Identification of 6-Hydroxy-*trans,trans*-2,4-hexadienoic Acid, a Novel Ring-Opened Urinary Metabolite of Benzene

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We studied the *in vivo* metabolism of benzene in mice to ring-opened compounds excreted in urine. Male CD-1 mice were treated intraperitoneally with benzene (110–440 mg/kg), [^{14}C]benzene (220 mg/kg) or *trans,trans*-muconaldehyde (MUC; 4 mg/kg), a microsomal, hematotoxic metabolite of benzene. Urine, collected over 24 hr, was extracted and analyzed by HPLC with a diode-array detector and by scintillation counting. In addition to *trans,trans*-muconic acid, previously the only known ring-opened urinary benzene metabolite, a new metabolite, 6-hydroxy-*trans,trans*-2,4-hexadienoic acid, was detected in urine of mice treated with either benzene or MUC. We identified the new metabolite based on coelution of metabolites and UV spectral comparison with authentic standards in unmethylated and methylated urine extracts. Results presented here are consistent with the intermediacy of MUC in the *in vivo* metabolism of benzene to ring-opened metabolites. *Key words:* benzene, 6-hydroxy-2,4-hexadienoic acid, muconaldehyde, muconic acid, ring-opened metabolite, urinary metabolite. *Environ Health Perspect* 101:310–312(1993)

The toxicity of benzene is generally thought to be mediated by metabolites formed in the liver (*1,2*). Benzene is metabolized *in vivo* to ring-hydroxylated intermediates as well as *trans,trans*-muconic acid (muconic acid, MA; Table 1), a ring-opened intermediate (*2*). We have hypothesized that reactive ring-opened intermediates play an important role in benzene toxicity (*3*). Our laboratory has identified one such compound, the dialdehyde *trans,trans*-muconaldehyde (muconaldehyde, MUC; Table 1), in mouse-liver microsomal incubations of benzene (*4*). Muconaldehyde is a potent electrophile (*5*), a cross-linking agent (*6*), and is hematotoxic when injected in mice (*7*). Muconaldehyde has not been directly detected in animals dosed with benzene, presumably due to its reactivity. *In vitro*, MUC is metabolized to a number of oxidized and reduced products of which MA and 6-hydroxy-*trans,trans*-hexadienoic acid (HHA, Table 1) are stable end products (*8,9*). We previously established that MUC is metabolized *in vivo* to MA (*10*). We undertook the present studies to identify HHA in the urine of CD-1 mice treated with MUC and benzene to provide further evidence for the intermediacy of MUC in the metabolism of benzene *in vivo*.

A procedure previously used in this laboratory for extracting and analyzing MA in mouse urine (*10*) was modified to simultaneously analyze HHA and MA. We added three volumes of methanol to one volume of urine. After cooling at -20°C for 2–3 hr, samples were spun in a microcentrifuge for 10 min and the pellet was discarded. We evaporated methanol from the supernatant at 37°C using a nitrogen stream and acidified the remaining sample to pH 2.5–3.0 with concentrated hydrochloric acid. Samples were extracted six times with two volumes of diethyl ether. We pooled the ether fractions, evaporated the ether at 37°C using a nitrogen stream, and redissolved the residues in methanol for analysis. Samples (10–20 µl) were analyzed for MA and HHA by analytical C18 reverse-phase HPLC using a flow rate of 1.0 ml/min, and absorbance was monitored at 260 nm. We determined the λ_{max} of chromatogram peaks using a diode-array detector. Samples were eluted isocratically using 10% methanol and 90% aqueous acetic acid (1%). Under these conditions HHA eluted between 9 and 10 min, and MA eluted between 13 and 14 min. We also reacted extracts with a diethyl ether solution of diazomethane (*11*) to convert HHA to the more hydrophobic methyl ester, Me-HHA (Table 1). We analyzed the methylated extracts by reverse-phase HPLC using 13% methanol and 87% aqueous acetic acid (1%) as the eluant. Under these conditions, Me-HHA eluted between 29 and 31 min.

