HEI HEALTH EFFECTS INSTITUTE

Development of Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry Methods for Determination of Urinary Metabolites of Benzene in Humans

Assieh A. Melikian, Min Meng, Ray O'Connor, Peifeng Hu, and Seth M. Thompson
American Health Foundation, Valhalla, NY

Includes the Commentary of the Institute's Health Review Committee

Research Report Number 87
June 1999

HEI HEALTH EFFECTS INSTITUTE

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate matter), and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 200 projects at institutions in North America and Europe.

Typically, HEI receives half its funds from the U.S. Environmental Protection Agency and half from 28 manufacturers and marketers of motor vehicles and engines in the United States. Occasionally, funds from other public or private organizations either support special projects or provide resources for a portion of an HEI study. Regardless of funding sources, HEI exercises complete autonomy in setting its research priorities and in reaching its conclusions. An independent Board of Directors governs HEI. The Institute's Research and Review Committees serve complementary scientific purposes and draw distinguished scientists as members. The results of HEI-funded studies are made available as Research Reports, which contain both the Investigators' Report and the Review Committee's evaluation of the work's scientific quality and regulatory relevance.

HEI Statement

Synopsis of Research Report Number 87

Development of New Methods to Measure Benzene Biomarkers

INTRODUCTION

Occupational exposure to benzene is associated with the development of leukemia and other blood disorders; however, the effects of exposure to low levels of benzene are not well understood. In 1994, the Health Effects Institute (HEI) initiated a research program (the HEI Air Toxics Program) to address uncertainties in the health effects resulting from exposure to ambient levels of toxic air pollutants including benzene. One of the goals of this program was to develop and validate biomarkers of exposure to benzene. There are a number of challenges in identifying suitable markers of recent benzene exposure that are appropriate for epidemiologic studies. First, benzene can be metabolized along a number of different pathways, and some products of benzene metabolism can also be derived from the metabolism of other compounds. Second, data on benzene metabolism in humans are limited, particularly in response to low-dose exposures, and it has been difficult to extrapolate results of controlled exposure studies in animals to the human situation. Third, it is difficult to detect and measure reproducibly the low levels of many benzene metabolites. In response to these concerns, HEI funded Dr. Melikian and colleagues to develop and validate a novel, practical method for assaying metabolites of benzene in humans.

STUDY DESIGN

Dr. Melikian and colleagues at the American Health Foundation developed methods using a technique known as Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry (LC-ESI-MS/MS) to measure benzene metabolites in human urine. The investigators measured *S*-phenylmercapturic acid (*S*-PMA) and *trans,trans*-muconic acid (*t,t*-MA) simultaneously; three other metabolites—*p*-hydroquinone (HQ), catechol (CAT), and 1,2,4-trihydroxybenzene (BT)— were assayed at the same time using a modification of the LC-ESI-MS/MS method. Because cigarette smokers have higher levels of some benzene metabolites than individuals not exposed, Dr. Melikian made preliminary assessments of the utility of her assays of benzene metabolites by measuring urinary levels in a group of smokers and in a control group of nonsmokers.

RESULTS AND INTERPRETATION

The LC-ESI-MS/MS assays that Dr. Melikian developed for all five metabolites were reproducible and sensitive. The assay for *S*-PMA was the most sensitive (down to 0.03 ng) and the assay for BT least sensitive (50 ng). The investigators found detectable levels of the five benzene metabolites in almost all of the analyzed urine samples from both smokers and nonsmokers. Mean levels of *S*-PMA and *t,t*-MA were approximately 2-fold higher in urine from smokers compared to nonsmokers: 2.1-fold and 1.9-fold, respectively. In contrast, the investigators found that mean levels of BT, HQ, and CAT were not significantly different in urine samples from the smokers and nonsmokers that were examined in the study.

Melikian and colleagues clearly demonstrated that their general methodological approach is sound. The LC-ESI-MS/MS method is analytically robust and has important advantages over existing methods. In particular, the investigators succeeded in developing a novel, sensitive, and selective method for measuring two minor metabolites of benzene, *S*-PMA and *t,t*-MA, in human urine. Their finding that these metabolites are detectable in the urine of both smokers and nonsmokers, coupled with the fact that *S*-PMA is a benzene-specific metabolite, indicates that *S*-PMA has great potential as a biomarker of low-level exposure to benzene.

More work is needed to validate these findings (such as determining the kinetics of appearance of *S*-PMA in urine after exposure to benzene) before the assay is generally recommended to the field. It is not clear that *t,t*-MA can be used as a unique marker of low-level benzene exposure because it can also be formed from sorbic acid in food, but it may still be useful as a marker for occupational studies in which exposure to benzene is high. Because Dr. Melikian's assays for HQ, CAT, and BT were not as sensitive as the assays of *S*-PMA or *t,t*-MA, and HQ, CAT, and BT can be derived from sources other than benzene, these metabolites are unlikely to be useful as biomarkers of low-level exposure to benzene. Further validation of Dr. Melikian's results in human subjects is being sought in an HEI-funded study in China, directed by Dr. Qingshan Qu of New York University.

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of a research project sponsored by HEI from September 1994. This study was conducted by Dr. Assieh A. Melikian and colleagues at the American Health Foundation, Valhalla, NY. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

Library of Congress Catalog Number for the HEI Report Series: WA 754 R432

The paper in this publication meets the minimum standard requirements of the ANSI Standard Z39.48-1984 (Permanence of Paper) effective with Report Number 21, December 1988, and with Report Numbers 25, 26, 32, 51, and 65 Parts IV, VIII, and IX excepted. These excepted Reports are printed on acid-free coated paper.

TABLE OF CONTENTS

Research Report Number 87

Development of Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry Methods for Determination of Urinary Metabolites of Benzene in Humans

Assieh A. Melikian, Min Meng, Ray O'Connor, Peifeng Hu, and Seth M. Thompson

(Continued on next page)

INTRODUCTION

Benzene and the Health Effects Institute's Air Toxics Program

In 1994, the Health Effects Institute (HEI) initiated a research program to address uncertainties about the health effects resulting from exposure to ambient levels of toxic air pollutants, including benzene. The following discussion is intended to provide background on HEI's benzene research program, which included the study presented in this report.

REGULATORY BACKGROUND

Regulation of benzene is addressed in several sections of the Clean Air Act Amendments (CAAA) of 1990. Section 112 lists benzene as one of the 188 hazardous air pollutants defined as compounds or groups of compounds "to which no ambient air standard is applicable and that . . . causes, or contributes to, air pollution which may reasonably be anticipated to result in an increase in morbidity or an increase in serious irreversible or incapacitating reversible illness." From this list of pollutants, the U.S. Environmental Protection Agency (EPA) Administrator was required to develop a strategy for identifying the 30 air pollutants most hazardous to human health. Sources accounting for 90% or more of these 30 pollutants are to be subject to regulation. In addition, cancer risk attributable to these substances is to be reduced by 75%. Sections 202 and 211 of the CAAA of 1990 also address the issue of regulating benzene as a mobile source air pollutant. Section 202 lists benzene, 1,3-butadiene, and formaldehyde as being among emissions that pose "the greatest risk to human health and about which significant uncertainties remain." Section 211 defines mobile source toxic air pollutants as aggregate emissions of benzene, 1,3-butadiene, polycyclic organic matter (POM), acetaldehyde, and formaldehyde, and the same section requires reduction in emissions of ozone-forming volatile organic compounds and emissions of toxic air pollutants.

In response to a suit filed by the Sierra Club against the EPA alleging failure to promulgate regulations to control hazardous air pollutants from motor vehicles and stationary sources, the EPA combined several regulatory efforts required by the Clean Air Act concerning air toxics into an Integrated Urban Air Toxics Strategy. This strategy is intended to address toxic emissions from all outdoor sources, including both stationary and mobile sources. In August 1998, the EPA released a draft Integrated Urban Air Toxics Strategy for public comment. The document outlines actions for reducing emissions of air toxics and hazardous air pollutants, as well as actions for developing better understanding of the health risks. The document also provides a list of categories of area sources that emit toxics, a draft list of the priority hazardous air pollutants (believed to pose the greatest threat to human health), and a work plan for actions to reduce substantially risks from air toxics from all urban area sources. Benzene is one of the 33 priority hazardous air pollutants on the draft list. The deadline for the final Integrated Urban Air Toxics Strategy is June 1999.

SCIENTIFIC BACKGROUND

Data from epidemiologic studies indicate that benzene is toxic to humans, especially to the bone marrow and bone marrow–derived cells. Exposure to benzene is associated with the development of acute nonlymphocytic leukemia (ANLL) (Ott et al. 1978; Rinsky et al. 1987; Aksoy 1989; Crump 1994) and other blood disorders, including pancytopenia, as well as chromosomal aberrations (Hite et al. 1980; Tice et al. 1990; Pirozzi-Chatterjee and Snyder 1991; Forni 1996). Although there is no established animal model for benzene-induced leukemia, benzene has been shown to cause other types of cancers in experimental animals. Benzene and its metabolites cause a number of changes in mouse bone marrow cells in culture (Hazel 1996; Irons 1996; Kalf 1996). Furthermore, bone marrow cells taken from benzene-exposed mice have impaired hematopoietic potential (Abraham 1996). Other reported changes in cells taken from benzene-exposed animals and grown in culture include reduction in reticulocyte number, reduction of B lymphocyte number in the spleen and bone marrow, and increased frequency of micronuclei in reticulocytes (Farris et al. 1996).

Despite findings of blood disorders in humans and animals and cancers at other sites in animals, the mechanism of benzene toxicity and the specific metabolite or metabolites that lead to disorders remain to be identified. (See Figure 1 in the Commentary of this report for metabolic pathways of benzene). Several mechanisms of benzene toxicity have been proposed. A widely held hypothesis is that certain benzene metabolites are transformed into reactive oxygen species that interact with cellular macromolecules, resulting in genotoxic events (Smith 1996).

Metabolites that could be transformed into reactive oxygen species include benzoquinone, hydroquinone, and transformation products of benzene triol (Smith 1996). It has also been suggested that a combination of ring-hydroxylated compounds may be the effective toxic agents (Smith 1996). Another hypothesis is that the precursor of *t,t*-muconic acid, *t,t*-muconaldehyde, possibly in combination with other metabolites, plays a role (Witz et al. 1996).

ASSESSMENT OF RISK

The occupational health literature has established that benzene is a leukemogen, and, as such it is regulated in the workplace. The Occupational Safety and Health Administration (OSHA) has established a workplace standard of 1 ppm as an 8-hour time-weighted average. In addition, an exposure of 5 ppm should not be exceeded in any 10-minute period. The American College of Government and Industrial Hygienists has recommended an airborne exposure limit of 10 ppm averaged over an 8-hour work shift. The National Institute of Occupational Safety and Health recommends an airborne exposure limit of 1 ppm not to be exceeded during any 60-minute period.

Workplace regulation is based on occupational epidemiology studies. Little direct information is available about the human health risk of exposure to typical environmental levels (generally 1 to 10 ppb). One approach to risk estimation is to extrapolate to humans the results from controlled low-dose exposure studies in different animal species. Species differ in their sensitivity to benzene, however. Although the overall metabolism appears to be qualitatively similar in a number of species, specific metabolites may differ in quantity, and it has been suggested that the differences in susceptibility among species may be due to the relative amounts of different metabolites formed (Henderson 1996). For example, mice (a more sensitive species) may form larger amounts of hydroquinone and its more labile sulfate conjugation product in the liver than do rats (a less sensitive species).

Another approach to risk estimation is high- to low-dose extrapolation. In the absence of data on low-dose exposures, such extrapolation assumes a linear relationship between exposure level, metabolite mixture and health outcome. It is possible that high- and low-dose benzene exposures result in different mixtures of metabolites. Experimental animals exposed to high levels of benzene form relatively less hydroquinone than when exposed to lower doses (Henderson 1996). These findings suggest that high- to low-dose extrapolation should consider metabolic patterns at various dose levels.

Recently, the EPA published "Carcinogenic Effects of Benzene: An Update," which includes a review of the relevant literature and an estimate of risk for environmental exposure to benzene (EPA 1998). After careful examination of the epidemiologic literature in conjunction with a consideration of other literature, the EPA determined that, at this time, "there is not sufficient evidence . . . to reject a linear dose-response curve for benzene in the low-dose region, nor is there sufficient evidence to demonstrate that benzene is, in fact, nonlinear in its effects." Thus, "the EPA default approach of using a model with low-dose linearity is still recommended. Of the various approaches employing a linear assumption, the [lifetime] risk at 1 ppm [of benzene in air] ranges from 7.1×10^{-3} to 2.5×10^{-2}, within which any calculated unit risk estimate would have equal scientific validity." Although the linear model is the present model for estimating risk, arguments exist for either a supra- or a sub-linear dose response model. Improving the accuracy of benzene risk assessment for the general population at environmental exposure levels will require more information about the mechanism of benzene-induced leukemia and other benzene-induced disorders. This includes information needed to characterize the specific mixture of benzene metabolites delivered from the liver to the bone marrow, the fate of these metabolites in the bone marrow, and, ultimately, the role of specific metabolites in the development of blood abnormalities. Finally, it requires more information about biomarkers of exposure as well as effect.

HEI RESEARCH PROGRAM

In 1992, HEI held a workshop that formed the basis of a publication issued in 1993, *Research Priorities for Mobile Air Toxics*. This document identified broad research needs related to several mobile source air toxics including benzene. As a response to specific research needs identified for benzene, HEI developed a program with two general goals. These were

- To develop sensitive methods to measure benzene metabolites formed after exposures to low levels of benzene with the goal of identifying markers of benzene exposure, uptake, and metabolism, and the relation between levels of different metabolites and benzene exposure in various species.
- To obtain information on the relation between different benzene metabolites and genetic changes. This will help elucidate the mechanism of benzene

toxicity and identify biomarkers of biologically effective dose and early effects in humans.

As a first step in its benzene program, HEI funded four benzene studies under RFA 93-1, *Novel Approaches to Extrapolation of Health Effects for Mobile Source Toxic Air Pollutants*. Three of the studies involved the development and validation of biomarkers of benzene exposure or effect in animal systems; the fourth involved the development of techniques to look at the metabolism of benzene at doses relevant for human urban air exposure. The four studies were

1. *S-Phenylcysteine in Albumin as a Benzene Biomarker*. William E. Bechtold, Lovelace Respiratory Research Institute, Albuquerque, NM (unpublished report).
2. *Quantification of Urinary Metabolites of Benzene by HPLC-MS Method: Assessment of the Relationship of Degree of Benzene Exposure to Differences in the Metabolic Activation Pathways in Humans*. Assieh A. Melikian, American Health Foundation, Valhalla, NY.
3. *Characterization and Mechanisms of Chromosomal Alterations Induced by Benzene in Mice and Humans*. David A. Eastmond, University of California, Riverside, CA.
4. *Benzene Metabolism at Doses Relevant for Human Urban Air Exposure*. Kenneth W. Turteltaub, Lawrence Livermore National Laboratory and University of California, San Francisco, CA.

The biomarkers developed by Drs. Bechtold, Eastmond, and Melikian were tested in animal systems. The investigators are now validating the most promising of these biomarkers in human subjects exposed to benzene. The study, *Validation of Biomarkers for Benzene in Human Populations*, is under the direction of Dr. Qingshan Qu of the Institute for Environmental Medicine of New York University, in collaboration with investigators from the Chinese Academy of Preventive Medicine. The study is divided into two phases:

- In Phase I, investigators who developed biomarkers under RFA 93-1 applied their techniques to the analysis of blood and urine samples taken from workers employed in shoe- and glue-manufacturing facilities (benzene exposures ranging from 100 ppm to less than 5 ppm) and from food-processing workers (benzene exposures below the limit of detection) in Tienjing, China. Dr. Qu and colleagues obtained personal exposure data from all participating subjects and reconstructed historical exposure data for all participants.
- Phase II, presently under way, is a dose-response study looking at biomarkers in blood and urine samples taken from subjects exposed to benzene concentrations ranging from less than 5 ppm to 15 ppm. Endpoints measured in Phase I are also being measured in Phase II. Personal exposure data are being obtained from personal samplers, and historical exposure profiles are being reconstructed for all participating subjects.

The Qu study will be completed in the year 2000, and should provide information about the suitability of biomarkers developed under RFA 93-1 for studies involving exposure to ambient levels of benzene. It is also expected to provide information about possible relationships between certain biomarkers of exposure and biomarkers of effect. The biomarkers developed by Dr. Melikian and discussed in this report are undergoing evaluation in the Qu study.

REFERENCES

Abraham NG. 1996. Hematopoietic effects of benzene inhalation assessed by long-term bone marrow culture. Environ Health Perspect 104 (Suppl 6):1277–1282.

Aksoy M. 1989. Hematotoxicity and carcinogenicity of benzene. Environ Health Perspect 82:193–197.

Crump KS. 1994. Risk of benzene-induced leukemia: A sensitivity analysis of the Pliofilm cohort with additional follow-up and new exposure estimates. J Toxicol Environ Health 42:219–242.

Farris GM, Robinson SN, Gaido KW, Wong BA, Wong VA, Leonard L, Shah R. 1996. Effects of low concentrations of benzene on mouse hematopoietic cells in vivo: A preliminary report. Environ Health Perspect 104 (Suppl 6):1274–1276.

Forni A. 1996. Benzene-induced chromosome aberrations: A follow-up study. Environ Health Perspect (Suppl 6) 104:1309–1312.

Hazel BA, O'Connor A, Niculescu R, Kalf GF. 1996. Induction of granulocytic differentiation in a mouse model by benzene and hydroquinone. Environ Health Perspect (Suppl 6) 104:1256–1264.

Health Effects Institute. 1993. Research Priorities for Mobile Air Toxics. HEI Communications Number 2. Health Effects Institute, Cambridge, MA.

Henderson RF. 1996. Species differences in the metabolism of benzene. Environ Health Perspect 104 (Suppl 6):1173–1175.

Hite M, Pecharo M, Smith I, Thornton S. 1980. The effect of benzene on the micronucleus test. Mutat Res 77:149–255.

Huff JE, Haseman JK, DeMarini DM, Eustis S, Maronpot RR, Peters AC, Persins RL, Chrisp CE, Jacobs AC. 1989. Multiple site carcinogenicity of benzene in Fischer rats and B6C3F1 mice. Environ Health Perspect 82:125–163.

Huff JE. 1992. Applicability to humans of rodent-specific sites of chemical carcinogenicity: Tumors of the forestomach and of the harderian, preputial, and Zymbal glands induced by benzene. J Occup Med Toxicol 1(2):109–141.

Irons RD, Stillman WS. 1996. Impact of benzene metabolites on differentiation of bone marrow progenitor cells. Environ Health Perspect 104:1247–1250.

Kalf GF, Renz JF, Niculescu R. 1996. *p*-Benzoquinone, a reactive metabolite of benzene, prevents the processing of pre-interleukins-1α and -1ß to active cytokines by inhibition of the processing enzymes, calpain and interleukin-1ß converting enzyme. Environ Health Perspect 104:1251–1256.

Ott MG, Townsend JC, Fishbeck WA, Langner RA. 1978. Mortality among individuals occupationally exposed to benzene. Arch Environ Health 33:3–10.

Pirozzi-Chatterjee S, Snyder R. 1991. Mechanisms of benzene toxicity. In: Molecular Aspects of Monooxygenases and Bioactivation of Toxic Compounds (Arine E, Schenkman JB, Hodgson E, eds.) pp. 387–414. Plenum Press, New York, NY.

Rinsky RA, Smith AB, Hornung R, Filloon TG, Young RJ, Okun AH, Landrigan PJ. 1987. Benzene and leukemia: An epidemiologic risk assessment. N Engl J Med 316:1044–1050.

Rothman N, Li GL, Dosemici M, Bechtold WE, Marti GE, Wang YZ, Linet M, Xi LQ, Lu W, Smith MT, Titenko-Holland N, Zhang LP, Blot W, Yin SN, Hayes RB. 1996. Hematotoxicity among Chinese workers heavily exposed to benzene. Am J Ind Med 29:236–246.

Schrenk D, Orzechowski A, Schwarz LR, Snyder R, Burchell B, Ingelman-Sundberg M, Bock KW. 1996. Phase II Metabolism of benzene. Environ Health Perspect 104 (Suppl 6):1183–1188

Smith MT. 1996. The mechanism of benzene-induced leukemia: a hypothesis and speculations on the causes of leukemia. Environ Health Perspect 104 (Suppl 6) 1219–1225.

Tice RR, Costa DL, Drew RT. 1996. Cytogenetic effects of benzene in murine bone marrow: Induction of sister chromatid exchanges, chromosomal aberrations and cellular proliferation inhibition in DBA/2 mice. Proc Natl Acad Sci USA 77:2148–2152.

U.S. Environmental Protection Agency. 1998. Carcinogenic Effects of Benzene: An Update. EPA/600P-97/001F. Office of Research and Development, Washington, DC.

Witz G, Zhang, Z, Goldstein BD. 1996. Reactive ring-opened aldehyde metabolites in benzene hematotoxicity. Environ Health Perspect 104 (Suppl 6):1195–1199.

Development of Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry Methods for Determination of Urinary Metabolites of Benzene in Humans

Assieh A. Melikian, Min Meng, Ray O'Connor, Peifeng Hu, and Seth M. Thompson

ABSTRACT

To investigate the ways in which different levels of exposure affect the metabolic activation pathways of benzene in humans, and to examine the relationship between urinary metabolites and other biological markers, we have developed two sensitive and specific liquid chromatography–tandem mass spectrometry (LC-MS/MS)* assays for quantitation of the benzene metabolites *trans,trans*-muconic acid (*t,t*-MA), *S*-phenylmercapturic acid (*S*-PMA), hydroquinone (HQ), catechol (CAT), and for estimation of 1,2,4-trihydroxybenzene (BT).

In our first assay, urinary *S*-PMA and *t,t*-MA were measured simultaneously by liquid chromatography–electrospray ionization–tandem mass spectrometry–selected reaction monitoring (LC-ESI-MS/MS-SRM) in the negative ionization mode. In this assay, the metabolites [$^{13}C_6$]-*S*-PMA and [$^{13}C_6$]-*t,t*-MA were used as internal standards. The efficacy of this specific assay was evaluated in human urine specimens from 28 smokers and 18 nonsmokers serving as the benzene-exposed and nonexposed groups, respectively. The coefficient of variation (CV) of analyses on different days (n = 8) for *S*-PMA was 7% for samples containing 9.4 µg/L urine, and for *t,t*-MA was 10% for samples containing 0.07 mg/L. The mean levels of *S*-PMA and *t,t*-MA in smokers were 1.9-fold (p = 0.02) and 2.1-fold (p = 0.03) higher, respectively, than those in nonsmokers.

The mean urinary concentration (± SE) was 9.1 ± 1.7 µg *S*-PMA/g creatinine (median 5.8 µg/g in a range from not detectable [1 of 28] to 33.4 µg/g creatinine) among the smokers. In nonsmokers' urine, the mean concentration was 4.8 ± 1.1 µg *S*-PMA/g creatinine (median 3.6 µg/g in a range from 1 to 19.6 µg/g creatinine). For *t,t*-MA in smokers' urine, the mean was 0.15 ± 0.03 mg *t,t*-MA/g creatinine (median 0.11 mg/g creatinine in a range from 0.005 to 0.54 mg/g creatinine); corresponding concentrations in nonsmokers' urine were 0.07 ± 0.02 mg *t,t*-MA/g creatinine (with a median of 0.03 mg/g creatinine in a range from undetectable [1 of 18] to 0.18 mg/g creatinine). There was a correlation between *S*-PMA and *t,t*-MA after logarithmic transformation (r = 0.41, p = 0.005).

In our second assay, the ring-hydroxylated metabolites, namely urinary BT, HQ, and CAT, after acid hydrolysis were quantitated simultaneously by LC-ESI-MS/MS-SRM in the negative ionization mode with [$^{13}C_6$]HQ and [$^{13}C_6$]CAT as internal standards. The coefficient of variation of analyses on different days for the specimens containing 0.7 mg BT/L urine (n = 7) was 20%. For HQ, the coefficient of variation was 12% (n = 7) for the sample containing 0.4 mg HQ/L and 4.6% (n = 7) for the sample with 1.9 mg CAT/L. The efficacy of this assay was evaluated by using urine specimens from both light cigarette smokers and nonsmokers. In the group of nonsmokers, the mean concentration of BT was estimated at 1.7 ± 0.3 mg/g creatinine (with a median of 1.5 mg/g in a range of 0.2 to 5.0 mg/g). Among smokers, the mean was 1.8 ± 0.5 mg/g creatinine (with a median of 1.7 mg/g in a range from 0.14 to 7.15 mg/g). The mean of HQ was 1.1 ± 0.3 mg/g creatinine (with a median of 0.7 mg/g in a range from 0.15 to 5.0 mg/g creatinine) in nonsmokers' urine, and 2.0 ± 0.6 mg/g creatinine (with a median of 1 mg/g creatinine in a range from 0.25 to 8.1 mg/g) in smokers. Corresponding CAT levels were 5.5 ± 1.4 mg/g creatinine (median = 3.1 mg/g creatinine in a range from 0.3 to 20.8 mg/g creatinine) in nonsmokers, and 5.0 ± 1.2 mg/g creatinine (with a median of 3.1 mg/g creatinine in a range from 1.6 to 17.5 mg/g creatinine) in smokers. In both groups combined, there was a correlation between CAT and BT (r = 0.54, p = 0.002) and

* A list of abbreviations appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute Research Report Number 87, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Assieh Melikian, American Health Foundation, One Dana Road, Valhalla, NY 10595.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R824835 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private-party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

between CAT and HQ ($r = 0.46$, $p = 0.01$). The LC-ESI-MS/MS methods appear to be sensitive and specific for assessing urinary metabolites of benzene, and they will enable the detection of various levels of benzene exposure in the general population.

INTRODUCTION

BENZENE EXPOSURE IN HUMANS

Benzene is a ubiquitous environmental pollutant. The major sources of this pollutant to the general population are automotive emissions and tobacco (Wallace 1989a,b) and various consumer products. About 400 out of 5,000 products tested by the National Aeronautics and Space Administration (NASA) emit benzene in amounts ranging from 0.01 to 140 µg/g (Ozkaynak 1987).

