Biotransformation of [^{12}C]- and [^{13}C]-*tert*-Amyl Methyl Ether and *tert*-Amyl Alcohol

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tert-Amyl methyl ether (TAME) is intended for use as a gasoline additive to increase oxygen content. Increased oxygen content in gasoline reduces tailpipe emissions of hydrocarbons and carbon monoxide from cars. Due to possible widespread use of TAME, the toxicity of TAME is under investigation. We studied the biotransformation of TAME in rats and one human volunteer after inhalation of ^{12}C- or ^{13}C-labeled TAME. In addition, the biotransformation of [^{13}C]-*tert*-amyl alcohol was studied in rats after gavage. Urinary metabolites were identified by GC/MS and ^{13}C NMR. Rats (two males and two females) were individually exposed to 2000 ppm [^{12}C]- or [^{13}C]TAME for 6 h, and urine was collected for 48 h. Free and glucuronidated 2-methyl-2,3-butanediol and a glucuronide of *tert*-amyl alcohol were identified by ^{13}C NMR, GC/MS, and LC/MS/MS as major urinary metabolites on the basis of the relative intensities of the ^{13}C NMR signals. The presence of several minor metabolites was also indicated by ^{13}C NMR; they were identified as *tert*-amyl alcohol, 2-hydroxy-2-methylbutyric acid, and 3-hydroxy-3-methylbutyric acid. One human volunteer was exposed to an initial concentration of 27 000 ppm [^{13}C]TAME by inhalation for 4 min from a 2 L gas sampling bag, and metabolites of TAME excreted in urine were analyzed by ^{13}C NMR. All TAME metabolites identified in rats were also present in the human urine samples. To study *tert*-amyl alcohol biotransformation, male rats ($n = 3$) were treated with 250 mg/kg [^{13}C]-*tert*-amyl alcohol dissolved in corn oil by gavage, and urine was collected for 48 h. ^{13}C NMR of the urine samples showed the presence of metabolites identical to those in the urine of [^{13}C]TAME-treated rats. Our results suggest that TAME is extensively metabolized by rats and humans to *tert*-amyl alcohol which may be further oxidized to diols and carboxylic acids. These reactions are likely mediated by cytochrome P450-dependent oxidations.

Introduction

Increased oxygen content in gasoline sold in certain areas of the United States is required by the 1990 Amendments to the Clean Air Act in areas that failed to meet the National Ambient Air Quality standard for carbon monoxide or ozone. The chemicals blended with gasoline hydrocarbons to meet the required oxygen content are termed "oxygenates" (*1*). At present, the oxygenates most often used are methyl *tert*-butyl ether and methanol. However, other ethers may be used in the future. *tert*-Amyl methyl ether (TAME)[1] is also used as an oxygenate due to relatively low production costs, the fact that its vapor pressure is lower than those of other ethers, and its ability to act as a high-octane gasoline blending compound (*2*, *3*).

Due to the potential widespread exposure of humans to oxygenates in fuel (*3*, *4*), studies on the toxicity of TAME are underway. The major toxic effect seen in rodents after inhalation of high concentrations of TAME is central nervous system depression. Subchronic inhalation studies with TAME in rats have found increased liver weights in rats exposed to 4000 ppm TAME for 4 weeks (6 h per day, 5 days per week). Significantly reduced body weights and relative increases in adrenal, kidney, testes, brain, and lung weights were seen in male rats exposed to 4000 ppm TAME, but there were no treatment-related histopathologic findings (*5*).

The biotransformation of TAME has apparently not been studied. *tert*-Amyl alcohol is a likely metabolite of TAME formed by cleavage of the ether bond; however, further metabolism of *tert*-amyl alcohol is likely as observed with other more lipophilic alcohols. For example, *tert*-butanol is extensively metabolized in rodents and humans (*6*).

In this study, we investigated the biotransformation of TAME in rats with the aim of identifying metabolites excreted in urine. Both [^{12}C]TAME and ^{13}C-labeled TAME were used for these studies. The use of ^{13}C-labeled TAME permits the detection of metabolites in urine by ^{13}C NMR without manipulations or chromatography and therefore may help in identifying metabolites with low stability or volatility and products formed by the possible reaction of TAME metabolites with endogenous chemicals (*7*−*9*).

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[1] Abbreviations: TAME, *tert*-amyl methyl ether; MS/MS, secondary ion mass spectrometry.

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Materials and Methods

Animals and Treatment. Male and female Fischer F344 rats were obtained from Harlan Winkelmann (Borchen, Germany). Animals were kept at constant humidity and temperature (21 °C) in the animal facility of the department with a 12 h light/dark cycle. Before the experiments, the animals were acclimated to the metabolic cages for 3 days, and control urine was collected during this time for 12 h before the exposure.

Exposure of One Human Volunteer to [^{13}C]TAME by Inhalation. A 2 L gas sampling bag (Linde, Germany) was filled with 99.9% oxygen, and 300 μL of [^{13}C]TAME was added with a microliter syringe through a septum to give a concentration of 27 000 ppm. The volunteer inhaled the contents of the bag for 4 min and exhaled into the bag to ensure maximum uptake of [^{13}C]TAME. Urine samples were taken in 6 h intervals for 48 h.

Chamber Design and Treatment. The design of the exposure chamber has been described in detail (*10*). The animals were introduced into the chamber at 10 a.m., and the calculated amounts of the ^{12}C- or ^{13}C-labeled ethers were introduced with a microliter syringe. The concentrations of the ethers in the gas phase of the chamber were monitored by an automatic gas sampling valve every 10 min. The gas phase (100 μL) was introduced into a capillary gas chromatograph (HP model 5970) and separated with a 40 m DB1-coated fused silica column (DB1, J&W Scientific, 40 m, 0.18 mm i.d., 0.4 μm film thickness). Ether concentrations in the air were quantified by flame ionization detection (FID). After the end of the 6 h exposure period, the animals were transferred to metabolic cages and the urine was collected on ice in 24 h intervals over the course of 48 h. Two male and two female rats were individually exposed to each of the two [^{13}C]- and [^{12}C]ethers.

