Comparison of the Levels of the Urinary Benzene Metabolite trans,trans-Muconic Acid in Smokers and Nonsmokers, and the Effects of Pregnancy

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Abstract
Urinary trans,trans-muconic acid, a ring-opened metabolite of benzene, was quantified in pregnant and nonpregnant smokers. The results of this study were compared with those reported previously in male smokers. The data clearly demonstrate that smoking increases the levels of trans, trans-muconic acid in urine. The mean levels of trans,trans-muconic acid in the urine of groups of male, female, and pregnant smokers were 3.6-, 4.8-, and 4.5-fold higher than those in the nonsmoking counterparts. Mean concentrations of trans,trans-muconic acid in groups of 42 men and 53 nonpregnant women were 0.22 ± 0.03 (SE) and 0.24 ± 0.02 mg/g creatinine, or 0.13 ± 0.06 and 0.13 ± 0.07 mg/mg cotinine, respectively. These data reveal that urinary trans,trans-muconic acid levels in female smokers are nearly the same as in male smokers. Mean levels of trans,trans-muconic acid in the urine of groups of 63 pregnant and 53 nonpregnant women were 0.27 ± 0.04 and 0.24 ± 0.02 mg/g creatinine, or 0.24 ± 0.06 and 0.13 ± 0.07 mg/mg cotinine, respectively. Mean concentrations of urinary cotinine in pregnant women were significantly lower than in the group of nonpregnant women (1.13 ± 0.12 versus 1.82 ± 0.14 mg/g creatinine). When levels of trans,trans-muconic acid were normalized against urinary cotinine, the mean concentration in urine of pregnant smokers was almost 2-fold greater than that in nonpregnant smokers. This could be due to an increased metabolism of benzene to urinary trans,trans-muconic acid during pregnancy. Alternatively, the percentage of benzene that is metabolized to urinary trans,trans-muconic acid may be greater at low exposure than it is at a high dose.

Introduction
Epidemiological studies have demonstrated a positive association between cigarette smoking and myeloid leukemia. This association has been reported both in large-scale prospective follow-up and case-control studies (1–13). Meta-analysis of seven prospective studies, conducted between 1970 and 1992, suggests a 30% higher risk of leukemia for people who smoked at some point in their lives, than for life-long nonsmokers (14). Numerous investigators also have shown a dose-response effect according to intensity (number of cigarettes/day) and/or duration of smoking (years smoked) (4–13).

Several constituents of tobacco smoke are suspected to play a role in enhancing the risk of leukemia in smokers. Among the chemicals tested, benzene is the strongest leukemogenic agent. Chronic exposure of humans to benzene is associated with myeloid leukemia (15). The concentration of benzene in mainstream cigarette smoke (smoke generated during puff drawing) is relatively high (54–73 µg benzene/cigarette) (16). Indeed, Wallace et al. (17) have demonstrated that tobacco smoke is a major source of benzene exposure for the cigarette-smoking population. In the United States, this affects 48 million smokers. The importance of benzene intake from cigarette smoke in regard to risk for leukemia ultimately can be ascertained only by reliable dosimetry of exposure.

Biochemical monitoring of benzene exposure usually has been based on the determination of benzene in exhaled air (18) and the measurement of urinary metabolites of benzene (18–25). Recently, quantifications of unmethylated urinary benzene (26) and serum protein adducts (27) have been applied to the monitoring of benzene exposure. Measurements of phenol, a major urinary metabolite of benzene, and assessments of derivatives of phenol, such as hydroquinone and catechol, have been used for high level benzene exposure. However, these compounds are not suitable as biomarkers for low level benzene exposure, since they can arise from various sources, such as occupational environments, food, cigarette smoke, etc. (28). Urinary trans,trans-muconic acid, a ring-opened metabolite of benzene (Fig. 1), appears to be a good candidate for monitoring benzene exposure and metabolism at low concentrations, such as benzene uptake from inhaled cigarette smoke (21–24). Indeed, quantification of this acid in the urine of smoking and nonsmoking males has indicated that levels in male smokers are about three times higher than in nonsmokers (24).

Some studies suggest that the risk of leukemia for women who smoke may be different from that for men, but there are insufficient data on women to verify this (9, 11). Several epidemiological studies also suggest that maternal smoking during pregnancy may increase the offspring’s risk of childhood leukemia (29–32). Thus, it is of interest to compare the uptake and metabolism of benzene from mainstream cigarette smoke in male smokers versus pregnant and...
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nonpregnant females. Therefore, the present pilot study was undertaken to investigate whether the uptake and activation of benzene to urinary trans,trans-muconic acid in pregnant and nonpregnant smokers are different than in male smokers.