About 50% of the benzene-related leukemias in the United States are attributed to tobacco smoking (Wallace 1989a,b). Personal daily uptake of benzene from mainstream cigarette smoke is about 50 to 60 µg per cigarette (1,800 µg from 30 cigarettes); exposure through passive smoking is up to 50 µg/day, while the intake of benzene from ambient air in the United States is estimated at about 430 to 1,530 µg/day (Wallace 1989a,b). Occupational benzene exposure occurs in the United States, where, of the 237,812 potentially exposed workers, 95% were found to be exposed to benzene levels below 16 mg/m^3 (5 ppm) for an 8-hour time-weighted average (TWA) (Weaver et al. 1983; Fishbein 1984). The threshold limit value (TLV) for the TWA of benzene has been lowered in the past decade in the United States; for the years 1996 and 1997, a TLV-TWA of 0.5 ppm (1.6 mg/m^3) has been suggested by the American Conference of Governmental Industrial Hygienists (ACGIH)(1996). In developing countries, benzene exposure can be several times higher. In Chinese facilities producing paint and manufacturing shoes, concentrations as high as 1,000 mg/m^3 (312 ppm) were found in more than 500 workplaces surveyed (Yin et al. 1987; Rothman et al. 1996).

TOXICITY AND CARCINOGENICITY OF BENZENE

Benzene produces several biological responses. Its acute effects at high doses can lead to central nervous system depression and even to loss of consciousness. Benzene suppresses the immune system in humans (Snyder and Kocsis 1975; Yardly-Jones et al. 1991). The International Agency for Research on Cancer (IARC) (1982) has concluded that a causal relationship exists between high levels of benzene exposure and anemia, leukopenia, thrombocytopenia, lymphocytopenia, aplastic anemia, leukemia, and especially acute myeloid leukemia (Aksoy et al. 1974; Vigliani and Forni 1976; Rinsky et al. 1981). Lung cancer, and possibly Hodgkin's disease and lymphoma, as well as multiple myeloma may also be related to benzene exposure (Aksoy 1980; Decouflé 1983; Yin et al. 1996). Studies of workers exposed to low levels of benzene are not conclusive; they either have null findings or point to only slightly elevated rates of either acute myeloid leukemia and all other leukemias, or multiple myeloma (Ott et al. 1978; Tsai et al. 1983; Bond et al. 1986; Wong 1987; Ireland et al. 1997).

In animal assays, chronic exposure to benzene by inhalation or via oral dosing (gavage) produces neoplasias at multiple sites. Most of the tumors are of epithelial origin and include tumors of the Zymbal gland, oro-nasal cavity, lung, liver, mammary gland, and skin. However, several types of lymphomas and leukemias have also been reported (Snyder et al. 1978, 1980, 1984, 1988; Goldstein et al. 1982; Cronkite et al. 1984, 1985, 1989; Huff et al. 1989; Maltoni et al. 1989; National Toxicology Program 1989a). The myelotoxicity of benzene in mice is more pronounced following a discontinuous dosing regimen than following continuous exposure (Tice et al. 1989).

Another type of biological response to benzene exposure is the production of clastogenic responses such as chromosome aberration (Philip and Jensen 1970; Forni et al. 1971), sister chromatid exchange (Tice et al. 1980), and induction of micronuclei (Hite et al. 1980; Siou et al. 1981). Benzene exposure has been associated with in vivo mutations to the *hprt* gene and to the *lacI* gene in the Big Blue mouse (Mullin et al. 1995).

METABOLISM OF BENZENE

Metabolism of benzene is complex; it is thought to take place primarily in the liver through the cytochrome P450 2E1 (CYP2E1) system (Johansson and Ingelman-Sundberg 1988; Koop et al. 1989; Nakajima et al. 1990; Chepiga et al. 1991; Gut et al. 1996a,b; Valentine et al. 1996) and, to a lesser extent, in such target tissues as the bone marrow (Irons et al. 1980; Kalf 1987; Lévay et al. 1992). The major metabolic routes consist of cytochrome P450–mediated oxidation of benzene to form a benzene-epoxide-oxepin intermediate (Figure 1), or interaction of benzene with a hydroxyl radical ($^{\bullet}OH$) to yield a hydroxycyclohexadienyl radical intermediate (Figure 2), or both (Dorfman et al. 1962; Johansson and Ingelman-Sundberg 1988). Benzene epoxide can react with glutathione and is excreted in urine as *S*-PMA. Benzene metabolism can also lead to the formation of three classes of compounds: ring-hydroxylated metabolites, ring-dimerized metabolites, and ring-

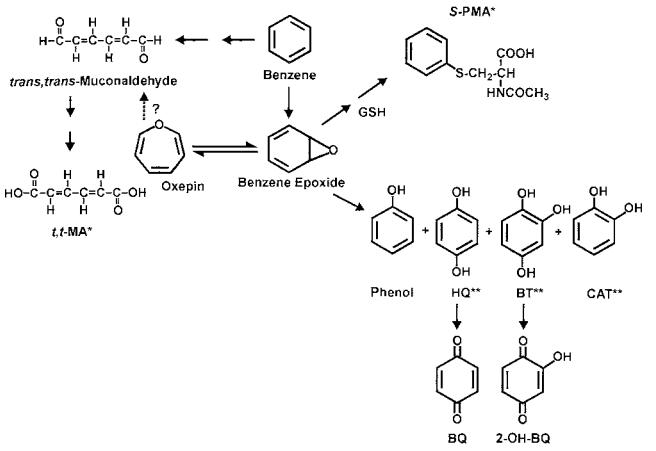

Figure 1. Metabolic activation of benzene via a benzene epoxide intermediate.

opened metabolites (Parke and Williams 1953; Snyder et al. 1987; Henderson et al. 1989; Sabourin et al. 1989, 1992; Henderson 1996; Schrenck et al. 1996; Snyder and Hedli 1996).

(1) Ring-hydroxylated metabolites, such as phenol, HQ, CAT, and BT, form predominantly sulfate and glucuronide conjugates that are excreted in urine. Some of these ring-hydroxylated metabolites can be further oxidized to corresponding semiquinones and benzoquinones and react with cellular macromolecules (Subrahmanyam et al. 1990, 1991). (2) Ring-dimerized metabolites, such as 2,2′-biphenol and 4,4′-biphenol, can form from phenol by a free radical mechanism (Figure 3) (Subrahmanyam et al. 1990, 1991). Further metabolism of these biphenols results in the formation of insoluble polymers that can bind noncovalently to cellular macromolecules (Smith et al. 1989). The hydroxycyclohexadienyl intermediate can also lead to the formation of biphenyl (Cohen et al. 1981), which can be further metabolized to biphenols and other dimers and polymeric products (Figure 2). (3) Ring-opened metabolites, such as urinary *t,t*-MA (Drummond and Finar 1938; Parke and Williams 1953), apparently are derived from *trans,trans*-muconaldehyde (Figure 2) (Zhang et al. 1995; Witz et al. 1996).

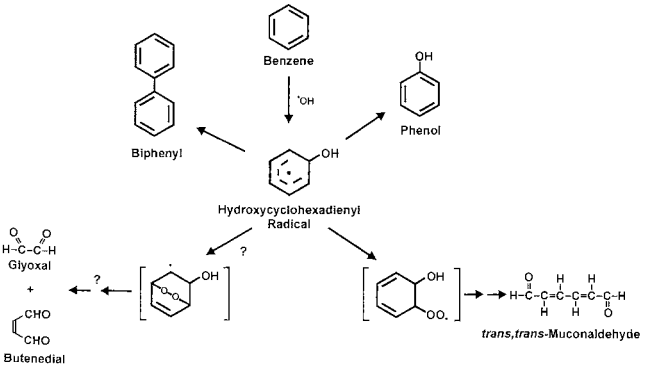

Figure 2. Metabolic activation of benzene via a hydroxycyclohexadienyl radical intermediate.

MECHANISM OF TOXICITY AND CARCINOGENICITY OF BENZENE

The molecular mechanisms responsible for benzene toxicity and carcinogenicity have not yet been fully elucidated. Several studies have indicated that metabolism of benzene to active intermediates is a prerequisite for its toxic and carcinogenic effects (Sammett et al. 1979; Sawahata et al. 1985; Gad-El Karim et al. 1986; Gaido and Wierda 1987; Kalf 1987). The ultimate toxic or carcinogenic metabolites of benzene are not clearly defined (Johnson and Lucier 1992; Snyder and Kalf 1994; Smith 1996a,b). Several hypotheses have been advanced regarding the active species.

Reactive Quinones

It has been proposed that reactive quinones formed from oxidation of ring-hydroxylated metabolites play an important role in benzene toxicity (Irons 1985; Sawahata et al. 1985; Smith et al. 1989; Subrahmanyan et al. 1991; Kalf and O'Connor 1993). This applies especially to *p*-benzoquinone (BQ), which is derived from peroxidase activation of HQ. The bone marrow, a target organ for the effects of benzene, has high levels of activity of peroxidases such as myeloperoxidase and eosinophil peroxidase, and of prostaglandin synthetase. The bone marrow is also relatively deficient in protective enzymes (Hanson et al. 1978; Dvorak and Ishizaka 1994; Ross 1996). Other target organs of benzene, such as the rodent Zymbal, Harderian, and preputial glands are also known to be rich in

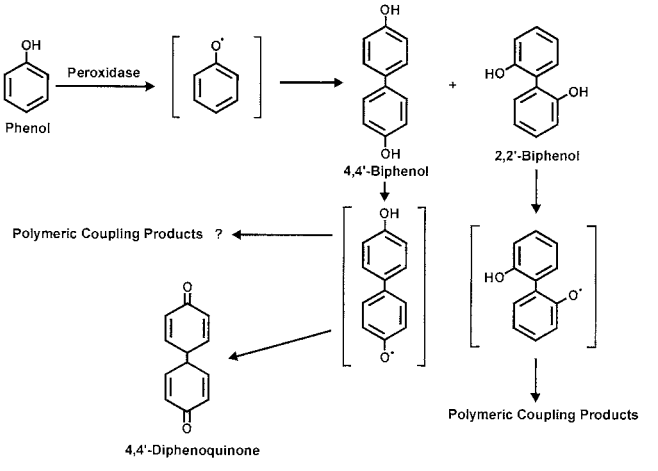

Figure 3. Peroxidase activation of phenol (adapted from reference Subrahmanyam et al. 1990, 1991).

peroxidase activity (Osborne et al. 1980; Strum and Shear 1982; De and Banarjee 1992). The toxicity associated with peroxidase activation of HQ to BQ is attributed to a reaction with highly nucleophilic thiols of tubulin, which damages the mitotic spindle and can play a role in benzene myelotoxicity (Hard et al. 1997). Hydroquinone is capable of inducing both hyperdiploidy and chromosomal breakage (Eastmond et al. 1994); it inhibits the protease involved in converting preinterleukin-1α and -1ß to mature cytokines (Niculescu et al. 1995). Hydroquinone may also contribute to the prevention of secretion of growth factor interleukin-1α, which is required for proliferation and differentiation of bone marrow stem cells (Carbonelle et al. 1995), and HQ inhibits apoptosis of myeloblasts (Hazel et al. 1996). *p*-Benzoquinone can react with cellular DNA and proteins (Melikian et al. 1992; McDonald et al. 1993; Chenna et al. 1995; Bodell 1996).

The hypothesis that quinones are the ultimate carcinogenic metabolites of benzene is in doubt because human exposure to precursors of quinones has not been associated with cancer development. The toxicity of HQ in laboratory animals has been extensively investigated. According to the National Toxicology Program (1989b) there is no evidence of tumorigenic activity of HQ in rats and mice. A study of the mortality of employees engaged in the manufacture and use of HQ revealed no significant excess of deaths attributable to kidney or liver cancers, or to leukemia (Pifer et al. 1995). Phenol and polyhydroxylated benzene do not exert a benzene-like action on bone marrow (Gad-El Karim et al. 1985a), and phenol is not known to induce leukemia (Deichmann 1983).

Benzene Epoxide

By analogy to known metabolic pathways of polycyclic aromatic hydrocarbons, benzene epoxide is suspected to be the active compound (Figure 1) (Daly et al. 1972). The strongest evidence for the formation of epoxide is that the addition of the enzyme epoxide hydrolase to microsomes causes the accumulation of benzene dihydrodiol (Tunek et al. 1978). Recently, Lindstrom and associates (1997) have detected and quantified benzene epoxide in the blood of rats after administration of benzene. Synthetic benzene epoxide does not show mutagenic activity and, to our knowledge, there is no published report that indicates its hematotoxicity. However, Busby and coworkers (1990) investigated the roles of benzene epoxide, enantiomers and racemates of benzene dihydrodiols, and diol epoxides in the induction of lung cancer in a newborn mouse model system after intraperitoneal administration. Lung tumor incidence and multiplicity increased after treatment with benzene epoxide, (±) benzene dihydrodiol, and (±)1α,2ß-dihydroxy-3ß, 4ß-epoxy-1,2,3,4-tetrahydrobenzene (*anti*-benzene diol epoxide). On the other hand, it has been shown that the major binding of benzene metabolites to cellular macromolecules is not derived from benzene epoxide; binding of synthetic [^{3}H]benzene epoxide with cellular macromolecules and with glutathione does not occur to any appreciable extent (Tunek et al. 1978; Tunek and Oesch 1982). Benzene epoxide can react with either the sulfhydryl group of cysteine of hemoglobin or serum albumin to form adducts, or can react with glutathione to form *S*-PMA, which is excreted in urine. Studies in humans and laboratory animals indicate that the formation of urinary *S*-PMA in humans exposed to benzene is comparatively low, about 1 to 2 orders of magnitude below the level observed for the minor benzene metabolite, *t,t*-MA. Similarly, *S*-phenylcysteine adducts of globin and serum albumin are formed at relatively low levels (Bechtold et al. 1992b; Melikian et al. 1992; McDonald et al. 1994; Yeowell-O'Connell 1996).

t,t-Muconaldehyde

t,t-Muconaldehyde (Figure 2), the precursor of urinary *t,t*-MA, is a toxic metabolite of benzene (Witz et al. 1996); it is a potent myelotoxin in CD-1 male mice, an active mutagen in V79 cells, and a potent clastogen in Chinese hamster ovarian cells (Glatt and Witz 1990; Witz et al. 1990). *t,t*-Muconaldehyde can form adducts with deoxyguanosine monophosphate (Latriano et al. 1986) and with glutathione (Kline et al. 1993) in vitro. It has been demonstrated that *t,t*-muconaldehyde increases gene expression of NF-κB and ap-1 binding activity as well as upregulation of c-*fos* and c-*jun*. It was suggested that this binding may play a role in the mechanism of benzene-related leukemogenesis (Ho and Witz 1997).

The major pathway for the formation of *t,t*-muconaldehyde, the precursor for *t,t*-MA, is not known. *t,t*-Muconaldehyde is obtained in low yield by air oxidation of benzene epoxide (Davies and Whitman 1977), and in high yield (together with phenol) during interaction of benzene with $^{\bullet}$OH radicals generated in irradiated water (Loeff and Stein 1963). These observations suggest that formation of ring-opened products from the hydroxycyclohexadienyl intermediate should be greater than from the benzene epoxide intermediate. Gorsky and Coon (1985) have demonstrated that activation via the hydroxycyclohexadienyl radical in benzene metabolism is a more important pathway at lower concentrations. In turn, it is anticipated that formation of *t,t*-MA will be shown to be enhanced at low level exposures of benzene.

Benzene Metabolite Combinations

It has been proposed that benzene-related hematotoxicity may result from activation of a combination of its metabolites, rather than from that of a single metabolite. Eastmond and coworkers (1987) have documented an interaction between phenol and HQ: coadministration of these metabolites produced more than additive myelotoxicity. They have also reported that the major increase in micronuclei induction by a phenol and HQ combination originated from breakage in the euchromatic region of the mouse chromosomes. The pattern of the induced micronuclei was different from that induced by HQ alone, but was almost identical to that seen in benzene-treated mice (Chen and Eastmond 1995). Marrazzini and coworkers (1994) have studied interaction of the three benzene metabolites, HQ, CAT, and phenol, in inducing micronuclei in the polychromatic erythrocytes of bone marrow in mice. Binary and ternary mixtures of two or three of these metabolites gave different results, causing considerable increase or decrease in micronuclei induction. Lévay and Bodell (1992) have also shown that the DNA adduct formation in HL-60 promyelocytic leukemia cells, treated with a combination of HQ and either CAT or BT, was three to six times greater than the sum of adduct formation produced by single agent treatments. Treatment with a combination of HQ and BT produced DNA adducts that were not detected after treatment with either of the metabolites alone. The synergistic interaction of benzene metabolites in generating DNA adducts may thus play an important role in the genotoxic effect of benzene in vivo.

Free Radicals

Finally, the free radical hypothesis of benzene-induced carcinogenesis is based on the following facts: Benzene exhibits specific toxicity to the bone marrow. Bone marrow contains myeloperoxidase, an enzyme that produces the superoxide radical $O_2^{\bullet-}$, and a relatively low concentration of its scavenger, superoxide dismutase (Hanson et al. 1978). Adding dismutase decreases the binding of [^{14}C]benzene to microsomal macromolecules (Tunek et al. 1980). Benzene produces chromosomal damage similar to that seen after ionization radiation (Forni and Vigliani 1972). Oxidative DNA damage and free radicals generated from benzene have also been suggested as important contributors to the carcinogenicity of benzene (Kalir et al. 1989). A significant increase of the levels of 8-hydroxydeoxyguanosine (8-OHdG) occurred in bone marrow DNA of mice 1 hour after administration of benzene, or a combination of phenol, CAT, and HQ (Kolachana et al. 1993). A dose-response effect between personal exposure to benzene and urinary concentration of 8-OHdG has been reported (Lagorio et al. 1994) and formation of 8-OHdG DNA adducts in peripheral blood lymphocytes correlated well with benzene exposure in humans (Liu et al. 1996). Benzene metabolites enhance the generation of reactive oxygen species in HL-60 human leukemia cells (Lévay and Bodell 1996; Shen et al. 1996).

EFFECT OF DOSE ON BENZENE METABOLISM

Prediction of risk due to low-level benzene exposure is hampered by many unknowns regarding the relationship between benzene metabolism and toxicity. Henderson and coworkers (1989) have demonstrated that the dose of benzene exposure affects the metabolic activation pathways in rodents. Formation of *t,t*-MA and HQ conjugates in rats and mice is favored at low concentrations of exposure. As the concentration increases, the metabolism of benzene is shifted toward production of phenyl conjugates and prephenyl mercapturic acid. Stommel and coworkers (1989) also observed that the ratio of urinary *S*-PMA to phenol in rats increases with rising concentration of exposure. On the other hand, the formation of *t,t*-MA increased when mice were exposed to lower doses of benzene (Witz et al. 1990). Using the physiological model, Medinsky and coworkers (1989) have observed a similar trend. These studies indicate that benzene is metabolized to HQ conjugates and to *t,t*-MA by a high-affinity, low-capacity enzyme system, and the phenol conjugates, as well as *S*-PMA, are formed by a low-affinity, and high-capacity enzyme system. Gorsky and Coon (1985) have demonstrated that at higher millimolar concentrations the activation of benzene via benzene epoxide is of much greater importance, whereas activation through the hydroxycyclohexadienyl radical is the dominant pathway at lower micromolar concentrations. Under these circumstances, cytochrome P450 is uncoupled and is known to generate hydrogen peroxide.

There are limited data on the metabolism of benzene in humans. At this time, it is not clear how the various doses of benzene exposure affect the metabolic activation pathways in humans.

BIOMARKERS OF BENZENE EXPOSURE AND TRANSITIONAL EPIDEMIOLOGY STUDIES

In recent years, advances in laboratory techniques have led to development of sensitive and specific biochemical and biological markers for environmental carcinogens. The definition of specificity and sensitivity of biomarkers varies across disciplines. From a laboratory standpoint, sensitivity is the minimum level of analyte that an assay can detect (Hulka 1991), and specificity is the ability to

detect a unique analyte in a group of closely related structures. In epidemiology, sensitivity and specificity are the measures of validity for a screening test or other procedure that requires evaluation (Hulka 1991).

The rationale for using biomarkers in transitional epidemiology research is as follows: (1) to improve accuracy in measurement of exposure by determining internal dose or biologically effective dose, instead of monitoring of environmental pollutant in ambient air; (2) to identify interindividual differences in susceptibility to disease; (3) to enhance our understanding of disease mechanisms; and (4) to provide opportunity for prevention and development strategies, including chemopreventive agents (Hulka 1991; Goldstein 1996).

The growing concern about benzene exposure and its health effects led to development of several biomarkers of internal dose, presented below.

Hemoglobin and Serum Albumin Adducts as Biomarkers of Exposure and Metabolic Activation

Benzene is metabolized to a number of electrophilic species that bind to proteins. Methods have been developed for quantitation of cysteine adducts of the benzene metabolites benzene epoxide, *o*-benzoquinone, and *p*-benzoquinone with hemoglobin and serum albumin in blood from humans or rodents (Bechtold et al. 1992a,b, 1996; Melikian et al. 1992; Rappaport et al. 1996).

Unmetabolized Benzene in Exhaled Breath, Blood, and Urine as Biomarkers of Exposure

Monitoring of unmetabolized benzene in different biological matrices has been investigated for use as a biomarker of exposure in recent years. Analysis of exhaled air has been developed and applied to the assessment of benzene exposure (Sherwood 1972; Berlin et al. 1980; Wallace 1984; Drummond et al. 1988; Perbellini et al. 1988; Brugnone et al. 1989, 1992; Angerer et al. 1992; Pekari et al. 1992; Popp et al. 1994; Yu and Weisel 1996). Money and Gray (1989) found a poor relationship between exhaled breath benzene at low benzene concentrations (< 0.9 ppm), yet a reasonable relationship at high levels of exposure (10 to 20 ppm). Similarly, Hotz and coworkers (1997) reported that this marker is not suitable for exposures of 0.1 to 1 ppm. This biomarker is not widely used because it requires immediate analysis.

Quantitation of benzene levels in blood has been proposed as a biomarker of benzene exposure (Berlin et al. 1980). This approach has been investigated in occupational settings and in smokers and nonsmokers (Jirka and Bourne 1982; Perbellini et al. 1988; Brugnone et al. 1989; Angerer et al. 1992; Pekari 1992; Kok and Ong 1994; Popp et al. 1994; Weisel et al. 1996). Hotz and coworkers (1997) found that this biomarker is not suitable at low exposures. One of the disadvantages of bloodborne biomarkers is that they are based on an invasive technique.

The relation of exposure with unmetabolized urinary benzene was also investigated (Ghittori et al. 1993; Ong et al. 1995). The main difficulty with urinary unmetabolized benzene as a biomarker is that it requires large volumes of sample, must be collected in a clean environment, and must immediately be plugged tightly to prevent evaporation (Ghittori et al. 1995).

Urinary Ring-Hydroxylated Metabolites: Phenol, HQ, CAT, and BT as Biomarkers of Benzene Uptake and Metabolic Activation

Traditionally, some of the ring-hydroxylated urinary metabolites of benzene after acid hydrolysis were used for monitoring the exposure of workers to high concentrations of benzene. These metabolites are not specific to benzene, however, but are also present in cigarette smoke (Brunnemann et al. 1976) and in foodstuffs. Phenol can be formed endogenously from metabolism of various aromatic amino acids; CAT, HQ, and BT can derive from phenol. Therefore, these metabolites are not useful as biomarkers of low exposures of benzene. The major metabolite, phenol, is by far the most widely investigated biomarker of benzene. This biomarker is also not specific for exposures below 10 ppm, however, as stated by the ACGIH (1994) (Ong et al. 1995; Hotz et al. 1997). Catechol does not distinguish between exposed and nonexposed subjects in occupational settings with low levels of benzene (Inoue et al. 1988; Ong et al. 1995; Hotz et al. 1997). Few studies have examined the relationship between benzene exposure and urinary excretion of HQ (Deisinger et al. 1996; Ong et al. 1996; Rothman et al. 1996; Hotz et al. 1997). The recent study by Hotz and coworkers (1997) suggests that this metabolite is not a suitable biomarker of exposure at low concentrations of benzene. Finally, only one study in the literature is reported to have quantified the concentrations of urinary BT in workers exposed to very high levels of benzene, and found a correlation with exposure (Inoue et al. 1989b).

Urinary *t,t*-MA as Biomarker of Benzene Uptake and Metabolic Activation

t,t-Muconic acid is a minor metabolite of benzene in humans. Literature data suggest that it is a fairly good indicator of benzene exposure in the 0.1 to 1 ppm range of exposure. Urinary muconic acid may not be specific to benzene exposure, because it is a minor metabolite of

sorbic acid ($CH_3CH=CHCH=CHCOOH$) in mice (Westöö 1964), and in humans (Ducos et al. 1990). Sorbic acid is a well-known food preservative that is used to inhibit the growth of mold, yeast, and bacteria (Luk 1990; Branen and Davidson 1998). It is found in fruit juices and wine, in dairy, bakery, meat, and fish products, as well as in mayonnaise, salad dressings, and various vegetables, and in cosmetics and pharmaceutical products.

Urinary *S*-PMA as Biomarker of Benzene Uptake and Metabolic Activation

S-phenylmercapturic acid is formed from the reaction of a benzene epoxide intermediate with glutathione. Urinary *S*-PMA was confirmed to be a sensitive and specific biomarker for measurement of low levels of benzene exposure, and it has been proposed by the ACGIH (1995) as a biological exposure index for benzene.

LC-ESI-MS/MS FOR MONITORING URINARY BENZENE METABOLITES

From a laboratory standpoint, the focus of the current study was on developing sensitive and specific assays that can measure simultaneously several urinary benzene metabolites at low concentrations. Liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) has, over the past decade, gained widespread acceptance for quantitation of drugs and their metabolites in biological matrices (Bruins 1985; Bowers 1989). This technique enhances the specificity and sensitivity of biomarkers from a laboratory standpoint. It has several advantages over gas chromatography–mass spectrometry (GC-MS) analysis: volatility of an analyte is not a requirement, and derivatization of the samples is not necessary for sample introduction. Also, several analytes that are structurally dissimilar, such as *t,t*-MA and *S*-PMA, can be measured in a single run. Selectivity and specificity of analytes can be further enhanced by using MS/MS and the selected reaction monitoring mode. Under MS/MS operational modes, only the ions with the *m/z* values of the protonated or deprotonated molecule species are selected by the first analyzer of the MS system. These ions are then passed through a collision cell in which they collide with collision gas to produce structure-specific product ions. One or more of these product ions are monitored by the second analyzer. The specificity of the MS/MS detection is achieved by a combination of the selection of specific precursor ions and specific detection of the product ions. It is possible that the biological matrix may produce some other ions that have the same *m/z* values as those of the molecular species of interest. However, the probability that these ions produce the same product ion is extremely low.

Application of LC-ESI-MS/MS in transitional epidemiology studies is novel and is in the experimental stage. The practicality and cost of such an application and its ultimate impact need to be evaluated.

SPECIFIC AIMS

The objectives of this project were to develop assays which were, from a laboratory standpoint, highly sensitive and specific, and could be applied to the assessment of effects of dose of benzene exposures on metabolic activation pathways in humans; and, further, to examine the relationship of the benzene metabolites to other biological markers, such as chromosomal damage, and to provide more information on mechanisms underlying the toxicity and carcinogenicity of benzene.

