Identification of Urinary Metabolites of Orally Administered N,N-Dimethyl-p-Toluidine in Male F344 Rats

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The metabolism of orally administered *N,N*-dimethyl-*p*-toluidine (DMPT) in male F344 rats was investigated. The rat urinary metabolite profile was determined by analytical reverse-phase high performance liquid chromatography (HPLC). Four radiolabeled peaks were observed, isolated, and purified by solid-phase extraction (SPE) and preparative HPLC methods. The 4 peaks were identified as *p*-(*N*-acetylhydroxyamino)hippuric acid (M1), DMPT *N*-oxide (M2), *N*-methyl-*p*-toluidine (M3), and parent DMPT. Metabolites M1 and M2 were identified by spectrometric and spectroscopic methods, including mass fragmentation pattern identification from both liquid chromatography/mass spectrometry and gas chromatography/mass spectrometry, and from chemical analysis of nuclear magnetic resonance spectra. Structural confirmation of metabolite M2 was accomplished by comparison with a synthetic standard. Peaks M3 and the peak suspected to be DMPT were identified by comparison of their HPLC retention times and mass fragmentation patterns with authentic standards of *N*-methyl-*p*-toluidine and DMPT, respectively. DMPT metabolism is similar to that reported for *N,N*-dimethylaniline.

INTRODUCTION

N,N-dimethyl-*p*-toluidine (DMPT) is a high-production-volume chemical used as a polymerization accelerator in the manufacture of bone cements and dental materials. DMPT is also found in industrial glues and artificial fingernail

preparations, and is an intermediate in the synthesis of dyes and pesticides. There is the potential for human exposure to DMPT in prosthesis users, individuals with dental plates, and occupational settings (Potter et al., 1988; Taningher, et al., 1993; Haddad et al., 1996). Human exposure to DMPT has resulted in methemoglobinemia and allergic responses (Pegam and Medhurst, 1971; Bunn and Forget, 1986; Potter et al., 1988; Tosti et al., 1990; Dutrée-Meulenberg et al., 1992; Haddad et al., 1995; Kao et al., 1997). Accidental ingestion of artificial fingernail solutions containing DMPT by a 16-month-old girl resulted in an acute cyanotic episode due to methemoglobinemia and a 5-month-old boy suffered from a less severe case after ingestion of an artificial fingernail solution containing DMPT (Potter et al., 1988; Kao et al., 1997).

The induction of methemoglobinemia produced by DMPT ingestion is suspected to be due to the formation of a toxic metabolite, *p*-methylphenylhydroxylamine. This metabolite is analogous to phenylhydroxylamine, which is likely responsible for methemoglobinemia observed after exposure to aniline (Potter et al., 1988; Bunn and Forget, 1986). Aniline, *N,N*-dimethylaniline (DMA), and toluidines (ortho-, meta-, and para-) are structurally similar to DMPT, and the known metabolism of these compounds may provide useful information on DMPT metabolism. It was reported that the primary metabolites of ortho-, meta-, and para-toluidine resulted from ring hydroxylation and subsequent conjugation (Cheever et al., 1980). Pathways of DMA metabolism include *N*-demethylation to form *N*-methylaniline and aniline; *N*-oxidation to form *N,N*-dimethylaniline *N*-oxide; and *N*-glucuronidation of *N*-methylaniline to form *N*-methylaniline *N*-glucuronide. In addition, ring hydroxylation in the para-position is another pathway involved in DMA metabolism and results in the formation of 4-aminophenol, *N*-methyl-4-aminophenol, and *N,N*-dimethyl-4-aminophenol (Gorrod and Gooderham, 1981; Gooderham and Gorrod, 1981; Sherrat and Damani, 1989). This study reports the identification of urinary metabolites of orally administered DMPT in F344 rats.

MATERIALS AND METHODS

Chemicals

DMPT and *N*-methyl-*p*-toluidine standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Radiolabeled DMPT (25.3 mCi/mmol of ^{14}C, uniformly ring labeled; PerkinElmer, Boston, MA) was provided by the National Institute of Environmental Health Sciences (NIEHS). High-performance liquid chromatography (HPLC)-grade solvents were purchased from Fisher Scientific Co. (Denver, CO) for column chromatography and HPLC. Deuterated nuclear magnetic resonance (NMR) solvents ($CDCl_3$ and CD_3OD) and tetramethylsilane (TMS), acetyl chloride, Diazald, and (trimethylsilyl)diazomethane were purchased from Sigma-Aldrich Chemical Co.

Animals

Adult male F344 rats weighing 180-227 g (10-11 weeks of age) on dose day were purchased from Charles River Laboratories (Raleigh, NC) and housed two to a cage during a 2-week quarantine. For 1 day prior to and following dosing, animals were housed individually in all-glass metabolism chambers. Animal studies were conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). Animals received a single oral dose of [^{14}C]DMPT (2.5 – 250 mg/kg) in 10% aqueous Alkamuls (Rhodia, Cranbury, NJ).