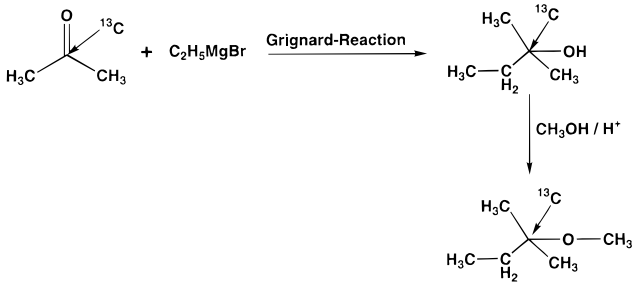
Metabolite Analysis in Urine. Urine obtained from the animals exposed to ^{13}C-labeled ethers or control urine (720 μL) was introduced into separate NMR tubes (5 mm i.d.), and 80 μL of D_2O was added. These samples were directly analyzed by ^{13}C NMR (*6*). Some of the urine samples were treated with β-glucuronidase (Sigma, G7846, lot no. 72H6836) for 30 min at 37 °C or sulfatase (Sigma, S9751, lot no. 621-07881) for 48 h at 37 °C (*11*). Aliquots of the treated urine samples were then also analyzed by NMR. For acid treatment, urine samples were acidified to pH 2 with concentrated hydrochloric acid and incubated for 1 h at 90 °C.

For gas chromatography/mass spectrometry, 0.5 mL of the urine samples was introduced into 1.5 mL sample vials, acidified with 2 N HCl to pH 4, and kept at 80 °C for 30 min. A volume (100 μL) of the headspace from the incubations was then injected into the gas chromatograph. To analyze less volatile metabolites, some samples (0.5 mL) were extracted with 0.5 mL of ethyl acetate, and 1 μL of the ethyl acetate layer was injected into the gas chromatograph.

To identify acidic metabolites, 0.5 mL of the urine samples was taken to dryness by lyophilization and the residues that were obtained were treated with 500 μL of BF_3/methanol (14%) at 60 °C for 30 min. Samples were then diluted with 250 μL of water and extracted with 1 mL of chloroform. The chloroform layers were dried over sodium sulfate, and 1 μL of the solution was injected into the gas chromatograph. Separation was performed on a 30 m × 0.25 mm fused silica column coated with DB-WAX (0.25 μm film thickness). For analysis, a temperature gradient from 35 to 230 °C with a heating rate of 10 °C/min was applied. Electron impact mass spectra (70 eV) were recorded and metabolite peaks identified by comparison of the chromatograms of the urines from treated rats with those of untreated controls.

Instrumental Analysis. ^{13}C NMR spectra were recorded with a Bruker 250 MHz spectrometer (Bruker AC 250) or a Bruker 600 MHz NMR spectrometer (Avance 600). Usually, 2000 scans were aquired for Fourier transformation (*6*). Gas chromatography/mass spectrometry was performed with a Fisons MD 800 mass spectrometer coupled to a Carlo Erba GC 8000 series gas chromatograph. Split injection (split ratio of 1:5) was used, and spectra were recorded from *m*/*z* 30 to 300 in 1 s intervals.

Scheme 1. Synthesis of 2-[^{13}C]-*tert*-Amyl Methyl Ether



Secondary ion mass spectrometry (MS/MS) was performed using a Finnigan TSQ-700 triple-quadropole mass spectrometer with electrospray ionization. Samples were introduced at a flow rate of 0.5 mL/min in water adjusted to pH 3 by addition of formic acid. LC/MS and LC/MS/MS spectra were obtained by automatically switching between positive- and negative-ion modes in alternating scans. The electrospray voltage was 4.5 kV, and the capillary temperature was 200 °C. MS/MS spectra were recorded with a collision energy of 30 eV. To isolate the nonvolatile metabolites of TAME, HPLC with an evaporative light scattering detector (Sedere 55, Knauer, Germany) was used due to the low UV absorption of the metabolites. Urine samples were separated on a 250 mm × 4 mm steel column filled with Partisil ODS-III using gradient elution. A linear gradient from 100% water (acidified with formic acid to pH 3) to 50% water/acetonitrile over the course of 25 min at a flow rate of 1 mL/min was used for separation.

Synthesis of 2-[^{13}C]-*tert*-Amyl Alcohol. A solution of 60 mmol of ethyl bromide (Aldrich, Steinheim, Germany) in diethyl ether was slowly added to equimolar amounts of magnesium turnings (1.5 g) covered by 5.0 mL of diethyl ether (Scheme 1). The Grignard reaction was initiated by the addition of traces of iodine (*12*, *13*). The mixture was stirred for 30 min at reflux; a solution of 50 mmol of 2-[^{13}C]acetone (lot no. P-7787, CIL Cambridge Isotope Laboratories, Andover, MA) in diethyl ether was added over the course of 30 min, and the mixture was kept at reflux for 2 h. After cooling, hydrolysis was performed with 30 mL of an ice-cold, saturated NH_4Cl solution. The layers were separated, and the aqueous layer was extracted five times with 10 mL of diethyl ether. The ether layers were combined and dried over K_2CO_3. After evaporation of the solvent, the residue was distilled to yield 2-[^{13}C]-*tert*-amyl alcohol (61% yield, 98% GC/FID purity). The structures of the reaction product was confirmed by GC/MS and ^{1}H and ^{13}C NMR.

