Urinary *trans,trans*-Muconic Acid as an Indicator of Exposure to Benzene in Cigarette Smokers

Assieh A. Melikian, Agasanur K. Pralahad, and Dietrich Hoffmann
American Health Foundation, Naylor Dana Institute for Disease Prevention, Valhalla, New York 10595

Abstract
Epidemiological studies have shown an association between cigarette smoking and increased risk of myeloid leukemia in smokers. In evaluating this link it is important to note that cigarette smoke contains benzene, among other carcinogens. Since chronic benzene exposure causes acute myeloid leukemia in humans, we aimed to determine the uptake and metabolic activation of benzene from cigarette smoke in smokers by measuring the levels of the urinary benzene metabolite, *trans,trans*-muconic acid (t,t-MA).

The method involved a clean-up procedure, followed by high-performance liquid chromatography with UV detection. The levels of urinary t,t-MA ranged from 0.02 to 1.3 mg/g creatinine, resulting in a mean of 0.29 ± 0.04 mg/g creatinine in 42 male smokers, and corresponding values in nonsmokers ranged from "nondetectable" to 0.52 mg/g creatinine with an average of 0.09 ± 0.02 mg/g creatinine. In the current study, the levels of t,t-MA in smokers were about 3 times higher than those in nonsmokers (*P* = 0.0001), and a significant correlation between concentration of t,t-MA and levels of cotinine in smokers was observed (*r* = 0.55; *P* = 0.0001; 95% confidence interval, 0.30–0.93), suggesting that urinary t,t-MA can be utilized as a biochemical marker to quantitate benzene exposure due to cigarette smoking.

Introduction
Although leukemia has not always been regarded as a smoking-related cancer, recent epidemiological studies, both case-control and large-scale prospective follow-up, have shown an association between cigarette smoking and leukemia (1–10). Relative risks were greater for myeloid leukemia than for other forms of leukemia in smokers. Risks of myeloid leukemia in smokers were 2–3 times greater than those in nonsmokers (3–5, 7–10). Furthermore, these studies have also pointed to a dose-response relationship, wherein myeloid leukemia showed the strongest dose response in terms of both levels of smoking (number of cigarettes per day) and duration of smoking (years smoked) (3–5, 7–10).

In evaluating the link between cigarette smoking and leukemia, one is drawn to the observation that cigarette smoke does contain benzene and its metabolites, among other carcinogens (11–13). Chronic exposure of humans to benzene is associated with acute myeloid leukemia (14), the same type of leukemia that is increased in smokers. The concentration of benzene in cigarette smoke is relatively high. The mainstream smoke of commercial cigarettes contains 54–73 μg (47–67 ppm) benzene per cigarette (11, 12). In the United States, the upper permissible limit for occupational exposure to benzene is 1 ppm, on the basis of an 8-h time-weighted average exposure. The Environmental Protection Agency’s TEAM study, which assessed the total exposure of 400 residents in 8 cities, indicated that the major source of exposure to benzene in the United States population is cigarette smoke (12). Thus, for more than 50 million smokers in the United States, the smoke (and especially the mainstream smoke generated during puff drawing) of their cigarette is the major source of benzene exposure (15). The benzene level in the exhaled breath of smokers is about 22–29 μg/m³ compared with levels of 5–8 μg/m³ in the breath of nonsmokers. Indoor air, polluted with tobacco smoke, also contained 37–107 μg benzene/m³ compared with 3–40 μg benzene/m³ in the air in non-smoking environments (16). Two studies have also shown that children of smokers have increased leukemia mortality (17–18).

The present work was undertaken to examine the uptake and metabolic activation of benzene from cigarette smoke in smokers. *trans,trans*-Muconic acid (Fig. 1) has been identified as a urinary metabolite of benzene in rabbits (19), mice, rats, and humans (20, 21). As early as 1916, it was reported that this acid was present in the urine of patients who had been treated with benzene to cure leukemia (22). Recently, quantitation of t,t-MA has been used for the biochemical monitoring of workers occupationally exposed to low levels of benzene (23–25). The pilot study described here compared levels of urinary t,t-MA in 42 male smokers with those in 42 nonsmokers and indicates that the mean level of urinary t,t-MA in smokers is about 3 times higher than that found in nonsmokers.