Materials and Methods

Sample Collection. Urine specimens from male and female smokers and nonsmokers were obtained from subjects who applied for life insurance. Aliquots of urine samples from pregnant subjects were kindly provided to us from the health promotion research program of the University of Vermont. Specimens from pregnant women were taken during weeks 7–35 of pregnancy. Subjects filled out a questionnaire on smoking history and other parameters, such as occupation. Daytime urine samples (approximately 50–70 ml) were collected on the spot from individuals in each group. The samples were protected from light and were stored at -20°C until analysis.

Analysis of Urinary trans,trans-Muconic Acid. All samples were analyzed by a technique described previously (22, 24). In brief, the samples were thawed and a 1-ml aliquot of each was applied to a 300-mg Prep Sep-Sax cartridge (Fisher Scientific, Fair Lawn, NJ) that had been preconditioned with 3 ml MeOH and 3 ml HO. The cartridge was washed with 3 ml of 1% aqueous acetic acid; then trans,trans-muconic acid was eluted with 3 ml of a 10% aqueous acetic acid solution. Twenty-µl fractions of the eluate were analyzed by HPLC

HPLC Analysis. Urine was analyzed for trans,trans-muconic acid by HPLC on a 10-µm LiChrosorb C18 (25 cm × 4.6 mm) column (EM-Science; Gibbstown, NJ) which was eluted isocratically at a flow rate of 1 ml/min with 1 volume of methanol:9 volumes of 1% aqueous acetic acid (system 1). Samples containing peaks that were not completely separated from the trans,trans-muconic acid peak were reanalyzed by HPLC, using a linear gradient from 100% solvent A (1% aqueous acetic acid) to 90:10 solvent A:MeOH over 10 min (system 2), at a flow rate of 1 ml/min. The eluates were monitored at 264 nm. The trans,trans-muconic acid peak appeared either 12.5 or 21 min after injection in HPLC system 1 or 2, respectively. Authenticity of trans,trans-muconic acid was confirmed further in a few samples by GC/MS after derivatization with pentafluorobenzyl bromide.

Derivatization of trans,trans-Muconic Acid. To reconfirm the identity of urinary trans,trans-muconic acid, both standard (about 10 ng) and urinary trans,trans-muconic acid peaks collected from HPLC were transferred separately to 0.6-µl reaction vials. The samples were dried at 40°C under a stream of N2 and were derivatized by adding 200 µl of acetonitrile containing 6 µl pentafluorobenzyl bromide (Pierce; Rockford, IL) and 1 µl triethylamine. The vials were capped and the mixture was allowed to react for 6 h in a 60°C heating block with vortex mixing for 30 s every 30 min. After cooling, 1 µl of the reaction mixture was analyzed by GC-MS.

GC-NICI/MS. GC-NICI/MS analyses of the derivatized trans,trans-muconic acid were performed on a Hewlett-Packard Model 5890 gas chromatograph, operating in the splitless mode, with the injection port at 250°C and an initial oven temperature of 50°C for 2 min, followed by a temperature gradient of 10°C/min to 300°C. The outlet of a SE-54 Econocap (Alltech; San Jose, CA) fused silica capillary column (30 m × 0.25 mm) was inserted directly into the ion

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1 The abbreviations used are: HPLC, high pressure liquid chromatography; GC, gas chromatography; MS, mass spectrometry; NICI, negative ion chemical ionization; m/z, mass per charge.
source of a Hewlett-Packard Model 5988A mass spectrometer. Methane gas was used for the NICI analysis. MS conditions were: ion source, 200°C; emission current, 304 μA; electron energy, 120 eV.

Stability of Urinary trans,trans-Muconic Acid under Storage Conditions. Two urine samples from male smokers were selected and each sample was divided into four parts kept in vials and in the dark at ~20°C till analysis. Every 3 months, an aliquot of samples was thawed (one vial from each sample) and analyzed. Levels of urinary trans,trans-muconic acid at 0, 3, 6, and 9 months after collection were 0.21, 0.19, 0.20, and 0.22 mg/g creatinine (sample 1) and 0.13, 0.12, 0.11, and 0.12 mg/g creatinine (sample 2), respectively.

Creatinine and Cotinine Determination. Urinary creatinine was determined with a Kodak Ektachem 500 Computer-Directed Analyzer as described previously (24). Cotinine was quantified by modification of the radioimmunoassay initially developed by Langone et al. (33), using a specific anti-serum (raised at the American Health Foundation) that is produced in rabbits by injection of trans-4-carboxycotinine bound to albumin (34).

