worn by 10 workers were analysed in two laboratories. Analytical results from these duplicate measurements were highly correlated (Spearman, r=0.98, p<0.0001). The workers exposed to benzene were monitored for personal exposure to benzene in air for 5 days. Workers in factory 1 worked about 5–6 hour work shifts; for almost all workers, most of the exposure to benzene occurred during a 2.5–3 hour period. A spot urine sample was collected at the end of this high exposure period. Workers in factories 2 and 3 worked 8 hour work shifts, and a spot urine sample was collected at the end of the work shift. Urine samples were immediately frozen on dry ice and stored for later shipment.

In factory 1, most workers used half face respirators fitted with activated charcoal cartridges that were changed when the workers could detect the odour of benzene (about every other day). The fit of the respirators was not tested. Respirators were not used in either factory 2 or 3.

Control subjects were enrolled from two workplaces. In the first workplace, which manufactured sewing machines, most subjects were monitored for exposure to benzene with passive dosimeters for one day on any of six different sampling days. Workers in the second control workplace, which was administrative, were not monitored. All control subjects provided a spot urine sample at a clinic.

MATERIALS
The reagents phenol, hydroquinone, catechol, and muconic acid (Aldrich Chemical, Milwaukee, WI, USA), ethyl acetate (Burdick and Jackson, Muskegon, MI, USA), sodium sulphate (Fisher Scientific, Pittsburgh, PA, USA), and N,N-bis (trimethylsilyl) trifluoracetamide (BSTFA) (Pierce Manufacturing, Appleton, WI, USA) used in this study were of the highest purities available. 12 Benzene metabolite standards were biosynthesised in rats as previously described, except that [13C6] benzene was used instead of [H6] benzene. In previous studies with deuterated metabolite standards, all samples containing internal standards were kept in liquid nitrogen until needed to avoid exchange of the labile deuterium. Because of the stability of the ring labelled carbon atoms, urine samples in this study were conveniently stored at -80°C until needed. Concentrations of the [13C6] benzene metabolites used as internal standards were measured against a primary standard solution.

ANALYSIS OF URINE FOR BENZENE METABOLITES
Samples of urine were analysed blinded with respect to exposure. They were thawed at room temperature, and 200 µl distilled water was added to vials containing 200 µl urine. Internal standard solution (100 µl) was added, and the contents were mixed with 100 µl concentrated HCl. The vials were capped, incubated at 100°C for 60 minutes, and allowed to cool. The solutions were extracted with 2 ml ethyl acetate. The ethyl acetate layer was removed and dried with a small scoop of sodium sulphate. The sample was decanted, and the drying agent was rinsed with 1 ml ethyl acetate. The ethyl acetate aliquots were combined and
evaporated to 200 µl under nitrogen. The analyses were derivatised by adding 100 µl BSTFA and incubated at 60°C for 30 minutes.

A Hewlett Packard 5890/5970B gas chromatography mass selective (GC-MS) detector was used for all analyses. This was equipped with a Restek RT-5 fused silica capillary column, 25 m (length) × 0.23 mm (internal diameter) with a 0.25 µm film thickness. The initial column temperature was 80°C for 1 minute, followed by a temperature gradient of 12°C/min to 265°C. All injections (1 µl) were split less with the splitter on at 30 s. Ions 166, 254, 254, and 265 respectively for phenol, catechol, hydroquinone, and muconic acid were 3.0, 9.0, 6.4, and 5.3 respectively. Standards curves were made by adding identical aliquots of internal standard solution to varying concentrations of analysed solutions in methanol (0.25-5.0 µg). The resulting mixtures were blown to dryness under a stream of dry nitrogen and were derivatised as described above. After analysis by gas chromatography mass spectroscopy (GC-MS), the absolute amounts of metabolite standard versus the ratio of integrated peak areas for analysed ions relative to respective internal standard ions were plotted. Standard curves were consistently linear with R² >0.99 for all metabolites. This same ratio was calculated for samples and amounts of metabolites computed from the standard curve. Urinary creatinine was measured by the Instrumentation Laboratories analyser. Test creatinine with a Monarch Instrument measured by the Instrumentation Laboratories standard curve. Urinary creatinine was quantified from subsequent analyses.

Therefore, data from this subject were deleted. Regression diagnostics, described below, showed that it was also a high influence point. The relation between exposure category and benzene concentrations in air and metabolite concentrations was tested by the Spearman rank order correlation test. One data point for urinary hydroquinone was twice as high as the next greatest value and a formal outlier (>1.5 times the interquartile range).