2-[^{13}C]-*tert*-Amyl alcohol: ^{1}H NMR (250 MHz, D_2O) δ 0.89 [3H, t, J = 8 Hz, J_{HC} = 4 Hz, CH_3CH$_2$C(CH$_3$)$_2$OH], 1.19 [6H, s, J_{HC} = 4 Hz, CH$_3$CH$_2$C(CH_3)$_2$OH], 1.52 [2H, q, J = 8 Hz, J_{HC} = 4 Hz, CH$_3$CH_2C(CH$_3$)$_2$OH]; ^{13}C NMR (63 MHz, D_2O) δ 10.7 [s, *C*H$_3$CH$_2$C(CH$_3$)$_2$OH], 30.0 [d, J_{CC} = 40 Hz, CH$_3$CH$_2$C(*C*H$_3$)$_2$OH], 38.1 [d, J_{CC} = 39 Hz, CH$_3$*C*H$_2$C(CH$_3$)$_2$OH], 75.0 [s, d with 0.5% of a satellite doublet J_{CC} = 39 Hz, CH$_3$CH$_2$*C*(CH$_3$)$_2$OH]; MS (70 eV) *m*/*z* (relative intensity) 74 ($M^+ - CH_3$, 85), 60 ($M^+ - C_2H_5$, 100), 56 (68), 44 (33), 43 (17), 42 (19), 41 (6), 40 (12).

Synthesis of 2-[^{13}C]TAME from 2-[^{13}C]-*tert*-Amyl Alcohol. A mixture of 60 mmol of methanol and 6 mL of 10% H_2SO_4 was heated to 65 °C, and then 20 mmol of 2-[^{13}C]-*tert*-amyl alcohol was added with a syringe (*14*). When the temperature of the mixture was increased to 100 °C, an azeotropic mixture of 2-[^{13}C]TAME, methanol, 2-methyl-2-butene, 2-methyl-1-butene, and *tert*-amyl alcohol were distilled off. Methanol was removed from the reaction mixture by extraction with water. Further purification was performed by slow distillation with dry ice cooling on a Büchi-GKR-51 Kugelrohr apparatus to yield 10% 2-[^{13}C]TAME. The GC/FID purity of the reaction product was

>98%. The structure of the reaction product was confirmed by GC/MS and ^{1}H and ^{13}C NMR.

2-[^{13}C]TAME: ^{1}H NMR (250 MHz, D_2O) δ 0.85 [3H, t, J = 8 Hz, J_{HC} = 4 Hz, C*H*$_3$CH$_2$C(CH$_3$)$_2$OCH$_3$], 1.17 [6H, s, J_{HC} = 4 Hz, CH$_3$CH$_2$C(C*H*$_3$)$_2$OCH$_3$], 1.55 [2H, q, J = 8 Hz, J_{HC} = 4 Hz, CH$_3$C*H*$_2$C(CH$_3$)$_2$OCH$_3$], 3.20 [3H, s, J_{HC} = 4 Hz, CH$_3$CH$_2$C(CH$_3$)$_2$OC*H*$_3$]; ^{13}C NMR (63 MHz, D_2O) δ 10.4 [s, *C*H$_3$CH$_2$C(CH$_3$)$_2$OCH$_3$], 26.5 [d, J_{CC} = 36 Hz, CH$_3$CH$_2$C(*C*H$_3$)$_2$OCH$_3$], 33.9 [d, J_{CC} = 38 Hz, CH$_3$*C*H$_2$C(CH$_3$)$_2$OCH$_3$], 51.2 [s, CH$_3$CH$_2$C(CH$_3$)$_2$OC*H*$_3$], 80.1 [s, d with 0.5% of a satellite doublet J_{CC} = 40 Hz, CH$_3$CH$_2$*C*(CH$_3$)$_2$OCH$_3$]; MS (70 eV) *m*/*z* (relative intensity) 88 (M$^+$ − CH$_3$, 75), 74 (M$^+$ − C$_2$H$_5$, 100), 72 (58), 56 (88), 46 (15), 44 (79), 42 (30), 41 (12), 40 (19).

Synthesis of 2-Methyl-2,3-butanediol. 2-Methyl-2,3-butanediol was prepared by reduction of 3-hydroxy-3-methyl-2-butanone (*13*). LiAlH$_4$ (30 mmol, 1,120 mg) was dissolved in 15 mL of diethyl ether followed by slow addition of 12 mmol of 3-hydroxy-3-methyl-2-butanone in 10 mL of diethyl ether at room temperature. The mixture was then stirred for 2 h at reflux. After the mixture had been cooled with ice, 2.5 mL of water and then 2 mL of a 20% solution of sodium hydroxide were added. After extraction with diethyl ether, the organic layers were combined and dried over K_2CO_3. After removal of the solvent, 2-methyl-2,3-butanediol was isolated by fractional distillation under reduced pressure.

2-Methyl-2,3-butanediol: ^{1}H NMR (250 MHz, D_2O) δ 1.15 [3H, d, J = 6 Hz, C*H*$_3$CH(OH)C(CH$_3$)$_2$OH], 1.17 [9H, s, CH$_3$-CH(OH)C(C*H*$_3$)$_2$OH], 3.63 [1H, q, J = 6 Hz, CH$_3$C*H*(OH)C(CH$_3$)$_2$-OH]; ^{13}C NMR (63 MHz, D_2O) δ 19.3 [s, *C*H$_3$CH(OH)C(CH$_3$)$_2$-OH], 25.9 [s, CH$_3$CH(OH)C(*C*H$_3$)$_2$OH], 27.0 [s, CH$_3$CH(OH)C-(*C*H$_3$)$_2$OH], 76.4 [s, CH$_3$CH(OH)*C*(CH$_3$)$_2$OH], 76.7 [s, CH$_3$*C*H-(OH)C(CH$_3$)$_2$OH]; MS (70 eV) *m*/*z* (relative intensity) 89 (M$^+$ − CH$_3$, 9), 71 (24), 59 (100), 45 (22), 43 (91), 41 (27).

Synthesis of 2-Methyl-1,2-butanediol. 2-Methyl-1,2-butanediol was prepared by reduction of 2-hydroxy-2-methylbutyric acid (Aldrich). LiAlH$_4$ (25 mmol, 960 mg) was dissolved in 12 mL of diethyl ether followed by slow addition of 12 mmol of 2-hydroxy-2-methylbutyric acid in 10 mL of diethyl ether at room temperature. The mixture was then stirred for 2 h at reflux. After the mixture had been cooled with ice, 2.5 mL of water followed by 2 mL of a 20% solution of sodium hydroxide was added. After extraction with diethyl ether, the organic layers were combined and dried over K_2CO_3. After removal of the solvent, 2-methyl-1,2-butanediol was isolated by fractional distillation under reduced pressure.