SPECIFIC AIM 1

The first aim was to develop a specific and sensitive LC-ESI-MS/MS assay for quantifying low levels of urinary metabolites of benzene in humans. The metabolites of interest were: (1) *t,t*-MA and *S*-PMA; and (2) ring-hydroxylated metabolites, namely, HQ, CAT, and BT after acid hydrolysis.

SPECIFIC AIM 2

The second aim was to develop a sensitive GC-MS method for quantifying unmetabolized urinary benzene, and possibly biphenyl, a metabolite that can be formed from dimerization of the hydroxycyclohexadienyl intermediate.

Specific Aim 2 was directed at investigating whether low-level benzene exposures favor the metabolism of benzene through a hydroxyradical intermediate. This Specific Aim was eliminated from the study because it required too high a volume of biological samples.

SPECIFIC AIM 3

The third aim was to validate the developed LC-ESI-MS/MS assays for determination of urinary metabolites of benzene, and to investigate the effect of dose in benzene exposures on metabolic activation pathways in humans.

This Specific Aim was not included here and is currently being addressed in a separate HEI-funded study.

METHODS AND STUDY DESIGN

CHEMICALS

[^{14}C]Benzene (112 mCi/mmol, > 98% pure by HPLC determination) was purchased from Chemsyn Science Laboratories (Lenexa, KS). Unlabeled benzene, obtained from Burdick and Jackson (Muskegon, MI), was used to dilute [^{14}C]benzene to 1 mCi/mmol. [$^{13}C_6$]Benzene ($^{13}C_6$, 99%), [$^{13}C_6$]aniline ($^{13}C_6$, 99%) and [$^{13}C_6$]phenol ($^{13}C_6$, 99%) were purchased from Cambridge Isotope Laboratory (Andover, MA). Sodium nitrate, *N*-acetyl-L-cysteine, urea, sodium sulfate, sodium bisulfite, copper sulfate, copper nitrate, *o*- and *p*-nitrophenol, HQ (> 99%), CAT (> 99%), BT (99%), BQ, zinc dust, 10% palladium/carbon (Pd/C) catalyst, and hydrochloric acid (HCl) were purchased from Aldrich (Milwaukee, WI). Whatman strong anion exchanger (SAX) cartridges (1,000 mg) were bought from Fisher Scientific Company (Fair Lawn, NJ). Monosodium and disodium phosphates were purchased from Sigma (St. Louis, MO). Unless specified, solvents were of HPLC grade from J. T. Baker (Phillipsburg, NJ).

ANIMALS

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

SYNTHESIS OF [$^{13}C_6$]S-PMA INTERNAL STANDARD

[$^{13}C_6$]*S*-PMA was prepared from [$^{13}C_6$]aniline by the Gattermann reaction as shown in Figure 4 (Rumpf 1981). In brief, [$^{13}C_6$]aniline (0.5 g; 5.3 mmol) was added to concentrated HCl (0.7 mL), then, after dilution with water (1.5 mL) and HCl (1 mL), a solution of sodium nitrite (1.4 mL; 6 mmol) was added to the resulting suspension while the temperature was kept below 5°C. While stirring, the formed phenyldiazonium salt mixture was added to the *N*-acetyl-L-cysteine solution (7.5 mL; 4.8 mmol), and the orange precipitate was centrifuged. The wet solid of *N*-acetyl-*S*-phenyldiazol-L-cysteine was dissolved in ethanol (6 mL) and, after adding freshly prepared copper (0.64 g) and water (14 mL), the suspension was heated under reflux for 1.5 hours at 80°C. The residue was filtered, washed with hot water, acidified, and extracted with chloroform. After removing the solvent, the crude [$^{13}C_6$]*S*-PMA residue was dissolved in 60% ethanol (2.5 mL); upon adding charcoal (0.25 g), it was boiled and filtered. The [$^{13}C_6$]*S*-PMA was crystallized from aqueous ethanol and characterized by NMR and MS. Both the NMR and mass spectra were similar to those reported for *S*-PMA.

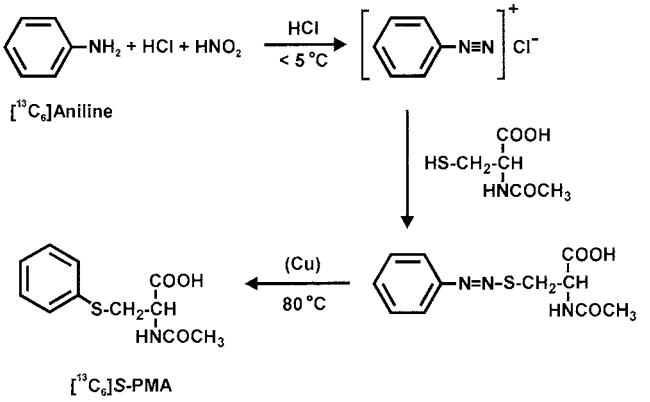

Figure 4. Synthesis of [$^{13}C_6$]*S*-PMA.

The 360 MHz NMR-spectrum and MS were ($CDCl_3$): δ 1.87 (3H, s, CH_3), 3.34 and 3.54 (2H, dd, cys_β and $cys_{\beta'}$ $J_{\beta\beta'}$ = 13.6 Hz), 4.77 to 4.82 (1H, m, cys_α), 6.2 (1H, d, NH), 7.21 to 7.43 (5H, m, aromatic), 12.8 (1H, broad, COOH); Electron Impact (EI)-MS at *m/z* 245, ($M^{+\cdot}$); 186 (M-59). Synthetic [$^{13}C_6$]*S*-PMA was more than 98% pure according to HPLC.

SYNTHESIS OF UNLABELED *S*-PMA

Unlabeled *S*-PMA was prepared according to a method described by Hanzlik and coworkers (1990). Thiophenol (1.8 g; 16.4 mmol) was suspended in 20 mL freshly distilled dioxane, along with acetamidoacrylic acid (1.94 g; 15 mmol) and 0.4 mL of piperidine; this suspension was flushed with N_2, and heated under reflux for 3 hours (Figure 5). The solvent was removed, and the residue was partitioned between ether and sodium bicarbonate ($NaHCO_3$) solution. The aqueous layer was neutralized, extracted with ether, and acidified to pH 1 to 2, whereby a precipitate of crude *S*-PMA was formed. The precipitate was crystallized from aqueous methanol and was characterized by its NMR and mass spectra (360 MHz NMR [d_6-dimethyl sulfoxide] δ 1.8 [3H, s, CH_3], 3.15 and 3.35 [2H, dd, cys_β and $cys_{\beta'}$ $J_{\beta\beta'}$ = 13.6 Hz], 4.35 [1H, m, cys_α], 7.25 to 7.35 [5H, m, aromatic], 8.3 [1H, d, NH], 12.9 [1H, broad, COOH]). The major components of the fragmentation pattern of the EI-MS were as follows: *m/z* 239 ($M^{+\cdot}$); 180 ($C_6H_5SCH_2CHCOOH$); 123 ($C_6H_5SCH_2$).

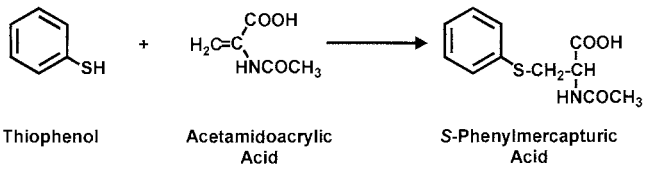

Figure 5. Synthesis of unlabeled *S*-PMA.

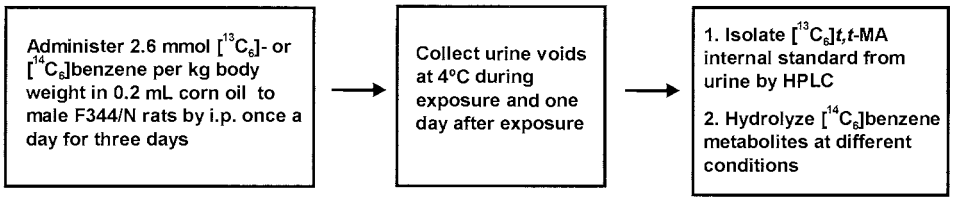

Figure 6. Biosynthesis of [$^{13}C_6$]*t,t*-MA and [^{14}C]-benzene metabolites.

BIOSYNTHESIS OF [^{14}C]*t,t*-MA, [^{14}C]*S*-PMA, AND [$^{13}C_6$]*t,t*-MA INTERNAL STANDARDS

[$^{13}C_6$]*t,t*-Muconic acid as an internal standard and [^{14}C]*S*-PMA and [^{14}C]*t,t*-MA, which were used for development of methodology and measurement of recoveries, have been prepared biosynthetically (Figure 6). Two groups of rats (three rats per group) were each given intraperitoneal injections, of either 2.6 mmol [$^{13}C_6$]benzene/kg body weight or of 2.6 mmol [^{14}C]benzene (1 mCi/mmol)/kg body weight in 0.2 mL corn oil once a day for three days. Using metabolic cages, the urine voids were collected at 4°C during exposure and after exposure on day four; they were then stored at −20°C until analysis. [^{14}C]- and [$^{13}C_6$]-labeled standards were isolated from the urine by the method described previously (Melikian et al. 1993). In brief, urine samples were subjected to solid-phase extraction cleanup procedures, which were followed by HPLC purification.

SYNTHESIS OF [$^{13}C_6$]CAT AND [$^{13}C_6$]HQ INTERNAL STANDARDS

[$^{13}C_6$]CAT and [$^{13}C_6$]HQ standards were prepared by a method described in the literature (Figure 7) (Chew and Heys 1981). [$^{13}C_6$]Phenol (0.8 mmol) was dissolved in 1 mL of glacial acetic acid and added to a suspension of copper nitrate ($Cu[NO_3]_2$:$3H_2O$; 0.5 mmol) in 1 mL of glacial acetic acid at room temperature. After stirring for 1 hour, ice chips were added and the products were extracted with 4 × 5 mL of ether:hexane (3:1). The solvents were removed from the combined extracts under vacuum and the *o*- and *p*-nitrophenol products were separated by TLC (hexane:acetone 5:2). The purity of the compounds was determined by HPLC analysis. The yields of *o*-nitrophenol and *p*-nitrophenol were 40% and 28%, respectively.

To prepare [$^{13}C_6$]CAT, [$^{13}C_6$]*o*-nitrophenol (0.2 mmol) was hydrogenated at 40 psi in 10 mL of ethanol with 25 mg of 10% palladium/carbon as the catalyst for 1 hour. The mixture was filtered into 1 mL of 4 N sulfuric acid and most of the ethanol was removed under vacuum. After cooling to 0° to 5°C, 1.1 mmol of sodium nitrite in 1 mL water was added and stirred at 0°C for 1 hour. Sufficient solid urea was used to destroy excess nitrous acid, and then 10 mL of a solution of copper sulfate (0.05 mol) and copper(1)oxide (0.3 mmol) were added. The mixture was stirred overnight at room temperature and then extracted with 4 × 10 mL ether; after removal of the solvent, the residue was dissolved in 1 mL methanol and purified by HPLC. The compound eluted at the correct retention time (compared with unlabeled standard), was collected from the HPLC, dried, dissolved in methanol, and characterized by UV, λ_{max} = 293 nm (log ε_{max} = 3.4), and by LC-MS in the negative ionization mode that showed molecular ion at *m/z* 115 $(M-1)^-$.

For the synthesis of [$^{13}C_6$]HQ, a [$^{13}C_6$]*p*-nitrophenol (0.2 mmol) sample was reduced at 40 psi with H_2 in 10 mL of ethanol with 15 mg of 10% palladium/carbon as the catalyst for 1.5 hours. The mixture was filtered off and the solvent was removed under vacuum. The residue was immediately dissolved in 2 mL of 6 N sulfuric acid and cooled to 5°C; then 0.6 mmol of sodium nitrite in 2 mL water was added and the mixture was stirred at 5°C for 3 hours. Sufficient solid urea was used to destroy excess nitrous acid, and then 15 mL of a solution of copper sulfate (0.07 mol) and copper(1)oxide (0.25 mmol) were

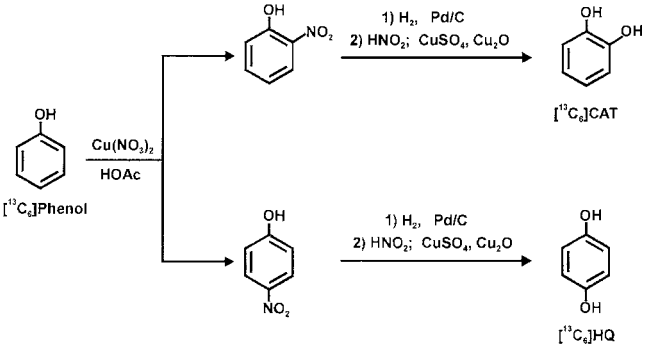

Figure 7. Synthesis of [$^{13}C_6$]CAT and [$^{13}C_6$]HQ.

added. Stirring was continued overnight at room temperature. The mixture was extracted with 4 × 10 mL ether; after removal of the solvent, the residue was dissolved in 1 mL methanol and purified by HPLC. The compound eluting at the correct retention time was collected from the

HPLC, dried, dissolved in methanol, and characterized by UV, λ_{max} = 225 nm (log ε_{max} = 3.7), and λ = 293 nm (log ε = 3.43), and by LC-MS in the negative ionization mode that showed molecular ion at *m/z* 115 $(M-1)^-$.

PURIFICATION OF URINE SAMPLES FOR *t,t*-MA AND *S*-PMA ANALYSIS BY LC-ESI-MS/MS

Optimal Purification Condition

The optimal condition for purification of urine samples by solid phase extraction was determined by spiking pooled smokers' urine with either [^{14}C]*t,t*-MA (100 ng/mL), or [^{14}C]*S*-PMA (120 ng/mL) alone, or with both together, followed by elution of the sorbed samples from the SAX cartridge with different combinations and volumes of eluates. One-mL fractions of each eluate were collected and the radioactivity of each fraction was measured. Fractions containing radioactive materials were combined and analyzed by HPLC as described previously (Melikian et al. 1993). The recoveries of [^{14}C]analytes from the cartridge were determined from HPLC radiograms. The analytical recoveries of spiked [^{14}C]*t,t*-MA (0.1 mg/L urine), and [^{14}C]*S*-PMA (120 ng/mL urine) from the anion exchange cartridge were about 85% and 75% (*n* = 2), respectively. The optimal condition for maximal recovery of both analytes from the SAX cartridge is shown in Figure 8 and was used for purification of human urine samples as described below.

Purification of Human Urine Samples

One-mL urine samples, spiked with 15 ng of [$^{13}C_6$]*S*-PMA and 30 ng of [$^{13}C_6$]*t,t*-MA as internal standards, were passed through a SAX cartridge that was preconditioned with 5 mL of MeOH and 5 mL of H_2O (Figure 8). The cartridge was eluted with 3 mL of H_2O, then with 3 mL of 5 mM phosphate buffer, pH 7, and 3 mL of 1% aqueous acetic acid; finally, the analytes were eluted with 4 mL of 10% aqueous acetic acid. Analytes eluted with 10% aqueous acetic acid were extracted with 3 × 5 mL ethyl acetate (EtOAc); extracts were combined and evaporated to dryness under N_2 at room temperature. The residue was dissolved in 150 µL of MeOH:1% aqueous acetic acid (20:80 v/v), and a 50-µL sample of this solution was analyzed by LC-ESI-MS/MS-SRM.

LC-ESI-MS/MS ANALYSIS OF *t,t*-MA AND *S*-PMA

HPLC Parameters

The HPLC features included a Waters Model 600 pump (Milford, MA), a Rheodyne model 7120 injector (Cotati, CA), and a Phenomenex Ultramex 5-µ C-18 narrow-bore column (250 × 2.0 mm) (Torrance, CA). A precolumn splitter was utilized to reduce the flow-rate from the 0.9 mL/minute HPLC pump to 200 µL/minute. A linear gradient from 80% solvent A (0.5% aqueous acetic acid), 20% solvent B (MeOH) to 100% solvent B over 5 minutes was employed in the elution program for analysis of *t,t*-MA and *S*-PMA.

Mass Spectrometry Parameters

HPLC was interfaced with a Finnigan TSQ 700 triple-stage quadrupole mass spectrometer (San Jose, CA) via an electrospray source. The mass spectrometer was operated in the negative ion mode. The spray voltage was 4.1 kV and the capillary temperature was 220°C. The liquid flow was introduced into the mass spectrometer at a rate of 200 µL/min without postcolumn splitting. For SRM, the first quadrupole (Q1) was used to select the precursor ion at the reaction, and then to pass it on to Q2 to produce fragmentation via collision with the collision gas (argon). The product ion at the reaction was monitored by Q3. The argon gas pressure in the Q2 collision cell was adjusted so that the precursor beam suppression was approximately 75%. [$^{13}C_6$]*t,t*-MA and [$^{13}C_6$]*S*-PMA were used as internal standards.

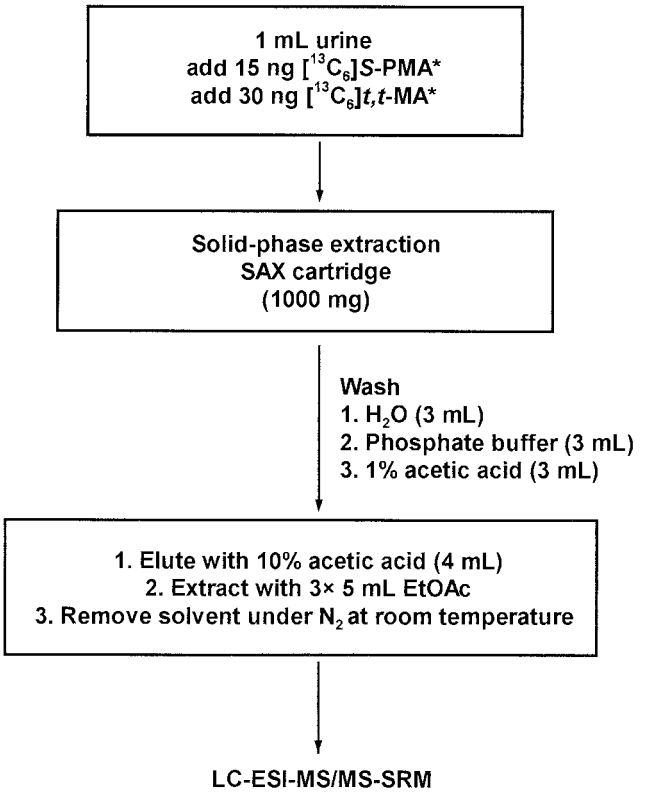

Figure 8. Outline of biological sample preparation prior to LC-ESI-MS/MS-SRM analysis for *t,t*-MA and *S*-PMA. * = internal standard.

Instrumentation control, data acquisition, and data processing were controlled by a DEC Station 5000-120 computer. Peak areas were utilized for the construction of calibration graphs and for quantification of the analytes.

HYDROLYSIS AND PURIFICATION OF URINE FOR BT, HQ, AND CAT ANALYSIS BY LC-ESI-MS/MS

Optimal Acid Hydrolysis Conditions

Hydrolysis of urine samples collected from rats treated with [^{14}C]benzene was examined under various conditions using 0.9 or 1.7 *N* HCl, 80°C to 95°C, 0.5 to 4 hours. Radioactive substances released after hydrolysis were extracted with EtOAc and quantified. Before acid hydrolysis, only 4.1% of the radioactive material present in rat urine was extractable with the organic solvent. After acid hydrolysis, using 0.9 *N* HCl and 80°C, the maximum of radioactive material recovered in the organic phase was 86.5%, reached after 2 hours of hydrolysis. Using 1.7 *N* HCl and 80°, 85°, and 90°C over 90 minutes, the maximally extractable radioactive material in the organic phase amounted to 90.3%, 89.6%, and 89.2%, respectively. The organic phases of samples were subjected to reverse-phase HPLC analysis before and after hydrolysis using a 5-µm Ultrasphere ODS C-18 column (250 × 4.6 mm) (Beckman, Fullerton, CA). Samples were eluted from the column with 100% H_2O for 20 minutes followed by a linear gradient from 100% H_2O to 100% MeOH over 40 minutes at a flow rate of 1 mL/minute. One-mL fractions were collected and the radioactivity of each fraction was measured (Figure 9).

Hydrolysis and Purification of Human Urine Samples Before LC-ESI-MS/MS Analysis

As summarized in Figure 10, 1-mL aliquots of samples were transferred into 7-mL vials; 50 µL of [$^{13}C_6$]CAT (50

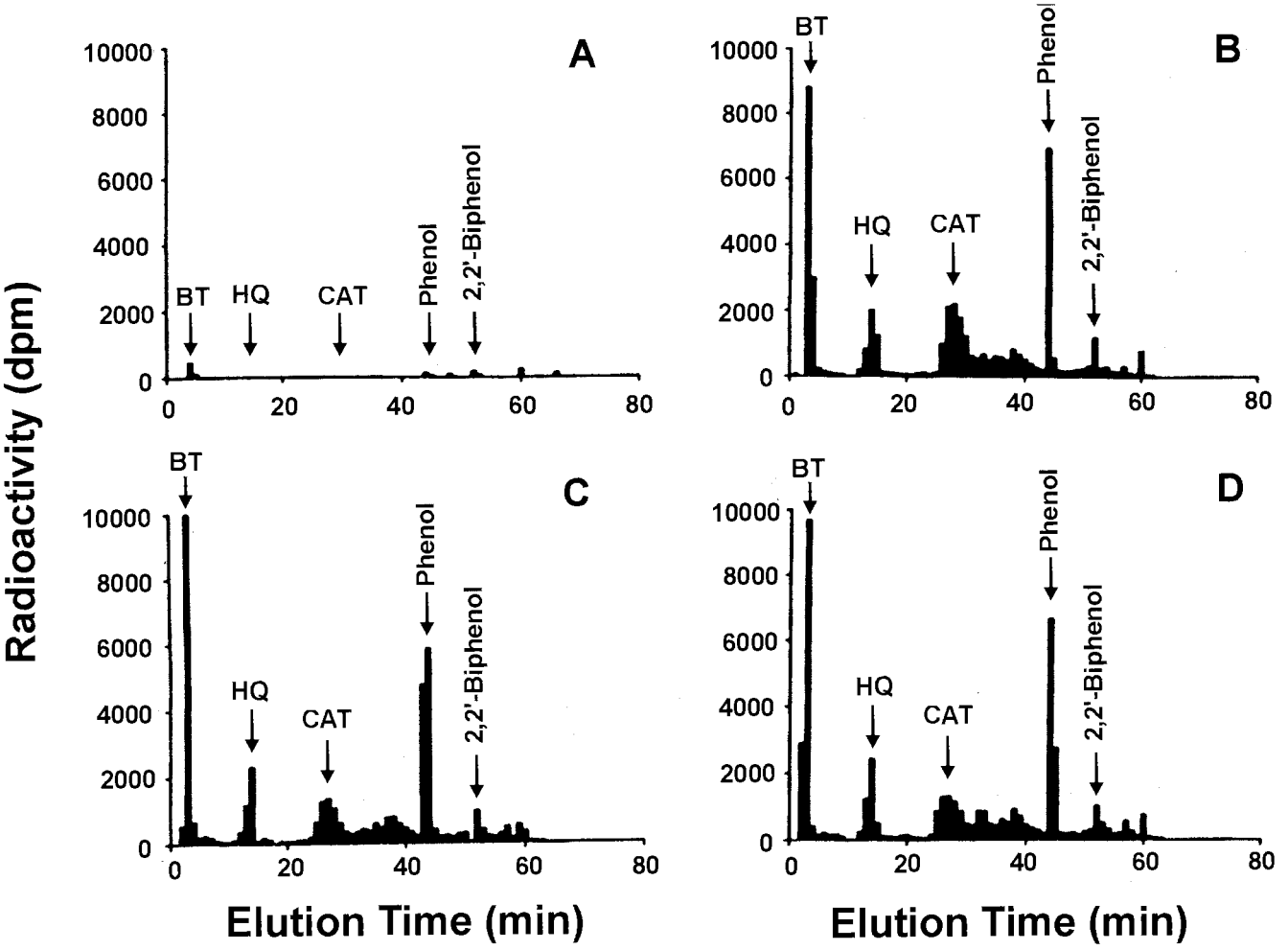

Figure 9. HPLC elution profiles of organic-phase extractable urinary [^{14}C]benzene metabolites in rats (A) before acid hydrolysis at 25°C, (B) after acid hydrolysis at 80°C, (C) after acid hydrolysis at 85°C, and (D) after acid hydrolysis at 90°C for 1.5 hours.

ng/μL) and 27 μL of [$^{13}C_6$]HQ (26 μg/μL) internal standard solutions were added; the liquids were vortex-mixed and subjected to acid hydrolysis. Three hundred fifty μL of 20% double-distilled HCl were added to the urine samples, which were then incubated at 90°C for 1.5 hours. After cooling, 300 mg of sodium sulfate was added (for enhancing extraction of analytes to organic phase) and mixtures were extracted twice with 2 mL of ether. The solvent was removed under a stream of N_2 at room temperature, and the samples were stored at –20°C until they were analyzed. For determination of levels of the analytes by LC-ESI-MS/MS-SRM, each residue was dissolved in 100 μL of MeOH; aliquots of this solution were mixed with equal volumes of sodium bisulfate (2 mg/mL), to prevent autooxidation of the analytes, and analyzed by HPLC interfaced with MS.

LC-ESI-MS/MS ANALYSIS OF BT, HQ, AND CAT

HPLC Parameters

The HPLC features included a Waters Model 600 pump, a Rheodyne model 7120 injector, and a Phenomenex Ultramex 5-μ C-18 narrow-bore column (250 × 2.0 mm). A precolumn splitter was utilized to reduce the flow rate from the HPLC pump to 200 μL/min. The HPLC program consisted of isocratic elution with MeOH:H_2O 50:50 for 10 minutes, followed by a linear gradient from 50% MeOH to 100% MeOH over 5 minutes, and kept at 100% MeOH for another 25 minutes.

Mass Spectrometry Parameters

BT, HQ, and CAT readily form noncovalent dimeric species under mild electrospray conditions (Figure 11). Relatively high capillary and tube lens values had to be applied to break the dimeric species and it was found that, under such conditions, the deprotonated HQ and CAT easily lose a hydrogen. The choice of the capillary and tube values was then balanced between breaking the dimeric species and keeping the monomeric species from losing a hydrogen. At the HPLC flow rate (about 200 μL/min), optimal capillary and tube lens values for the aqueous methanol solution were 45 and 115 V, respectively. With these settings, the intensities of the $(M-1)^-$ ions for HQ and CAT were maximized, although some dimeric species were still present and some hydrogen loss products were also observed.

