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Metabolism of Ether Oxygenates Added to Gasoline

Human Cytochrome P450 Isozymes in Metabolism and Health Effects of Gasoline Ethers

Jun-Yan Hong, Yong-Yu Wang, Sandra N Mohr, Flordeliza Y Bondoc, and Chenjun Deng

Biotransformation of MTBE, ETBE, and TAME After Inhalation or Ingestion in Rats and Humans

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MTBE Inhaled Alone and in Combination with Gasoline Vapor: Uptake, Distribution, Metabolism, and Excretion in Rats

Janet M Benson, Edward B Barr, and Jennifer R Krone

Includes a Commentary by the Institute's Health Review Committee

HEI

The Health Effects Institute, established in 1980, is an independent and unbiased source of information on the health effects of motor vehicle emissions. HEI studies all major pollutants, including regulated pollutants (such as carbon monoxide, ozone, nitrogen dioxide, and particulate matter) and unregulated pollutants (such as diesel engine exhaust, methanol, and aldehydes). To date, HEI has supported more than 200 projects at institutions in North America and Europe and has published over 100 research reports.

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

HEI STATEMENT

Synopsis of Research Report 102

Metabolism of Ether Oxygenates Added to Gasoline

INTRODUCTION

The Clean Air Act Amendments of 1990 required use of oxygenated fuels in areas that exceeded the National Ambient Air Quality Standards for carbon monoxide and in areas with very high ozone levels. Adding oxygenates, such as MTBE (methyl *tert*-butyl ether), to gasoline promotes more efficient combustion and reduces emission of carbon monoxide, ozone-forming hydrocarbons, and some air toxics by increasing the oxygen content of the fuel. On the other hand, some oxygenates may increase emission of toxic compounds such as formaldehyde or acetaldehyde. Increased use of MTBE in fuel in the early 1990s led to complaints of unpleasant odor, headaches, and burning of eyes and throat. After reviewing the literature, HEI issued a request for applications to fund research on the comparative metabolism of ether oxygenates, such as MTBE, ETBE (ethyl *tert*-butyl ether), and TAME (*tert*-amyl methyl ether). The three studies funded are presented in this Research Report.

APPROACH

The studies reported here were initiated to increase our knowledge of the metabolism of ether oxygenates in humans and other species. Dr Jun-Yan Hong (the University of Medicine and Dentistry of New Jersey and the Robert Wood Johnson Medical School) used rat and human liver cells to determine the relative contribution of different members of a family of liver enzymes (cytochrome P450 [CYP] isozymes) to the metabolism of MTBE, ETBE and TAME. Blood samples from human volunteers who reported that they were sensitive to the health effects of MTBE were examined by Hong and colleagues in order to determine whether genetic variants of CYP isozymes were present. Dr Wolfgang Dekant (University of Würzburg) exposed rats and human volunteers by inhalation to two concentrations of MTBE, ETBE or TAME in order to provide detailed data for interspecies comparison. He also exposed human volunteers by ingestion to MTBE or TAME to compare metabolic pathways after

inhalation and ingestion of these compounds. Dr Janet Benson (Lovelace Respiratory Research Institute) exposed rats by inhalation to several concentrations of MTBE alone or to MTBE in combination with gasoline vapors in order to determine how the presence of gasoline affects the uptake, kinetics, metabolism and excretion of MTBE.

RESULTS AND IMPLICATIONS

These three studies have advanced our understanding of the metabolism of gasoline ethers after inhalation. The study by Dr Hong identified one particular CYP isozyme, CYP2A6, as a major enzyme involved in metabolism of MTBE, ETBE and TAME at the concentrations studied. Although the relative importance of this isozyme over others (such as CYP2E1, which was found to be important in previous studies) remains undetermined, the results invite research into the involvement of these and other isozymes in the health effects of ethers. Dr Hong also found several genetic variants of CYP2A6 in some human volunteers who reported sensitivity to MTBE. Further research should evaluate a larger group of sensitive individuals to identify the prevalence of such isozymes in the general population and to determine whether expression of these isozymes may contribute to the reported sensitivity.

The study by Dr Dekant provides a detailed characterization of metabolites of MTBE, ETBE and TAME. The pathways for metabolism of MTBE and ETBE were found to be similar, whereas the metabolism of TAME followed a slightly different pathway with more steps involved and the formation of more metabolites. For all three ethers the pathways of metabolism in rats and humans were similar, and the blood levels were not significantly different although the rate of metabolism was more rapid in rats. The metabolic pathway after ingestion of MTBE and TAME in humans was almost identical to the pathway after inhalation. No first pass effect—in which the liver metabolizes a compound before it enters into the general circulation—was observed after ingestion, and rates of metabolism were

similar for both exposure routes. These data can be used, therefore, in extrapolating results across species and routes of exposure for the human health risk assessment of ether exposure by inhalation or ingestion.