Sample Collection

Urine was collected separately from each animal into receivers cooled over dry ice. Samples were stored in tightly capped containers in the dark at −22 °C until analysis. The collection-interval composites of rat urine from the 250-mg/kg dose group were prepared by combining 10% by weight of the total urinary output from 4 rats to yield composites in 5 different hourly collections (6, 12, 24, 48, and 72 hr). Samples were analyzed for total radioactivity using a Packard Model 2500 TR Liquid Scintillation Analyzer (Packard Instrument Co. Inc., Meridien, CT). Samples were assayed for ^{14}C by directly dissolving them in Ultima Gold scintillation cocktail (Packard Instrument Co. Inc.).

HPLC Analysis of Urine Samples

Analytical HPLC was performed using an Agilent Model 1100 HPLC system (Agilent Technologies, Palo Alto, CA) coupled with a β-RAM-Model 3 radioactivity detector (IN/US Systems, Tampa, FL) attached to a Phenomenex (Torrance, CA) Luna C-18 column (5 μm, 150 × 4.6 mm internal diameter [i.d.]). ChemStation (version A.09.01 or A.09.03, Agilent Technologies) was used for system control and data acquisition. Signals were monitored at 254 nm and the column was maintained at 40°C. Preparative HPLC was carried out on the same HPLC system with the exception of a Phenomenex Luna semi-preparative column (5 μm, 250 × 10 mm i.d.). The HPLC mobile phase was 5% acetonitrile in H_2O for 5 min, then linearly increasing acetonitrile content to 95% over 30 min. The flow rate was 1 mL/min for the analytical HPLC and 5 mL/min for semi-preparative HPLC. Four radiolabeled peaks were present as shown in Figure 1.

Isolation and Purification of Radioactive Peaks

Prior to HPLC analysis, urine samples were cleaned using an Eppendorf 5417C microcentrifuge for 10 min at 10,000 RCF (relative centrifugal force) to pellet any particulate
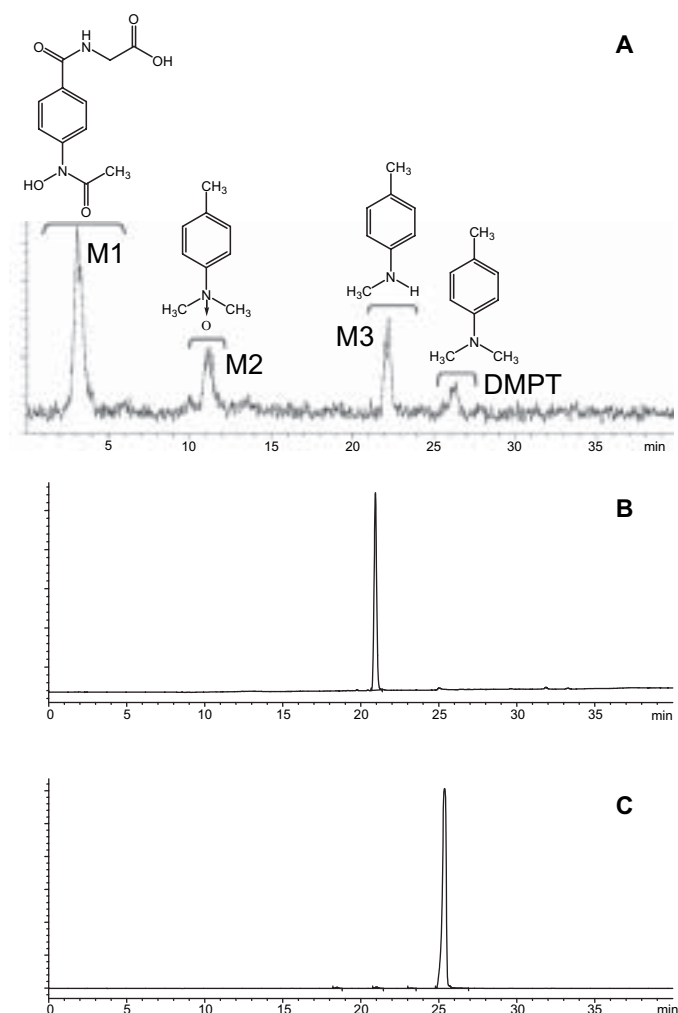

FIG. 1. Radiochromatogram of radiolabeled components (M1, M2, M3, and parent DMPT) isolated from urine of [^{14}C]DMPT-treated male F344 rats (A), nonradiolabeled standards of *N*-methyl-*p*-toluidine (B) and *N,N*-dimethyl-*p*-toluidine (C). The spectra were obtained on the HPLC system; radiochromatogram signals commonly appear approximately 0.5 min after the UV signals.

