ABSTRACT:

α-Methylstyrene (AMS) is a volatile hydrocarbon used primarily in the production of specialty polymers and resins. In the present study, the tissue distribution, metabolism, and excretion of [14C]AMS was investigated in male rats after i.v. administration (11 mg/kg). Over 90% of AMS administered intravenously to rats was excreted in 72 h. Urinary excretion accounted for 86% of the administered dose, volatile breath and feces accounted for 2.2 and 1.9%, respectively, and elimination as carbon dioxide was negligible. Metabolites were isolated from rat urine following a high oral dose of AMS (1000 mg/kg) and characterized using gas chromatography/mass spectrometry and NMR spectrometry. The metabolites were 2-phenyl-1,2-propanediol (3% of urinary radioactivity) and its glucuronide (50%), atrolactic acid (27%), S-(2-hydroxy-2-phenylpropyl)-N-acetylcysteine (13%), and 2-phenylpropionic acid (1%); the glucuronides and mercapturates were each conjugated on the methylene carbon beta to the ring. The presence of both of the diastereomeric isomers of the mercapturates and of the glucuronides suggested that the initial epoxidation of AMS was not stereoselective and proceeded with addition of active oxygen to yield enantiomeric epoxides. Incubation of AMS with human liver slices produced the same metabolites as those excreted in rat urine, with 2-phenyl-1,2-propanediol present as the predominant metabolite after 5 h of incubation.

α-Methylstyrene (AMS; 2-phenylpropylene) (CAS 83-98-9) is a volatile aromatic hydrocarbon (b.p. 165°C) used primarily in the production of specialty polymers and resins. The majority of the AMS used industrially is in the manufacture of acrylonitrile-butadiene-styrene copolymers, which are lightweight and have good heat-distortion properties at high temperatures (Lewis et al., 1983). AMS is less reactive than styrene and moderates the polymerization rate, resulting in coatings and resins with improved clarity. The National Occupational Exposure Survey released by NIOSH in 1976 indicated heavy construction contractors, paper and allied products, and other business services comprise industries with the largest number of workers potentially exposed to AMS.

AMS is not highly toxic, and lethality probably arise mostly from oversedation. The oral LD_{50} for AMS in rats is 4.9 g/kg (Wolf et al., 1956). The toxicity of AMS to B6C3F1 mice and Fischer 344 rats following exposure by inhalation of concentrations ranging from 125 to 1000 ppm for 6 h/day for 12 days has been recently reported (Morgan et al., 1999). Mortality was observed in female mice after the first exposure at concentrations greater than 600 ppm; no male mice died, but both male and female mice were sedated at these levels.

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1 Abbreviations used are: AMS, α-methylstyrene; GC/MS, gas chromatography/mass spectrometry; HMBC, heteronuclear multiple bond correlation; TBR, tissue/blood ratio; TMS, trimethylsilylame.
1,2-propanediol was prepared at Research Triangle Institute (Triangle Park, North Carolina) by the reduction of atracolactic acid using the method of Shore and Yuen (1972). The identity of nonradionabeled AMS was confirmed by proton NMR using CDCl3 as a solvent. [14C]AMS, uniformly radioabeled with carbon-14 in the phenyl ring, was obtained from Wizard Laboratories, Inc. (West Sacramento, CA) at a specific activity of 1.0 mCi/mmol. The radiochemical purity of [14C]AMS (≥98%) was established using a Microsorb-MV phenyl column (4.6 × 250 mm, 5-μm particle size; Varian, Palo Alto, CA) and an isocratic mobile phase of methanol/water (v/v); the flow rate was 1 ml/min. β-Glucuronidase (prepared from Escherichia coli), sulfatase (prepared from Aerobacter aerogenes), and acylase (prepared from porcine kidney) were purchased from the Sigma Chemical Company (St. Louis, MO). Bis(trimethylsilyl)trifluoroacetamidine was purchased from Supelco, Inc. (Bellefonte, PA). Emulphor EL-620 was obtained from the GAF Chemical Corporation (Wayne, NJ). Mass spectra were determined by GC/MS analysis on a Hewlett Packard (Palo Alto, CA) 5890 series 2 gas chromatograph and a HP-5989A mass spectrometer using electron impact. Metabolites and standards were dissolved in CD3OD or CDCl3 before obtaining NMR spectra on a Bruker (Newark, DE) AMX-500 MHz instrument.