Because the parent ion and the product ion used differ in mass by only one dalton, it is important that the mass spectrometer has at least unit resolution for both precursor selection by Q1 and product ion detection by Q3. The use of less than unit resolution on either Q1 or Q3 will result in loss of specificity. The loss of selectivity when using less than unit resolution on Q1 is obvious because ions with one amu less than that of the precursor will pass through Q1 and be detected by Q3, which is set to detect product ions. The unit resolution for Q3 is also absolutely necessary, even if Q1 has sufficient resolution to select only the ion with the *m/z* value of the precursor ion. It is probable that the complex urine matrix may produce ions with the same *m/z* values as the precursor ion of interest. These ions will be indiscriminately passed through Q1. If on Q3 there is less than unit resolution, some of these matrix-derived ions will be detected just like the sample signal. Thus, for this study, both Q1 and Q3 were set to operate under unit resolution.

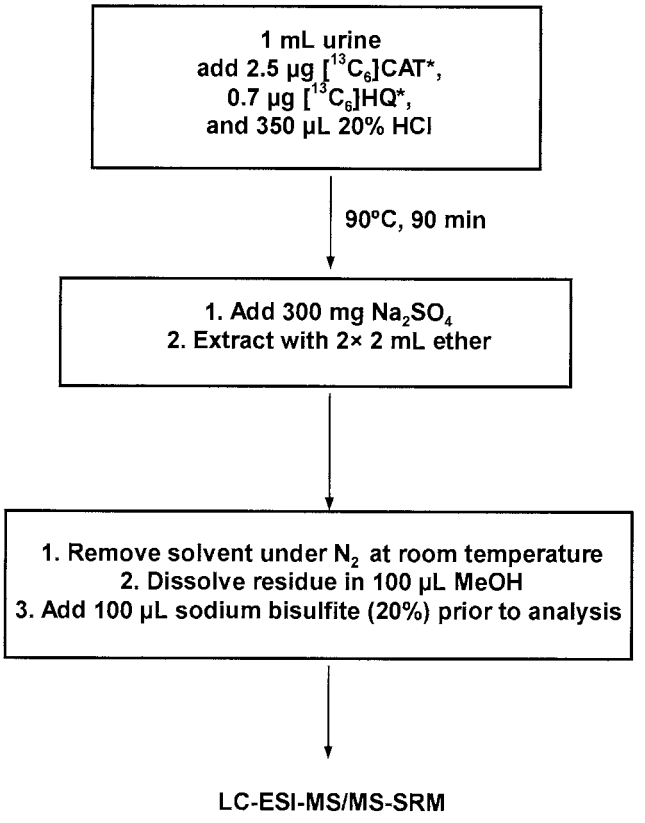

Figure 10. Outline of biological sample preparation prior to LC-ESI-MS/MS-SRM analysis for BT, HQ, and CAT. * = internal standard.

Instrumentation control, data acquisition, and data processing were controlled by a DEC Station 5000-120 computer. Peak areas were utilized for the construction of calibration graphs and for quantification of the analytes.

STANDARD CALIBRATION GRAPHS

Stock standard solutions of *t,t*-MA were prepared by dissolving 1 mg *t,t*-MA in 10 mL of 10:90 MeOH:0.5%

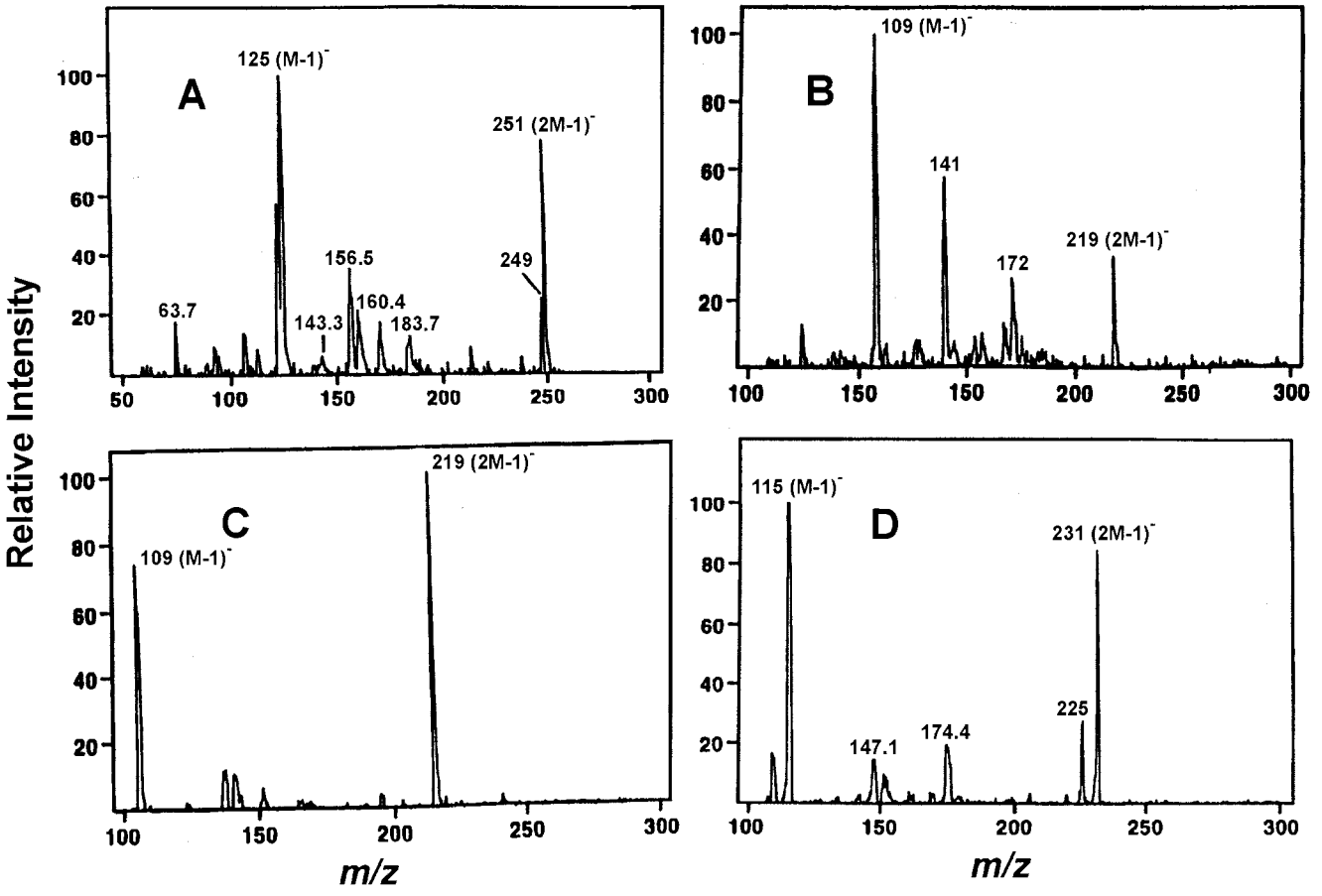

Figure 11. ESI-MS spectra of standards in the negative ionization mode (A) BT, (B) HQ, (C) CAT, and (D) [$^{13}C_6$]CAT.

aqueous acetic acid. *S*-phenylmercapturic acid solutions were prepared by dissolving 1 mg compound in 10 mL MeOH. The solutions were further diluted with MeOH and spiked with internal standards to give a series of working standard solutions with 0.1, 1, 5, 50, 100, or 1,000 µg/L *S*-PMA; 0.005, 0.05, 0.1, 0.5, 5, or 10 mg/L *t,t*-MA; and 15 µg/L [$^{13}C_6$]*S*-PMA and 30 µg/L [$^{13}C_6$]*t,t*-MA internal standards. Stock standard solutions of BT, HQ, and CAT were prepared by dissolving 1 mg of each standard in 1 mL MeOH. These solutions were further diluted with MeOH and spiked with the internal standards, 2.5 mg/L of [$^{13}C_6$]CAT and 0.7 mg/L of [$^{13}C_6$]HQ, to give a series of working standard solutions within the range of biological samples. The purity of each standard sample was checked by HPLC and all standard samples were prepared on the day of analysis; they were stored at –20°C until analyzed.

Standard calibration graphs were constructed by peak area ratios of analytes to internal standards, measured at each nominal concentration; the corresponding nominal concentrations were used to obtain the equation of the analyte calibration graph:

$$y = a + bx$$

where x = nominal urinary concentration of analyte, y = peak area ratio of each analyte to the internal standard, a = intercept, and b = slope of the calibration graph. A non-weighted least square linear regression procedure was used to obtain the equation of calibration curves, along with the value of the regression coefficient (r^2).

REPRODUCIBILITY OF ASSAYS

The intra-run precision and accuracy of the assays were determined by eight replicate analyses of pooled smokers' urine spiked with internal standards. With all runs, a blank sample containing 1 mL H_2O and the internal standard were also subjected either to solid-phase extraction for the *t,t*-MA and *S*-PMA analysis followed by LC-ESI-MS/MS, or to acid hydrolysis for BT, HQ, and CAT, followed by LC-ESI-MS/MS analysis.

HUMAN SPECIMENS

Urine samples for the *t,t*-MA and *S*-PMA assay were from male and female subjects, who ranged from nonsmokers to heavy smokers (> 20 cigarettes/day). These specimens were kindly provided to us by the Metropolitan Life Insurance Laboratories, Elmsford, NY. They were collected on the spot and contained one tablet of a preservative that is generally used in clinical laboratories (each tablet contained 84 mg potassium acid phosphate, 42 mg sodium benzoate, 54 mg benzoic acid, 8 mg sodium bicarbonate, 0.66 mg mercuric oxide, 1.5 mg polyvinylpyrrolidone, and 1.7 mg magnesium stearate). Upon arrival at the American Health Foundation (AHF), all samples were immediately protected from light and stored at −20°C until analysis. Urine samples for the measurement of BT, HQ, and CAT were collected from male and female AHF employees who ranged from nonsmokers to light smokers (approximately 10 cigarettes/day). These specimens were frozen immediately at −20°C without any preservative.

CREATININE DETERMINATION

Urinary creatinine was determined with the Kodak Ektacheme 500 Computer-Directed Analyzer at the American Health Foundation's Clinical Biochemistry Facilities as described previously (Melikian et al. 1993). Generally, urinary content of substances is normalized against excretion of creatinine because urine flow changes unpredictably during the day, but total creatinine excretion is generally constant.

COTININE DETERMINATION

Urinary cotinine was quantified by radioimmunoassay at the American Health Foundation's Clinical Biochemistry Facility as described previously (Haley et al. 1983). Cotinine, a metabolite of nicotine, is commonly used as an indicator of smoking.

STATISTICAL METHODS AND DATA ANALYSIS

The interrelationship between *t,t*-MA and *S*-PMA was assessed using linear regression version 6.12 of the Statistical Analysis System (SAS), including 95% group and individual confidence limits (SAS 1990). Since the two measures were found to be highly skewed and nonnormally distributed, the logarithmic (base 10) transformation was used on the data points. Prior to transformation, each measure was adjusted for levels of creatinine. Log-normal distributions are preferred when the range of values is closed at the lower end but open at the higher end, as in the case of materials in body fluids (Ducos et al. 1992). For variables found below the limit of detection, zero was imputed as a data point. To see whether urinary BT, HQ, and CAT, adjusted for creatinine, differed between smokers and nonsmokers, the independent-samples *t*-test was used. The CV is given for the reproducibility of assays. All statistical analyses (determination of mean, median, standard errors, and linear regression for calibration graphs) were performed on a personal computer with Microsoft Excel Software; all reported *p*-values are two-tailed.

RESULTS

CALIBRATION GRAPHS, REPRODUCIBILITY, AND DETECTION LIMITS

The calibration curves for each analyte consisted of six calibration levels (Figures 12 and 13). All points were the mean of at least three measurements. The calibration curves were linear at concentrations of 0.033 ng to 333 ng/injection for *S*-PMA; 1.67 ng to 3,300 ng/injection for

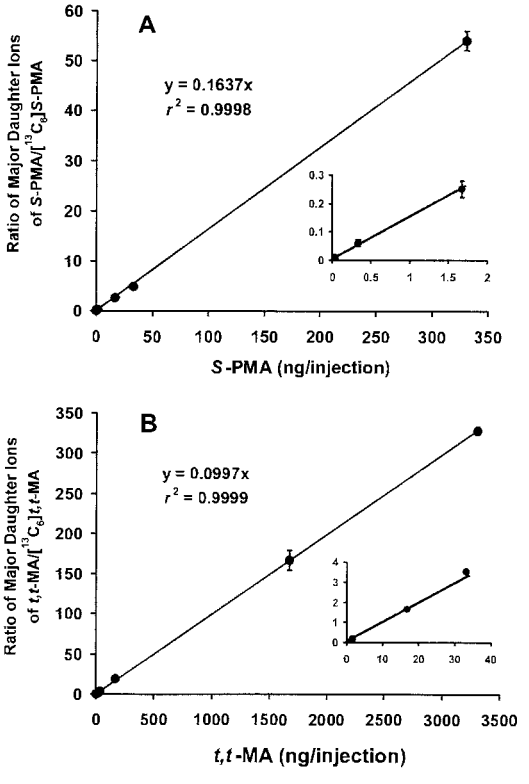

Figure 12. Calibration curves for (A) *S*-PMA, and (B) *t,t*-MA.

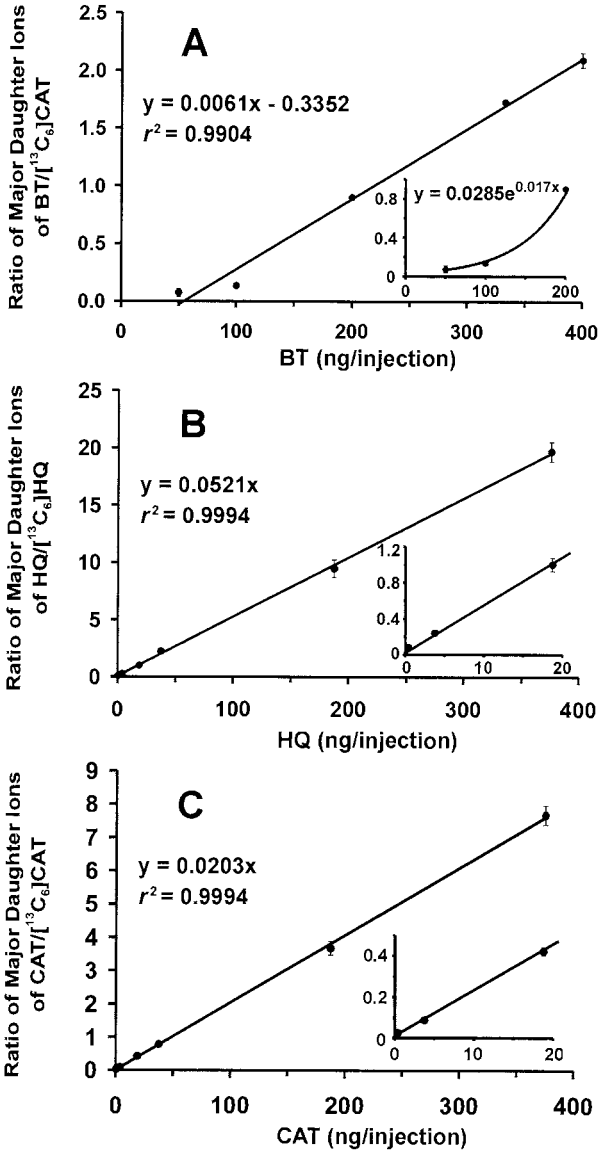

Figure 13. Calibration curves for (A) BT, (B) HQ, and (C) CAT.

t,t-MA; 200 to 400 ng/injection for BT; 18 to 400 ng/injection for HQ; and 5 to 400 ng/injection for CAT.

Pooled urine samples from smokers were used to determine the reproducibility of the assays between runs on different days. The CV between different runs (n = 8) for *S*-PMA was 7% for the sample containing 9.4 µg *S*-PMA/L; it was 10% for the sample containing 0.07 mg *t,t*-MA/L; 20% for a sample containing 0.7 mg BT/L; 12% for a sample containing 0.4 mg HQ/L; and 4.6% for a sample containing 1.9 mg CAT/L, as summarized in Table 1.

The detection limit for *S*-PMA standard samples was 0.03 ng/injection, and for *t,t*-MA was 2 ng/injection. At these concentrations, the ratio of signal to noise was greater than 5 to 1. For *S*-PMA, the lowest value quantified so far was 0.4 µg/L urine and the highest was 1,950 µg/L (unpublished data from the current study for validation of the assay). For *t,t*-MA, the lowest concentration measured in this study was 0.003 mg/L urine. The detection limit for BT was 50 ng/injection; for HQ, 4 ng/injection; and for CAT, 0.4 ng/injection. (BT standard solutions are not stable; they change color to red immediately after preparation.) Blank samples subjected to the entire analytical procedure showed no detectable analytes. The mean total recoveries of spiked [$^{13}C_6$]*t,t*-MA (0.03 mg/L urine) in samples analyzed by LC-MS/MS-SRM were 65%, n = 28 (ranging from 40% to 107%); the recoveries of spiked [$^{13}C_6$]*S*-PMA (15 µg/L urine) were 43%, n = 28 (ranging from 27% to 55%); corresponding recoveries for [$^{13}C_6$]CAT (2.5 mg/L urine) were 41%, n = 30 (ranging from 26% to 59%); and recoveries for [$^{13}C_6$]HQ (0.7 mg/L urine) were 39%, n = 30 (ranging from 20% to 100.5%).

DETERMINATION OF URINARY *S*-PMA AND *t,t*-MA BY LC-ESI-MS/MS-SRM IN THE NEGATIVE IONIZATION MODE

The HPLC system provided separation of analytes within 12 minutes. *t,t*-Muconic acid eluted at 9.3 minutes

Table 1. Reproducibility and Limits of Detection of Urinary Benzene Metabolites by LC-ESI/MS/MS Analysis

Metabolite[a]	n[b]	Coefficient of Variation (%)	Concentration of Metabolite in Urine	Limits of Detection of Standard Samples per Injection (ng)
S-PMA	8	7	9.4 µg/L	0.03
t,t-MA	8	10	0.07 mg/L	1.0
BT	7	20	0.7 mg/L	50.0
HQ	7	12	0.4 mg/L	4.0
CAT	7	4.6	1.9 mg/L	0.4

[a] For *S*-PMA, concentrations are in µg/g creatinine; for all other metabolites, concentrations are in mg/g creatinine.

[b] n = Number of runs on different days.

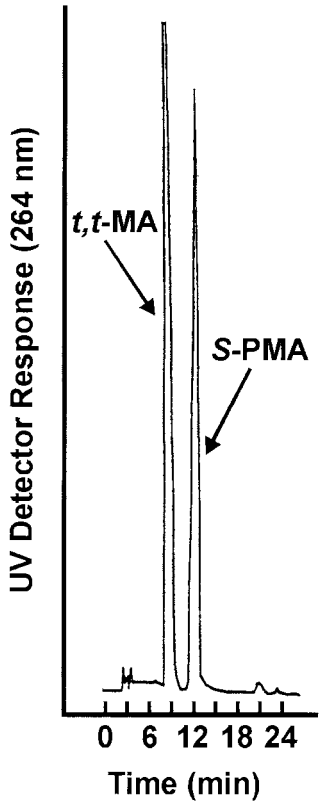

Figure 14. HPLC profile of *t,t*-MA and *S*-PMA.

and *S*-PMA at 12 minutes (Figure 14). The HPLC gradient is such that within 9.3 minutes *t,t*-MA is eluted from the column, and, when it is introduced into the mass spectrometer, the analytes are predominantly in MeOH.

The $(M-1)^-$ ion of *S*-PMA produces a major ion by losing CO_2 and CH_2=CH-NHCOCH$_3$. This reaction was thus chosen for detection of *S*-PMA. The $(M-1)^-$ ion of *t,t*-MA readily loses a CO_2 molecule. This reaction was, therefore, chosen for the detection of *t,t*-MA. Figures 15 and 16 show the analytes' typical tandem mass spectra in the SRM mode as obtained from a human urine sample. In this mode, $(M-1)^-$ ions at *m/z* 238 for *S*-PMA and *m/z* 244 for the internal standard [$^{13}C_6$]*S*-PMA are selected in the Q1 analyzer; product ions, at *m/z* 109 and 115, respectively, are monitored by the second analyzer (Q3, Figure 15). Similarly, ions at *m/z* 141 for *t,t*-MA and *m/z* 147 for the internal standard [$^{13}C_6$]*t,t*-MA were selected in Q1, and daughter ions, at *m/z* 97 and 102, respectively, were monitored in Q3 (Figure 16).

Urine samples collected from women and men, both smokers and nonsmokers, were analyzed by the newly developed method. Figures 17 and 18 show the concentrations of *S*-PMA and *t,t*-MA in the urine of smokers and nonsmokers, respectively. In smokers, the concentration of *S*-PMA was 9.1 ± 1.7 µg/g creatinine (mean ± SE), and the median was 5.8 µg/g creatinine; values ranged from not detectable (1 of 28) to 33.4 µg/g creatinine. The mean ± SE of *t,t*-MA was 0.15 ± 0.03 mg/g creatinine, with a median of 0.11 mg/g creatinine in a range from 0.005 to 0.54 mg/g. In nonsmokers, the corresponding values were 4.8 ± 1.1 µg *S*-PMA/g creatinine, (median = 3.6 µg/g creatinine, ranging from 1.0 to 19.6 µg/g creatinine). The mean of *t,t*-MA was 0.07 ± 0.02 mg/g creatinine, (median = 0.03), ranging from not detectable (1 of 18) to 0.18 mg/g creatinine. The levels of urinary *S*-PMA and *t,t*-MA in smokers were significantly higher than in nonsmokers, at $p = 0.02$ and 0.03, respectively (Table 2). The mean ± SE concentration of urinary cotinine in smokers was 4.6 ± 0.5 µg cotinine/g creatinine; ranging from 0.56 to 15.5 µg/g creatinine (median = 3.4 µg/g). In nonsmokers, corresponding data were 0.02 ± 0.009 µg cotinine/mg creatinine, ranging from not detectable to 0.14 µg/g creatinine (median = 0.009).

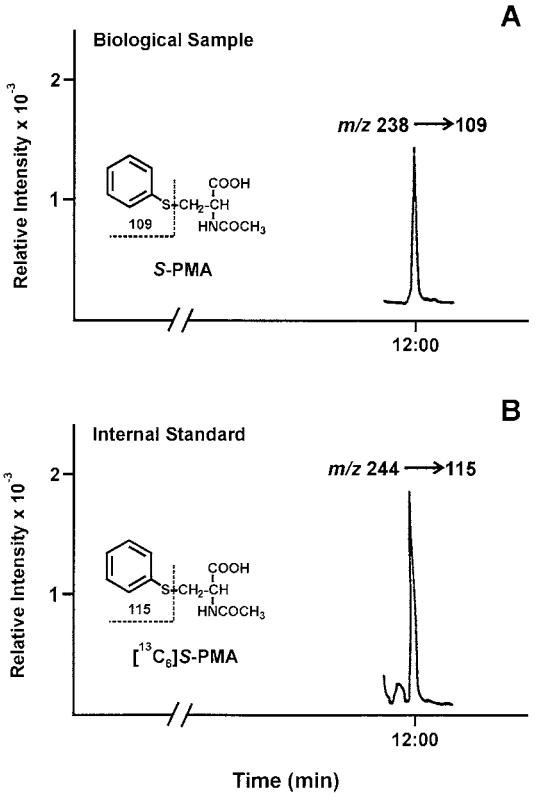

Figure 15. LC-ESI-MS/MS-SRM spectra of human urine sample in the negative ionization mode (A) *S*-PMA, and (B) [$^{13}C_6$]S-PMA internal standard.

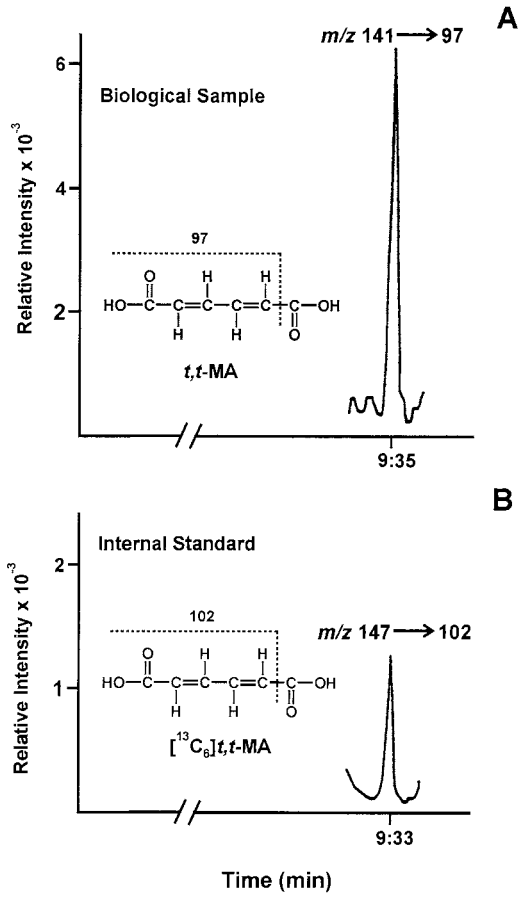

Figure 16. LC-ESI-MS/MS-SRM spectra of human urine sample in the negative ionization mode (A) *t,t*-MA, and (B) [$^{13}C_6$]*t,t*-MA internal standard.

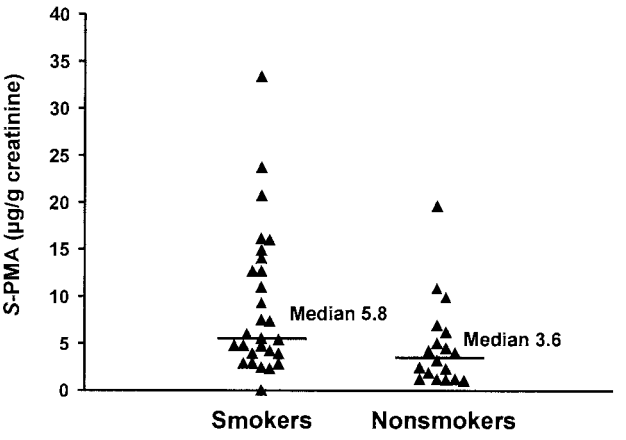

Figure 17. Concentration of *S*-PMA in the urine of smokers and nonsmokers.