material. Prior to semi-preparative HPLC, solid-phase extraction (SPE) was performed for crude purification of metabolites using Sep-Pak reversed-phase (C_{18}) cartridges (3 mL, 500 mg sorbent; Waters, Milford, MA). The C_{18} SPE cartridge was preconditioned with 3 mL of H_2O prior to sample loading. Urine (3 mL) from rats was loaded onto the conditioned cartridge. Urine was washed through the cartridge with no elution solvent and collected as fraction 1. The cartridge was then washed with 3 mL of purified water (fraction 2) followed by 3 mL of acetonitrile (fraction 3). Fraction 1 contained M1 and M2 with M1 as the major peak. Fraction 2 also contained M1 and M2 but with M2 as the major peak. Fraction 3 contained M3 and what appeared to be parent DMPT based on chromatographic retention time. Although there were only 4 signals in the radiochromatograms, multiple peaks were detected in the ultraviolet (UV) chromatogram indicating the fractions contained contaminants. The three SPE fractions were purified by semi-preparative HPLC to yield peaks M2, M3, and DMPT as pure samples. M1 still contained multiple UV peaks and additional purification steps were carried out by SPE using Sep-Pak reversed phase (C_{18}, 3 mL, 500 mg sorbent) and Strata ™ X-C (Phenomenex) cation exchange (3 mL, 200 mg sorbent) cartridges. The Strata X-C cartridge was conditioned with 2 mL methanol before equilibration with 2 mL H_2O. Partially purified M1 in a 1-mL H_2O solution was acidified with 20 μL of H_3PO_4, loaded onto the cartridge, and eluted with 2 mL 0.1% aqueous H_3PO_4. The cartridge was dried for 2 min, eluted with 2 mL of 100% methanol and again with 2 mL of 5% aqueous NH_4OH. The methanol fraction containing M1 was further purified by reversed-phase SPE (Sep-Pak C_{18}). The sample was dissolved in 2 mL aqueous 1% NH_4OH. The Sep-Pak was conditioned with 2 mL methanol then 2 mL H_2O. The sample solution was loaded and eluted with 2 mL H_2O then 2 mL of methanol to yield pure M1 in both the radioactive and UV chromatograms.

NMR and MS Analysis

NMR spectra (1D and 2D) were recorded in $CDCl_3$ or CD_3OD using a Bruker AVANCE™ DRX 500 spectrophotometer (500 MHz; Bruker BioSpin Corp., Billerica, MA) with TMS as an internal standard. Liquid chromatography/mass spectrometry (LC/MS) was performed on an Agilent 1100 Series LC system with photodiode array (PDA) detection coupled with an Applied Biosystems MDS Sciex API 4000 G-TRAP Triple Quad MS/MS (Foster City, CA). LC/MS data were acquired with Analyst software (version 1.4.1, Agilent Technologies). The gas chromatography/mass spectrometry (GC/MS) system consisted of an Agilent 6890N Network GC system, a 5973 inert mass selective detector with electron ionization (at 70 eV) or chemical ionization using methane, and a 7683 series injector. Data were acquired with MDS ChemStation software (version D.01.02.16, Agilent Technologies).

Chemical Synthesis

DMPT N-oxide was synthesized according to a method described by Seto and Guengerich (1993) and purified by silica gel column chromatography. The product was analyzed by HPLC, LC/MS, and NMR.

p-Acetaminohippuric acid was synthesized by reaction of p-aminohippuric acid with acetyl chloride. p-Aminohippuric acid was stirred in a solution of sodium hydroxide (1.3 mL, 2N in water) in methanol (7.7 mL) at 0°C. Acetyl chloride (1.0 mL) was added and the mixture stirred for 10 min. Hydrochloric acid (25 mL, 8% in water) was added and the mixture stirred for an additional 30 min. The resulting precipitate was filtered and washed with hydrochloric acid (25 mL, 8% in water) and water (100 mL). The product was analyzed by HPLC, LC/MS, and NMR.

M1 and p-aminohippuric acid were derivatized using a solution of diazomethane in diethyl ether. The diazomethane was generated from Diazald using a Diazald kit (Sigma-Aldrich) to give an approximate concentration of 2N in ethyl ether. Diazomethane (3 mL, 2N) was added to a solution of either M1 or p-aminohippuric acid (39.1 mg) in ethanol (1 mL) and the solutions stirred for 1 hr at room temperature. The solvents were removed under a stream of nitrogen and the products were analyzed by HPLC, LC/MS, and NMR.

M1 and p-aminohippuric acid were also derivatized with trimethylsilyldiazomethane in methanol. Trimethylsilyldiazomethane (100 μL, 2N in diethyl ether) was added to a solution of either M1 or p-aminohippuric acid (38 mg) in methanol (1 mL) and the solutions stirred for 30 min at room temperature. The solvents were removed under a stream of nitrogen and the products were analyzed by HPLC, LC/MS, and NMR.