### Table 1  Correlation between benzene exposure category and urinary phenol, catechol, hydroquinone, and muconic acid in workers occupationally exposed to benzene and controls studied in Shanghai, China, 1992

<table>
<thead>
<tr>
<th>Group</th>
<th>Benzene 8h TWA (ppm)</th>
<th>Phenol (ng/g creatinine)</th>
<th>Catechol (ng/g creatinine)</th>
<th>Hydroquinone (ng/g creatinine)</th>
<th>Muconic acid (ng/g creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=17):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>ND</td>
<td>17.3</td>
<td>3.2</td>
<td>1.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Range</td>
<td>3.7–55.3</td>
<td>1.6–9.7</td>
<td>0.9–6.7</td>
<td>0.1–0.8</td>
<td></td>
</tr>
<tr>
<td>25–75 Percentile</td>
<td>8.7–27.0</td>
<td>2.8–3.7*</td>
<td>1.3–3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exposed (1–&lt;25 ppm, n=20):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>9.5</td>
<td>55.6</td>
<td>7.2</td>
<td>16.4</td>
<td>14.6</td>
</tr>
<tr>
<td>Range</td>
<td>0.8–25.1</td>
<td>16.5–487.2</td>
<td>2.5–84.2</td>
<td>3.8–82.2</td>
<td>2.6–63.4</td>
</tr>
<tr>
<td>25–75 Percentile</td>
<td>4.7–25.1</td>
<td>32.7–102.0</td>
<td>5.2–14.6†</td>
<td>9.8–31.9</td>
<td>6.4–28.1</td>
</tr>
<tr>
<td>Exposed (&gt;25ppm, n=18):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>74.0</td>
<td>350.6</td>
<td>67.3</td>
<td>65.3</td>
<td>49.0</td>
</tr>
<tr>
<td>Range</td>
<td>33.1–331.7</td>
<td>27.7–516.7</td>
<td>7.0–81.4</td>
<td>8.3–196.6</td>
<td>5.3–103.4</td>
</tr>
<tr>
<td>25–75 Percentile</td>
<td>51.4–146.1</td>
<td>246.3–397.1</td>
<td>50.0–74.4</td>
<td>39.7–83.9</td>
<td>37.0–73.1</td>
</tr>
<tr>
<td>Spearman R</td>
<td>0.82</td>
<td>&lt;0.0001</td>
<td>0.82</td>
<td>0.86</td>
<td>0.86</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*p=16. †n=19.

Evaporated to 200 µl under nitrogen. The analyses were derivatised by adding 100 µl BSTFA and incubated at 60°C for 30 minutes. A Hewlett Packard 5890/5970B gas chromatography mass selective (GC-MS) detector was used for all analyses. This was equipped with a Restek RT-5 fused silica capillary column, 25 m (length) × 0.23 mm (internal diameter) with a 0.25 µm film thickness. The initial column temperature was 80°C for 1 minute, followed by a temperature gradient of 12°C/min to 265°C. All injections (1 µl) were split less with the splitter on at 30 s. Ions 166, 254, 254, and 265 respectively monitored for phenol, catechol, hydroquinone, and muconic acid, respectively, while ions 172, 260, 260, and 277 were monitored for the isotopically labelled metabolites.

Standard curves were made by adding identical aliquots of internal standard solution to varying concentrations of analysed solutions in methanol (0.25-5.0 µg). The resulting mixtures were blown to dryness under a stream of dry nitrogen and were derivatised as described above. After analysis by gas chromatography mass spectroscopy (GC-MS), the absolute amounts of metabolite standard versus the ratio of integrated peak areas for analysed ions relative to respective internal standard ions were plotted. Standard curves were consistently linear with $R^2 >0.99$ for all metabolites. This same ratio was calculated for samples and amounts of metabolites computed from the standard curve. Urinary creatinine was measured by the Instrumentation Laboratories analyser. Test creatinine with a Monarch Instrument measured by the Instrumentation Laboratories standard curve. Urinary creatinine was quantified from subsequent analyses.

Therefore, data from this subject were deleted. Regression diagnostics, described below, showed that it was also a high influence point. The relation between exposure category and benzene concentrations in air and metabolite concentrations was tested by the Spearman rank order correlation test. One data point for urinary hydroquinone was twice as high as the next greatest value and a formal outlier (>1.5 times the interquartile range). Regression diagnostics, described below, showed that it was also a high influence point. Therefore, data from this subject were deleted from subsequent analyses.