We validated quantification of MA and HHA by this method using spiked mouse urine. [HHA was prepared by from 6-oxo-*trans,trans*-hexadienoic acid (*5*) by reduction with sodium borohydride (*9*)]. Extraction, HPLC analyses, and peak integration of MA/HHA spiked mouse urine indicated a linear response in unmethylated extracts for MA in the range of 5–50 µg/ml urine (r^2 = 0.956) and HHA in the range of 4–10 µg/ml urine (r^2 = 0.975). Below 4 µg/ml, quantification of HHA was compromised due to the presence of endogenous peaks present in some urines eluting close to HHA. Recoveries of both MA and HHA were 40% after the extraction. Reproducibility was 18% relative standard deviation (RSD) for HHA and 19% RSD for MA at 10 µg/ml (N=6). Diode-array spectra of peaks coeluting with HHA and MA exhibited maxima in the range of 258–259 nm and 264–265 nm, respectively, as did the chromatographed standards of HHA and MA in methanol. In methylated spiked-urine extracts, Me-HHA exhibited diode-array spectral maxima in the range of 260–264 nm. Dose response of Me-HHA integration was linear from 1 to 10 µg HHA/ml (r^2 = 0.964). Methylation of HHA in urine extracts was quantitative.

We treated male CD-1 mice, 10 weeks old, intraperitoneally with benzene, [^{14}C]benzene, or MUC. MUC was custom-synthesized by Calbiochem (San Diego, CA) by a procedure of Kossmehl and Bohn (*12*) previously used in our laboratory (*3*). One day before treatment, we transferred animals to metabolism cages (three mice per cage) and collected urine over 24 hr in traps containing 2 ml of 0.2% (w/v) ascorbic acid. These urines served as no-treatment controls. We transferred each group of mice to clean metabolism cages and injected them intraperitoneally with 0.1 ml of corn oil solutions containing either benzene (110, 220, or 440 mg/kg), [^{14}C]benzene (220 mg/kg), or MUC (4 mg/kg). The urine from each treated group was collected over 24 hr in traps containing 2 ml 0.2% ascorbic acid. We determined total urine volume and stored samples at -70°C until analysis. Scintillation counting of aliquots of urine collected from [^{14}C]benzene-treated mice indicated that at least 68% of administered label was excreted in the urine after 24 hr. After extraction, 15% of this radioactivity partitioned into the ether phase. One-half of the ether extract from each urine was

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Received 9 Nov 1992; accepted 28 May 1993.

subjected to methylation using diazomethane, and extracts were analyzed by HPLC and, for radioactive samples, scintillation counting. We spiked a portion of the extracts from unmethylated samples with MA and reanalyzed HHA to establish coelution of peaks. Similarly, portions of extracts from methylated samples were spiked with Me-HHA and reanalyzed.

Figure 1A shows the chromatogram of extracted urine from mice treated intraperitioneally with 220 mg/kg [^{14}C]benzene, which exhibits the presence of two peaks eluting at 9.39 min and 13.39 min. These peaks co-chromatographed with HHA and MA, respectively, and exhibited diode-array spectra identical to the authentic compounds (λ_{max}: HHA, 259 nm; MA 264 nm). Radioactive peaks were also observed in the radiochromatogram at retention times of HHA and MA (Fig. 1A), indicating the presence of benzene metabolites. Corresponding HHA and MA coeluting peaks were also seen in chromatograms of urine extracts from mice treated with 110, 220, and 440 mg/kg benzene and 4 mg/kg muconaldehyde. These peaks were absent in chromatograms from extracted urines of the same mice collected 24 hr before benzene treatment (data not shown).

The chromatogram of a methylated ether extract from urine of mice treated with 220 mg/kg [^{14}C]benzene (Fig. 1B) reveals the presence of a compound that coelutes with HHA methyl ester (t_R = 29.3 min) and exhibits a diode-array spectrum consistent with Me-HHA (λ_{max} = 259 nm). (A shoulder on the Me-HHA peak in the chromatogram shown in Figure 1B was not present in chromatograms of methylated urine extracts from mice treated with unlabeled benzene). A radioactive peak also coeluting with Me-HHA was observed in the radiochromatogram (Fig. 1B). Total counts of radioactivity coeluting with HHA in the unmethylated extract (Fig. 1A) and Me-HHA in the methylated extract (Fig. 1B) were the same. A peak coeluting with unmethylated HHA is absent from the chromatogram and radiochromatogram (as is MA). The corresponding coeluting peak was also seen in chromatograms of methylated urine extracts from mice treated with 110, 220, and 440 mg/kg benzene and 4 mg/kg muconaldehyde and was absent in chromatograms from methylated urine extracts of the same mice collected 24 hr before benzene treatment (data not shown). The dimethyl ester of muconic acid (Me-MA; Table 1) was also present in methylated extracts of urines from benzene and MUC-treated animals (data not shown). This compound, being more hydrophobic than Me-HHA, did not elute under the HPLC conditions described.