The study by Dr Benson and coworkers has provided detailed data on the metabolism and disposition of MTBE and its metabolites in rats after inhalation of MTBE alone and of MTBE with gasoline vapors. The investigators showed that MTBE was rapidly taken up into the blood and distributed evenly over body compartments (such as liver, kidney, and lungs). The uptake and metabolism were not linear between 4 and 400 ppm, suggesting that saturation may have occurred at the highest dose. These results indicate that caution is needed in using linear extrapolation of high doses to low doses for human health risk assessment of MTBE exposure. Inhalation of MTBE in combination with gasoline vapor (200 ppm) reduced the total amount of MTBE taken up into the body and increased the amount of MTBE and metabo-

lites exhaled in breath, suggesting that the toxic effects of MTBE during refueling may be lower compared to exposure to MTBE by itself.

In conclusion, the investigators successfully addressed the relative importance of certain CYP isozymes, the metabolic pathways after ether inhalation and ingestion, and the effects of coexposure to gasoline vapors on ether metabolism; some results will require further research to understand the range of their implications. Some avenues of needed research include: investigating the prevalence of different CYP isozymes in the general population, and determining whether the lack of a specific enzyme correlates with increased susceptibility to the health effects of oxygenates; further research into the toxicity of ether metabolites; and further research into the effects of exposure to mixtures (including gasoline vapors) on metabolism and the health effects of exposure to individual compounds, such as oxygenates.

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HEI STATEMENT

This Statement is a nontechnical summary of the Investigators' Report and the Health Review Committee's Commentary.

PREFACE

INVESTIGATORS' REPORTS

When an HEI-funded study is completed, the investigators submit a final report. The Investigators' Report is first examined by three outside technical reviewers and a biostatistician. The report and the reviewers' comments are then evaluated by members of the HEI Health Review Committee, who had no role in selecting or managing the project. During the review process, the investigators have an opportunity to exchange comments with the Review Committee and, if necessary, revise the report.

Human Cytochrome P450 Isozymes in Metabolism and Health Effects of Gasoline Ethers

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Biotransformation of MTBE, ETBE, and TAME After Inhalation or Ingestion in Rats and Humans

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COMMENTARY   Health Review Committee

The Commentary about the Investigators' Report is prepared by the HEI Health Review Committee and staff. Its purpose is to place the study into a broader scientific context, to point out its strengths and limitations, and to discuss the remaining uncertainties and the implications of the findings for public health.

RELATED HEI PUBLICATIONS

PREFACE

INTRODUCTION

The 1990 Clean Air Act Amendments mandate the use of oxygenates in gasoline for two purposes:

1. to reduce carbon monoxide (CO*) emissions in areas of the United States where the National Ambient Air Quality Standards (NAAQS) for CO were being exceeded; and

2. to decrease emissions of ozone-forming hydrocarbons in areas of the United States with the highest ozone levels.

Oxygenates include ethers, such as methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE), *tert*-amyl methyl ether (TAME), and diisopropyl ether (DIPE), and alcohols, such as ethanol or *tert*-butyl alcohol (TBA). Adding oxygenates to gasoline reduces emission of CO and possibly some air toxics, such as benzene, but may also increase toxic aldehydes, such as formaldehyde or acetaldehyde.

MTBE, the most frequently used oxygenate at the present time, has been added to gasoline in low concentrations since the 1970s to enhance the octane content. However, its introduction at higher concentrations in the late 1980s and early 1990s led to complaints from workers and the general public regarding unpleasant odor, headaches, burning of the eyes and throat, and other discomforts. In response to a request by the US Environmental Protection Agency (EPA), HEI assembled the Oxygenates Evaluation Committee to assess possible health effects of MTBE, other ethers, and ethanol. The findings are published in the HEI Special Report, *The Potential Health Effects of Oxygenates Added to Gasoline: A Review of the Current Literature* (HEI 1996). To address some of the research needs identified in this report, a Request for Applications, *Comparative Metabolism and Health Effects of Ethers Added to Gasoline to Increase Oxygen Content*, was issued in 1996 and led to funding of three studies on the comparative metabolism of ethers.