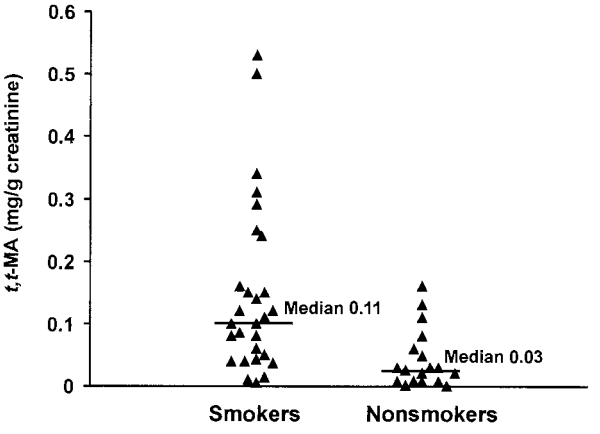

Figure 18. Concentration of *t,t*-MA in the urine of smokers and nonsmokers.

CORRELATION OF URINARY EXCRETION OF *S*-PMA WITH *t,t*-MA

Figure 19 illustrates the plot of *S*-PMA concentrations against urinary *t,t*-MA in the same sample after logarithmic transformation from both smokers and nonsmokers ($r = 0.41$, $p = 0.005$, $n = 46$).

DETERMINATION OF URINARY BT, HQ, AND CAT AFTER ACID HYDROLYSIS BY LC-ESI-MS/MS-SRM IN THE NEGATIVE IONIZATION MODE

Hydroquinone and CAT are isomers differing in the relative positions of the two hydroxyl groups. Upon collisional activation, the $(M-1)^-$ ion at *m/z* 109 of both HQ and CAT loses a hydrogen to produce a quinone molecule impregnated with an electron (*m/z* 108). The reaction dominates the collision-activated decomposition (CAD) of both compounds (Figures 20 and 21). Because HQ and CAT are chromatographically separated in this work, the same reaction was used to monitor both compounds. Similarly, the $(M-1)^-$ ion of [$^{13}C_6$]HQ and [$^{13}C_6$]CAT at *m/z* 115

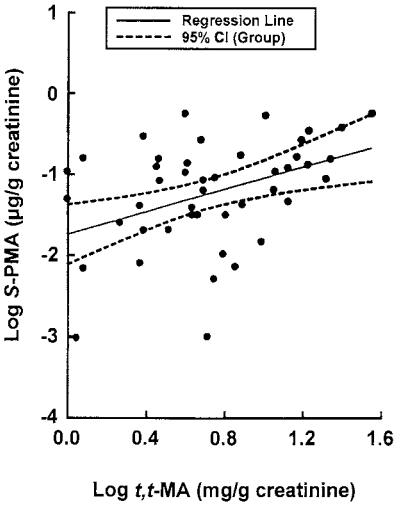

Figure 19. Relationship of concentration of urinary *S*-PMA to *t,t*-MA in smokers and nonsmokers after logarithmic transformation ($r = 0.41$, $p = 0.005$).

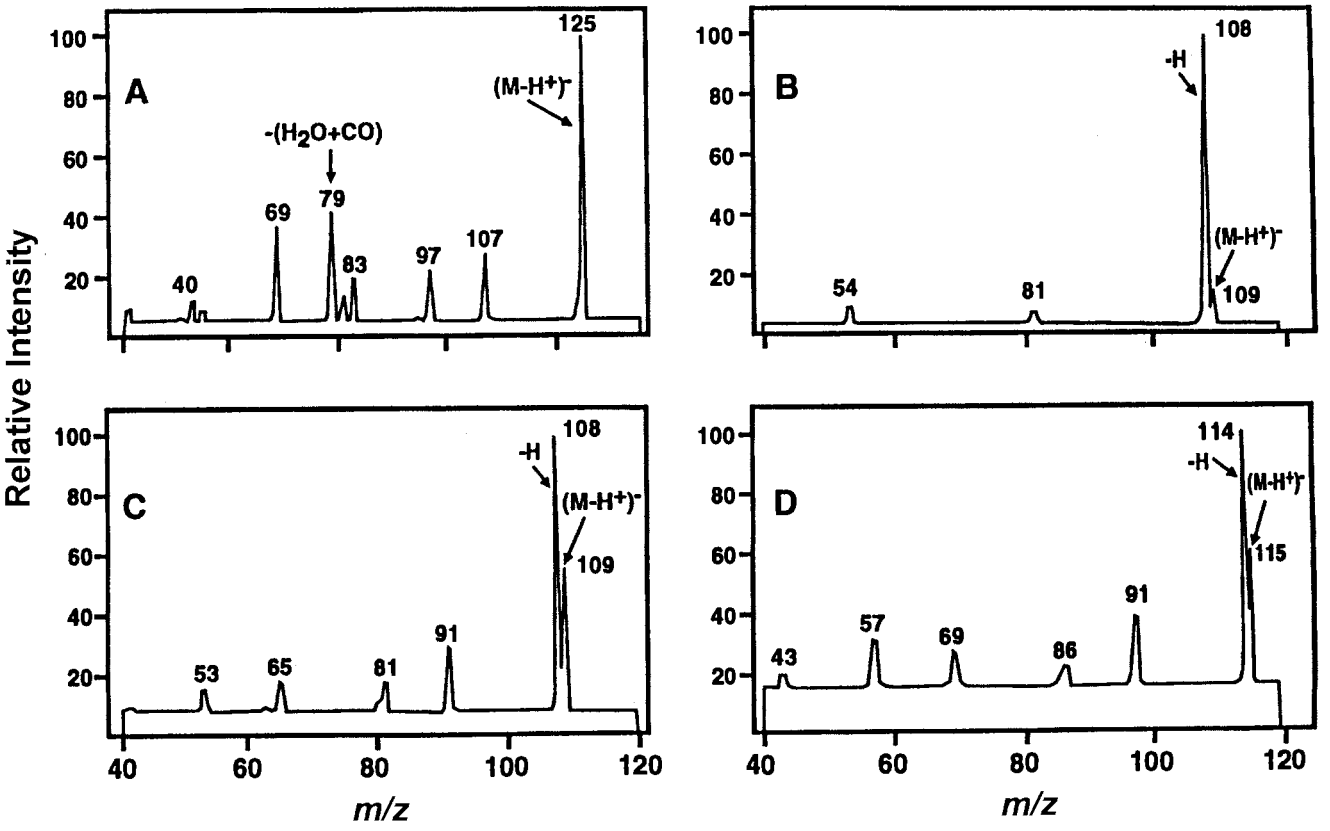

Figure 20. ESI-MS/MS spectra of standards in the negative ionization mode (A) BT, (B) HQ, (C) CAT, and (D) [$^{13}C_6$]CAT.

Table 2. Concentrations of Urinary Benzene Metabolites in Smokers and Nonsmokers Quantitated by LC-ESI-MS/MS

	Nonsmokers				Smokers			
Metabolite[a]	n[b]	Mean ± SE	Median	Range[c]	n[b]	Mean ± SE	Median	Range[c]
S-PMA	18	4.8 ± 1.1[d]	3.6	1.0–19.6	28	9.1 ± 1.7[d]	5.8	ND–33.4
t,t-MA	18	0.07 ± 0.02[e]	0.03	ND–0.18	28	0.15 ± 0.03[e]	0.11	0.005–0.54
BT	15	1.7 ± 0.3[f]	1.5	0.2–5.0	15	1.8 ± 0.5[f]	1.7	0.14–7.1
HQ	15	1.1 ± 0.3[g]	0.66	0.15–5.0	15	2.0 ± 0.6[g]	0.96	0.25–8.1
CAT	15	5.5 ± 1.4[h]	3.1	0.3–20.8	15	5.0 ± 1.2[h]	3.1	1.6–17.5

[a] For *S*-PMA, concentrations are in μg/g creatinine; for all other metabolites, concentrations are in mg/g creatinine.

[b] n = Number of samples.

[c] ND = Not detected.

[d] $p = 0.02$.

[e] $p = 0.03$.

[f] $t = -0.24$, $p = 0.82$ (unpaired t-test).

[g] $t = 1.37$, $p = 0.19$ (unpaired t-test).

[h] $t = 0.28$, $p = 0.78$ (unpaired t-test).

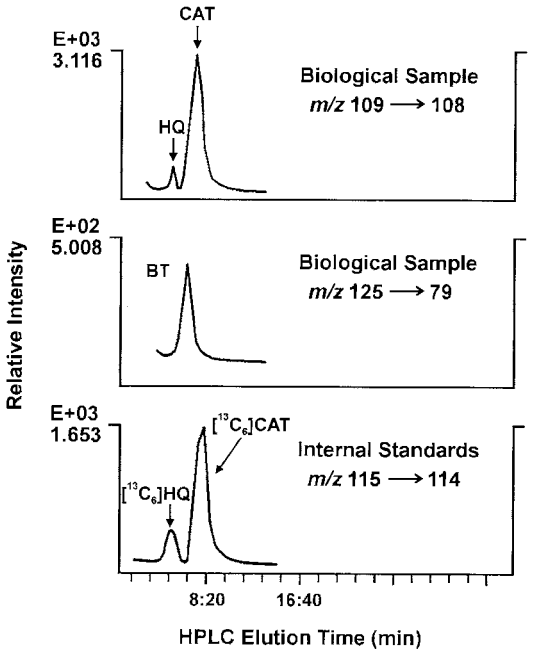

Figure 21. LC-ESI-MS/MS-SRM spectra of HQ and CAT, BT, $[^{13}C_6]$HQ, and $[^{13}C_6]$CAT internal standards in a biological sample.

was selected in the Q1 analyzer; product ions at *m/z* 114 were monitored by the second analyzer.

Benzene triol decomposes extensively upon collision activation. Losses of H_2O, CO, and a combination of these two are the major fragmentation pathways. The most abundant ion, under typical CAD conditions, is produced by the loss of H_2O and CO. The transition corresponding to this reaction, from the $(M\text{-}H)^-$ ion (125) to *m/z* 79, is used to monitor BT.

The HPLC system provided separation of BT, HQ, CAT, and $[^{13}C_6]$ CAT within 7 minutes. For further confirmation, the HPLC was also equipped with a UV detector at 290 nm. A delay of about 0.5 minutes between liquid chromatography–selected ion monitoring–mass spectrometry (LC-SIM-MS) and the HPLC-UV detector was observed because of the movement of eluant between the UV detection and the MS.

Urine samples collected from smokers and nonsmokers, both men and women, were analyzed for BT, HQ, and CAT by LC-MS/MS-SRM methods. Figures 22, 23, and 24 show the concentrations of these analytes in the biological samples. As summarized in Table 2, the mean concentration (± SE) of BT in groups of 15 nonsmokers was 1.7 ± 0.3 mg/g creatinine, with a median of 1.5 mg/g in a range of 0.2 to 5.0 mg/g. In a group of 15 smokers, the mean (± SE) was 1.8 ± 0.5 mg/g creatinine, with a median of 1.7 mg/g in a range of 0.14 to 7.1 mg/g. In the same groups, the mean (± SE) of HQ was 1.1 ± 0.3 mg/g creatinine, with a median of

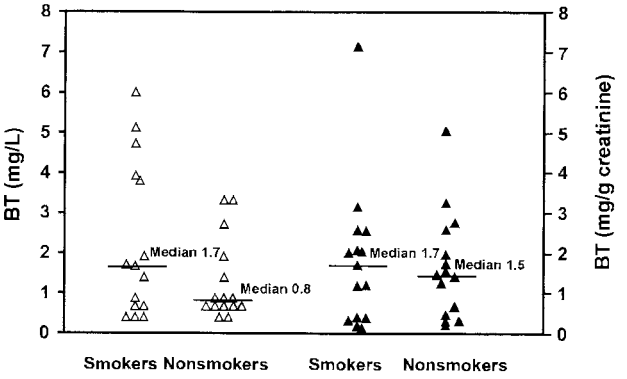

Figure 22. Concentration of BT (△) as mg/L and (▲) as mg/g creatinine in the urine of smokers and nonsmokers.

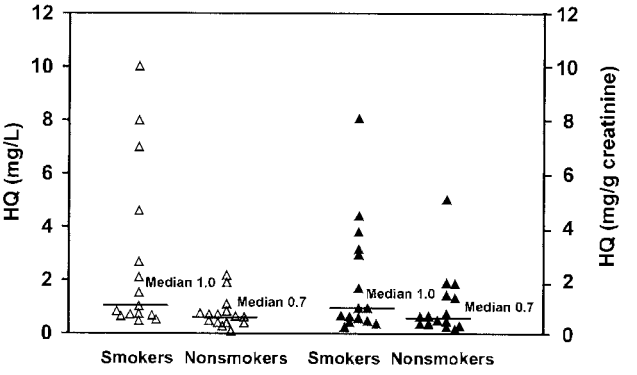

Figure 23. Concentration of HQ (△) as mg/L and (▲) as mg/g creatinine in the urine of smokers and nonsmokers.

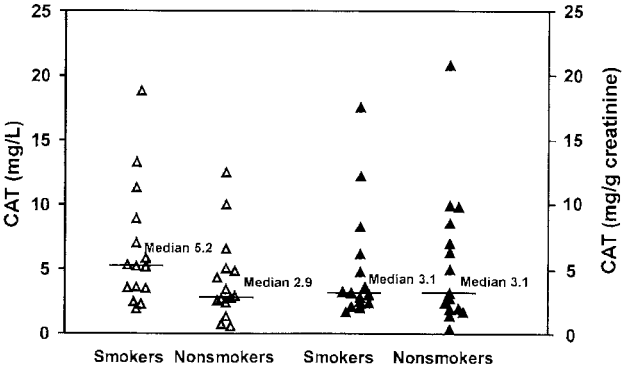

Figure 24. Concentration of CAT (△) as mg/L and (▲) as mg/g creatinine in the urine of smokers and nonsmokers.

0.7 mg/g in a range of 0.15 to 5.0 mg/g creatinine in nonsmokers, and 2.0 ± 0.6 mg/g creatinine, with a median of 1 mg/g creatinine in a range of 0.25 to 8.1 mg/g in smokers. Corresponding values for CAT were 5.5 ± 1.4 mg/g creatinine (median = 3.1 mg/g creatinine in a range of 0.3 to 20.8 mg/g creatinine) in nonsmokers, and 5.0 ± 1.2 mg/g creatinine (median = 3.1 mg/g creatinine in a range of 1.6 to 17.5 mg/g creatinine) in smokers. As shown in Table 3, in both smoker and nonsmoker groups combined, there was a correlation between CAT and BT ($r = 0.54$, $p = 0.002$) and between CAT and HQ ($r = 0.46$, $p = 0.01$).

DISCUSSION

Two sensitive and specific LC-MS/MS assays have been developed for measuring five urinary benzene metabolites that have been suggested as markers for biological monitoring of benzene exposure. These assays facilitate the simultaneous determination of *S*-PMA and *t,t*-MA in urine and the measurement of BT, HQ, and CAT in a single analysis. The feasibility and efficacy of these assays have been examined in the present pilot studies using cigarette smokers as subjects with low benzene exposure and nonsmokers as the control group.

RATIONALE FOR DEVELOPMENT OF LC-ESI-MS/MS METHODS

During the past decades, several new analytical methods have been employed for measurement of urinary *t,t*-MA and *S*-PMA. Several relatively simple HPLC-based assays and GC-MS have been employed for quantitating *t,t*-MA (Gad-El Karim et al. 1985b; Inoue et al. 1989a,b; Ducos et al. 1990; Bechtold et al. 1991; van Sittert et al. 1993; Lee et al. 1993a,b; Melikian et al. 1993, 1994; Schäfer et al. 1993; Bartczak et al. 1994; Lauwerys et al. 1994; Ong and Lee 1994; Ong et al. 1994; Popp et al. 1994; Boogaard and van Sittert 1995; Ghittori et al. 1995, 1996; Ruppert et al. 1995, 1997; Weaver et al. 1996; Yu and Weisel 1996; Hotz et al. 1997). Since *S*-PMA is a minor metabolite of benzene, most analytical methods are not sensitive enough to determine this biomarker in the urine of persons with low benzene exposure. The available methods reported thus far for this biomarker are based on HPLC with fluorescence detection, or on GC-MS (Jongeneelen et al. 1987; Stommel et al. 1989; Popp et al. 1994; Boogaard and van Sittert 1995; Eining and Dehnen 1995). There are limited assays on monitoring ring-hydroxylated metabolites of benzene, especially BT in humans (Greenlee et al. 1981; Inoue et al. 1988, 1989b; Risner and Cash 1990; Schad et al. 1992; Deisinger et al. 1996; Ong et al. 1996; Rothman et al. 1996; Hotz et al. 1997). In many cases, these polar analytes are measured by an HPLC-UV method. Lee and coworkers (1993b) have developed an HPLC method with variable wavelength fluorometric detection for simultaneous measurement of HQ, CAT, and phenol. Hotz and coworkers (1997) measured urinary HQ and CAT by GC after derivatization. Inoue and coworkers (1989b) quantified urinary BT in workers by HPLC-UV detection.

HPLC-UV chromatograms of urinary extracts, especially from smokers, generally contain several compounds that elute in the vicinity of *t,t*-MA, and also other analytes that can interfere with the exact measurement of these compounds. The use of an internal standard is also hampered in HPLC-UV methods because of the presence of these unidentified confounders. The GC-MS method eliminates these problems, and allows the quantification of low levels of *S*-PMA and *t,t*-MA. Derivatization of each analyte to more volatile compounds, however, makes the GC-MS method less attractive for large transitional epidemiology studies. Because volatility of an analyte is not a requirement, and derivatization of the samples is not necessary in the LC-MS technique, methods can be developed in such a way that several structurally dissimilar analytes, such as *t,t*-MA and *S*-PMA, can be measured in a single analysis.

In any quantitative method, sensitivity is a major consideration. The electrospray interface, typically considered to act as a concentration-dependent detector, has recently gained much interest in analytical chemistry.

Table 3. Correlation[a] Between Ring-Hydroxylated Metabolites in Smokers and Nonsmokers

	Smokers ($n = 15$)		Nonsmokers ($n = 15$)		Combination of Smokers and Nonsmokers ($n = 30$)	
Metabolites	r	p	r	p	r	p
CAT, BT	0.34	0.22	0.64	0.01	0.54	0.002
CAT, HQ	0.28	0.31	0.68	0.005	0.46	0.01
BT, HQ	−0.17	0.54	0.35	0.2	0.08	0.67

[a] Spearman correlation coefficients.

Despite the success of the electrospray method in quantitative analysis, this technique does have certain limitations. One problem with the electrospray in quantitative analysis is the suppression of a monitored ion analyte by coeluting matrix components. Hence, ion suppression may adversely affect both the sensitivity and the reproducibility of a particular assay (Buhrman et al. 1996). Eliminating the confounding matrix components by means of improved extraction methods may allow for quantitation at lower concentrations. Biological samples analyzed by LC-MS usually require a cleanup prior to analysis.

DETERMINATION OF *t,t*-MA AND *S*-PMA

The optimal LC-MS/MS condition for the *t,t*-MA and *S*-PMA assay was obtained in the negative ionization mode. Operating in this mode, both *t,t*-MA and *S*-PMA and their corresponding internal standards generated predominantly deprotonated molecular ions $(M-1)^-$; these were selected as parent ions. The optimal HPLC condition for *t,t*-MA and *S*-PMA was assessed with an elution gradient from an acidic to an organic solvent; thus, the complete elution of *t,t*-MA from the HPLC column was achieved by acidic solvent elution and, when this analyte was introduced into the mass spectrometer, it was observed predominantly in MeOH. Methanol was suited for ionization and deprotonation of both *t,t*-MA and *S*-PMA.

Stable isotope isomers of $[^{13}C_6]$*t,t*-MA and $[^{13}C_6]$*S*-PMA were used as internal standards for quantifying of urinary *t,t*-MA and *S*-PMA. These internal standards were selected because they have nearly identical physicochemical properties, and their HPLC retention times are similar to those of the urinary analytes, while their molecular weights are 6 mass units higher.

The efficacy of the newly developed LC-ESI-MS/MS assay was evaluated in humans by analyzing urine specimens from 28 smokers and 18 nonsmokers in benzene-exposed and nonexposed groups, respectively. The number of samples analyzed was based on the availability of specimen at the time of the experiments. Urinary cotinine was used to assess the intensity of smoking. The mean concentration of cotinine in the urine of smokers was 4.6 μg/g creatinine, suggesting that the subjects in this study were heavy tobacco smokers (Vine et al. 1993). This pilot study indicated that the mean concentrations of *t,t*-MA and *S*-PMA in the urine of smokers were, respectively, 2.1-fold ($p = 0.03$) and 1.9-fold ($p = 0.02$) higher than those of nonsmokers (Figures 14 and 15; Table 2).

The concentrations reported in the literature (Boogaard and van Sittert 1995; Melikian et al. 1992, 1993; Ruppert et al. 1995, 1997; Ong and Lee 1994; Ong et al. 1994; Ghittori et al. 1996; Hotz et al. 1997) of urinary *t,t*-MA in nonsmokers range from 0.05 to 0.21 mg/g creatinine and those in smokers range from 0.14 to 0.61 mg/g creatinine. The mean concentrations of 0.07 mg/g creatinine in nonsmokers and 0.15 mg/g in smokers observed for *t,t*-MA in the current study is in agreement with literature data. Similarly, levels of *S*-PMA reported by Boogaard and van Sittert (1995) and by Ghittori and coworkers (1995) were 1.99 and 1.5 μg *S*-PMA/g creatinine for nonsmokers, and 3.61 and 9.4 μg *S*-PMA/g creatinine for smokers in subjects who were not occupationally exposed to benzene. The mean concentration of *S*-PMA in nonsmokers quantified by LC-MS/MS in the present study was 4.8 μg/g creatinine and, in smokers, 9.1 μg/g creatinine.

Concentration of *t,t*-MA in workers exposed to 0.5 ppm benzene has been reported in the range of 0.39 to 1.1 mg/g creatinine (Popp et al. 1994; Boogaard and van Sittert 1995; Ghittori et al. 1995; Hotz et al. 1997). Similarly, the predicted excretion of *S*-PMA ranged from 7.2 to 25 μg/g creatinine for 0.5 ppm benzene exposure (Popp et al. 1994; Boogaard and van Sittert 1995; Ghittori et al. 1995; Hotz et al. 1997). On the basis of the literature data, and assuming that the uptake of benzene by smokers is 1.8 mg/day at a respiratory rate of 10 L air/minute, the levels of *t,t*-MA excreted from cigarette smoking are estimated to range from 0.09 to 0.26 mg/g creatinine, and *S*-PMA from 1.63 to 5.9 μg/g creatinine. The levels of *t,t*-MA and *S*-PMA in smokers are higher than those found in nonsmokers (0.15 versus 0.07 mg/creatinine for *t,t*-MA and 9.1 versus 4.8 for *S*-PMA, respectively). These findings are in good agreement with the previously estimated ranges.

A linear regression analysis of logarithmic transformation pointed to a correlation between *S*-PMA and *t,t*-MA of $r = 0.41$, $p = 0.005$ (Figure 18).

There was inter-subject variation from 1 to 146 in the ratio of urinary excretion of *t,t*-MA to *S*-PMA. This inter-individual variation may be due to differences in detoxification of benzene epoxide by glutathione, or these metabolites may be derived from different intermediates or, alternatively, they may stem from varying amounts of ingested sorbic acid.

Sorbic acid is present in foods at about 0.2 to 3.0 g/kg (Pezzagno and Maestri 1997). Recently, Pezzagno and Maestri (1997) have studied the rate of transformation of sorbic acid into *t,t*-MA in humans, and found that 0.3 to 0.5 g ingested sorbic acid would suffice to lead to excretion of 1 mg of *t,t*-MA, a quantity corresponding to that derived from benzene exposure at levels greater than 1 ppm. Similarly, Ruppert and coworkers (1997) have reported that dietary supplementation of eight nonsmokers with 500 mg sorbic acid significantly increased

the urinary *t,t*-MA excretion from 0.08 to 0.88 mg/24 hours. About 0.12% of the sorbic acid dose was excreted in urine as *t,t*-MA. The investigators stated that typical dietary intake of 6 to 30 mg/day sorbic acid accounts for 10% to 50% of background *t,t*-MA excretion in nonsmokers, and for 5% to 25% in smokers. These recent findings are in agreement with earlier studies by Ducos and coworkers (1990). *t,t*-MA may partly reflect differences in dietary habits that contribute, to some extent, to the background levels. About 75% of *t,t*-MA was excreted within 6 hours after ingestion of sorbic acid. Thus, only urine samples collected many hours after the last meal can minimize the effect of ingested sorbic acid on the total excretion of *t,t*-MA.

Studies are in progress to validate LC-MS/MS assays for determination of urinary benzene metabolites in occupationally exposed subjects. In the course of these studies it has been noted that there is sometimes a carryover of analytes from one run to the next when biological samples are very concentrated. To eliminate this problem, solvent is being injected between sample runs.

DETERMINATION OF BT, HQ, AND CAT AFTER ACID HYDROLYSIS

The optimal conditions for simultaneous measurement of BT, HQ, and CAT by ESI-MS or by ESI-MS/MS in the negative ionization mode were developed. The effects of various solvents on optimal detection limits were also investigated. The detection limit of HQ was enhanced when 100% MeOH was used as solvent. However, for the best chromatographic separation of CAT and HQ, which have a similar molecular ion, 1:1 MeOH:H_2O was optimal. The variation of response versus flow rate and use of a split system for HPLC-ESI-MS were also explored.

Stable isotope isomers of [$^{13}C_6$]HQ were used for quantitation of HQ, and [$^{13}C_6$]CAT for measurement of CAT and BT. [$^{13}C_6$]Benzene triol was not utilized as an internal standard because it can easily oxidize before it is added to biological samples. The use of urine samples from rats treated with [^{13}C]benzene as an internal standard is under investigation.

Prior to development of LC-ESI-MS/MS, the LC-MS method was used for quantifying ring-hydroxylated benzene metabolites in the urine of smokers and nonsmokers. In this assay, [$^{13}C_6$]CAT was the only internal standard that could be utilized, because it appears that human urine contains an agent that has chromatographic characteristics similar to those of HQ and produces ions similar to the [$^{13}C_6$]HQ molecular ion $(M-1)^-$ at *m/z* 115. Analysis by LC-MS is less selective than LC-MS/MS, especially for ring-hydroxylated metabolites of benzene. These compounds are polar molecules of very small size, which give more background interference than larger molecules.