Among the subgroup of workers with urinary phenol >50 ng/g creatinine (n=27), concentrations of benzene in air and urinary

### Table 2  Ratio of each benzene metabolite to total benzene metabolites by exposure level in workers exposed to benzene with >50 ng/g creatinine urinary

<table>
<thead>
<tr>
<th>Group</th>
<th>Phenol (ng/g creatinine)</th>
<th>Catechol (ng/g creatinine)</th>
<th>Hydroquinone (ng/g creatinine)</th>
<th>Muconic acid (ng/g creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–&lt;25 ppm (n=11)*</td>
<td>20.5</td>
<td>4.8–25.1</td>
<td>11.7–21.7</td>
<td>0.61 (0.04)</td>
</tr>
<tr>
<td>&gt;25 ppm (n=16)†</td>
<td>78.0</td>
<td>40.3–331.7</td>
<td>57.4–178.2</td>
<td>0.65 (0.05)</td>
</tr>
</tbody>
</table>

*p=9 subjects with <50 ng/g creatinine urinary phenol deleted.
†One subject with <50 ng/g creatinine urinary phenol and one subject with an outlying value of hydroquinone deleted.
metabolites in workers exposed to ≤25 ppm were compared with concentrations in workers exposed to >25 ppm by the t-test. Subjects with phenol concentrations <50 ng/g were excluded, as phenol is not specific for exposure to benzene at lower concentrations. The relation between total urinary metabolites (used to estimate internal benzene dose) and the ratio of each metabolite to total metabolites was evaluated by multiple linear regression. Both linear and quadratic relations between total metabolites and each ratio were tested, and potential confounding by age, sex, current alcohol consumption, tobacco use, and body mass index (a measure of obesity calculated as weight (kg)/height (m^2)) was evaluated. Confounding was considered to be present if the regression coefficients for total urinary metabolites changed by >15% in either direction. The appropriateness of final models was evaluated by analysis of residuals and tests to identify high influence values. Two-sided tests were calculated throughout; p values <0.05 were considered adequate to reject the null hypothesis. Statistical analyses were performed with SAS for personal computer version 6.04.

Results
Summary measures of urinary concentrations of metabolites of benzene are presented in table 1 for controls, workers exposed to ≤25 ppm benzene, and workers exposed to >25 ppm benzene, and were highly correlated with exposure category (Spearman R²=0.80, p<0.0001, for all metabolites). The proportion of total metabolites made up by each specific metabolite is presented for workers exposed to ≤25 ppm and workers exposed to >25 ppm in table 2. Comparisons of the percentage change occurring in this proportion were made between the lower and higher exposed groups; phenol increased by 6% (p=0.04), catechol increased by 22% (p=0.007), hydroquinone decreased by 19% (p=0.04), and muconic acid decreased by 27% (p=0.006).

Individual concentrations of benzene in air, as continuous measures, could not be confidently used to provide more detailed insight into the nature of the relation between dose of benzene and metabolite ratio because exposure to benzene received by workers who wore respirators could only be approximated by the air monitoring data. Therefore, we used the sum of the concentrations of the major metabolites as a surrogate measure of air exposure. Studies in rats and mice have shown that these metabolites, as their conjugates, represent the overwhelming majority of the metabolites found in urine. Scatter plots are presented for...
As total metabolites increased, the proportion made up by phenol increased ($p=0.02$, fig 3 A) whereas the proportion made up by hydroquinone and muconic acid decreased ($p=0.002$ and $0.003$, respectively, fig 3 B, C), similar to the change in metabolite patterns found as a function of air concentration shown in table 2. The shift in the proportion of total metabolites made up by catechol showed a more complex, quadratic relation, initially increasing and then falling (fig 3 D). Multiple linear regression analysis showed that there was no confounding of these relations by age, sex, body mass index, current alcohol consumption, or current tobacco use.

As noted previously, samples of urine were collected at the end of the high exposure period in factory 1 and at the end of a full 8 hour work shift in factories 2 and 3. To determine if the difference in collection times influenced the results, we stratified the analysis of urinary metabolite ratios among lower and higher exposed workers presented in table 2 by factory (factory 1 v factories 2 and 3, combined). The direction of the change in metabolite proportions from lower to higher exposed workers was the same in each group (phenol 2% increase in factory 1 and 12% increase in factories 2 and 3; catechol 26% and 29% increase, respectively; hydroquinone 12% and 22% decrease, respectively; muconic acid 17% and 32% decrease, respectively). Also, a factory variable (factory 1 v factories 2 and 3) was not significant in the regression analysis of any metabolite outcome and did not confound the associations presented in figure 3 A–D.

Discussion
We analysed urinary benzene metabolites in a cross sectional study of workers exposed to a wide range of benzene concentrations and unexposed controls. Compared with workers exposed to $\leq 25$ ppm, the relative amounts of muconic acid and hydroquinone decreased among more highly exposed workers, whereas the relative amounts of catechol increased. The relative amounts of phenol increased as well, but to a smaller extent (6%). Linear regression analysis of these outcomes with total metabolites as a measure of internal dose showed that these relations were linear for muconic acid, hydroquinone, and phenol, and quadratic for catechol.