Animal Studies. Adult male Fischer 344 rats were purchased from Charles River Laboratories, Inc. (Raleigh, NC) and given Purina (St. Louis, MO) Rodent Chow (#5002) and water ad libitum. At dosing, rats were 79 to 85 days old and weighed 241 to 263 g. Intravenous dose formulations contained 21 to 24 μCi of [14C]AMS and an appropriate amount of Emulphor EL-620 and phosphate-buffered saline (1:20) in a single dose to deliver a volume of 1 ml/kg. An oral dose formulation was prepared for dosing one rat that contained 2% excreted in the

### TABLE 1

<table>
<thead>
<tr>
<th>End of Collection Period</th>
<th>Cumulative Percentage of Dose Recovered in</th>
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<tbody>
<tr>
<td>h</td>
<td>Urine</td>
</tr>
<tr>
<td>6</td>
<td>34.1 ± 3.9</td>
</tr>
<tr>
<td>12</td>
<td>53.0 ± 7.3</td>
</tr>
<tr>
<td>24</td>
<td>76.4 ± 2.1</td>
</tr>
<tr>
<td>48</td>
<td>84.9 ± 1.6</td>
</tr>
<tr>
<td>72a</td>
<td>86.2 ± 1.4</td>
</tr>
</tbody>
</table>

F-344, Fischer 344.

a Includes cage rinse.

Aliquots of urine from the i.v. study were incubated with β-glucuronidase, sulfatase, or acylase to aid in the characterization of any conjugates that may have been present. β-Glucuronidase incubations contained urine (50–100 μl) and 200 μl (ca. 500 units) of enzyme solution. Sulfatase incubations contained 50 μl of urine, 250 μl of TRIZMA [tris(hydroxymethyl)aminomethane] buffer (pH 7.6), and 250 μl of sulfatase solution (10–20 units/ml). Controls were prepared with heat-deactivated and flash-frozen enzyme. The incubations were performed at 37°C for 1 h. Alternately, urine samples (100 μl) were incubated at 37°C for 6 h with 100 μl of acylase solution (ca. 1400 units in 0.9 M potassium phosphate buffer, pH 7.4). Incubation mixtures were analyzed as described above and the profiles compared with those from untreated urine.

Urine collected 6 to 24 h post dosing from a rat administered AMS by oral gavage (1000 mg/kg) was used for isolation and purification of metabolites by HPLC. Urine from this experiment was chromatographed and observed to display a similar metabolite profile as the urine from the intravenous study. Eluant fractions containing the respective metabolites were manually collected from the radioactivity detector following multiple chromatographic runs. The organic solvent was removed by rotary evaporation, and the residue (except for metabolite D) was brought to dryness by lyophilization and then reconstituted in methanol. Residue containing metabolite D was basified to pH 10 with 1 N NaOH before lyophilization. Metabolites C, D, E, and F were further purified by use of a Waters (Milford, MA) C18 Sep-Pak Plus extraction column before analysis by GC/MS. After treatment of metabolite B with β-glucuronidase, a new peak appeared in the HPLC radiochromatogram that was collected and concentrated to dryness. The trimethylsils (TMS) derivatives of metabolite C, the aglycone of metabolite B, 2-phenyl-1,2-propanediol, metabolite D, metabolite E, and atracolactic acid were prepared and then analyzed by GC/MS. 2-Phenylpropionic acid and metabolite F were analyzed by GC/MS. A separate sample of metabolite B was further purified using a Microsorb-MV phenyl analytical column with an isocratic mobile phase of 10% acetonitrile/aqueous 1% acetic acid (v/v) using a flow rate of 1 ml/min. The samples of metabolite B and metabolite E were analyzed by NMR using 1H, 13C, correlation spectroscopy, heteronuclear multiple quantum correlation, and heteronuclear multiple bond correlation (HMBC) spectrometry.

Liver Slices. Human liver slices were received from the International Laboratory for the Advancement of Medicine (Exton, PA). The donor was a 45-year-old black male that died from a gunshot wound to the leg. He had renal cancer and used cocaine. The medications received during his hospital stay were DDAAVP [1-(3-mercapto propionic acid)-8-d-arginine vaspressin], cephalin, sodium, and dopamine. Human liver slices were incubated with AMS (1 mM in medium) as previously described (Mathews et al., 1996).