2-Methyl-1,2-butanediol: ^{1}H NMR (250 MHz, D_2O) δ 0.89 [3H, t, J = 8 Hz, C*H*$_3$CH$_2$C(CH$_3$)(OH)CH$_2$OH], 1.13 [3H, s, CH$_3$-CH$_2$C(C*H*$_3$)(OH)CH$_2$OH], 1.52 [2H, q, J = 8 Hz, CH$_3$C*H*$_2$C(CH$_3$)-(OH)CH$_2$OH], 3.43 [2H, s, CH$_3$CH$_2$C(CH$_3$)(OH)C*H*$_2$OH]; ^{13}C NMR (63 MHz, D_2O) δ 9.9 [s, *C*H$_3$CH$_2$C(CH$_3$)(OH)CH$_2$OH], 24.5 [s, CH$_3$CH$_2$C(*C*H$_3$)(OH)CH$_2$OH], 32.8 [s, CH$_3$*C*H$_2$C(CH$_3$)(OH)CH$_2$-OH], 70.8 [s, CH$_3$CH$_2$C(CH$_3$)(OH)*C*H$_2$OH], 76.4 [s, CH$_3$CH$_2$*C*-(CH$_3$)(OH)CH$_2$OH]; MS (70 eV) *m*/*z* (relative intensity) 89 (M$^+$ − CH$_3$, 41), 75 (89), 73 (98), 71 (54), 58 (42), 57 (94), 55 (83), 53 (30), 45 (73), 42 (100), 41 (79).

Chemicals. 2-Hydroxy-2-methylbutyric acid and 3-hydroxy-3-methyl-2-butanone were purchased from Aldrich, and 3-hydroxy-3-methylbutyric acid and 2-methyl-2,4-butanediol were obtained from Tokyo Kasei Kogyo Ltd. (Tokyo, Japan). All other chemicals were obtained from commercial suppliers at the highest purity available.

Results

Synthesis of ^{13}C-Labeled *tert*-Amyl Alcohol and TAME. The ^{13}C-labeled compounds were synthesized for identification of TAME and *tert*-amyl alcohol metabolites without the need for ^{14}C-label and detection of nonvolatile metabolites. The use of ^{13}C-labeled compounds permits metabolite identification and may provide information about the relative quantities of metabolites that are present. The ^{13}C-labeled compounds were synthesized by standard procedures (Scheme 1); the purity of TAME and *tert*-amyl alcohol used for the exposures was >98% as checked by GC/MS.

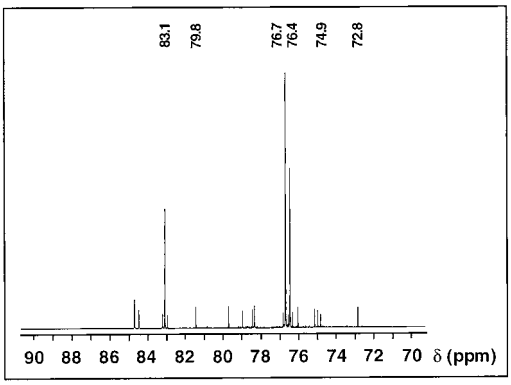


Figure 1. ^{13}C NMR spectrum (151 MHz, 2000 scans) of a 24 h urine sample from a male Fisher F344 rat exposed to 2000 ppm 2-[^{13}C]TAME for 6 h. The following structural assignments for the NMR signals were made: δ 72.8, 3-hydroxy-3-methylbutyric acid; δ 74.9, *tert*-amyl alcohol; δ 76.4, 2-methyl-2,3-butanediol; δ 76.7, glucuronide of 2-methyl-2,3-butanediol; δ 79.8, 2-hydroxy-2-methylbutyric acid; and δ 83.1, glucuronide of *tert*-amyl alcohol.

Biotransformation of TAME. Two male and two female rats were individually exposed by inhalation to 2000 ppm (initial concentration) [^{12}C]- or [^{13}C]TAME. Monitoring of the exposure chamber air for TAME indicated a continuous decrease in the air concentrations of TAME due to uptake of the ethers by the rats and biotransformation to less volatile metabolites (data not shown). At the end of the exposure period of 6 h, the TAME concentrations in the chamber were less than 300 ppm, indicating extensive uptake and biotransformation of TAME by the rats. Analysis of the chamber air by gas chromatography did not reveal the formation of volatile and exhaled metabolites of TAME that could be detected by the flame ionization detector. At the end of the exposure period, the animals were transferred to metabolic cages and urine samples were collected in 24 h intervals for 48 h and analyzed by GC/MS and ^{13}C NMR spectroscopy to identify metabolites.

A typical NMR spectrum of a urine sample from a male rat exposed to [^{13}C]TAME is shown in Figure 1. The ^{1}H-decoupled ^{13}C NMR spectrum exhibited several resonances which were also present in the spectra of urine from control animals (data not presented). The structures of these endogenous compounds were assigned to urea, creatinine, hippurate, and glucose by comparison with literature data (*15*) and reference spectra (*6*). The ^{13}C atom in [^{13}C]TAME metabolites was expected to give resonances in the range of chemical shifts between 70 and 90 ppm. In control urine, only one resonance was observed in that region of the ^{13}C NMR spectra (δ 78.3). Urine from animals exposed to [^{13}C]TAME exhibited several signals in the region between 70 and 90 ppm, indicative of three major and at least three minor metabolites. The structures of metabolites were elucidated by a combination of ^{13}C NMR spectroscopy, GC/MS, and LC/MS/MS.