Materials and Methods
Chemicals. [14C]Benzene (112 mCi/mmol) was purchased from Chemsyn Science Laboratories (Lenexa, KS).

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2 To whom requests for reprints should be addressed.
Reverse-phase HPLC determined it to be >98 pure. Unlabeled benzene, obtained from Burdick and Jackson (Muskegon, MI), was used to dilute [14C]benzene to the desired specific activity. t,t-MA, >98% pure, was purchased from Aldrich Chemical Co. (Milwaukee, WI). A PrepSep-SAX cartridge filled with 300 mg strong anionic exchange silica was bought from Fisher Scientific Co. (Fair Lawn, NJ).

Animals. Male F344/N rats were supplied by Charles River Breeding Laboratories (North Wilmington, MA). They were 12 weeks old at the onset of the assay.

Animal Treatment. Ten rats were given i.p. injections of a single dose of 10 mmol [14C]benzene/kg body weight in 0.5 ml corn oil. Twenty-four-h urine voids were collected and were stored at -20°C prior to analysis.

Analysis of Urinary t,t-MA. About 50-70 ml daytime urine samples were collected from smokers and nonsmokers. Both human and rat urine samples were first subjected to a solid-phase extraction clean-up procedure, developed by Ducos et al. (24), followed by HPLC analysis (20). Frozen urine samples were thawed immediately before analysis, a 1-ml aliquot of the test sample was applied to a PrepSep-SAX cartridge preconditioned with 3 ml methanol and 3 ml H2O. The cartridge was washed with 3 ml of a 1% aqueous acetic acid solution; then t,t-MA was eluted with 3 ml of a 10% aqueous acetic acid solution. The eluates were monitored at 264 nm. The t,t-MA peak appeared 12.5 or 21 min after injection in HPLC systems 1 and 2, respectively. The mean recovery rates were 98, 93, 90, and 80% when t,t-MA-free urine samples were spiked with 0.1, 0.2, 0.4, and 1.0 mg/liter of unlabeled t,t-MA; 75% of the radioactivity was recovered when the urine samples had been spiked with 10 mg/liter (78,000 dpm/ml) of [14C]t,t-MA obtained from the urine of rats treated with [14C] benzene and purified by HPLC. Twenty-five % of the radioactivity was lost during the clean-up on the PrepSep-SAX cartridge. All recovery analyses were done in triplicate. All concentrations reported in Fig. 2 and Table 1 were corrected for a 92% recovery. The detection limit of the method is sensitive enough to measure urinary t,t-MA at a concentration of 0.02 mg/liter.

Analysis of Urinary Cotinine. Cotinine was quantified by modification of the radioimmunoassay initially devel-
opened by Langone et al. (26), using a specific antiserum (raised at American Health Foundation) that is produced in rabbits by injection of trans-4-carboxycotinine bound to albumin (27).

Creatinine Determination. Urinary creatinine was determined with a Kodak Ektachem 500 Computer-Directed Analyzer (Eastman Kodak Company, Clinical Products Division) (28). Briefly, a 10-μl urine specimen is deposited on the Kodak Ektachem Clinical Chemistry Slide (CREA). This slide contains dry, multilayered analytical elements coated on a clear polyester support. Creatinine diffuses to the gel layer and is hydrolyzed by amidinohydrolase to creatine. The creatine thus formed is converted to sarcosine and urea by creatine amidinohydrolase. In the presence of sarcosine oxidase sarcosine is oxidized to glycine, formaldehyde, and hydrogen peroxide. The final reaction involves the peroxidase-catalyzed oxidation of triarylboradazole leuco dye by hydrogen peroxide to produce a colored product, which is read at 670 nm.