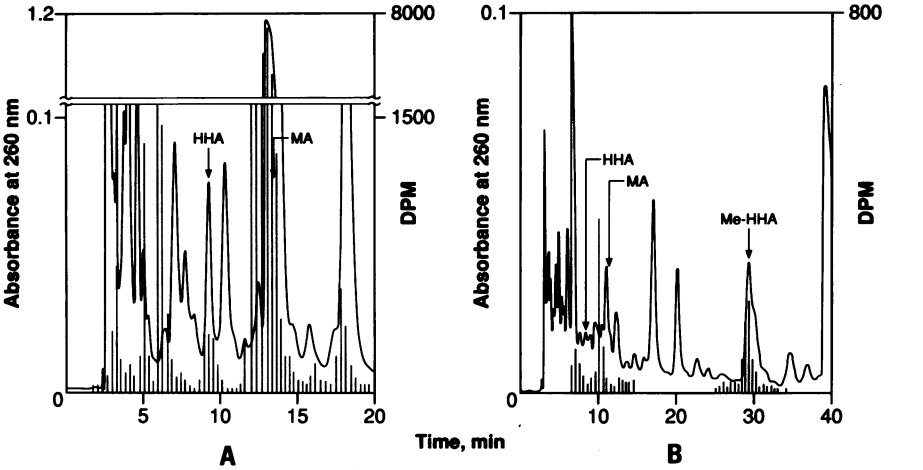
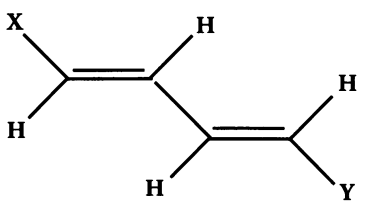


Figure 1. Chromatograms and radiochromatograms of (A) unmethylated and (B) methylated urine extracts from mice treated with [^{14}C]benzene. See text for details of methods. The unmethylated and methylated extract were each dissolved in 200 µl of methanol, and 10 µl was analyzed on reverse-phase HPLC using elution with 1% acetic acid:methanol (unmethylated, 90:10; methylated, 87:13). Fractions of eluent (unmethylated, 0.3 ml; methylated, 0.5 ml) were quantitfied by scintillation counting. (Line) absorbance; (vertical bar) dpm.

Table 1. Structure of benzene metabolites



Compound	X	Y
MA	CO_2H	CO_2H
MUC	CHO	CHO
HHA	CH_2OH	CO_2H
Me-HHA	CH_2OH	CO_2Me
HA1	CH_2OH	CHO
AA1	CO_2H	CHO

Abbreviations: MA, *trans,trans*-muconic acid; MUC, *trans,trans*-muconaldehyde; HHA, 6-hydroxy-*trans,trans*-2,4-hexadienoic acid; Me-HHA, 6-hydroxy-*trans,trans*-2,4-hexadienoic acid methyl ester; Me-MA, *trans,trans*-muconic acid dimethyl ester; HA1, 6-hydroxy-*trans,trans*-2,4-hexadienal; AA1, 6-oxo-*trans,trans*-hexadienoic acid.

The excretion of urinary MA and HHA by benzene- and MUC-treated mice is summarized in Table 2. Muconic acid was excreted in significantly greater amounts than HHA in MUC-treated mice and in benzene-treated animals at all doses. Ratios of HHA to MA at all benzene doses in this study (7.1, 15.3, and 11.1) were similar to the HHA to MA ratio obtained after MUC administration (5.2; Table 2).