The NMR signal at δ 72.8 was identical in chemical shift to C-2 (which carries the ^{13}C-label) of 3-hydroxy-3-methylbutyric acid. The presence of this compound in the urine of both [^{12}C]- and [^{13}C]TAME-treated rats was also confirmed by GC/MS. Gas chromatographic separation

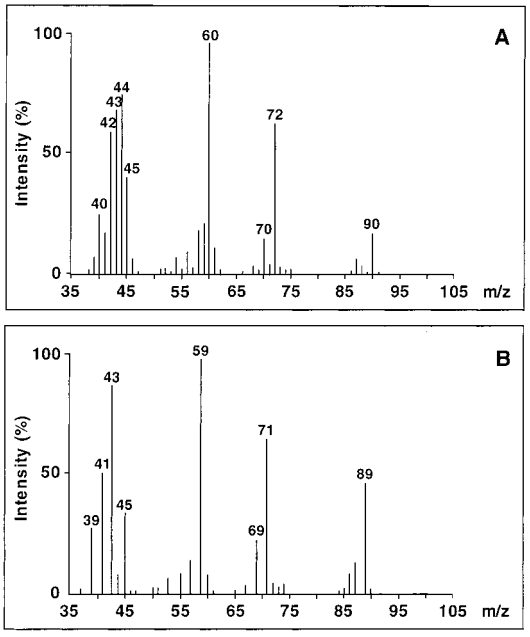


Figure 2. Mass spectrum of 2-methyl-2,3-butanediol present in the urine (collected for 24 h after the end of exposure) of a male Fischer F344 rat exposed to 2-[^{13}C]TAME (2000 ppm) for 6 h (A) and to [^{12}C]TAME (2000 ppm) for 6 h (B).

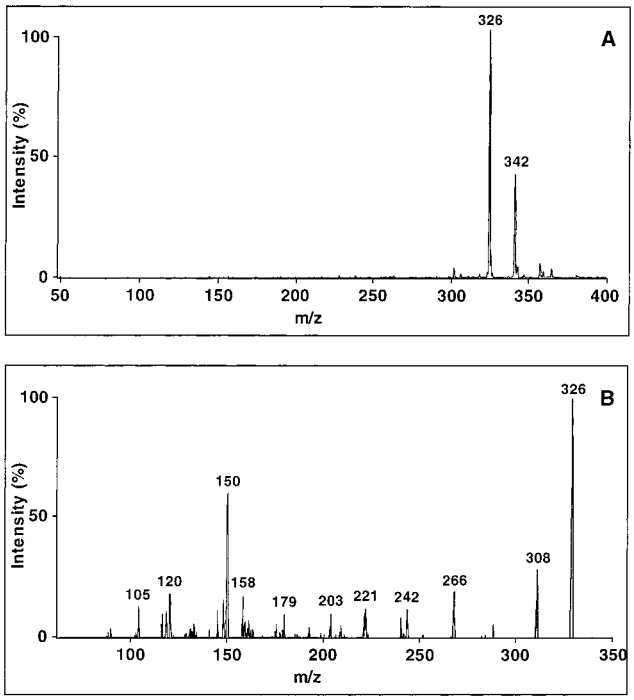


Figure 3. Electrospray mass spectrum (A) and further fragmentation (*m/z* 326) (B) of the isolated metabolite with a ^{13}C resonance at δ 76.7 at a collision energy of 30 mV. Spectra were recorded after direct loop injection of the metabolite isolated by preparative HPLC.

of the urine from those animals showed peaks not present in urine from control animals. The mass spectra of one of these peaks from [^{12}C]TAME-treated animals were identical to those of authentic 3-hydroxy-3-methylbutyric acid (data not shown). In the mass spectra of this peak obtained by separation of urine from [^{13}C]TAME-treated animals, several fragments were shifted by 1 mass unit, suggesting the presence of ^{13}C in the molecule (data not presented). These observations confirm 3-hydroxy-3-methylbutyric acid as a minor urinary metabolite of TAME in rats.

The minor signal in the NMR spectrum of urine from [^{13}C]TAME-treated animals at δ 74.9 was identical in chemical shift to that of C-2 in *tert*-amyl alcohol. This compound was also identified as a minor TAME metabolite by GC/MS (data not shown).

The signal at δ 76.4 ppm represents a major TAME metabolite in the rat. The C-2 atom of synthetic 2-methyl-2,3-butanediol also exhibited a resonance at δ 76.4. Moreover, mass spectra recorded from a peak present in the urine of [^{12}C]TAME-treated rats, but not in the urine of control animals, were identical to that of 2-methyl-2,3-butanediol (Figure 2). Again, in GC/MS of urine samples from [^{13}C]TAME-treated rats, several fragments of the compound were shifted by 1 mass unit due to the high abundance of ^{13}C in the molecule (Figure 2). These observations confirm 2-methyl-2,3-butanediol as a major metabolite of TAME in rat urine.

The most abundant signal in the ^{13}C NMR spectrum from urine of [^{13}C]TAME-treated rats at δ 76.7 was identified as a glucuronide of 2-methyl-2,3-butanediol on the basis of the following observations. (1) Electrospray MS/MS of the metabolite isolated from the urine of rats exposed to [^{13}C]TAME by HPLC showed signals at *m/z* 326 and 342 (Figure 3). (2) The metabolite from [^{12}C]-TAME had signals at *m/z* 325 and 341, indicating the addition of two sodium ions or one sodium and one potassium ion to the molecular ion of 2-methyl-2,3-butanediol glucuronide (Figure 3). The ion at *m/z* 326