The combination of LC-ESI-MS/MS in the negative mode is more specific, from a laboratory standpoint, than other available techniques such as HPLC-UV, GC, or LC-MS. Our pilot study indicated that the mean urinary concentrations of BT, HQ, and CAT in smokers were not significantly different from those in nonsmokers (1.1-fold [$t = 0.24$, $p = 0.82$]; 1.8-fold [$t = 1.37$, $p = 0.19$]; and 0.9-fold [$t = 0.28$, $p = 0.78$], respectively). The statistical evaluations are summarized in Tables 2 and 3. The mean concentration of HQ was 2.0 mg/g creatinine in smokers versus 1.1 mg/g creatinine in nonsmokers; that of BT was 1.8 mg/g creatinine in smokers versus 1.7 mg/g creatinine in nonsmokers; CAT was found in the urine of smokers at 5.0 mg/g creatinine and in nonsmokers' urine at 5.5 mg/g. In smokers, the mean concentration of cotinine was 0.60 ± 0.11 µg/g creatinine, which indicates that the subjects in this group were light smokers, in contrast to the smokers enrolled in the *t,t*-MA and *S*-PMA analyses.

The levels of urinary HQ were lower than those of CAT in the subjects analyzed. This confirms the earlier findings that the urinary CAT concentration is generally higher than that of HQ (Inoue et al. 1988; Lee et al. 1993b; Ong et al. 1995). Catechol can be formed by the metabolism of phenol through further oxidation. Moreover, catechol is present in certain foods (Luh et al. 1967), in coffee (0.42 to 0.74 mg/6-oz cup) (Carmella and Hecht, unpublished data), and in hair dyes. Catechol is the most abundant phenol in mainstream cigarette smoke (0.09 to 0.2 mg/cigarette) (Brunnemann et al. 1976). We were unable to show an increase of catechol in smokers. This is not surprising. Ong and coworkers (1995) were likewise unable to demonstrate any significant correlation between urinary catechol and benzene at low exposures. Inoue and coworkers (1988), however, reported a significant correlation between urinary catechol and environmental benzene exposure ($r = 0.85$) in a study in which the subjects were exposed to high concentrations of benzene (up to 200 ppm) while correlation below 10 ppm was not assessed. There are only a few studies that have dealt with the correlation of benzene exposure with urinary HQ. Apparently, this biomarker can distinguish those who were exposed to less than 1 ppm of benzene from those who were unexposed.

Concentrations of BT were comparable with HQ. Both CAT and HQ could be precursors of BT. It has been shown that rats metabolize both HQ and phenol to BT, but BT formation from HQ was greater than from phenol (Inoue et al. 1989b). The only study in which urinary BT was measured in occupational settings was based on the HPLC-UV

method (Inoue et al. 1989b). A drawback of this method was that only benzene exposure above 10 ppm could be detected.

In studies of urinary biomarkers of benzene exposure in humans, coexposure to high levels of toluene resulted in marked reduction of urinary phenol, HQ, and BT with small reduction of CAT (Inoue et al. 1988). These observations are supported by the finding that coadministration of benzene and toluene to laboratory animals results in suppression of metabolism (Ikeda et al. 1972). Brondeau and coworkers (1992) have shown that exposure of rats to benzene and toluene simultaneously lowered the excretion of urinary *t,t*-MA on average by 28%, 44%, and 85%; exposures were to combinations of 100, 200, or 1,000 ppm toluene with 20 ppm benzene. Thus, measurement of *t,t*-MA and other urinary metabolites of benzene may underreport the true exposure to benzene in cases where toluene was also present. Since toluene generally occurs together with benzene in environmental pollutants, monitoring both chemicals may be necessary to determine any confounding effect of toluene on the quantitation of benzene metabolites as biomarkers of exposure. The use of antioxidants such as vitamins, as well as consumption of tea, which contains polyphenols such as epigallocatechin gallate, may prevent metabolism of benzene to active metabolites and could also affect the levels of urinary benzene metabolites.

CONCLUSIONS

We have developed assays for quantitative assessment of five biomarkers that have been suggested for biological monitoring. The developed HPLC separation, coupled with electrospray interface MS/MS methods to determine benzene metabolites, is sensitive and specific from a laboratory standpoint, and enable us to detect the background levels of benzene exposure in the general population. This pilot study confirms that quantitation of *t,t*-MA and *S*-PMA provides very reliable biomarkers for monitoring chronic benzene exposure at low doses. Among the three phenolic metabolites of benzene, HQ seems to be the most reliable. These biomarkers of benzene remain to be validated in experiments with known benzene exposures, which are in progress.

ACKNOWLEDGMENTS

The authors greatly acknowledge the Health Effects Institute for supporting this study. They also acknowledge the cooperation of Dr. Nancy Haley of the Metropolitan Life Insurance Testing Laboratories in providing urine samples, Dr. J. Ni for MS analysis of some standard samples, and Ms. Ilse Hoffmann for manuscript editing.

REFERENCES

Aksoy M, Erdem S, DinCol G. 1974. Leukemia in shoeworkers exposed chronically to benzene. Blood 44:837–841.

Aksoy M. 1980. Different types of malignancies due to occupational exposure to benzene: A review of recent observations in Turkey. Environ Res 23:181–190.

American Conference of Governmental Industrial Hygienists. 1994. Threshold Limit Values and Biological Exposure Indices for 1994. American Conference of Governmental Industrial Hygienists, Cincinnati, OH.

American Conference of Governmental Industrial Hygienists. 1995. Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices for 1995. American Conference of Governmental Industrial Hygienists, Cincinnati, OH.

American Conference of Governmental Industrial Hygienists. 1996. Threshold Limit Values and Biological Exposure Indices for 1996. American Conference of Governmental Industrial Hygienists, Cincinnati, OH.

Angerer J, Horsch B. 1992. Determination of aromatic hydrocarbons and their metabolites in human blood and urine. J Chromatogr 580:229–255.

Bartczak A, Kline SA, Yu R, Weisel CP, Goldstein BD, Witz G, Bechtold WE. 1994. Evaluation of assays for the identification and quantitation of muconic acid, a benzene metabolite in human urine. J Toxicol Environ Health 42:245–258.

Bechtold WE, Lucier G, Birnbaum LS, Yin SN, Li GL, Henderson RF. 1991. Muconic acid determinations in urine as a biological exposure index for workers occupationally exposed to benzene. Am Ind Hyg Assoc J 52:473–478.

Bechtold WE, Strunk MR. 1996. *S*-Phenylcysteine in albumin as a benzene biomarker. Environ Health Perspect (Suppl 6) 104:1147–1149.

Bechtold WE, Sun JD, Birnbaum LS, Yin SN, Li GL, Kasicki S, Lucier G, Henderson RF. 1992a. *S*-Phenylcysteine formation in hemoglobin as a biological exposure index to benzene. Arch Toxicol 66:303–309.

Bechtold WE, Willis JK, Sun JD, Griffith WC, Reddy TV. 1992b. Biological markers of exposure to benzene: *S*-Phenylcysteine in albumin. Carcinogenesis 13:1217–1220.

Berlin M, Gage JC, Gullberg B, Holm S, Knutsson P, Eng C, Tunek A. 1980. Breath concentration as an index of the health risk from benzene. Scand J Work Environ Health 6:104–111.

Bodell WJ, Pathak DN, Lévay G, Ye Q, Pongracz K. 1996. Investigation of the DNA adducts formed in B6C3F$_1$ mice treated with benzene: Implications for molecular dosimetry. Environ Health Perspect (Suppl 6) 104:1189–1193.

Bond GG, McLaren EA, Baldwin CL, Cook RR. 1986. An update of mortality among chemical workers exposed to benzene. Br J Ind Med 43:685–691.

Boogaard PJ, van Sittert NJ. 1995. Biological monitoring of exposure to benzene: A comparison between *S*-phenylmercapturic acid, *trans,trans*-muconic acid, and phenol. Occup Environ Med 52:611–620.

Bowers LD. 1989. High performance liquid chromatography/mass spectrometry: State of the art for the drug analysis laboratory. Clin Chem 35:1282–1287.

Brondeau MT, Ducos P, Gaudin R, Morel G, Bonnet P, de Ceaurriz J. 1992. Evaluation of the interaction of benzene and toluene on the urinary excretion of *t,t*-muconic acid in rats. Toxicol Lett 61:311–316.

Brugnone F, Perbellini L, Faccini GB, Pasini F, Danzi B, Maranelli G, Romeo L, Gobbi M, Zedde A. 1989. Benzene in the blood and breath of normal people and occupationally exposed workers. Am J Ind Med 16:385–399.

Brugnone F, Perbellini L, Maranelli G, Romeo L, Guglielmi G, Lombardini F. 1992. Reference values for blood benzene in the occupationally unexposed general population. Int Arch Occup Environ Health 64:179–184.

Bruins AP. 1985. Developments in interfacing microbore high-performance liquid chromatography with mass spectrometry. J Chromatogr 323:99–111.

Brunnemann KD, Lee HC, Hoffmann D. 1976. Chemical studies on tobacco smoke: XLVII. On the quantitative analysis of catechols and their reduction. Anal Lett 9:939–955.

Buhrman DL, Price PI, Rudewicz PJ. 1996. Quantitation of SR 27417 in human plasma using electrospray liquid chromatography–tandem mass spectrometry: A study of ion suppression. J Am Soc Mass Spectrom 7:1099–1105.

Busby WF Jr, Wang JS, Stevens EK, Padykula RE, Aleksejczyk RA, Berchtold GA. 1990. Lung tumorigenicity of benzene oxide, benzene dihydrodiols and benzene diol epoxides in the BLUH: A newborn mouse assay. Carcinogenesis 11:1473–1478.

Carbonnelle P, Lison D, Leroy JY, Lauwerys R. 1995. Effect of the benzene metabolite, hydroquinone, on interleukin-1 secretion by human monocytes in vitro. Toxicol Appl Pharmacol 132:220–226.

Chen H, Eastmond DA. 1995. Synergistic increase in chromosomal breakage within the euchromatin induced by an interaction of the benzene metabolites phenol and hydroquinone in mice. Carcinogenesis 16:1963–1969.

Chenna A, Hang B, Rydberg B, Kim E, Pongracz K, Bodell WJ, Singer B. 1995. The benzene metabolite *p*-benzoquinone forms adducts with DNA bases that are excised by a repair activity from human cells that differs from an ethenoadenine glycosylase. Proc Natl Acad Sci USA 92:5890–5894.

Chepiga TA, Yang CS, Snyder R. 1991. Benzene metabolism by two purified, reconstituted rat hepatic mixed function oxidase systems. In: Biologically Reactive Intermediates: IV. Molecular and Cellular Effects and Their Impact on Human Health (Witmer CM, Snyder R, Jollow DJ, Kalf GF, Kocsis JJ, Sipes IG, eds.). Plenum Press, New York, NY.

Chew EH, Heys JR. 1981. Preparation of uniformly ^{14}C-labeled and ^{13}C-enriched catechol and hydroquinone via phenol. J Label Compd Radiopharm XVIII 4:525–533.

Cohen G, Sinet PM. 1981. Oxygen consumption during a Fenton-type reaction between hydrogen peroxide and iron-chelate Fe(1I-DTPA). In: Oxygen and Oxy-Radicals in Chemistry and Biology (Rogers MAJ, ed.). Academic Press, Orlando, FL.

Cronkite EP, Bullis JE, Inoue T, Drew RT. 1984. Benzene inhalation produces leukemia in mice. Toxicol Appl Pharmacol 75:358–361.

Cronkite EP, Drew RT, Inoue T, Bullis JE. 1985. Benzene hematotoxicity and leukemogenesis. Am J Ind Med 7:447–456.

Cronkite EP, Drew RT, Inoue T, Hirabayashi Y, Bullis JE. 1989. Hematotoxicity and carcinogenicity of inhaled benzene. Environ Health Perspect 82:97–108.

Daly JW, Jerina DM, Witkop B. 1972. Arene oxides and NIH shift: The metabolism, toxicity and carcinogenicity of aromatic compounds. Experientia 28:1129–1149.

Davies SG, Whitham GH. 1977. Benzene oxide-oxepin: Oxidation to muconaldehyde. J Chem Soc [Perkin 1] I:1346–1347.

De PK, Banerjee RK. 1992. Purification and characterization of a soluble peroxidase of rat preputial gland: Com-

parison with lactoperoxidase. Biochim Biophys Acta 1120(2):167–172.

Decouflé P, Blattner WA, Blair A. 1983. Mortality among chemical workers exposed to benzene and other agents. Environ Res 30:16–25.

Deichmann WB. 1983. Phenols and phenolic compounds. In: Encyclopedia of Occupational Health and Safety, Vol. 2, 3rd ed. (Parmeggiani L, ed.). International Labor Organization, Geneva, Switzerland.

Deisinger PJ, Hill TS, English JC. 1996. Human exposure to naturally occuring hydroquinone. J Toxicol Environ Health 47:31–46.

Dorfman LM, Taub IA, Buhler RE. 1962. Pulse radiolysis studies. I. Transient spectra and reaction-rate constants in irradiated aqueous solutions of benzene. J Chem Phys 36:3051–3061.

Drummond JC, Finar IL. 1938. Muconic acid as a metabolic product of benzene. Biochemistry 32:79–84.

Drummond L, Luck R, Afacan AS, Wilson HK. 1988. Biological monitoring of workers exposed to benzene in the coke oven industry. Br J Ind Med 45:256–261.

Ducos P, Gaudin R, Bel J, Maire C, Francin JM, Robert A, Wild P. 1992. *trans,trans*-Muconic acid, a reliable biological indicator for detection of individual benzene exposure down to the ppm level. Int Arch Occup Environ Health 64:309–313.

Ducos P, Gaudin R, Robert A, Francin JM, Maire C. 1990. Improvement in HPLC analysis of urinary *trans,trans*-muconic acid, a promising substitute for phenol in the assessment of benzene exposure. Int Arch Occup Environ Health 62:529–534.

Dvorak AM, Ishizaka T. 1994. Human eosinophils in vitro: An ultrastructural morphology primer. Histol Histopathol 9:339–374.

Eastmond DA, Rupa DS, Hasegawa LS. 1994. Detection of hyperdiploidy and chromosome breakage in interphase human lymphocytes following exposure to the benzene metabolite hydroquinone using multicolor fluorescence in situ hybridization with DNA probes. Mutat Res 322:9–20.

Eastmond DA, Smith MT, Irons RD. 1987. An interaction of benzene metabolites reproduces the myelotoxicity observed with benzene exposure. Toxicol Appl Pharmacol 91:85–95.

Einig T, Dehnen W. 1995. Sensitive determination of the benzene metabolite *S*-phenylmercapturic acid in urine by high-performance liquid chromatography with fluorescence detection. J Chromatogr A 697:371–375.

Fishbein L. 1984. An overview of environmental and toxicological aspects of aromatic hydrocarbons: I. Benzene. Sci Total Environ 40:189–218.

Forni AM, Cappellini A, Pacifico E, Vigliani EC. 1971. Chromosome changes and their evolution in subjects with past exposure to benzene. Arch Environ Health 23:385–391.

Forni A, Vigliani EC. 1972. Chemical leukemogenesis in man. Ser Haematol 7:212–223.

Gad-El Karim MM, Sadagopa Ramanujam VM, Ahmed EA, Legator MS. 1985a. Benzene myeloclastogenicity: A function of its metabolism. Am J Ind Med 7:475–484.

Gad-El Karim MM, Sadagopa Ramanujam VM, Legator MS. 1985b. *trans,trans*-Muconic acid, an open-chain urinary metabolite of benzene in mice: Quantification by high-pressure liquid chromatography. Xenobiotica 15:211–220.

Gad-El Karim MM, Sadagopa Ramanujam VM, Legator MS. 1986. Correlation between the induction of micronuclei in bone marrow by benzene exposure and the excretion of metabolites in urine of CD-1 mice. Toxicol Appl Pharmacol 85:464–477.

Gaido KW, Wierda D. 1987. Suppression of bone marrow stromal cell function by benzene and hydroquinone is ameliorated by indomethacin. Toxicol Appl Pharmacol 89:378–390.

Ghittori S, Fiorentino ML, Maestri L, Cordioli G, Imbriani M. 1993. Urinary excretion of unmetabolized benzene as an indicator of benzene exposure. J Toxicol Environ Health 38:233–243.

Ghittori S, Maestri L, Fiorentino ML, Imbriani M. 1995. Evaluation of occupational exposure to benzene by urinalysis. Int Arch Occup Environ Health 67:195–200.

Ghittori S, Maestri L, Rolandi L, Lodola L, Fiorentino ML, Imbriani M. 1996. The determination of *trans,trans*-muconic acid in urine as an indicator of occupational exposure to benzene. Appl Occup Environ Hyg 11:187–191.

Glatt H, Witz G. 1990. Studies on the induction of gene mutations in bacterial and mammalian cells by the ring-opened benzene metabolites *trans,trans*-muconaldehyde and *trans,trans*-muconic acid. Mutagenesis 5:263–266.

Goldstein BD. 1996. Biological markers and risk assessment. Drug Metab Rev 28:225–233.

Goldstein BD, Snyder CA, Laskin S, Brombert I, Albert RE. Nelson N. 1982. Myelogenous leukemia in rodents inhaling benzene. Toxicol Lett 13:169–173.

Goldstein BD, Witz G. 1992. Benzene. In: Environmental Toxicants, Human Exposure and Their Health Effects (Lippman M, ed.). Van Nostrand Reinhold, New York, NY.

Gorsky LD, Coon LJ. 1985. Evaluation of the role of free hydroxyl radicals in the cytochrome P450-catalyzed oxidation of benzene and cyclohexanol. Drug Metab Dispos 13:169–174.

Greenlee WF, Chism JP, Rickert DE. 1981. A novel method for the separation and quantitation of benzene metabolites using high-pressure liquid chromatography. Anal Biochem 112:367–370.

Gut I, Nedelcheva V, Soucek P, Stopka P, Tichavská B. 1996a. Cytochromes P450 in benzene metabolism and involvement of their metabolites and reactive oxygen species in toxicity. Environ Health Perspect (Suppl 6) 104:1211–1218.

Gut I, Nedelcheva V, Soucek P, Stopka P, Vodicka P. Gelboin HV, Ingelman-Sundberg M. 1996b. The role of CYP2E1 and 2B1 in metabolic activation of benzene derivatives. Arch Toxicol 71:45–56.

Haley NJ, Axelrad CM, Tilton KA. 1983. Validation of self-reported smoking behavior: Biochemical analyses of cotinine and thiocyanate. Am J Public Health 73:1204–1207.

Hanson SK, Anders MW, Montgomery MR. 1978. Oxygen metabolism in rabbit bone marrow and liver. Biochem Biophys Res Commun 85:783–787.

Hanzlik RP, Weller PE, Desai J, Zheng J, Hall LR, Slaughter DE. 1990. Synthesis of mercapturic acid derivatives of putative toxic metabolites of bromobenzene. J Org Chem 55:2736–2742.

Hard GC, Whysner J, English JC, Zang E, Williams GM. 1997. Relationship of hydroquinone-associated rat renal tumors with spontaneous chronic progressive nephropathy. Toxicol Pathol 25:132–143.

Hazel BA, Baum C, Kalf GF. 1996. Hydroquinone, a bioreactive metabolite of benzene, inhibits apoptosis in myeloblasts. Stem Cells 14:730–742.

Henderson RF. 1996. Species difference in the metabolism of benzene. Environ Health Perspect (Suppl 6) 104:1173–1175.

Henderson RF, Sabourin PJ, Bechtold WE, Griffith WC, Medinsky MA, Birnbaum LS, Lucier GW. 1989. The effect of dose, dose rate, route of administration, and species on tissue and blood levels of benzene metabolites. Environ Health Perspect 82:9–17.

Hite M, Pecharo M, Smith I, Thornton S. 1980. The effect of benzene in the micronucleus test. Mutat Res 77:149–155.

Ho T-Y, Witz G. 1997. Increased gene expression in human promyeloid leukemia cells exposed to *trans,trans*-muconaldehyde, a hematotoxic benzene metabolite. Carcinogenesis 18(4):739–744.

Hotz P, Carbonelle P, Haufroid V, Tschopp JP, Buchet JP, Lauwerys R. 1997. Biological monitoring of vehicle mechanics and other workers exposed to low concentrations of benzene. Int Arch Occup Environ Health 70:29–40.

Huff JE, Haseman JK, DeMarini DM, Eustis S, Maronpot RR, Peters AC, Persing RL, Chrisp CE, Jacobs AC. 1989. Multiple-site carcinogenicity of benzene in Fischer 344 rats and $B6C3F_1$ mice. Environ Health Perspect 82:125–163.

Hulka BS. 1991. Epidemiological studies using biological markers: Issues for epidemiologists. Cancer Epid Biomark Prevention 1:13–19.

Ikeda M, Otsuji H, Imamura T. 1972. In vivo suppression of benzene and styrene oxidation by co-administered toluene in rats and effects of phenobarbital. Xenobiotica 2:101–106.

Inoue O, Seiji K, Kasahara M, Nakatsuka H, Watanabe T, Yin SG, Li GL, Cai SX, Jin C, Ikeda M. 1988. Determination of catechol and quinol in the urine of workers exposed to benzene. Br J Ind Med 45:487–492.

Inoue O, Seiji K, Nakatsuka H, Watanabe T, Yin S, Li GL, Cai SX, Jin C, Ikeda M. 1989a. Excretion of 1,2,4-benzenetriol in the urine of workers exposed to benzene. Br J Ind Med 46:559–565.

Inoue O, Seiji K, Nakatsuka H, Watanabe T, Yin SN, Li GL, Cai SX, Jin C, Ikeda M. 1989b. Urinary *t,t*-muconic acid as an indicator of exposure to benzene. Br J Ind Med 46:122–127.

International Agency for Research on Cancer. 1982. Evaluation of the Carcinogenic Risk of Chemicals to Humans: Vol. 29. Benzene. International Agency for Research on Cancer, Lyons, France.

Ireland B, Collins JJ, Buckley CF, Riordan SG. 1997. Cancer mortality among workers with benzene exposure. Epidemiology 8:318–320.

Irons RD. 1985. Quinones as toxic metabolites of benzene. J Toxicol Environ Health 16:673–678.

Irons RD, Wierda D, Pfeifer RW. 1980. The immunotoxicity of benzene and its metabolites. In: Carcinogenicity

and Toxicity of Benzene (Mehlman MA, ed.). Princeton Scientific Publishers, Princeton, NJ.

Jirka AM, Bourne S. 1982. Gas chromatographic analysis for benzene in blood. Clin Chem 28:1492–1494.

Johansson I, Ingelman-Sundberg M. 1988. Benzene metabolism by ethanol-, acetone-, and benzene-inducible cytochrome P-450(IIE1) in rat and rabbit liver microsomes. Cancer Res 48:5387–5390.

Johnson ES, Lucier G. 1992. Perspectives on risk assessment impact of recent reports on benzene. Am J Ind Med 21:749–757.

Jongeneelen FJ, Dirven HA, Leijdekkers CM, Henderson PT, Brouns RM, Halm K. 1987. *S*-phenyl-*N*-acetylcysteine in urine of rats and workers after exposure to benzene. J Anal Toxicol 11:100–104.

Kalf GF. 1987. Recent advances in the metabolism and toxicity of benzene. CRC Crit Rev Toxicol 18:141–159.

Kalf GF, O'Connor A. 1993. The effects of benzene and hydroquinone on myeloid differentiation of HL-60 promyelocytic leukemia cells. Leuk Lymphoma 11(5–6):331–338.

Kalir A, Bessler H, Braun R, Gitter S, Djaldetti M. 1989. Benzene-derived free radical: A possible mediator of its carcinogenicity. I. Ultrastructural and functional studies on the effect of benzene on human white blood cells and its possible prevention. Isr J Med Sci 25:421–428.

Kline SA, Xiang Q, Goldstein BD, Witz G. 1993. Reaction of (E,E)-muconaldehyde and its aldehydic metabolites (E,E)-6-oxohexadienoic acid and (E,E)-6-hydroxyhexa-2,4-dienal, with glutathione. Chem Res Toxicol 6:578–583.

Kok PW, Ong CN. 1994. Blood and urinary benzene determined by headspace gas chromatography with photoionization detection: Application in biological monitoring of low level nonoccupational exposure. Int Arch Occup Environ Health 66:195–201.

Kolachana P, Subrahmanyam VV, Meyer KB, Zhang L, Smith MT. 1993. Benzene and its phenolic metabolites produce oxidative DNA damage in HL60 cells in vitro and in the bone marrow in vivo. Cancer Res 53:1023–1026.

Koop DR, Laethem CL, Schnier GG. 1989. Identification of ethanol-inducible P-450 isozyme 3a (P450IIE1) as a benzene and phenol hydroxylase. Toxicol Appl Pharmacol 98:278–288.

Lagorio S, Tagesson C, Forastiere F, Iavarone I, Axelson O, Carere A. 1994. Exposure to benzene and urinary concentrations of 8-hydroxydeoxyguanosine, a biological marker of oxidative damage to DNA. Occup Environ Med 51:739–743.

Latriano L, Goldstein BD, Witz G. 1986. Formation of muconaldehyde, an open-ring metabolite of benzene in mouse liver microsomes: An additional pathway for toxic metabolites. Proc Natl Acad Sci USA 83:8356–8360.

Lauwerys RR, Buchet JP, Andrien F. 1994. Muconic acid in urine: A reliable indicator of occupational exposure to benzene. Am J Ind Med 25:297–300.

Lee BL, New AL, Kok PW, Ong HY, Shi CY, Ong CN. 1993a. Urinary *trans,trans*-muconic acid determined by liquid chromatography: Application in biological monitoring of benzene exposure. Clin Chem 39:1788–1792.

Lee BL, Ong HY, Shi CY, Ong CN. 1993b. Simultaneous determination of hydroquinone, catechol and phenol in urine using high-performance liquid chromatography with fluorimetric detection. J Chromatogr 619:259–266.

Lévay G, Bodell WJ. 1992. Potentiation of DNA adduct formation in HL-60 cells by combinations of benzene metabolites. Proc Natl Acad Sci USA 89:7105–7109.

Lévay G, Bodell WJ. 1996. Role of hydrogen peroxide in the formation of DNA adducts in HL-60 cells treated with benzene metabolites. Biochem Biophys Res Commun 222(1):44–49.

Lindstrom AB, Yeowell-O'Connell K, Waidyanatha S, Golding BT, Tornero-Velez R, Rappaport SM. 1997. Measurement of benzene oxide in the blood of rats following administration of benzene. Carcinogenesis 18:1637–1641.

Liu L, Zhang Q, Feng J, Deng L, Zeng N, Yang A, Zhang W. 1996. The study of DNA oxidative damage in benzene-exposed workers. Mutat Res 370:145–150.

Loeff I, Stein G. 1963. The radiation and photochemistry of aqueous solutions of benzene. J Chem Soc 1963:2623–2633.

Luh BS, Hsu ET, Stachowicz K. 1967. Polyphenolic compounds in canned cling peaches. J Food Sci 32:251–258.

Luk E. 1990. Food application of sorbic acid and its salts. Food Addit Contam 7:711–715.