Analysis of Cigarette Smoke Particulate Matter for t,t-MA. Commercially available cigarettes were smoked on a 30-port Borgwaldt smoking machine (Heinrich Borgwaldt, Hamburg, Germany), and the mainstream smoke was collected on a Cambridge filter (9 cm in diameter). The total particulate matter from 10 cigarettes, smoked under similar conditions, was collected on a Cambridge filter (9 cm in diameter). The particulate matter from 10 cigarettes including the filter was suspended in 2 ml NH4OH (150 ml) and was then extracted with equal volumes of ethyl ether. Upon separating the organic acids and neutral constituents of the tar by partitioning with ethyl ether; the solvent was evaporated, and the residue was suspended in 1 ml H2O.

Table 1. t,t-MA and cotinine in the urine of smokers and nonsmokers

<table>
<thead>
<tr>
<th>Urinary metabolites</th>
<th>Smokers</th>
<th>Nonsmokers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/liter urine</td>
<td>mg/g creatinine</td>
</tr>
<tr>
<td>t,t-MA</td>
<td>0.41 ± 0.05</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>Cotinine</td>
<td>2.3 ± 0.33</td>
<td>1.6 ± 0.30</td>
</tr>
</tbody>
</table>

* Mean ± SE for n = 42.

HPLC analysis of urine samples from rats revealed that the clean-up extraction procedure used in the current study selectively removes those urinary metabolites of [14C]benzene that might not be completely resolved from the [14C]t,t-MA peak under the HPLC elution conditions. The single radioactive peak eluting at 12.5 min (Fig. 3A) co-eluted with authentic t,t-MA. No t,t-MA could be detected in the urine of untreated control animals. When samples from treated rats were analyzed by HPLC without passing through the extraction cartridge, several other urinary metabolites of [14C]benzene eluted from the HPLC column without a complete resolution from the t,t-MA peak (data not shown); thus, they could interfere in the measurement of t,t-MA. This clean-up procedure (24) discriminates the t,t-MA HPLC-UV signal from natural background and eliminates some of the other metabolites of benzene, thereby increasing the sensitivity of detection.

Human urine samples that contained high levels of t,t-MA, or agents that were not completely resolved from t,t-MA, were reanalyzed using HPLC elution system 2, a longer program, which separated t,t-MA from other urine constituents. A HPLC profile of 24-h urine voids from rats treated with [14C]benzene is illustrated in Fig. 3A, and typical HPLC profiles of urine samples from a non-smoker and smoker are shown in Fig. 3, B and C. All HPLC profiles shown in Fig. 3 are obtained with HPLC system 1; profiles from HPLC system 2 are not shown.

Level of t,t-MA in Mainstream Cigarette Smoke. Total particulate matter from 10 cigarettes, smoked under standardized machine smoking conditions, was trapped on a Cambridge filter and analyzed for t,t-MA (13). The concentrations of t,t-MA in two popular cigarettes smoked in the United States were lower than 0.01 μg/cigarette.

Cotinine, a major metabolite of nicotine, was used to distinguish the urine of smokers from that of non-smokers.
smokers. Table 1 compares mean urinary concentrations of t, t-MA and cotinine in nonsmokers and smokers both uncorrected and normalized against creatinine. Levels of urinary cotinine in nonsmokers were almost negligible, except for 4 of 42 individuals who had somewhat elevated levels. The mean concentration of cotinine and the SE in the urine of smokers was 1.6 ± 0.30 mg/g creatinine (ranging from 0.3 to 1.2 mg/g creatinine); for nonsmokers it was 0.006 ± 0.003 mg/g creatinine (ranging from "not detectable" to 0.08 mg/g creatinine). Urinary t, t-MA versus cotinine for the smokers is plotted in Fig. 4 (the correlation coefficient is $r = 0.55; P = 0.0001; 95\%$ confidence interval, 0.30-0.93).

Discussion

Urinary phenol has been frequently measured to assess benzene exposure in industrial settings. A major drawback of this approach is that there are other sources of phenol in the environment, so that this analyte becomes unspecific at low levels of benzene exposure (29, 30). Recent studies suggest that urinary t, t-MA, a ring-opened metabolite of benzene (Fig. 1), is a potential candidate for monitoring benzene exposure at levels down to 1 or 2 ppm (23-25). Our pilot study confirms these findings and suggests that quantitation of this acid in the urine of smokers can be used as an indicator of uptake and activation of benzene that was inhaled as a cigarette smoke constituent.