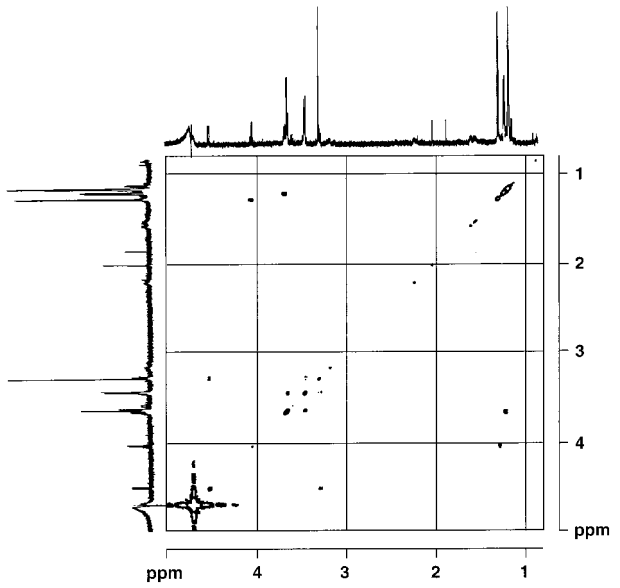


Figure 4. COSY-NMR spectrum of the isolated metabolite with a ^{13}C resonance at δ 76.7. For recording NMR spectra, the metabolite was isolated by preparative HPLC and dissolved in D_2O.

produced a prominent secondary ion at *m/z* 150 (glucuronic acid) and several minor fragments indicative of the consecutive loss of water and/or ketene. COSY-NMR spectra (Figure 4) of the isolated peak exhibited a set of resonances typical for glucuronic acids and their derivatives (*16*) (δ 3−5) and the presence of the 2-methyl-2,3-butanediol moiety containing ^{13}C (δ 1−1.2, 3.6). Moreover, the presumed glucuronide was slowly cleaved by glucuronidase to give 2-methyl-2,3-butanediol. Taken together, the data conclusively identify the major urinary metabolite of TAME as a glucuronide of 2-methyl-2,3-butanediol.

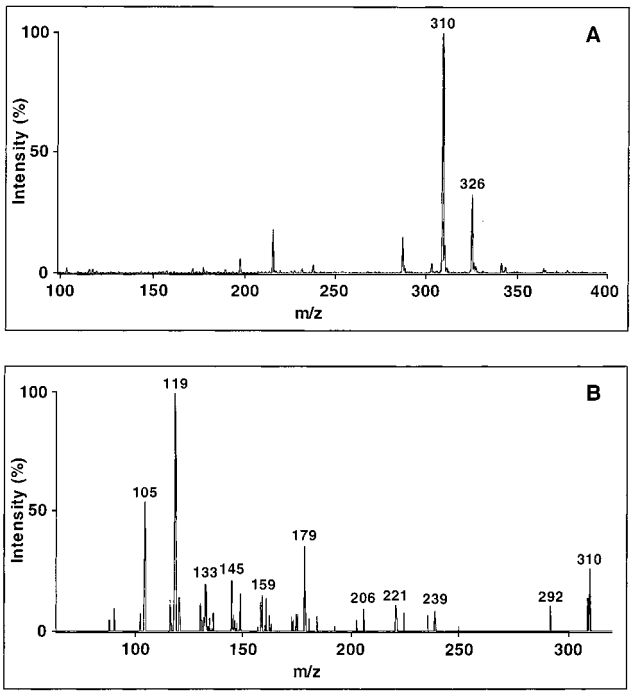


Figure 5. Electrospray mass spectrum (A) and further fragmentation (*m*/*z* 310) (B) of the isolated metabolite with a ^{13}C resonance at δ 83.1 at a collision energy of 30 mV. Spectra were recorded after direct loop injection of the metabolite isolated by preparative HPLC.

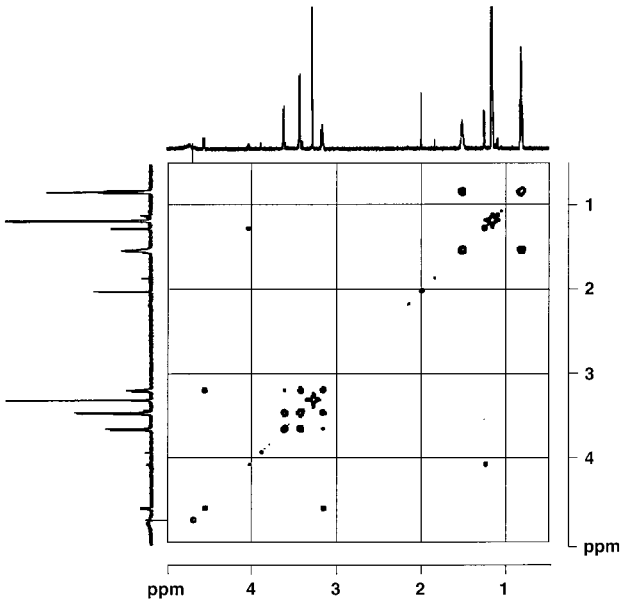


Figure 6. COSY-NMR spectrum of the isolated metabolite with a ^{13}C resonance at δ 83.1. For recording NMR spectra, the metabolite was isolated by preparative HPLC and dissolved in D_2O.

The third intense signal (δ 83.1) in the ^{13}C NMR spectra of urine samples from [^{13}C]TAME-treated rats also slowly disappeared after acid (or glucuronidase) treatment of the urine. This observation suggests that the compound also represents a glucuronide. This assumption is supported by electrospray mass spectra (Figure 5) and COSY-NMR spectra (Figure 6) of the isolated compound. The mass spectra that were recorded were very similar to those of the glucuronide of 2-methyl-2,3-butanediol with a difference of *m*/*z* 16 in major fragments suggesting a glucuronide of *tert*-amyl alcohol.