Results

Urinary trans,trans-muconic acid was quantified by HPLC techniques after solid phase extraction as reported previously (22, 24). A few selected urine samples were collected from HPLC and further analyzed by GC-NICI/MS. Derivatives of both reference trans,trans-muconic acid and urinary trans,trans-muconic acid samples collected from HPLC yielded a single peak at 24.5 min on GC analysis (data not shown) and gave the MS shown in Fig. 2, A and B, respectively. Characteristic features of these spectra are the molecular ion of the trans,trans-muconic acid derivative at m/z 502 and the major fragment at m/z 321, which is formed by loss of C2F5CH2. There is no significant change in the levels of urinary trans,trans-muconic acid when specimens are stored in the dark at ~20°C over a period of 9 months.

Table 1 shows the mean levels of trans,trans-muconic acid and cotinine in the urine for the groups of pregnant and nonpregnant smokers and nonsmokers and compares these data with corresponding values in men that were reported previously (24). Urinary trans,trans-muconic acid and cotinine are normalized against creatinine and cotinine in Table 1. The mean values of trans,trans-muconic acid in the groups of male, female, and pregnant smokers were 3.6-, 4.8-, and 4.5-fold higher than the mean concentration of this acid in their nonsmoking counterparts (Table 1). The differences in the mean trans,trans-muconic acid concentrations between smoking and nonsmoking groups were significant (P = 0.0001, P = 0.0001, and P = 0.002 in male, nonpregnant, and pregnant smokers, respectively). The distribution of levels of trans,trans-muconic acid in the groups of male, female, and pregnant smokers is shown in Fig. 3, A–C, respectively. Corresponding data for cotinine are displayed in Fig. 4, A–C. In these figures the concentration of trans,trans-muconic acid (Fig. 3, A–C) or levels of urinary cotinine (Fig. 4, A–C) are plotted versus frequency (the percent of individuals having the same concentration of this acid, or of cotinine, in each group). Comparison of Fig. 4, A–C indicates that urinary cotinine distribution is similar in men and in nonpregnant women, while most of the pregnant women (Fig. 4C) showed low levels of cotinine. Indeed, a majority of the pregnant women reported light smoking.

Discussion

Urinary trans,trans-muconic acid, a ring-opened metabolite of benzene, has been used for quantitative comparisons of benzene uptake and metabolism from mainstream cigarette smoke in male, female, and pregnant smokers. The data clearly demonstrate that smoking increased the levels of trans,trans-muconic acid in urine about 4- to 5-fold in both men and women. Mean concentrations of trans,trans-muconic acid in groups of men and women smokers were 0.13 ± 0.06 and 0.13 ± 0.07 mg/mg cotinine (mean ± SE), respectively. Similarly, the distribution of levels of urinary trans,trans-muconic acid and urinary cotinine was about the same in the above two groups (Fig. 3, A and B, and Fig. 4, A and B). Thus, analysis of these data reveals that urinary trans,trans-muconic acid in female smokers is nearly the same as in male smokers.

In the present study urinary cotinine is used to assess smoking levels. Mathai et al. (35) have shown that levels of urinary cotinine are increased slightly with advancing gestation. Therefore, one would expect that at the same levels of smoking, pregnant smokers will have slightly higher urinary cotinine levels. In our pilot study, levels of urinary cotinine are 1.13 ± 0.12 and 1.82 ± 0.14 (SE) mg cotinine/g creatinine in pregnant and nonpregnant smoking groups, respectively. Comparison of Fig. 4, B and C also indicates that the percentage of subjects with low levels of urinary cotinine is higher in the pregnant group. Thus, the mean levels of trans,trans-muconic acid were about two times greater (Table 1) for these subjects than for nonpregnant smokers (0.24 ± 0.06 versus 0.13 ± 0.07 mg trans,trans-muconic acid/mg cotinine). This may suggest that the metabolism of benzene to urinary trans,trans-muconic acid increases during pregnancy or that at low levels of benzene exposure, the
percentage of benzene that is metabolized to urinary trans,trans-muconic acid is greater than at a higher dose, i.e., that it is more efficient. Animal model studies have shown that at lower levels of benzene exposure the concentration of trans,trans-muconic acid represents a larger fraction of total benzene metabolized than at higher exposure levels (36). Further dose-response studies are required to confirm this finding.