In addition to benzene, cigarette smoke contains the metabolites of benzene such as phenol, catechol, and hydroquinone in significant amounts (13). To assess exposure to benzene but not exposure to the metabolites of benzene, one has to monitor specific metabolites of benzene or adducts other than those derived from phenol or its derivatives.

The initial step in benzene metabolism is believed to be oxidation to benzene oxide and/or formation of a hydroxycyclohexadienyl free radical by insertion of a hydroxyl radical (31-34). Benzene oxide may react with glutathione, with the resulting conjugate being converted to urinary 5-phenylmercapturic acid (5-phenyl-N-acetyl-cysteine) (35), or benzene oxide is converted to phenol and several hydroxylated species (Fig. 1). Benzene may also be converted to t, t-muconaldehyde, which is oxidized to t, t-MA and excreted in the urine (36-38). Although the mode of formation of muconaldehyde is unknown, it has been shown that phenol, catechol, and hydroquinone are not precursors to urinary t, t-MA (20, 39). Thus, urinary t, t-MA was selected as a biochemical marker of benzene exposure in smokers because of the practicality of the analytical procedure with high sensitivity and because t, t-MA is formed directly from benzene but not from hydroxylated metabolites of benzene.

This study has revealed that only 12 of 42 nonsmokers were free from detectable levels of this acid. The presence of t, t-MA in the other individuals in this group may reflect uptake of small amounts of benzene from the environment. About 4 subjects in the nonsmoking group had relatively high levels of urinary cotinine, which suggests that they had probably been exposed to environmental tobacco smoke. In addition to benzene, sorbic acid (trans, trans-2,4-hexadienoic acid), a food additive and preservative in certain cosmetics and pharmaceuticals, is a known precursor to t, t-MA (40). Thus, sorbic acid may also be a source of t, t-MA. However, the metabolic conversion of sorbic acid to t, t-MA is rather low, about 0.13-0.18% of the dose in humans and 0.2-0.6% in mice (24).

Levels of t, t-MA were significantly higher in the urine of smokers than in that of nonsmokers (Table 1; Fig. 2). There was also a significant correlation between concentrations of urinary t, t-MA and cotinine (Fig. 4). About a 3-fold higher excretion of this acid in smokers than nonsmokers indicates that benzene from cigarette smoke is bioavailable and is indeed metabolized in humans. Undoubtedly, differences in the types of cigarettes smoked, in individual smoking habits, and in metabolism of benzene among individuals contribute to variations in the amount of urinary t, t-MA. The concentration of t, t-MA in mainstream cigarette smoke is less than 0.01 µg/cigarette. Thus, for a smoker of 20 cigarettes/day (12 h), urinary excretion of t, t-MA from cigarettes would not exceed 0.006 mg/g creatinine, which is lower than the detection limit; thus, most of the urinary t, t-MA in smokers is derived from the benzene present in the cigarette smoke they inhaled.

The mechanism(s) of leukemia induction by benzene are not yet fully understood. Unlike benzene exposure, administration of phenol, a major metabolite of benzene, to rats does not induce hematotoxicity (41), whereas Witz et al. (42) have demonstrated that trans, trans-muconaldehyde, a precursor of t, t-MA (Fig. 1), is myelotoxic in mice. It has been hypothesized that combinations of metabolites of benzene, such as hydroquinone and phenol, or muconaldehyde and hydroquinone, may be involved in the hematotoxicity and carcinogenicity of benzene (43). Since cigarette smoke also contains metabolites of benzene, such as phenol, hydroquinone, and catechol, these, as well as other smoke constituents, may synergistically enhance the toxicity and carcinogenicity of benzene in smokers.

In conclusion, levels of urinary t, t-MA in smokers are significantly higher than in nonsmokers. Concentrations of this acid correlate significantly with levels of urinary cotinine. Further studies are required to confirm this observation.
Acknowledgments

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References


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