RESULTS AND DISCUSSION

Four radiolabeled peaks were observed by analytical HPLC in urine from [^{14}C]DMPT-treated rats (Figure 1). Off-column recovery of injected radioactivity was >95%. Urine samples were stored frozen and analyzed periodically over a period of 2 – 3 years. The same chromatographic profile was reproduced over the 2- to 3-yr storage period, indicating that the metabolites are stable in the urine matrix under these conditions. These peaks were separated and purified by SPE and semi-preparative HPLC. The purified peaks were analyzed by analytical HPLC, LC/MS, GC/MS, and NMR and compared to authentic standards to identify their structures. HPLC retention times and NMR data are shown in Table 1.

M3 and a peak suspected to be DMPT were tentatively identified as N-methyl-p-toluidine and parent DMPT by comparing analytical HPLC retention times of M3 (20.9 min) and the suspected DMPT (25.2 min) to authentic standards (Figure 1). The mass fragmentation patterns of M3 and DMPT obtained from GC/MS confirmed M3 as N-methyl-p-toluidine (Figure 2) and the fourth peak as DMPT (Figure 3). This is consistent with P450 2B1-mediated N-dealkylation of N,N-dialkylarylamines reported by Seto and Guengerich (1993).

TABLE 1
HPLC retention times and corresponding ^{1}H NMR and MS data

Analyte	T_R (min)	^{1}H NMR (δ)	MS Fragmentation Pattern (m/z)
M1 (p-(N-acetylhydroxyamino) hippuric acid)	2.5	2.50 (s, 3H), 4.09 (s, 2H), 7.67 (d, 2H, J=8.5 Hz), 7.96 (d, 2H, J=8.9 Hz)	77, 94, 120, 134, 162, 180, 254
p-Acetaminohippuric acid	1.7	2.15 (s, 3H), 4.09 (s, 2H), 7.67 (d, 2H, J=8.5 Hz), 7.82 (d, 2H, J=8.5 Hz)	58, 65, 92, 120, 162, 192, 237
M1-methyl ester (p-(N-acetylhydroxyamino) hippuric acid methyl ester)	16.1	2.17 (s, 3H), 3.61 (s, 3H), 4.13 (s, 2H), 7.51 (m, 2H), 7.93 (m, 2H)	65, 77, 93, 108, 120, 162, 194, 251, 268
p-Acetaminohippuric acid methyl ester	12.1	2.16 (s, 3H), 3.75 (s, 3H), 4.12 (s, 2H), 7.67 (d, 2H, J=8.5 Hz), 7.83 (d, 2H, J=9.0 Hz)	65, 92, 120, 162, 192, 219, 251
M2 (N,N-dimethyl-p-toluidine N-oxide)	10.4	2.40 (s, 3H), 3.68 (s, 6H), 7.26 (m, 2H), 7.80 (m, 2H)	120, 135, 152
N,N-dimethyl-p-toluidine N-oxide	10.4	2.32 (s, 3H), 3.52 (s, 6H), 7.19 (d, 2H, J=9.0 Hz), 7.73 (d, 2H, J=8.5 Hz)	120, 135, 152
M3 (N-methyl-p-toluidine)	20.9	N/A	51, 59, 65, 77, 91, 106, 120, 121
N-Methyl-p-toluidine	20.9	N/A	51, 59, 65, 77, 91, 106, 120, 121
DMPT-parent (N,N-dimethyl-p-toluidine)	25.2	N/A	51, 65, 77, 91, 105, 119, 134, 135
N,N-Dimethyl-p-toluidine	25.2	2.14 (s, 3H), 2.67 (s, 6H), 6.87 (d, 2H, J=8.5 Hz), 7.08 (d, 2H, J=8.3 Hz)	51, 65, 77, 91, 105, 119, 134, 135

In the mass spectra of M2, obtained by LC/MS, the molecular ion peak at m/z 152 [M+H] is 16 amu larger than the parent peak of DMPT (m/z 135), indicating the metabolite may be oxygenated. DMA, a DMPT analog, is metabolized to DMA N-oxide. Therefore, the hypothesized structure of M2 was DMPT N-oxide. Formation of DMPT N-oxide was previously reported by Seto and Guengerich (1993) and is likely mediated by the flavin-containing monooxygenaeses as reported for N,N-dimethylaniline and other N,N-dialkylarylamines (Sherratt and Damani, 1989; Agosin and Ankley, 1987; Gorrod and Gooderham, 1981; Gold and Ziegler, 1973; Hamill and Cooper, 1984; and Grothusen et al., 1996).

The proton NMR spectra of M2 show signals for the 4 sets of protons in M2 and DMPT, indicating that the core structure of DMPT is present in M2. However, the chemical shifts for the 4 signals in M2 are shifted downfield by δ 0.26 – 1.01 ppm from DMPT depending on the distance from the proposed N-oxide. For instance, DMPT displays a signal at δ 2.67 ppm for the methyl protons associated with the amine, and M2 displays a signal at δ 3.68 ppm also for the methyl protons associated with the amine bearing the proposed oxygen. These results suggested that DMPT N-oxidation occurred to yield M2. To confirm this observation, DMPT N-oxide was synthesized. The analytical HPLC retention time of the synthesized DMPT N-oxide (10.2 min) was compared with the retention time of metabolite M2 (10.4 min) and the retention times were

consistent. The proton NMR spectra of the synthesized DMPT N-oxide and M2 confirmed the identification of M2 as DMPT N-oxide. The mass fragmentation pattern was also consistent with the findings of Seto and Guengerich (1993).