Exposure to benzene received by workers who wore respirators could only be approximated by the air monitoring data. Therefore, total internal dose of benzene was characterised by the sum of the four metabolites in urine. As an alternative to normalising the concentrations of individual metabolites to the total metabolites, individual metabolites were calculated as a ratio to phenol. Others have used this approach for normalising the urinary concentrations of other metabolites from exposure to benzene. This analysis led to essentially the same conclusions as with total metabolites. Phenol excretion has been shown to be linear with breath zone concentrations of benzene between 10 and 200 ppm as a time weighted average, although the scatter in these data sets may not have distinguished any subtle shifts in metabolism. Although we found that the relative amount of total metabolites as indicated by phenol increased in more highly exposed workers, as noted previously, this overall increase was small.
We note that the compositional nature of our outcome variable may have increased the possibility of artifactual or chance findings. If the ratio of one metabolite to total metabolites increases, then the relative amount of one or more other metabolites to total metabolites must necessarily decrease. If the four measured metabolites comprised almost all the metabolites of benzene in urine, then conclusions drawn from relative increases or decreases in metabolite ratios would be appropriate. If, however, the decrease in one metabolite resulted in the formation of a second, unmeasured metabolite, then relative changes found could be, in part, artifactual. This is unlikely because, as previously noted, studies in rodents have shown that these metabolites, as their conjugates, represent the overwhelming majority of the metabolites found in urine. Also, we had strong initial hypotheses, based on previous animal and theoretical studies, that the proportion of total benzene metabolites made up of one or more of the toxic benzene metabolites would decrease as internal dose increased. The consistency between these previous observations and the data reported here reduces the probability that the results from this study are artifactual or chance observations.

Benzene metabolism has been extensively studied in various animal models, in vitro incubations, and by theoretical calculations. The exact pathways and enzymatic systems involved remain unclear (fig 4). However, the trends found in our study are consistent with the model previously described by Schlosser et al, based on benzene and phenol metabolism by mouse and rat liver microsomes. In this model, all of the metabolic steps were assumed to be catalysed by the same set of enzymes, probably cytochrome P-450. Furthermore, benzene and its ring hydroxylated metabolites were all assumed to reversibly compete for the same reaction site on the cytochrome. Competitive binding would suggest that as benzene concentration increases, metabolism of phenol to hydroquinone should decrease. Therefore, the ratio of hydroquinone to phenol would decrease. In support of the model, at higher concentrations of catechol, hydroquinone relative to phenol, as well as total hydroquinone and other metabolites including 1,2,4-benzenetriol and particularly 1,4-benzoquinone, are highly toxic. However, other studies have shown synergistic effects between combinations of hydroquinone and other metabolites including phenol and catechol. Thus, although the latest evidence points to hydroquinone having a central role in benzene toxicity, a role for other metabolites cannot be ruled out.

Urinary metabolites do not necessarily reflect metabolite concentrations in target tissues. However, the use of urinary metabolites of benzene to gain insight into the in vivo metabolism of benzene is supported by the finding that 87% of metabolites derived from benzene are excreted in the urine of rodents. If we assume that the concentrations of benzene metabolites in urine reflect concentrations encountered in relevant tissues, our results suggest that the risk for adverse health outcomes due to exposure to benzene may have a complex relation with level of exposure. In particular, if the most important toxic benzene metabolites are assumed to derive from hydroquinone, ring opened precursors to muconic acid, or both, the results presented here suggest...
Urinary phenol, catechol, hydroquinone, and muconic acid and exposure to benzene

that risk for adverse health outcomes due to benzene may show a supralinear relation with external dose. Indeed, we have recently reported that there was a markedly smaller increase in risk for myelodysplastic syndromes and acute non-lymphocytic leukemia with exposure to increasing concentrations of benzene in a retrospective cohort study of 74 828 workers exposed to benzene in China.1 This dose-response relation was particularly evident for the 22 cases identified from a large subcohort of workers who were exposed to constant (fixed) concentrations of benzene over their working lifetimes.1

In summary, as exposure to benzene increased among workers exposed to a wide range of benzene concentrations in Shanghai, China, the proportion of benzene metabolites excreted as muconic acid and hydroquinone decreased whereas the proportion excreted as catechol and phenol increased. These complex patterns suggest that linear extrapolation of the toxic effects of benzene from highly exposed workers to less exposed populations may be inappropriate, to the extent that compounds related to hydroquinone and muconic acid play critical parts in benzene toxicity, and could lead to an underestimation of risk.

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