Results

Distribution and Excretion. Intravenous doses of AMS (11 mg/kg) were mainly excreted in the urine with 76 ± 2% excreted in the first 24 h post dosing and 86 ± 1% excreted after 72 h (Table 1). Fecal elimination accounted for 2% of the dose. Exhalation as volatile organics and carbon dioxide accounted for only 2 and 0.02% of the dose, respectively. The profiles of metabolites present (see Metabolite Identification below for characterization) in each urinary collection interval up to 48 h post dosing for one rat are shown in Table 2, and a typical HPLC radiochromatogram is displayed in Fig. 1. Unfortu-
nately, the diol glucuronide had partially hydrolyzed to the aglycone upon storage, but data from the analysis of urine freshly collected from one rat indicated that the majority of the diol metabolites were excreted as the glucuronide. The most abundant metabolite was 2-phenyl-1,2-propanediol glucuronide (41% of dose), followed by atrolactic acid; the most abundant metabolite was 2-phenyl-1,2-propanediol.

At sacrifice (72 h), only 0.3% of the administered radioactivity was present in the tissues sampled (Table 3). The highest concentrations were found in spleen, with a tissue/blood ratio (TBR) of 17, followed by kidney (TBR of 12), bladder (TBR of 7), and lung (TBR of 6).

Metabolite Identification. AMS was administered orally to one rat at a dose of 1000 mg/kg to obtain greater quantities of metabolites for structural characterization. The percentage of radioactivity excreted in urine and the urinary metabolite profile from this experiment (data not shown) was similar to that from the intravenous study. Five of the metabolites were isolated from the urine and identified by GC/MS.

Treatment of metabolite B with β-glucuronidase yielded a peak that coeluted with metabolite C. The spectra of TMS derivatives of metabolite B and the aglycone of metabolite B were identical to that of 2-phenyl-1,2-propanediol glucuronide, and metabolite C was 2-phenyl-1,2-propanediol.
gradient enhanced HMBC spectrum optimized to provide correlations between protons and carbons that are separated by three bonds, although some two-bond correlations may be detected. The position of the glucuronide was evident from the correlation of the methylene protons (b) with the anomeric carbon of the glucuronide conjugate, the α-methyl carbon and the quaternary carbon of the aromatic ring. If the glucuronide were attached to the α-carbon, the interaction between the methylene protons and the anomeric carbon of the glucuronide would be not be possible. Additionally, there was clear evidence of the presence of diastereomeric pairs in the carbon resonances of the 13C spectrum roughly corresponding to a 2:1 ratio of stereoisomeric products.

Metabolite D was identified as atrolactic acid by comparison of GC/MS data for its bis-TMS derivative with that of the standard, with peaks at m/z 295 (loss of a methyl), 193 (loss of CO2-TMS), and 147 (data not shown).

Treatment with acylase converted metabolite E to a new component that eluted between metabolites A and B. The 1H NMR spectrum of metabolite E is consistent with an N-acetylcysteine conjugate resulting from reaction of glutathione with the epoxide of AMS, followed by further metabolism to the mercapturate. Analogous metabolites are found for the metabolism of styrene (Seutter-Berlage et al., 1978) and the vinyltoluenes (Bergemalm-Rynell and Steen, 1982). The HMBC spectrum (Fig. 3) also supports this assignment and defines the position of attachment of the sulfur on the β-carbon, as evidenced by the correlation of the β-methylene protons with the methylene carbon of the mercapturate. In contrast to the glucuronides, there was no evidence of diastereomeric pairs in the NMR spectra. TMS derivatives of metabolite E were prepared for GC/MS analysis, and two di-derivatized and two tri-derivatized products of each were present in roughly equal amounts in the gas chromatogram (data not shown). The fragmentation patterns (in particular the ion at m/z 193) in these spectra are also consistent with the formation of both diastereomers of just one of two possible positional isomers for the mercapturate (data not shown).