Due to the difficulty of separating peaks with early HPLC retention times, M1 was chemically modified to its methyl ester using trimethylsilyldiazomethane. Mass fragmentation patterns obtained by LC/MS indicated the possible presence of a glycine conjugate. The proton NMR of derivatized M1 showed two major signals (δ 7.51 and 7.93 ppm) in the aromatic region, which is consistent with DMPT. These signals integrated for two protons in each of two different environments on the aromatic ring. This indicated that the ring was not more substituted than DMPT. The signal at δ 4.13 ppm also integrated for two protons which correlated to a methylene moiety, possibly from glycine. Signals at δ 2.17 and 3.61 ppm integrated for three protons each, one signal for the methyl protons of the methyl ester of the hippuric acid (derivatized M1) and the other signal for methyl protons of a possible N-acetyl group. Heteronuclear multiple bond correlation (HMBC) NMR studies indicated a three-bond correlation between a set of methylene protons and a carbonyl carbon, which is additional evidence that M1 is a glycine conjugate. HMBC two-bond correlation studies indicated a set of methyl protons adjacent to a carbonyl carbon indicating the presence of an N-acetyl group, which was confirmed by the LC/MS mass fragmentation
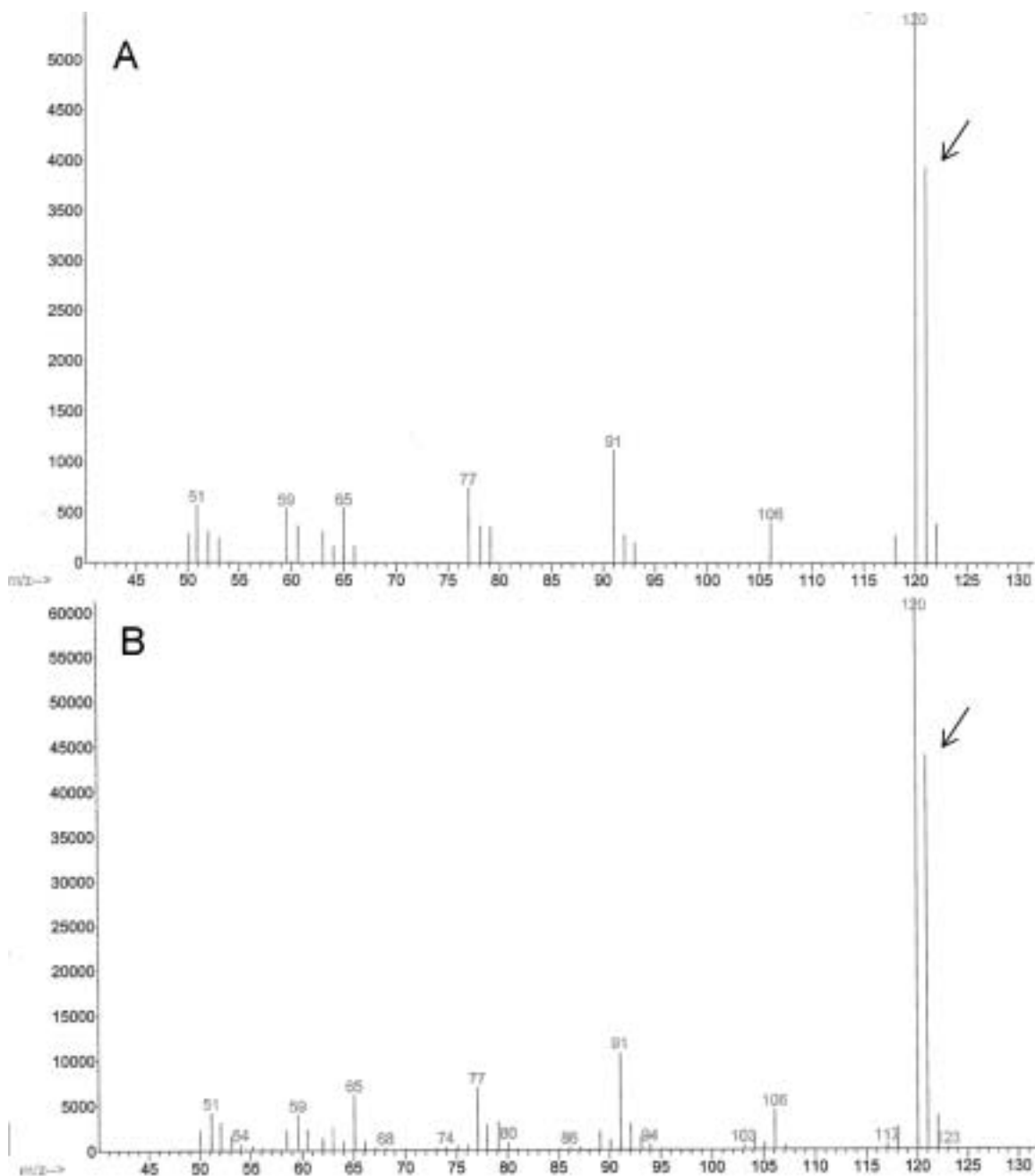
FIG. 2. Mass spectra of M3 (A) and *N*-methyl-*p*-toluidine (B). The molecular ions are identified with arrows.