Maltoni C, Ciliberti A, Cotti G, Conti B, Belpoggi F. 1989. Benzene, an experimental multipotential carcinogen: Results of the long-term bioassays performed at the Bologna Institute of Oncology. Environ Health Perspect 82:109–124.

Marrazzini A, Chelotti L, Barrai I, Loprieno N, Barale R. 1994. In vivo genotoxic interactions among three phenolic benzene metabolites. Mutat Res 341:29–46.

McDonald TA, Waidyanatha S, Rappaport SM. 1993. Measurement of adducts of benzoquinone with hemoglobin and albumin. Carcinogenesis 14:1927–1932.

McDonald TA, Yeowell-O'Connell K, Rappaport SM. 1994. Comparison of protein adducts of benzene oxide and benzoquinone in the blood and bone marrow of rats and mice exposed to [$^{14}C/^{13}C_6$]benzene. Cancer Res 54:4907–4914.

Medinsky MA, Sabourin PJ, Lucier GW, Birnbaum LS, Henderson RF. 1989. A physiological model for simulation of benzene metabolism by rats and mice. Toxicol Appl Pharmacol 99:193–206.

Melikian AA, Prahalad AK, Coleman S. 1992. Isolation and characterization of the two benzene-derived hemoglobin adducts in vivo in rats. Cancer Epidemiol Biomarkers Prev 1:307–313.

Melikian AA, Prahalad AK, Hoffmann D. 1993. Urinary *trans,trans*-muconic acid as an indicator of exposure to benzene in cigarette smokers. Cancer Epidemiol Biomarkers Prev 2:47–51.

Melikian AA, Prahalad AK, Secker-Walker RH. 1994. Comparison of levels of the urinary benzene metabolite *trans,trans*-muconic acid in smokers and nonsmokers, and the effects of pregnancy. Cancer Epidemiol Biomarkers Prev 3:239–244.

Money CD, Gray CN. 1989. Exhaled breath analysis as measure of workplace exposure to benzene ppm. Ann Occup Hyg 33:257–262.

Mullin AH, Rando R, Esmundo F, Mullin DA. 1995. Inhalation of benzene leads to an increase in the mutant frequencies of a *lacI* transgene in lung and spleen tissues of mice. Mutat Res 327(1–2):121–129.

Nakajima T, Elovaara E, Park SS, Gelboin HV, Hietanen E, Vainio H. 1990. Monoclonal antibody-directed characterization of benzene, ethoxy-resorufin and pentoxyresorufin metabolism in rat liver microsomes. Biochem Pharmacol 40:1255–1261.

National Toxicology Program. 1989a. Fifth Annual Report on Carcinogens. NTP 89-239. U.S. Department of Health and Human Services, Public Health Service, National Toxicology Program, Research Triangle Park, NC.

National Toxicology Program. 1989b. Toxicology and Carcinogenesis Studies of Hydroquinone (CAS No. 123-31-9) in F344/N rats and B6C3F$_1$ mice (gavage studies). NIH Publication 90-2821, NTP-TR-366. National Toxicology Program, Research Triangle Park, NC.

Niculescu R, Bradford HN, Colman RW, Kalf GF. 1995. Inhibition of the conversion of pre-interleukins-1α and 1ß to mature cytokines by *p*-benzoquinone, a metabolite of benzene. Chem Biol Interact 98:211–222.

Ong CN, Kok PW, Lee BL, Shi CY, Ong HY, Chia KS, Lee CS, Luo XW. 1995. Evaluation of biomarkers for occupational exposure to benzene. Occup Environ Med 52:528–533.

Ong CN, Kok PW, Ong HY, Shi CY, Lee BL, Phoon WH, Tan KT. 1996. Biomarkers of exposure to low concentrations of benzene: A field assessment. Occup Environ Med 53:328–333.

Ong CN, Lee BL. 1994. Determination of benzene and its metabolites: Application in biological monitoring of environmental and occupational exposure to benzene. J Chromatogr B 660:1–22.

Ong CN, Lee BL, Shi CY, Ong HY, Lee HP. 1994. Elevated levels of benzene-related compounds in the urine of cigarette smokers. Int J Cancer 59:177–180.

Osborne JC, Metzler M, Neumann HG. 1980. Peroxidase activity in the rat Zymbal gland and its possible role in the metabolic activation of aminostilbenes in the target tissue. Cancer Lett 8(3):221–226.

Ott MG, Townsend JC, Fishbeck WA, Langner RA. 1978. Mortality among individuals occupationally exposed to benzene. Arch Environ Health 33:3–10.

Ozkaynak H, Ryan PB, Wallace LA, Nelson WC, Behar JV. 1987. Sources and emission rates of organic chemical vapors in homes and buildings. In: Proceedings of the 4th International Conference on Indoor Air Quality and Climate, August 17–21, 1987. Institute for Water, Soil, and Air Hygiene, West Berlin, Germany.

Parke DV, Williams RT. 1953. Studies in detoxification: 44. The metabolism of benzene containing ^{14}C-benzene. Biochem J 54:231–238.

Pekari K, Vainiotalo S, Heikkila P, Palotie A, Luotamo M, Riihimaki V. 1992. Biological monitoring of occupational exposure to low levels of benzene. Scand J Work Environ Health 18:317–322.

Perbellini L, Faccini GB, Pasini F, Cazzoli F, Pistoia S, Rossellini A, Valsecchi M, Brugnone F. 1988. Environmental and occupational exposure to benzene by analysis of breath and blood. Br J Ind Med 45:345–352.

Pezzagno G, Maestri L. 1997. The specifics of *trans,trans*-muconic acid as a biological indicator for low levels of environmental benzene. Indoor Environ 6:12–18.

Philip P, Jensen MK. 1970. Benzene induced chromosome abnormalities in rat bone marow cells. Acta Pathol Microbiol Scand A78:489–490.

Pifer JW, Hearn FT, Swanson FA, O'Donoghue JL. 1995. Mortality study of employees engaged in the manufacture and use of hydroquinone. Int Arch Occup Environ Health 67:267–280.

Popp W, Rauscher D, Müller G, Angerer J, Norpoth K. 1994. Concentrations of benzene in blood and *S*-phenylmercapturic and *t,t*-muconic acid in urine in car mechanics. Int Arch Occup Environ Health 66:1–6.

Rappaport SM, McDonald TA, Yeowell-O'Connell K. 1996. The use of protein adducts to investigate the disposition of reactive metabolites of benzene. Environ Health Perspect (Suppl 6) 104:1235–1237.

Rinsky RA, Young RJ, Smith AB. 1981. Leukemia in benzene workers. Am J Ind Med 2:217–245.

Risner CH, Cash SL. 1990. A high-performance liquid chromatographic determination of major phenolic compounds in tobacco smoke. J Chromatogr Sci 28:239–244.

Ross D. Metabolic basis of benzene toxicity. 1996. Eur J Haematol Suppl 60:111–118.

Rothman N, Li GL, Dosemeci M, Bechtold WE, Marti GE, Wang YZ, Linet M, Xi LQ, Lu W, Smith MT, Titenko-Holland N, Zhang LP, Blot W, Yin SN, Hayes RB. 1996. Hematotoxicity among Chinese workers heavily exposed to benzene. Am J Ind Med 29:236–246.

Rumpf P, Darstellung RP. 1981. Charakteriserung und Trennung der Merkaptursäuren von Benzol, Toluol, Aethylbenzol und Xylolen durch GC-MS Analyse (Ph.D. Thesis). University of Hamburg, Hamburg, Germany.

Ruppert T, Scherer G, Tricker AR, Adlkofer F. 1997. *trans,trans*-Muconic acid as biomarker of non-occupational environmental exposure to benzene. 1997. Int Arch Occup Environ Health 69:247–251.

Ruppert T, Scherer G, Tricker AR, Rauscher D, Adlkofer F. 1995. Determination of urinary *trans,trans*-muconic acid by gas chromatography-mass spectrometry. J Chromatogr B 666:71–76.

Sabourin PJ, Bechtold WE, Griffith WC, Birnbaum LS, Lucier G, Henderson RF. 1989. Effect of exposure concentration, exposure rate, and route of administration on metabolism of benzene by F344 rats and B6C3F$_1$ mice. Toxicol Appl Pharmacol 99:421–444.

Sabourin PJ, Muggenburg BA, Couch RC, Lefler D, Lucier G, Birnbaum LS, Henderson RF. 1992. Metabolism of [^{14}C]benzene by cynomolgus monkeys and chimpanzees. Toxicol Appl Pharmacol 114:277–284.

Sammett D, Lee EW, Kocsis JJ, Snyder R. 1979. Partial hepatectomy reduces both metabolism and toxicity of benzene. J Toxicol Environ Health 5:785–792.

SAS. 1990. SAS/STAT User's Guide, Version 6. SAS Institute, Cary, NC.

Sawahata T, Rickert DE, Greenlee WF. 1985. Metabolism of benzene and its metabolites in bone marrow. In: Toxicology of the Blood and Bone Marrow (Irons RD, ed.). Raven Press, New York, NY.

Schad H, Schäfer F, Weber L, Seidel HJ. 1992. Determination of benzene metabolites in urine of mice by solid-phase extraction and high-performance liquid chromatography. J Chromatogr 593:147–151.

Schäfer F, Schad H, Weber L. 1993. Determination of phenylmercapturic acid in urine of benzene-exposed BDF-1 mice. J Chromatogr 620:239–242.

Schrenck D, Orzechowski A, Schwarz LR, Snyder R, Burchell B, Ingelman-Sundberg M, Bock KW. 1996. Phase II metabolism of benzene. Environ Health Perspect (Suppl 6) 104:1183–1188.

Shen Y, Shen HM, Shi CY, Ong CN. 1996. Benzene metabolites enhance reactive oxygen species generation in HL60 human leukemia cells. Hum Exp Toxicol 15:422–427.

Sherwood RJ, 1972. Benzene: The interpretation of monitoring results. Ann Occup Hyg 15:409–421.

Siou G, Conan L, el Haitem M. 1981. Evaluation of the clastogenic action of benzene by oral administration with 2 cytogenetic techniques in mouse and Chinese hamster. Mutat Res 90:273–278.

Smith MT. 1996a. Mechanistic studies of benzene toxicity: Implications for risk assessment. Adv Exp Med Biol 387:259–266.

Smith MT. 1996b. The mechanism of benzene-induced leukemia: A hypothesis and speculations on the causes of leukemia. Environ Health Perspect (Suppl 6) 104:1219–1225.

Smith MT, Yager JW, Steinmetz KL, Eastmond DA. 1989. Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. Environ Health Perspect 82:23–29.

Snyder CA, Goldstein BD, Sellakumar AR, Albert RE. 1984. Evidence for hematotoxicity and tumorigenesis in rats exposed to 100 ppm benzene. Am J Ind Med 5:429–434.

Snyder CA, Goldstein BD, Sellakumar AR, Bromberg I, Laskin S, Albert RE. 1980. The inhalation toxicology of benzene: Incidence of hematopoietic neoplasms and hematotoxicity in AKR/J and C57BL/6J mice. Toxicol Appl Pharmacol 54:323–331.

Snyder CA, Goldstein BD, Sellakumar AR, Wolman I, Eelichman MN, Laskin S. 1978. Hematotoxicity of inhaled benzene to Sprague-Dawley rats and AKR mice at 300 ppm. J Toxicol Environ Health 4:605–618.

Snyder CA, Sellakumar AR, James DJ, Albert RE. 1988. The carcinogenicity of discontinuous inhaled benzene exposures in CD-1 and C57B1/6 mice. Arch Toxicol 62:331–335.

Snyder R, Hedli CC. 1996. An overview of benzene metabolism. Environ Health Perspect (Suppl) 104:1165–1175.

Snyder R, Jowa L, Witz G, Jakf G, Rushmore T. 1987. Formation of reactive metabolites from benzene. Arch Toxicol 60:61–64.

Snyder R, Kalf GF. 1994. A perspective on benzene leukemogenesis. Crit Rev Toxicol 24(3):177–209.

Snyder R, Kocsis JJ. 1975. Current concepts of chronic benzene toxicity. CRC Crit Rev Toxicol 3:265–288.

Stommel P, Müller G, Stücker W, Verkoyen C, Schöbel S, Norpoth K. 1989. Determination of *S*-phenylmercapturic acid in the urine: An improvement in the biological monitoring of benzene exposure. Carcinogenesis 10:279–282.

Strum JM, Shear CR. 1982. Harderian glands in mice: Fluorescence, peroxidase activity and fine structure. Tissue Cell 14(1):135–148.

Subrahmanyam VV, McGirr LG, O'Brien PJ. 1990. Peroxidase/hydrogen peroxide– or bone marrow homogenate/hydrogen peroxidase–mediated activation of phenol and binding to protein. Xenobiotica 20:1369–1378.

Subrahmanyam VV, Ross D, Eastmond DA, Smith MT. 1991. Potential role of free radicals in benzene-induced myelotoxicity and leukemia. Free Radic Biol Med 11:495–515.

Tice RR, Costa DL, Drew RT. 1980. Cytogenetic effects of inhaled benzene in murine bone marrow: Induction of sister chromatid exchanges, chromosomal aberrations and cellular proliferation inhibition in DBA/2 mice. Proc Natl Acad Sci (USA) 77:2148–2152.

Tice RR, Luke CA, Drew RT. 1989. Effect of exposure route, regimen, and duration on benzene-induced genotoxic and cytotoxic bone marrow damage in mice. Environ Health Perspect 82:65–74.

Tsai SP, Wen CP, Weiss NS, Wong O, McClelland WA, Gibson RL. 1983. Retrospective mortality and medical surveillance studies of workers in benzene areas of refineries. J Occup Med 25:685–692.

Tunek A, Oesch F. 1982. Multi-step metabolic activation of benzene in rat-liver microsomes. Adv Exp Med Biol 136A:319–329.

Tunek A, Platt KL, Bentley P, Oesch F. 1978. Microsomal metabolism of benzene to species irreversibly binding to microsomal protein and effects of modifications of this metabolism. Mol Pharmacol 14:920–929.

Tunek A, Platt KL, Przybylski M, Oesch F. 1980. Multistep metabolic activation of benzene: Effect of superoxide dismutase on covalent binding to microsomal macromolecules and identification of glutathione conjugates using HPLC and FDMS. Chem Biol Interact 33:1–17.

Valentine JL, Lee SS, Seaton MJ, Asgharian B, Farris G, Corton JC, Gonzalez FJ, Medinsky MA. 1996. Reduction of benzene metabolism and toxicity in mice that lack CYP2E1 expression. Toxicol Appl Pharmacol 141:205–213.

van Sittert NJ, Boogaard PJ, Beulink GDJ. 1993. Application of the urinary *S*-phenylmercapturic acid test as a biomarker for low levels of exposure to benzene in industry. Br J Ind Med 50:460–469.

Vigliani EC, Forni A. 1976. Benzene and leukemia. Environ Res 11:122–127.

Vine MF, Hulka BS, Morgolin YK, Truong YK, Hu PC, Schramm MM, Griffith JD, McCann M, Everson RB. 1993. Cotinine concentrations in semen, urine, and blood of smokers and nonsmokers. Am J Public Health 83:1335–1338.

Wallace LA. 1989a. Major sources of benzene exposure. Environ Health Perspect 82:165–169.

Wallace LA. 1989b. The exposure of the general population to benzene. Cell Biol Toxicol 5:197.

Wallace LA, Pellizzari E, Hartwell T, Rosenzweig M, Erikson M, Sparacino C, Zelon H. 1984. Personal exposure to volatile organic compounds. Environ Res 35:293–319.

Weaver VM, Davoli CT, Heller PJ, Fitzwilliam A, Peters HL, Sunyer J, Murphy SE, Goldstein GW, Groopman JD. 1996. Benzene exposure, assessed by urinary *trans,trans*-muconic acid, in urban children with elevated blood lead levels. Environ Health Perspect 104:318–323.

Weaver NK, Gibson RL, Smith CW. 1983. Occupational exposure to benzene in the petroleum and petrochemical industries. Adv Mod Environ Toxicol 4:63–75.

Weisel C, Yu R, Roy A, Georgopoulos P. 1996. Biomarkers of environmental benzene exposure. Environ Health Perspect 104:1141–1146.

Westöö G. 1964. On the metabolism of sorbic acid in the mouse. Acta Chem Scand 18:1373–1378.

Witz G, Gad SC, Tice RR, Oshiro Y, Piper CE, Goldstein BD. 1990. Genetic toxicity of the benzene metabolite *trans,trans*-muconaldehyde in mammalian and bacterial cells. Mutat Res 240:295–306.

Witz G, Maniara WM, Mylavarapu VJ, Goldstein BD. 1990. Comparative metabolism of benzene and *trans,trans*-muconaldehyde to *trans,trans*-muconic acid in DBA/2N and C57BL/6 mice. Biochem Pharmacol 40:1275–1280.

Witz G, Zhang Z, Goldstein BD. 1996. Reactive ring-opened aldehyde metabolites in benzene hematotoxicity. Environ Health Perspect 104:1195–1199.

Wong O. 1987. An industry wide mortality study of chemical workers occupationally exposed to benzene: II. Dose response analyses. Br J Ind Med 44:382–395.

Yardley-Jones A, Anderson D, Parke DV. 1991. The toxicity of benzene and its metabolism and molecular pathology in human risk assessment. Br J Ind Med 48:437–444.

Yeowell-O'Connell K, McDonald TA, Rappaport SM. 1996. Analysis of hemoglobin adducts of benzene oxide by gas chromatography–mass spectrometry. Anal Biochem 237(1):49–55.

Yin S-N, Hayes RB, Linet MS, Li GL, Dosemeci M, Travis LB, Li CY, Zhang ZN, Li DG, Chow WH, Wacholder S, Wang YZ, Jiang ZL, Dai TR, Zhang WY, Chao XJ, Ye PZ, Kou QR, Zhang XC, Lin XF, Meng JF, Ding CY, Zho JS, Blot WJ. 1996. A cohort study of cancer among benzene-exposed workers in China: Overall results. Am J Ind Med 29:227–235.

Yin S-N, Li G-L, Tain F-D, Fu Z-I, Jin C, Chen Y-J, Luo S-J, Ye P-Z, Zhang J-Z, Wang G-C, Zhang X-C, Wu H-N, Zhong Q-C. 1987. Leukaemia in benzene workers: A retrospective cohort study. Br J Ind Med 44:124–128.

Yu R, Weisel CP. 1996. Measurement of the urinary benzene metabolite *trans,trans*-muconic acid from benzene exposure in humans. J Toxicol Environ Health 48:453–477.

Zhang Z, Xiang Q, Glatt H, Platt KL, Goldstein BD, Witz G. 1995. Studies on pathways of ring opening of benzene in Fenton system. Free Radic Biol 18:411–419.

ABOUT THE AUTHORS

Assieh A. Melikian is head of the Carcinogenic Biomarkers Facility at the American Health Foundation. She received her M.S. in chemical engineering from Teheran University, Iran, and her doctorate in bioorganic chemistry from New York University. Her research interests are mechanisms of chemical carcinogenesis and development of carcinogen biomarkers in human studies.

Min Meng received her B.S. and M.S. in organic chemistry from Beijing Normal University, China, and her doctorate in bioanalytical chemistry from the University of Maryland.

Ray O'Connor received his B.S. in chemistry and M.S. in analytical chemistry from University College, Cork, Ireland.

Peifeng Hu has a B.S. in pharmacy from Nanjing College of Pharmacy and an M.S. from the Institute of Medicine, China. He received his doctorate in chemistry from the University of Nebraska and had postdoctoral training at Parke-Davis Pharmaceutical Company.

Seth M. Thompson is head of the Biostatistics and Computer Facility at the American Health Foundation. He received his undergraduate degree in mathematics and chemistry from Bowdoin College in 1986 and his doctorate in biostatistics from Columbia University in 1995. His research interests include item response theory and measurement error in logistic regression models.

PUBLICATIONS RESULTING FROM THIS RESEARCH

Melikian AA, O'Connor R, Prahalad AK, Hu P, Li H, Kagan M, Thompson S. 1999. Determination of the urinary benzene metabolites *S*-phenylmercapturic acid and *trans,trans*-muconic acid by liquid chromatography–tandem mass spectrometry. Carcinogenesis 20(4):719–726.

ABBREVIATIONS

ACGIH	American Conference of Governmental Industrial Hygienists
BQ	*p*-benzoquinone
BT	benzene triol (systematic name: 1,2,4-trihydroxybenzene)
CAD	collision-activated decomposition
CAT	catechol (systematic name: 1,2-dihydroxybenzene)
CID	collision-induced dissociation
CV	coefficient of variation
CYP2E1	cytochrome P-450 2E1
EI-MS	electron impact–mass spectrometry
EtOAc	ethyl acetate
ESI	electrospray ionization
GC-MS	gas chromatography–mass spectrometry
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
HQ	*p*-hydroquinone [hydroquinone or 1,4-dihydroxybenzene]
IARC	International Agency for Research on Cancer
LC-ESI-MS/MS	liquid chromatography–electrospray ionization–tandem mass spectrometry
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LC-SIM-MS	liquid chromatography–selected ion monitoring–mass spectrometry
MeOH	aqueous methanol
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass/charge
NASA	National Aeronautics and Space Administration
NMR	nuclear magnetic resonance
$^{\bullet}$OH	hydroxyl radical
8-OHdG	8-hydroxydeoxyguanosine
Pd/C	palladium/carbon
PVP	polyvinylpyrrolidone
Q1, . . . , Q4	first quadrupole, . . . , fourth quadrupole
r^2	regression coefficient
SAX	strong anion exchanger
SIM	selected ion monitoring
S-PMA	*S*-phenylmercapturic acid (or *N*-acetyl-*S*-phenyl-L-cysteine)
SRM	selected reaction monitoring
TLV	threshold limit value
t,t-MA	*trans,trans*-muconic acid
TWA	time-weighted average

INTRODUCTION

Simple, sensitive, and specific analytical methods are required to evaluate which benzene metabolite or metabolites may be useful as biomarkers of exposure in humans. Previously, quantifying benzene metabolites has involved labor-intensive, multistep assays. The Health Effects Institute (HEI) funded Dr. Melikian and colleagues to develop and validate a new method for assaying metabolites of benzene in human urine using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS).* This approach, if successful, would have a number of advantages over existing methodologies, with the specificity of analyte analyses being most important. Dr. Melikian proposed to measure urinary levels of five different benzene metabolites, derived from different metabolic pathways, and to test the discrimination of her analytical method. To do this, she compared levels of metabolites of benzene in smokers and nonsmokers, since cigarette smoke contains benzene (Wallace 1989a,b).

Dr. Melikian's study was funded under RFA 93-1, *Novel Approaches to Extrapolation of Health Effects for Mobile Source Toxic Air Pollutants*. Her draft Investigators' Report underwent external peer review under the direction of the HEI Health Review Committee, which discussed the Report and the reviewers' critiques and prepared this Commentary. The Commentary is intended to aid HEI sponsors and the public by highlighting the strengths of the study, pointing out alternative interpretations, and placing the report into scientific perspective. During the review of Melikian's study, the HEI Review Committee and the investigators exchanged comments and clarified issues in the Investigators' Report and in the Commentary.

SCIENTIFIC BACKGROUND

BENZENE METABOLISM

Benzene is metabolized primarily in the liver via a number of alternative pathways (reviewed in Snyder and Hedli 1996). Figure 1 depicts the major pathways and metabolites formed. One of the key early intermediates in benzene metabolism is benzene oxide, a highly reactive epoxide, which can be converted to several ring-hydroxylated compounds. These include phenol (formed by the addition of a hydroxyl group to the benzene ring), *p*-hydroquinone (HQ) and catechol (CAT) (both dihydroxylated compounds), and 1,2,4-trihydroxybenzene (benzene triol, or BT). Benzene oxide can also react to form *S*-phenylmercapturic acid (*S*-PMA), via reaction with glutathione and ring-opened compounds, including *trans,trans*-muconic acid (*t,t*-MA). An alternative pathway of benzene metabolism involves the generation of a free radical, leading to the formation of biphenyl. All these products of benzene metabolism are excreted in urine. The factors that control the balance among these distinct pathways are currently under investigation. Because benzene is intrinsically unreactive but is metabolized rapidly, it is likely that a metabolite or combination of metabolites is responsible for benzene's toxic effects. Identification of these metabolites and the determination of how they may exert their toxicological effects has been an area of intense research (see the accompanying Introduction to this Research Report).

BIOMARKERS OF HUMAN BENZENE EXPOSURE

Because all the compounds described above can be produced as the result of benzene metabolism, they may serve as potential biomarkers of recent benzene exposure. Determining which metabolite may be an informative marker of human exposure to benzene, however, particularly at low, nonoccupational exposure levels, is difficult. One of the key problems is identifying a benzene-specific metabolite in biological samples: some products of benzene metabolism can also be derived from the metabolism of other compounds, such as amino acids, and thus may not be solely indicative of exposure to benzene. A second major concern is that information concerning benzene metabolism in humans is limited, particularly in response to low-dose exposures. It is difficult to extrapolate results of animal studies to humans because different species exposed to the same level of benzene produce different levels of benzene metabolites (Henderson et al. 1989; Henderson 1996), and the pattern of metabolites is dif-

* A list of abbreviations appears at the end of the Investigators' Report.

Dr. Assieh A. Melikian's two-year study, *Development of Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry Methods for Determination of Urinary Metabolites of Benzene in Humans*, began in September 1994 and had total expenditures of $256,618. The Investigators' Report from Dr. Melikian and colleagues was received for review in October 1997. A revised report, received in March 1998, was accepted for publication in April 1998.