The GC/MS of metabolite F matched that of 2-phenylpropionic acid with both spectra containing a molecular ion at m/z 150 and signals at m/z 105 (loss of COOH) and 77 (data not shown). An analogous urinary metabolite was identified after administration of 4-isopropenyltoluene to rabbits (Matsumoto et al., 1994).

Metabolism by Human Liver Slices. In investigations of the metabolism of AMS by human liver slices, the same metabolites were produced as those identified in rat urine. As observed in rats, the major metabolite was 2-phenyl-1,2-propanediol (25% of the radioactivity) after 5 h of incubation (data not shown); atrolactic acid and 2-phenylpropionic acid each accounted for approximately 1% of the radioactivity, and the remainder of the metabolites accounted for less than 0.3% of the radioactivity.
Discussion

AMS was readily metabolized by rats to form products that were chiefly excreted in the urine. There was little accumulation of radiolabeled equivalents in the tissues. In the present study, 96% of the urinary radioactivity was characterized, and all of the metabolites were products of oxidation of the vinyl group; no ring oxidized metabolites were found (Fig. 4). Metabolism of AMS to atrolactic acid has been reported in humans and other mammals, and this metabolite has been suggested as an appropriate marker of exposure to this hydrocarbon (Aizvert, 1975). Five metabolites were identified in the present study. The predominant metabolite, 2-phenyl-1,2-propanediol glucuronide (accounting for 50% of the urinary radioactivity), was present in roughly twice the amount of that of atrolactic acid in rat urine. Only 3% of the urinary radioactivity was present as 2-phenyl-1,2-propanediol. 2-Phenyl-1,2-propanediol was the predominant metabolite present in the media of human liver slices incubated with AMS, and it and its glucuronide may be the major human urinary metabolites. Accordingly, the diol, or more likely its glucuronide metabolite, may also deserve consideration as a biomarker for exposure of humans to AMS.

The mercapturate metabolite (E), formed after the reaction of the epoxide with glutathione, was most abundant in the early urine collections. It was the next most abundant metabolite and ultimately composed 13% of the urinary radioactivity. Its formation is consistent with the marked depletion of liver glutathione observed in inhalation studies with AMS (Morgan et al., 1999). Small amounts of 2-phenylpropionic acid (1% of urinary radioactivity) were also formed. It is likely that this derives from 2-phenylpropionaldehyde, formed from a 1,2-hydride shift during the transfer of active oxygen to the vinyl group, as has been proposed for the cytochrome P450-mediated oxidation of styrene to form phenylacetaldehyde (Ortiz de Montellano, 1995).

The presence of both diastereomeric forms of mercapturates and glucuronides suggests that the initial epoxidation of AMS is not stereoselective and proceeds with the addition of active oxygen to yield enantiomeric epoxides. Both enzymatic hydrolysis and glutathione conjugation of epoxides is known to proceed by S_N2 reactions. Therefore, enzymatic hydrolysis can yield enantiomeric diols. Further oxidation of these diols to atrolactic acid does not affect the chiral center at the benzyl position, and the potential products are enantiomers. However, conjugation with a chiral molecule such as glutathione or glucuronic acid would produce diastereomeric metabolites from the enantiomeric products, as was the case with the mercapturates and glucuronides characterized in these studies. In contrast to the reaction with styrene oxide (Seutter-Berlage et al., 1978; Sumner and Fennell, 1994), conjugation of glutathione with the epoxide of AMS occurs only at the less hindered $\beta$-carbon. Seutter-Berlage et al. (1978) demonstrated the intermediacy of an enantiomeric epoxide of

![NMR spectra were obtained on a Bruker AMX-500 MHz instrument.](image-url)
styrene from the diastereomeric mercapturates formed at the \( \alpha \)-carbon, but apparently the isochronicity of the NMR resonances of the \( \beta \)-carbon mercapturates did not allow assessment of the relative contributions of mercapturate diastereomers formed at this position. Similarly, the \( \beta \)-carbon mercapturates formed from AMS epoxide lent no NMR evidence of the formation of diastereomers, presumably due also to isochronous resonances, while the GC/MS data did.

This work is the first comprehensive study of the metabolism of AMS in rats and includes the first report of sulfur-containing AMS metabolites. The data are currently being used in support of the toxicological risk assessment of AMS exposure by inhalation and other routes of administration and in the construction of physiologically based pharmacokinetic models for this hydrocarbon.

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