pattern of the derivatized M1 (*m/z* 120 and *m/z* 162) as shown in Figure 4. With this data at hand, the derivatized M1 was tentatively identified as *p*-acetaminohippuric acid methyl ester, which suggested that M1 is *p*-acetaminohippuric acid. To confirm this, *p*-acetaminohippuric acid methyl ester was synthesized. However, when the retention time of *p*-acetaminohippuric acid methyl ester (12.1 min) was compared with the retention time of derivatized M1 (16.1 min) by analytical HPLC, the retention times were significantly different, indicating that M1 was not *p*-acetaminohippuric acid. The synthesized compound showed a similar proton with NMR as metabolite M1. However, M1 displayed a mass fragment at *m/z* 180 (Figure 4), which was absent in *p*-acetaminohippuric acid (Figure 4). The derivatized M1 and *p*-acetaminohippuric acid methyl ester showed common fragmentation patterns by LC/MS (*m/z* 120, 162) but derivatized M1 displayed an additional mass fragment at *m/z* 194 (Figure 4), suggesting the possible presence of an additional hydroxyl group. The mass fragmentation pattern along with the proton NMR spectrum allowed localization of the hydroxyl group as attached to the *N*-acetyl nitrogen. The
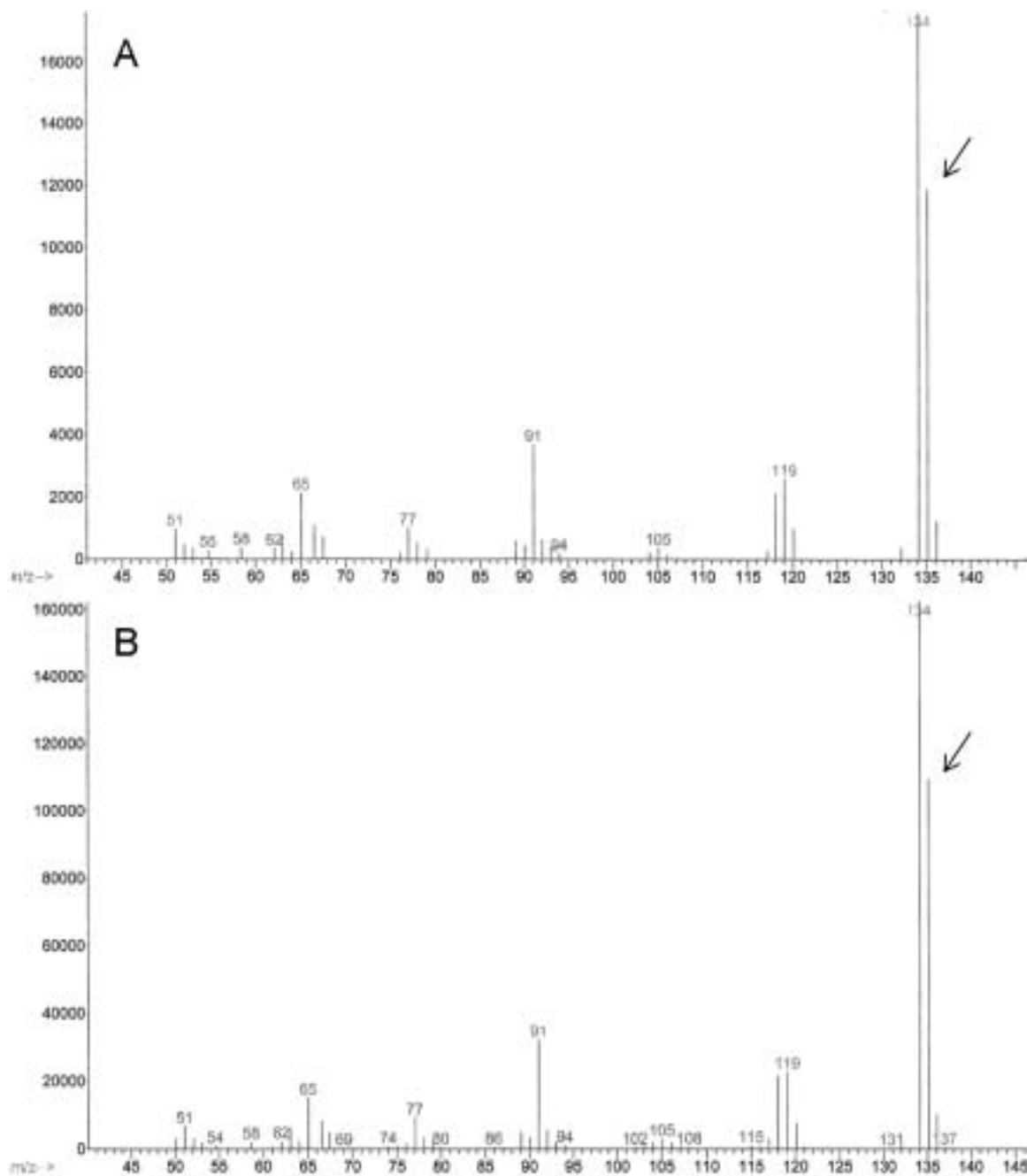
FIG. 3. Mass spectra of DMPT-parent (A) and *N*,*N*-dimethyl-*p*-toluidine (B). The molecular ions are identified with arrows.