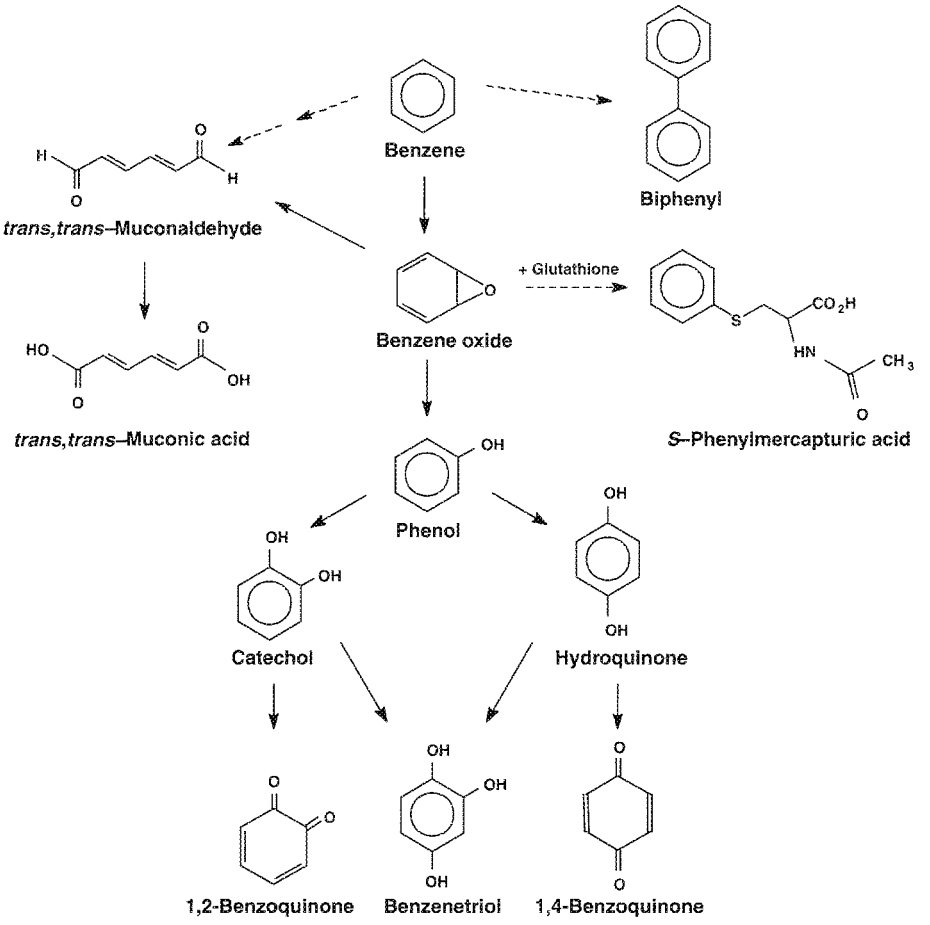

Figure 1. Pathways of benzene metabolism.

ferent at different levels of benzene exposure (Sabourin et al. 1987, 1988, 1989; Stommel et al. 1989; Witz et al. 1990). For example, in rats and mice a smaller fraction of benzene is converted to HQ and ring-opened metabolites at higher than at lower benzene exposures (Sabourin et al. 1987, 1988, 1989). It is possible that similar changes in metabolic pathways occur in humans in response to different levels of exposure. A further important issue is the difficulty in detecting and measuring reproducibly the low levels of many benzene metabolites.

A number of metabolites have been suggested as potentially useful biomarkers of human exposure to benzene (see Figure 1). Phenol is a major benzene metabolite and has been widely studied as a potential biomarker. It can also be formed endogenously by the metabolism of some amino acids, however, and appears not to be specific for low-level benzene exposures (Ong et al. 1995; Hotz et al. 1997). A number of the ring-hydroxylated derivatives have also been considered, including HQ, CAT, and BT. These compounds are also not specific for benzene exposure, however, and, in addition, are difficult to assay with the necessary sensitivity. Researchers have recently focused attention on *S*-PMA and the ring-opened metabolite *t,t*-MA as biomarkers of benzene exposure. Both *S*-PMA and *t,t*-MA are minor metabolites of benzene and have been difficult to detect by standard assays. *S*-PMA is believed to be a specific metabolite of benzene, but *t,t*-MA is not: it can also be generated from sorbic acid, which is used as a preservative in a number of food products (Pezzagno and Maestri 1997; Ruppert et al. 1997). Finally, unmetabolized benzene in blood, urine, or exhaled breath has also been suggested as a potential marker of benzene exposure, but its usefulness has not been clearly demonstrated (Ghittori et al. 1993; Ong et al. 1995; Weisel et al. 1996; Hotz et al. 1997). Dr. Melikian and colleagues proposed to develop methods for the sensitive and reproducible measurement of urinary benzene and selected benzene metabolites. Their experimental approaches are described in the sections below.

TECHNICAL EVALUATION: AIMS AND ATTAINMENT OF STUDY OBJECTIVES

Dr. Melikian's proposed study had three specific aims:

1. To develop and validate sensitive and specific analytical high-performance liquid chromatographic–mass spectrometric (LC-MS) methods to measure the metabolites of benzene, *S*-PMA, *t,t*-MA, HQ, CAT, and BT, in urine for potential use as markers of exposure to benzene in humans.
2. To develop a sensitive gas chromatographic–mass spectrometric method for quantifying urinary biphenyl and unmetabolized benzene.
3. To validate in humans the LC-MS assays developed for urinary metabolites of benzene, and to investigate the effect of benzene exposure dose on benzene metabolite levels in humans.

Following discussions with members of HEI's Research Committee and staff, Dr. Melikian did not pursue Aim 2 because of technical issues that arose in developing methods for assaying unmetabolized benzene and biphenyl in urine. In addition, Aim 3, the validation of the biomarker assays in humans, was dropped before Melikian's study started. HEI and Dr. Melikian agreed to validate all the proposed benzene biomarkers in humans in a single HEI-funded study, conducted by Dr. Qingshan Qu in China (see the HEI Research Program section of the Overview). Thus, Melikian and colleagues' report focused on Aim 1, the development of assays for benzene metabolites in human urine.

METHODS AND STUDY DESIGN

LIQUID CHROMATOGRAPHY–ELECTROSPRAY IONIZATION–TANDEM MASS SPECTROMETRY

The methods described in this report are based on LC-ESI-MS/MS with sample introduction by liquid chromatography. Using this technique, samples are initially separated by liquid chromatography, and pumped into the first of two linked mass spectrometers (tandem mass spectrometry). In the first spectrometer, the materials to be analyzed (analytes) are sprayed with a stream of electrons. Ions produced in the source region are separated on the basis of mass-to-charge ratio, and then fragmented by bombardment with neutral gas atoms. The fragmented (daughter) ions are mass analyzed in the second spectrometer. The specificity of MS/MS detection derives from the combination of the selection of specific precursor ions and detection of the product ions.

The compounds Melikian studied are all polar substances that are well suited for analysis by liquid chromatography. In addition, electrospray is currently considered the most versatile method for ionizing liquid samples for mass spectrometry. The use of tandem instruments generally enhances the selectivity and sensitivity needed for analyzing compounds present at low concentrations in complex biological fluids such as urine. Moreover, for most of the metabolites analyzed, Melikian's quantitation was based on comparisons with isotopically labeled analogs as internal standards. This technique is generally considered to be the best for analyses of this nature. Thus, Melikian's analytical approach was appropriate for this investigation.

Melikian and colleagues developed two independent methods to measure levels of five potentially important benzene metabolites in urine. They simultaneously assayed the metabolites *S*-PMA and *t,t*-MA, based on an LC-ESI-MS/MS method using selected reaction monitoring. To monitor recovery, they spiked urine samples with [$^{13}C_6$]-*S*-PMA and [$^{13}C_6$]-*t,t*-MA as internal standards. Recoveries were 43% and 65%, respectively, for these two analytes. The investigators used similar techniques to measure simultaneously the ring-hydroxylated metabolites HQ, CAT, and BT: prior to analysis by LC-ESI-MS/MS, samples were hydrolyzed in acid, extracted with ether, and reduced with sodium bisulfite. To monitor recovery of the ring-hydroxylated metabolites, they spiked urine samples with [$^{13}C_6$]-CAT and [$^{13}C_6$]-HQ, but did not use isotopically labeled BT as an internal standard because it is easily oxidized, making determinations of the recovery of BT more difficult. Recoveries of these analytes were low. This fact, coupled with the inability to use an internal standard for BT, suggests that this approach may not be optimal for quantifying levels of these metabolites in urine.

ASSAY VALIDATION

As an initial attempt to test the analytical robustness of the assay in benzene-exposed individuals, the investigators evaluated human urine specimens from two sets of cigarette smoking and nonsmoking individuals. For the analysis of *S*-PMA and *t,t*-MA, Melikian and colleagues treated the urine derived from one of the sets with a preservative containing benzoate and polyvinylpyrrolidone before freezing. For analysis of BT, CAT, and HQ they used the urine from the second set of samples without preservative. It is not clear whether the use of preservative in some but not all samples affected the results of the study. Com-

Table 1. Summary of Melikian's Findings for Benzene Metabolites in Human Urine by LC-ESI-MS/MS Analysis

Metabolite	Assay Reproducibility (% Coefficient of Variation)	Limits of Detection (ng)	Assay Linearity range (ng)	Level in Nonsmokers[a]	Level in Smokers[a]
S-PMA	7	0.03	0.033–33	4.8 ± 1.1	9.1 ± 1.7
t,t-MA	10	1	1.67–3,300	0.07 ± 0.02	0.15 ± 0.03
BT	20	50	200–400	1.7 ± 0.3	1.8 ± 0.5
HQ	12	4	18–400	1.1 ± 0.3	2.0 ± 0.6
CAT	4.6	0.4	5–400	5.5 ± 1.4	5.0 ± 1.2

[a] For *S*-PMA, concentrations expressed are in μg/g creatinine; for all other metabolites, concentrations are in mg/g creatinine.

paring levels of metabolites in identically processed samples from a single large group of smokers with those from nonsmokers would be more appropriate in future studies, however. As described previously, validation of this assay is now under way in the HEI-funded study of benzene-exposed workers in China, performed by Dr. Qu, and due to be completed in 2000.

RESULTS

Melikian and colleagues were successful in developing new analytical methods for measuring selected benzene metabolites in human urine as possible biomarkers of benzene exposure. The investigators' key results are summarized in Table 1.

REPRODUCIBILITY, SENSITIVITY, AND LINEARITY

Based on the analysis of samples of urine from smokers tested on eight separate occasions, assays of the five metabolites were reproducible. The assay for *S*-PMA was the most sensitive (able to detect levels down to 0.03 ng), and the assay for BT the least sensitive (50 ng). Limits of detection for HQ, CAT, and *t,t*-MA were in the nanogram range. Assays for *S*-PMA and *t,t*-MA were linear over a four-log range; assays for BT, HQ, and CAT were linear over a two-log or lesser range.

METABOLITE LEVELS IN THE URINE OF SMOKERS AND NONSMOKERS

The investigators found detectable levels of the five selected benzene metabolites in almost all of the analyzed urines of both smokers and nonsmokers. These levels are comparable to those found in urine by other methods (Boogaard and van Sittert 1995; Ghittori et al. 1995; Hotz et al. 1997; Inoue et al. 1988; Lee et al. 1993; Ong et al. 1995; Popp et al. 1994). As shown in Table 1 of the Commentary and in more detail in Table 2 of the Investigators' Report, analysis of specimens from the first cohort, 28 smokers and 18 nonsmokers, indicated that mean levels of *S*-PMA and *t,t*-MA were significantly higher in urine from smokers compared to nonsmokers: 2.1-fold ($p = 0.03$) and 1.9-fold ($p = 0.01$), respectively. Further analysis, such as separating values from male and female smokers, might have shown larger differences between smokers and nonsmokers.

In contrast, the investigators found that mean levels of the ring-hydroxylated metabolites were not significantly different when evaluated in a second cohort consisting of 15 smokers and 15 nonsmokers. The mean levels of BT, HQ, and CAT were 1.1-fold ($p = 0.82$), 1.8-fold ($p = 0.19$), and 0.9-fold ($p = 0.78$) higher, respectively, in smokers than in nonsmokers.

There were noteworthy minor issues with the data presented. The investigators found a significant correlation between levels of *S*-PMA and *t,t*-MA after logarithmic transformation ($r = 0.41$, $p = 0.005$), and also found correlations between levels of CAT and BT, and of CAT and HQ. Melikian and colleagues measured *S*-PMA and *t,t*-MA from one study cohort and the ring-hydroxylated metabolites from the other cohort, however. For this reason, it was not possible to evaluate relationships among all five metabolites; data for all five metabolites in each individual would have been helpful in assessing these potential interrelationships. In addition, the investigators reported that some urine samples contained undetectable levels of metabolites; given the reported assay sensitivity, however, the reasons for such "non-detects" are not apparent. They may be due to the presence in urine of substances that interfere with the assay; for example, Melikian and colleagues have detected an as-yet-uncharacterized agent in human urine with chromatographic properties similar to HQ that suppresses its ionization and yields product ions with a mass-to-charge ratio similar to HQ.

DISCUSSION

Melikian and colleagues have clearly demonstrated that their general methodological approach is sound. The LC-ESI-MS/MS method has important advantages over existing techniques: it avoids the tedious cleanup procedures and derivatization steps required in standard gas chromatographic–mass spectrometric (GC-MS) methods, and provides specificity lacking in HPLC. LC-ESI-MS/MS has other advantages over GC-MS: volatility of an analyte is not required, and several analytes that are structurally dissimilar can be measured in a single run. In addition, the use of MS/MS and the selected reaction monitoring mode further enhance the selectivity and specificity of analyte detection.

POTENTIAL BIOMARKERS OF LOW-LEVEL EXPOSURE TO BENZENE

S-PMA and *t,t*-MA

The use of LC with ESI-MS/MS with human biofluids is novel for *S*-PMA and *t,t*-MA and would appear to yield substantive improvements in existing analytical specificity and sensitivity. The use of isotopically labeled analogs as internal standards gives the method quantitative rigor. The specificity associated with *S*-PMA as a unique product derived from benzene metabolism, coupled with the specificity associated with its quantitative measurement, makes it a prime candidate for use in benzene exposure studies. The pilot studies using urine of smokers and nonsmokers demonstrates that the assay has sufficient analytical sensitivity to be of practical value in human biomonitoring. *S*-PMA could be detected in the urine of all but one of the 46 participants. Although its general feasibility has been established, more work is needed to establish the robustness of the method. An excellent coefficient of variation of 7% was observed with repeated, independent measures of a sample with a concentration of 9.4 µg *S*-PMA/mg creatinine. In only a few of the 46 cohort specimens, however, did the *S*-PMA levels exceed this concentration; questions thus remain about the reproducibility of the *S*-PMA assay for monitoring benzene at ambient exposures (in subjects such as children in urban environments). Assessment of higher occupational exposures to benzene should not be problematic. This issue can be readily addressed with continued development and application of the assay. Much work remains to be done in the validation of the assay, including assessments in populations with known benzene exposures. It is also difficult to know whether there is *predictive* value in measuring *S*-PMA levels, because it is not clear how accurately levels of urinary *S*-PMA reflect levels of the active metabolites of benzene that are responsible for its toxic effects. Further research is needed to clarify this issue.

All of the analytical hallmarks that engender enthusiasm for the assay of *S*-PMA hold true for the assay of *t,t*-MA. *t,t*-MA lacks one critical feature embodied in *S*-PMA, however—it does not appear to be uniquely derived from benzene. An expanding literature demonstrates that dietary sources can lead to the excretion of *t,t*-MA; in particular, ingestion of the food additive sorbic acid can confound the interpretation of measurements of this benzene metabolite (Pezzagno and Maestri 1997; Ruppert et al. 1997). Thus, the use of this biomarker in assessing relationships between lifestyle and environmental exposures to benzene and risk of disease may be limited. It is worth noting, though, that in the Melikian pilot study a strong association was seen between levels of *t,t*-MA and *S*-PMA in the urine samples of both smokers and nonsmokers. Although this finding suggests that sorbic acid is unlikely to affect the levels of *t,t*-MA, more rigorous tests are required to exclude interference from sorbic acid.

Ring-Hydroxylated Metabolites of Benzene: BT, HQ, and CAT

Melikian and colleagues also established LC-ESI-MS/MS methods for evaluating the ring-hydroxylated metabolites of benzene. Again, there are many strengths to the analytical approach used for their measurement—particularly specificity. Measurement of these analytes, however, is unlikely to prove suitable for human biomonitoring for several reasons. First, the methods do not appear to be particularly sensitive, although these metabolites were detected in virtually all of the human urine samples. The limit of detection is about 10 to 1,000 times higher than that of *S*-PMA, depending upon the specific metabolite. Second, the analytic robustness of the assay was not established for all the metabolites measured. For example, for BT, the CV was 20%. Third, these metabolites are not unique to benzene, but can be derived from a variety of dietary and occupational sources. Fourth, the assay appears to be susceptible to interference by preservatives used routinely in the collection of urine samples, making the widespread use of the assay, especially in banked samples of human fluids, problematic. Finally, the lack of effect of smoking status on levels of any of these putative biomarkers suggests problems with their application to either exposure assessments or risk analyses. High background levels from other sources apparently may mask any contributions of benzene from smoking.

CONCLUSIONS

Dr. Melikian and colleagues have succeeded in developing a new, sensitive, and selective method for measuring the minor metabolites of benzene, *S*-PMA and *t,t*-MA, in human urine. Their finding that levels of the metabolites are detectable in the urine of both smokers and nonsmokers suggests that the developed analytical methodology will be appropriate for detecting low levels of these benzene metabolites in the general population. Their findings also indicate, however, that this approach may not be suitable or analytically sensitive enough to quantify the ring-hydroxylated benzene metabolites BT, CAT, and HQ. Further studies are required to validate the use of *S*-PMA as a monitor of ambient benzene exposures, and to assess its potential utility in risk assessments.

ACKNOWLEDGMENTS

The Health Review Committee wishes to thank the ad hoc reviewers for their help in evaluating the scientific merit of the Investigators' Report and Dr. Geoffrey Sunshine for assisting the Committee in preparing its Commentary. The Committee also acknowledges Thomas Atwood, Julia Campeti, Virgi Hepner, Joann Merriman, Hope Steele, and Quentin Sullivan for their roles in publishing this report.

REFERENCES

Boogaard PJ, van Sittert NJ. 1995. Biological monitoring of exposure to benzene: A comparison between *S*-phenylmercapturic acid, *trans,trans*-muconic acid, and phenol. Occup Environ Med 52:611–620.

Ghittori S, Fiorentino ML, Maestri L, Cordioli G, Imbriani M. 1993. Urinary excretion of unmetabolized benzene as an indicator of benzene exposure. J Toxicol Environ Health 38:233–243.

Ghittori S, Maestri L, Fiorentino ML, Imbriani M. 1995. Evaluation of occupational exposure to benzene by urinalysis. Int Arch Occup Environ Health 67:195–200.

Henderson RF. 1996. Species difference in the metabolism of benzene. Environ Health Perspect (Suppl 6) 104: 1173–1175.

Henderson RF, Sabourin PJ, Bechtold WE, Griffith WC, Medinsky MA, Birnbaum LS, Lucier GW. 1989. The effect of dose, dose rate, route of administration, and species on tissue and blood levels of benzene metabolites. Environ Health Perspect 82:9–17.

Hotz P, Carbonelle P, Haufroid V, Tschopp JP, Buchet JP, Lauwerys R. 1997. Biological monitoring of vehicle mechanics and other workers exposed to low concentrations of benzene. Int Arch Occup Environ Health 70:29–40.

Inoue O, Seiji K, Kasahara M, Nakatsuka H, Watanabe T, Yin SG, Li GL, Cai SX, Jin C, Ikeda M. 1988. Determination of catechol and quinol in the urine of workers exposed to benzene. Br J Ind Med 45:487–492.

Lee BL, Ong HY, Shi CY, Ong CN. 1993. Simultaneous determination of hydroquinone, catechol and phenol in urine using high-performance liquid chromatography with fluorimetric detection. J Chromatogr 619:259–266.

Ong CN, Kok PW, Lee BL, Shi CY, Ong HY, Chia KS, Lee CS, Luo XW. 1995. Evaluation of biomarkers for occupational exposure to benzene. Occup Environ Med 52:528–533.

Pezzagno G, Maestri L. 1997. The specificity of *trans,trans*-muconic acid as a biological indicator for low levels of environmental benzene. Indoor Environ 6:12–18.

Popp W, Rauscher D, Müller G, Angerer J, Norpoth K. 1994. Concentrations of benzene in blood and *S*-phenylmercapturic and *t,t*-muconic acid in urine in car mechanics. Int Arch Occup Environ Health 66:1–6.

Ruppert T, Scherer G, Tricker AR, Adlkofer F. 1997. *trans,trans*-Muconic acid as biomarker of non-occupational environmental exposure to benzene. Int Arch Occup Environ Health 69:247–251.

Sabourin PJ, Chen BT, Lucier G, Birnbaum LS, Fisher E, Henderson RF. 1987. Effect of dose on the absorption and excretion of [^{14}C] benzene administered orally or by inhalation in rats and mice. Toxicol Appl Pharmacol 87: 325–336.

Sabourin PJ, Bechtold WE, Birnbaum LS, Lucier G, Henderson RF. 1988. Differences in the metabolism and disposition of inhaled [^{3}H] benzene by F344/N rats and B6C3F$_1$ mice. Toxicol Appl Pharmacol 94:128–140.

Sabourin PJ, Bechtold WE, Griffith WC, Birnbaum LS, Lucier G, Henderson RF. 1989. Effect of exposure concentration, exposure rate, and route of administration on metabolism of benzene by F344 rats and B6C3F$_1$ mice. Toxicol Appl Pharmacol 99: 421–444.

Snyder R, Hedli CC. 1996. An overview of benzene metabolism. Environ Health Perspect (Suppl 6) 104:1165–1171.

Stommel P, Müller G, Stücker W, Verkoyen C, Schöbel S, Norpoth K. 1989. Determination of *S*-phenylmercapturic

acid in the urine—An improvement in the biological monitoring of benzene exposure. Carcinogenesis 10:279–282.

Wallace LA. 1989a. Major sources of benzene exposure. Environ Health Perspect 82:165–169.

Wallace LA. 1989b. The exposure of the general population to benzene. Cell Biol Toxicol 5:197.

Weisel C, Yu R, Roy A, Georgopoulos P. 1996. Biomarkers of environmental benzene exposure. Environ Health Perspect 104:1141–1146.

Witz G, Maniara WM, Mylavarapu VJ, Goldstein BD. 1990. Comparative metabolism of benzene and *trans,trans*-muconaldehyde to *trans,trans*-muconic acid in DBA/2N and C57BL/6 mice. Biochem Pharmacol 40:1275–1280.

RELATED HEI PUBLICATIONS: AIR TOXICS

Report No.	Title	Principal Investigator	Publication Date
Research Reports			
45	The Effects of Exercise on Dose and Dose Distribution of Inhaled Automotive Pollutants	M. T. Kleinman	1991
49	Mechanisms of Aldehyde-Induced Bronchial Reactivity: Role of Airway Epithelium	G. D. Leikauf	1992
51	Effects of Formaldehyde on Xenotransplanted Human Respiratory Epithelium	A. J. P. Klein-Szanto	1992
53	Use of Physical Chemistry and in Vivo Exposure to Investigate the Toxicity of Formaldehyde Bound to Carbonaceous Particles in the Murine Lung	G. J. Jakab	1992
67	Development of Methods for Measuring Biological Markers of Formaldehyde Exposure	T. R. Fennell	1994
HEI Communications			
	Communication Number 2: Research Priorities for Mobile Air Toxics		1993
	Communication Number 6: A Partnership to Examine Emerging Health Effects: EC/HEI Workshop on 1,3-Butadiene		1999

HEI

The Board of Directors

Archibald Cox *Chairman*
Carl M. Loeb University Professor (Emeritus), Harvard Law School

Douglas Costle
Chairman of the Board and Distinguished Senior Fellow, Institute for Sustainable Communities

Alice Huang
Senior Councilor for External Relations, California Institute of Technology

Donald Kennedy
President (Emeritus) and Bing Professor of Biological Sciences, Stanford University

Susan B. King
Fellow, Sanford Institute of Public Policy, Duke University

Richard B. Stewart
Professor, New York University School of Law

Robert M. White
President (Emeritus), National Academy of Engineering, and Senior Fellow, University Corporation for Atmospheric Research

Health Research Committee

Bernard D. Goldstein *Chairman*
Director, Environmental and Occupational Health Sciences Institute

Glen R. Cass
Professor of Environmental Engineering and Mechanical Engineering, California Institute of Technology

Seymour J. Garte
Professor of Environmental and Community Medicine, Environmental and Occupational Health Sciences Institute

Rogene Henderson
Senior Scientist, Lovelace Respiratory Research Institute

Meryl H. Karol
Professor of Environmental and Occupational Health, University of Pittsburgh Graduate School of Public Health

Stephen I. Rennard
Larson Professor, Pulmonary and Critical Care Medicine Section, Department of Internal Medicine, University of Nebraska Medical Center

Jonathan M. Samet
Professor and Chairman, Department of Epidemiology, School of Hygiene and Public Health, Johns Hopkins University

Robert F. Sawyer
Class of 1935 Professor of Energy (Emeritus), Professor of the Graduate School, University of California, Berkeley

Frank E. Speizer
Edward H. Kass Professor of Medicine, Channing Laboratory, Harvard Medical School, Department of Medicine, Brigham and Women's Hospital

Gerald van Belle
Professor, Departments of Environmental Health and Statistics, University of Washington

Health Review Committee

Daniel C. Tosteson *Chairman*
Professor of Cell Biology, Dean Emeritus, Harvard Medical School

John C. Bailar III
Professor, Department of Health Studies, Biological Sciences Division, The University of Chicago

A. Sonia Buist
Professor of Medicine and Physiology, Oregon Health Sciences University

Ralph D'Agostino
Professor of Mathematics/Statistics and Public Health, Boston University

Thomas W. Kensler
Professor, Division of Toxicological Sciences, Department of Environmental Sciences, Johns Hopkins University

Edo D. Pellizzari
Vice President for Analytical and Chemical Sciences, Research Triangle Institute

Donald J. Reed
Distinguished Professor of Biochemistry, Department of Biochemistry and Biophysics, and Environmental Health Sciences Center, Oregon State University

David J. Riley
Professor of Medicine, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School

Sverre Vedal
Professor of Medicine, University of British Columbia

Officers and Staff

Daniel S. Greenbaum *President*
Richard M. Cooper *Corporate Secretary*
Howard E. Garsh *Director of Finance and Administration*
Kathleen M. Nauss *Director for Scientific Review and Evaluation*
Robert M. O'Keefe *Director of Program Strategy*
Jane Warren *Director of Research*
Aaron J. Cohen *Senior Scientist*
Maria G. Costantini *Senior Scientist*
Alison Geyh *Staff Scientist*
Bernard Jacobson *Staff Scientist*
Debra A. Kaden *Senior Scientist*
Diane J. Mundt *Staff Scientist*
Martha E. Richmond *Staff Scientist*
Geoffrey H. Sunshine *Staff Scientist*
Gail V. Allosso *Office and Contracts Manager*
Thomas Atwood *Manager of Publications and Information*
Julia F. Campeti *Publications Assistant*
Terésa Fasulo *Senior Administrative Assistant*
L. Virgi Hepner *Senior Scientific Editor*
Darlene Jones *Senior Administrative Assistant*
Judith Lopez *Receptionist*
Francine Marmenout *Senior Executive Assistant*
Teresina McGuire *Accounting Assistant*
Beverly Morse *Administrative Assistant*
Jacqueline C. Rutledge *Controller*
Hope Steele *Acting Managing Editor*

HEI HEALTH EFFECTS INSTITUTE 955 Massachusetts Avenue, Cambridge, MA 02139 (617) 876-6700

Research Report Number 87 **June 1999**