The mechanism(s) responsible for the induction of bone marrow toxicity and leukemia by benzene have not been elucidated. It is generally accepted that benzene exerts its leukemogenic action upon metabolic activation. In the first step, benzene is metabolized to benzene oxide and/or cyclohexadienyl radical intermediates. These intermediates may react in several ways to lead to phenol, di-, and tri-hydroxylated benzene derivatives, biphenyl, some un-

### Table 1

<table>
<thead>
<tr>
<th>Subjects</th>
<th>No.</th>
<th>mg trans,trans-muconic acid/g creatinine</th>
<th>mg cotinine/g creatinine</th>
<th>mg trans,trans-muconic acid/mg cotinine</th>
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<tbody>
<tr>
<td>Male smoker</td>
<td>42</td>
<td>0.22 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.61 ± 0.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.13 ± 0.06</td>
</tr>
<tr>
<td>Female smoker&lt;sup&gt;d&lt;/sup&gt;</td>
<td>53</td>
<td>0.24 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.82 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.13 ± 0.07</td>
</tr>
<tr>
<td>Pregnant smoker</td>
<td>63</td>
<td>0.27 ± 0.04&lt;sup&gt;k&lt;/sup&gt;</td>
<td>1.13 ± 0.12&lt;sup&gt;h&lt;/sup&gt;</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td>Male nonsmoker</td>
<td>42</td>
<td>0.06 ± 0.03&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.006 ± 0.01&lt;sup&gt;h&lt;/sup&gt;</td>
<td>10 ± 1.9</td>
</tr>
<tr>
<td>Female nonsmoker&lt;sup&gt;e&lt;/sup&gt;</td>
<td>37</td>
<td>0.05 ± 0.007&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.008 ± 0.003</td>
<td>6.25 ± 1.5</td>
</tr>
<tr>
<td>Pregnant nonsmoker</td>
<td>34</td>
<td>0.06 ± 0.02&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.01 ± 0.003</td>
<td>6 ± 1.9</td>
</tr>
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</table>

<sup>a</sup> Mean ± SE.
<sup>b</sup> Data from Ref. 24.
<sup>c</sup> P = 0.0001; male smokers versus nonsmokers.
<sup>d</sup> Nonpregnant smokers and nonsmokers.
<sup>e</sup> P = 0.0001; female smokers versus nonsmokers.
<sup>f</sup> P = 0.002; pregnant smokers versus nonsmokers.
<sup>g</sup> P = 0.93; nonpregnant smokers versus pregnant smokers.
<sup>h</sup> P = 0.004; nonpregnant smokers versus pregnant smokers.

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**Fig. 3.** Levels of urinary trans,trans-muconic acid versus frequency (%). A, group of 42 male smokers; B, group of 53 female nonpregnant smokers; C, group of 63 pregnant smokers.

**Fig. 4.** Levels of urinary cotinine versus frequency (%). A, group of 42 male smokers; B, group of 53 female nonpregnant smokers; C, group of 63 pregnant smokers.
known metabolites, as well as ring-opened trans,trans-
muconaldehyde and trans,trans-muconic acid (Fig. 1)
(37–46). The latter has been detected in both bone mar-
row and urine after administration of benzene to mice; it is
also a urinary metabolite of benzene in humans. Although
the exact mechanism of formation of trans,trans-muconic
acid from benzene is not known, it has been documented
that phenol, catechol, or hydroquinone administration
to mice does not lead to detectable levels of this metabo-
lite in the urine (46). Since cigarette smoke contains high
levels of phenol, catechol, and hydroquinone, along with
benzene, it appears that urinary trans,trans-muconic acid
is a good candidate for monitoring low-level benzene
exposure in smokers. Witz et al. (47) have demonstrated that
trans,trans-muconaldehyde, presumably a precursor of
trans,trans-muconic acid (Fig. 1) is myelotoxic in mice. This
aldehyde has not yet been detected in vivo, but it has
been shown that mouse liver microsomes can metabo-
lize benzene to muconaldehyde (44). It also has been hy-
pothesized that combinations of benzene metabolites,
such as hydroquinone and phenols or muconaldehyde
and hydroquinone may be involved in the overall toxicity
and leukemogenicity of benzene (48). Since cigarette
smoke also contains derivatives of benzene, such as phae-
nol, catechol, and hydroquinone in significant amounts
(39), these may have a synergistic effect and thereby
enhance the toxicity of benzene in smokers.

In conclusion, levels of trans,trans-muconic acid were
significantly higher in the urine of male and female smokers
than in nonsmokers. It appears that the levels of this ben-
zeine metabolite increase during pregnancy. However, fur-
ther dose-response studies are required to confirm this
observation.

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urine samples from the pregnant subjects. We are also indebted to
them dose-response studies are required to confirm this

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