N-oxidation in M1 was confirmed by synthesis. Derivatization of M1 and *p*-acetaminohippuric acid to their respective methyl esters using both diazomethane and (trimethylsilyl)diazomethane would distinguish if the hydroxyl group was located on the aromatic ring or on the *N*-acetyl nitrogen. Diazomethane is a potent methylating agent and methylates an aromatic hydroxyl group, whereas (trimethylsilyl)diazomethane is a weaker methylating agent and may not readily methylate an aromatic hydroxyl group. When these two reagents were employed, the M1 derivatization products displayed the same HPLC chromatogram retention times and LC/MS fragmentation patterns, indicating that no aromatic hydroxyl group was present in M1. Therefore, the data indicate that the structure of M1 is *p*-(*N*-acetylhydroxyamino)hippuric acid. A proposed metabolism scheme for DMPT is shown in Figure 5.
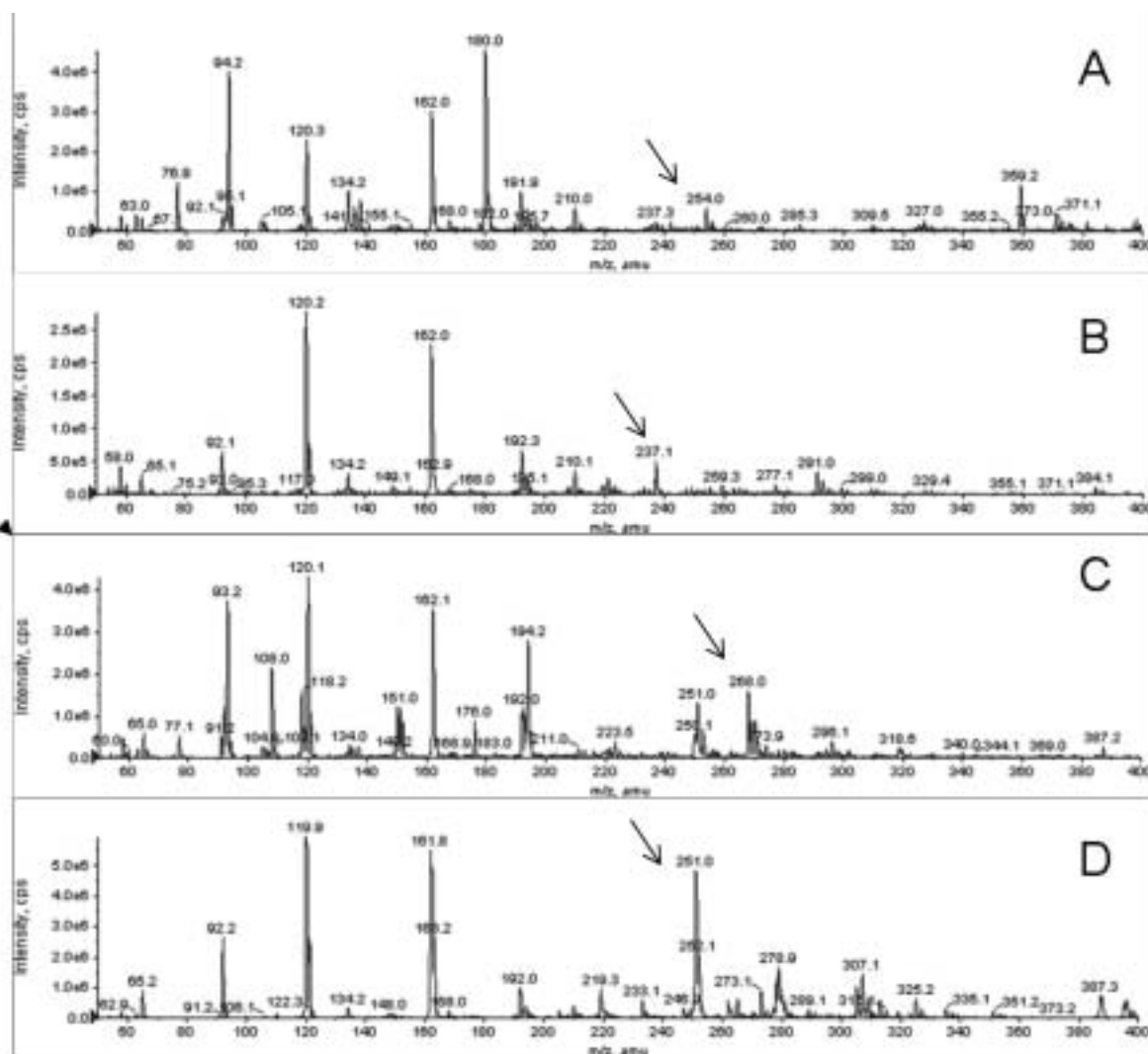
FIG. 4. Mass spectra of M1: (A) metabolite M1, (B) *p*-acetaminohippuric acid, (C) metabolite M1 methyl ester, and (D) *p*-acetaminohippuric acid methyl ester. The molecular ions are identified with arrows.