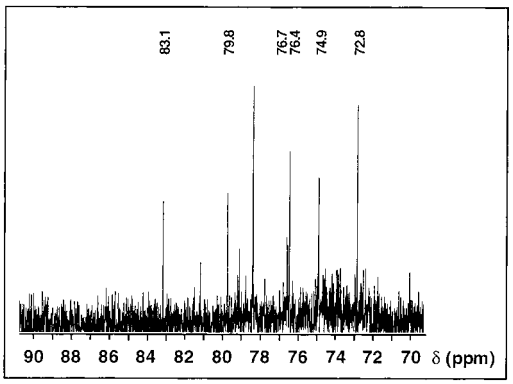


Figure 7. ^{13}C NMR spectrum (151 MHz, 32 000 scans) of a 6 h urine sample from a male human volunteer exposed to 27 000 ppm TAME (in a 2 L gas sampling bag) for 4 min. The following structural assignments for the NMR signals were made: δ 72.8, 3-hydroxy-3-methylbutyric acid; δ 74.9, *tert*-amyl alcohol; δ 76.4, 2-methyl-2,3-butanediol; δ 76.7, glucuronide of 2-methyl-2,3-butanediol; δ 79.8, 2-hydroxy-2-methylbutyric acid; and δ 83.1, glucuronide of *tert*-amyl alcohol.

Moreover, the NMR spectra also were consistent with the presence of the glucuronic acid moiety (δ 3−5) and the *tert*-amyl alcohol moiety in the molecule.

Of the other minor metabolites that were present, the compound giving the signal at δ 79.8 was identified as 2-hydroxy-2-methylbutyric acid by comparison of the ^{13}C NMR spectra of a synthetic reference and by GC/MS (data not presented).

No major differences in the intensity of the ^{13}C NMR signals of the individual metabolites were observed when comparing the NMR spectra of urines from male and female rats exposed to [^{13}C]TAME. Moreover, the kinetics of excretion of the metabolites with the urine was similar in male and female rats.

Identical metabolites were also present in rat urine samples collected between 24 and 48 h after [^{13}C]TAME exposure. Relative concentrations of these metabolites, however, were different in the samples between 24 and 48 h compared to those in the 0−24 h sampling period, indicating differences in excretion kinetics of the individual metabolites (data not shown).

To confirm that human metabolism of TAME is identical to TAME metabolism in rats, one human volunteer was exposed to [^{13}C]TAME. To be able to use small amounts of the expensive ^{13}C-labeled material, [^{13}C]-TAME (300 μL of liquid) was added to a 2 L gas sampling bag filled with 2 L of pure oxygen. The volunteer inhaled the TAME-containing oxygen for 4 min and exhaled into the sampling bag. This procedure ensured that most of the TAME present in the sampling bag was taken up by the volunteer. No major discomfort or symptoms of toxicity were associated with the procedure. Urine of the volunteer was collected in 6 h fractions for 48 h and analyzed by ^{13}C NMR. The presence of identical metabolites of TAME observed in rats was indicated by the recorded ^{13}C NMR spectra (Figure 7). In contrast to the case in rats, free [^{13}C]-*tert*-amyl alcohol was present in significant amounts in human urine after [^{13}C]TAME inhalation and the glucuronide of 2-methyl-2,3-butanediol was only a minor excretion product. All other detected metabolites were present in the human urine samples at concentrations similar to those seen in rat urine (based on relative signal intensities in ^{13}C NMR). Metabolites of [^{13}C]TAME were also detected in the

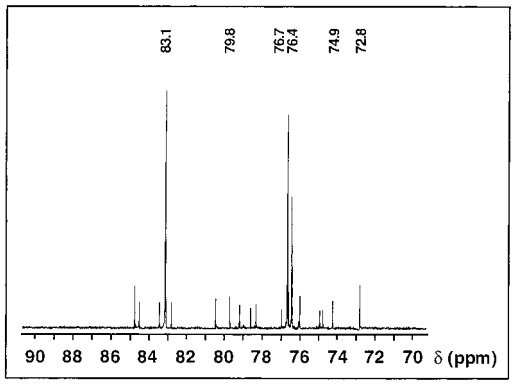


Figure 8. ^{13}C NMR spectrum (63 MHz, 2000 scans) of a 24 h urine sample from a male Fischer F344 rat treated orally with 250 mg/kg 2-[^{13}C]-*tert*-amyl alcohol. The following structural assignments for the NMR signals were made: δ 72.8, 3-hydroxy-3-methylbutyric acid; δ 74.9, *tert*-amyl alcohol; δ 76.4, 2-methyl-2,3-butanediol; δ 76.7, glucuronide of 2-methyl-2,3-butanediol; δ 79.8, 2-hydroxy-2-methylbutyric acid; and δ 83.1, glucuronide of *tert*-amyl alcohol.

human urine sample collected up to 48 h after TAME inhalation.

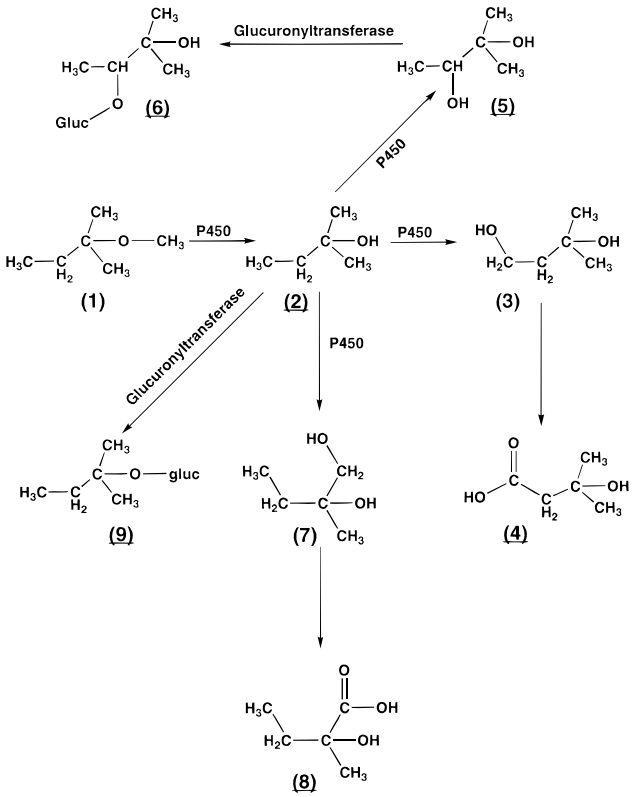
Biotransformation of *tert*-Amyl Alcohol in Rats. Studies on the metabolism of *tert*-amyl alcohol were included in this study to confirm structures of metabolites "downstream" from the formation of *tert*-amyl alcohol and to determine if any of the minor metabolites represent products formed by oxidation of TAME at other sites in the molecule leaving the methyl ether function intact. Moreover, [^{13}C]-*tert*-amyl alcohol was available from the synthesis of [^{13}C]TAME. Male rats ($n = 3$) were treated with 250 mg/kg [^{13}C]TAME (dissolved in corn oil) by gavage. Urine was collected in 24 h intervals for 48 h and analyzed by ^{13}C NMR.