Trans,trans-muconic acid was first definitely identified as an *in vivo* metabolite of benzene in the urine of rabbits (*13*). HHA is the second ring-opened metabolite of benzene that has been detected *in vivo*. The evidence for identification of this compound is based on the following: 1) the presence of a compound in extracts of urine from benzene- and MUC-treated mice that coelutes with authentic HHA on reverse-phase HPLC and which has a UV diode-array spectrum consistent with HHA. This compound is absent in extracts of urine obtained from the same mice immediately before treatment; 2) the presence of a peak in methylated urine extracts from benzene- and MUC-treated mice that coelutes with authentic HHA methyl ester. This compound has a UV diode-array spectrum consistent with HHA methyl ester and is not present in control urine extracts; 3) coelution of radioactivity with HHA and Me-HHA in extracted urine (unmethylated and methylated) from mice exposed to [^{14}C]benzene. Total radioactive counts eluting with HHA and Me-HHA were the same in unmethylated and methylated extracts.

Detection of HHA as a metabolite of MUC *in vivo* extends observations made *in vitro* (*8,9*). Detection of HHA in urine of benzene-treated animals is consistent with the hypothesis that benzene is metabolized *in vivo* to ring-opened metabolites via MUC (Fig. 2). Similar MA:HHA ratios in benzene *versus* MUC-treated animals strengthen this hypothesis. MUC is a benzene metabolite in mouse microsomal

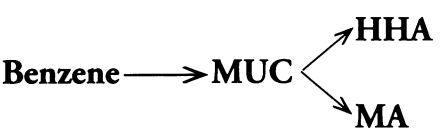


Figure 2. Possible metabolism of benzene to 6-hydroxy-*trans,trans*-hexadienoic acid (HHA) and *trans,trans*-muconic acid (MA) via *trans,trans*-muconaldehyde (MUC).

Table 2. Excretion of muconic acid (MA) and 6-hydroxy-*trans,trans*-2,4-hexadienoic acid (HHA) by CD-1 mice treated with benzene or *trans,trans*-muconaldehyde (MUC)

Compound	µmol/kg Injected (mg/kg)	Mean ± SE excreted in urine (µmol/kg; $n = 3$) MA	HHA
Benzene	1500 (110)	12.2 ± 0.2	1.73 ± 0.16
Benzene	2800 (220)	36.6 ± 3.7	2.31 ± 0.27
Benzene	5600 (440)	16.9 ± 1.5	1.52 ± 0.06
MUC	36 (4)	8.1 ± 0.8	1.56 ± 0.51

Mice, three per cage, one cage per dose, were treated intraperitoneally with compound dissolved in corn oil. Urines were collected for 24 hr and volume was determined. Portions of the urines were extracted and analyzed as described in the text. Urinary MA concentration was calculated from the integrated area of the MA coeluting peak in unmethylated urine extracts using a standard curve generated by extraction and analysis of mouse urine spiked with MA. Urinary HHA concentration was determined from the integrated area of the Me-HHA coeluting peak in methylated urine extracts using a standard curve generated by extraction, methylation, and analysis of mouse urine spiked with HHA.

incubations, but its reactivity probably precludes its direct detection *in vivo*.

Although MUC is a potential intermediate in the metabolic conversion of benzene to ring-opened products, other compounds may also be considered. Conversion of MA to HHA is unlikely because carboxylic acids are not reduced by dehydrogenases. The mixed-hydroxy aldehyde analogue of MUC (HAl; Table 1) is converted to HHA by yeast aldehyde dehydrogenase (*8*) and mouse liver cytosol (*9*). However, HAl is not converted to MA in these systems (*8,9*). Another MUC analogue, the carboxylic acid aldehyde (AAl; Table 1) is metabolized to MA and to a small amount of HHA by mouse liver cytosol (*9*). In incubations of NADH-fortified alcohol dehydrogenase, the carboxylic acid aldehyde is not reduced to HHA (*8*). Thus, HAl, as well as MUC, is a common precursor of HHA and MA *in vitro*. However, there is no evidence to rule out either of these compounds as precursors of HHA and MA formed from benzene *in vivo*.

In conclusion, we have established the presence of a novel ring-opened metabolite in the urine of benzene-treated mice. This metabolite is also present in the urine of mice after treatment with muconaldehyde. The formation of HHA as an *in vivo* metabolite of MUC confirms results obtained *in vitro* (*9*). The formation of this compound from benzene *in vivo* provides further evidence for the intermediacy of MUC in benzene metabolism. The detection and quantification of HHA in the urine of humans with known exposure to benzene is a goal of our research, particularly in view of recent attempts to use human urinary muconic acid as a biological marker relevant to the risk assessment of benzene (*14*).

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