In summary, 4 major radiolabeled peaks in [^{14}C]DMPT-treated rats were isolated and identified as DMPT-parent, *N*-methyl-*p*-toluidine, DMPT *N*-oxide, and *p*-(*N*-acetylhydroxylamino)hippuric acid. *N*-demethylation and *N*-oxidation are known cytochrome P450-mediated metabolic pathways for DMPT (Seto and Guengerich, 1993; MacDonald et al., 1989). Based on the metabolism of DMA to phenylhydroxlamine, which produces methemoglobinemia, the metabolite putatively responsible for DMPT-induced methemoglobinemia is *p*-methylphenylhydroxylamine. *p*-Methylphenylhydroxylamine was not identified in urine. However, *p*-(*N*-acetylhydroxyamino) hippuric acid, which is the glycine conjugate of *N*-acetylated *p*-methylphenylhydroxylamine was present. Therefore, it is reasonable to speculate that *p*-methylphenylhydroxylamine is formed from DMPT *in vivo*. Overall DMPT metabolism is consistent with the metabolism of DMA.

Spectral Data

NMR spectra were recorded in CDCl$_3$ or CD$_3$OD on a Bruker Avance DRX 500 spectrophotometer (500 MHz) with TMS as an internal standard. Chemical shifts are reported in parts per million (Table 1). Retention times (T_R) were obtained by analytical HPLC on an Agilent Model 1100 HPLC system coupled with a β-RAM-Model 3 radioactivity detector attached to a Phenomenex Luna C-18 column. Retention times are recorded in minutes. Mass spectra for M1 and M2 were obtained by LC/MS on an Agilent 1100 Series LC system with photodiode assay detection coupled with an Applied Biosystems MDS Sciex API 4000 G-TRAP Triple Quad MS/MS. Mass Spectra for M3 and DMPT were obtained by GC/MS on an Agilent 6890N Network GC system, a 5973 inert mass selective detector with electron ionization, and a 7683 series injector.
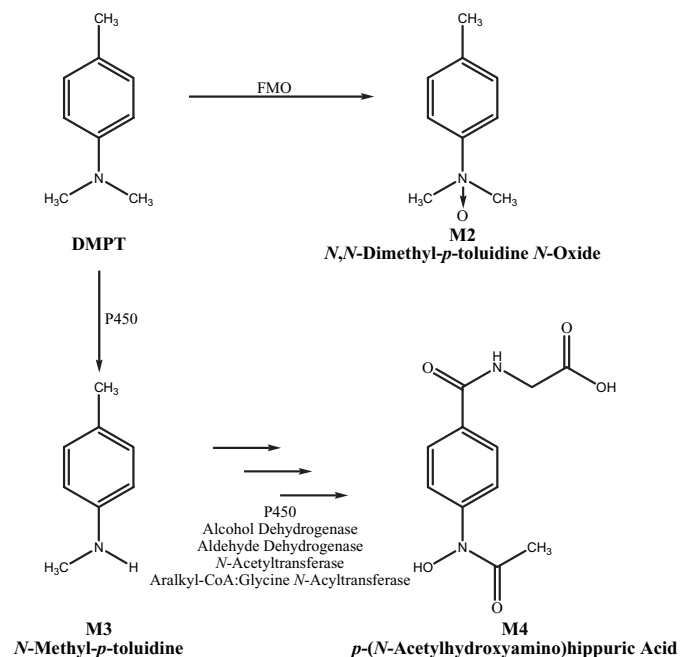
FIG. 5. Proposed metabolism scheme for DMPT. FMO=flavin-containing monooxygenase; P450=cytochrome P450.

ACKNOWLEDGMENTS

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