The ^{13}C NMR spectra of the urine samples (Figure 8) collected after treatment of rats with [^{13}C]-*tert*-amyl alcohol were very similar to those obtained from rats treated with [^{13}C]TAME, suggesting that all the metabolites formed from [^{13}C]-*tert*-amyl alcohol are identical to those formed from [^{13}C]TAME. On the basis of relative signal intensities in ^{13}C NMR, *tert*-amyl alcohol glucuronide and 2-methyl-2,3-butanediol and its glucuronide are also major metabolites of [^{13}C]-*tert*-amyl alcohol excreted in urine; free *tert*-amyl alcohol, 2-hydroxy-2-methylbutyric acid, and 3-hydroxy-3-methylbutyric acid were identified by ^{13}C NMR as minor metabolites. In the urine samples taken 48 h after exposure, only 2-methyl-2,3-butanediol and its glucuronide were present, suggesting rapid elimination of the *tert*-amyl alcohol glucuronide.

Discussion

The biotransformation of TAME and the presumed major metabolite *tert*-amyl alcohol was studied in rats. In addition, to confirm an identical metabolism, biotransformation of [^{13}C]TAME was also studied in one human volunteer after TAME inhalation. The aim of the study was to identify metabolites formed and excreted to prepare for a study on the kinetics of excretion of TAME and its metabolites in rats and humans. The use of ^{13}C-labeled ethers permitted the detection and identification of metabolites by ^{13}C NMR and identification of metabolites by mass spectrometry due to differences of 1 mass unit in selected fragments of ^{13}C-containing metabolites.

Scheme 2. Biotransformation of TAME in Rats[a]



[a] Metabolites found in urine are underlined: (1) TAME, (2) *tert*-amyl alcohol, (3) 2-methyl-2,4-butanediol, (4) 3-hydroxy-3-methylbutyric acid, (5) 2-methyl-2,3-butanediol, (6) glucuronide of 2-methyl-2,3-butanediol, (7) 2-methyl-1,2-butanediol, (8) 2-hydroxy-2-methylbutyric acid, and (9) glucuronide of *tert*-amyl alcohol.

The structures of the TAME metabolites that have been delineated suggest a complex biotransformation of TAME (Scheme 2). The first and major step in the biotransformation of TAME is oxidation of the methyl group to give an unstable hemiacetal which decomposes to *tert*-amyl alcohol. The low concentrations of *tert*-amyl alcohol recovered in the urine of rats exposed to both TAME and *tert*-amyl alcohol suggest extensive further metabolism of this alcohol by conjugation and by further oxidation like that for *tert*-butanol (*6*, *17*, *18*). Glucuronidation of *tert*-amyl alcohol seems to be a major step in *tert*-amyl acohol biotransformation, resulting in the excretion of a glucuronide. In addition, *tert*-amyl alcohol is oxidized to several diols in reactions likely involving cytochrome P450-catalyzed CH-based oxidation. The major pathway of *tert*-amyl alcohol oxidation occurs at the C-3 atom to give 2-methyl-2,3-butanediol, which, including the glucuronide formed, is the major product of TAME and *tert*-amyl alcohol biotransformation excreted in the urine of rats exposed to TAME. Oxidation of the carbon atom at the 3-position to the alcohol moiety seems to be a minor process resulting in 2-methyl-2,4-butanediol as an intermediate, which is further oxidized to 3-hydroxy-3-methylbutyric acid.

Oxidation of the methyl group next to the alcoholic function in *tert*-amyl alcohol also represents a minor pathway which results in the intermediate formation of 2-methyl-1,2-butanediol which may be further oxidized to give 2-hydroxy-2-methylbutyric acid. In humans, biotransformation of TAME after inhalation seems to be qualitatively similar to that of rats; however, both the

24 and 48 h urine samples exhibited differences in the relative signal intensities for the individual metabolites, suggesting possible quantitative differences in routes of TAME biotransformation between humans and rats. In rats, on the basis of metabolite structures and semiquantitative comparison of signal intensities, glucuronidation or further oxidation of *tert*-amyl alcohol is the almost exclusive pathways of TAME biotransformation. In the human volunteer, conjugation of the *tert*-amyl alcohol that is formed or oxidation to the 2,3-diol seems to be less efficient than the mechanism of rats, and metabolites formed by oxidations in other parts of the molecule are also excreted in larger amounts. These differences may be due to differences in substrate specificity of P450s and P450 profiles between rats and humans.

The structures of the metabolites that have been elucidated and the delineated mechanisms of their formation indicate that the formation of reactive intermediates in TAME metabolism is unlikely. Thus, the chronic toxicity of TAME should be low based on predictions made from the metabolism structures. Indeed, the available data for the toxicity of TAME support this prediction. Rats exposed for 4 weeks to concentrations of up to 4000 ppm TAME (6 h per day, 5 days per week) showed only increased relative liver weights without treatment-related histopathological findings. The major toxic effects were severe CNS depression which is not likely to be caused by metabolites (*5*).

In summary, the results of this study show that TAME is extensively metabolized in rats. The structures of the metabolites excreted in urine suggest a major role for cytochrome P450 enzymes in TAME biotransformation. Moreover, the results obtained demonstrate the feasibility of using ^{13}C-labeled chemicals and ^{13}C NMR for metabolism studies.

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