BACKGROUND

ETHERS: SOURCES AND USE

Two types of fuel reformulation involve increasing the oxygen content by adding compounds containing oxygen (called oxygenates). Oxygenated gasoline (or *oxyfuel*) is conventional gasoline to which a minimum of 2.7% oxygen by weight has been added. Increasing the oxygen content of fuel improves combustion and decreases CO emissions resulting from incomplete combustion of fuel, particularly in cold weather. Introduction of oxyfuel was required by section 211(m) of the Clean Air Act Amendments of 1990, starting in the fall of 1992 in areas that exceeded the 8-hour NAAQS for CO. Reformulated gasoline (RFG) is a significantly modified form of conventional gasoline that contains at least 2% oxygen (by weight) and has a reduced content of benzene and other aromatic compounds. Introduction of RFG was required by section 211(k) of the 1990 Amendments, starting in January of 1995, in nine areas with the highest ozone levels. These regulations led to increased public exposure to oxygenates, predominantly MTBE.

Oxygenates are compounds known as aliphatic ethers that contain one oxygen atom within a chain of carbon with hydrogen atoms. They are derivatives of hydrocarbons (which are made of carbon and hydrogen), in which an oxygen atom is connected to two carbon atoms (C–O–C). Because the carbon–oxygen bond is not readily cleaved, ethers are not very reactive with other compounds. Preface Figure 1 shows the chemical structures of the ether oxygenates, some of which are used as gasoline additives (MTBE, ETBE, TAME, and DIPE).

The ethers discussed in this report are all colorless, flammable liquids, but they have a distinct odor, with odor-detection thresholds ranging from 13 to 53 parts per billion (ppb). ETBE is the most odorous ether, followed by
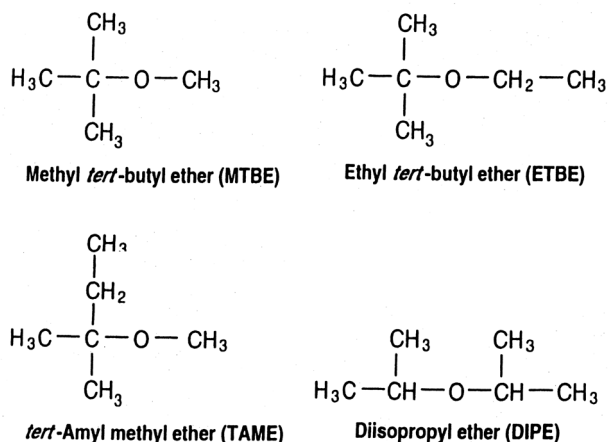

Figure 1. Structure of the ethers MTBE, ETBE, TAME and DIPE.

TAME and MTBE. Ethers also have a very low taste threshold when mixed with water: MTBE and ETBE can be detected at concentrations of approximately 50 ppb. Ethers are only partially soluble in water, with MTBE having the greatest water solubility and DIPE having the lowest solubility. Their solubility in water has consequences for how the ethers travel with groundwater after contamination (eg, after gasoline leaks from underground storage tanks). MTBE travels much faster with groundwater than the slower, toxic BTEX (benzene, toluene, ethyl benzene, and xylene) plume associated with groundwater contamination from gasoline spills. In addition, MTBE is not biodegraded as rapidly as other gasoline components, causing long-term contamination problems in groundwater.

Recently, questions concerning the use of MTBE have arisen due to groundwater contamination associated with gasoline spills and leaks from underground storage tanks, and these questions have led to closure of drinking water wells. Several states are reconsidering their oxygenated fuel programs, with California being the first state to phase out MTBE by the end of 2002. As a result, EPA announced that it will work with Congress to reduce the use of MTBE in the national fuel supply (Environmental Science and Technology 1999; MTBE Blue Ribbon Panel 1999). In spite of the proposed reduction of MTBE usage in the US, however, its use may actually increase in other countries, most notably in Europe and developing countries.
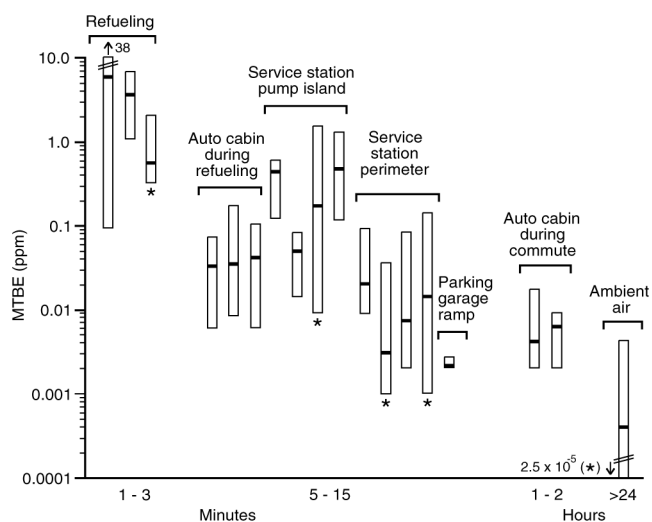
Figure 2. Time-weighted average exposures of general public to MTBE. Only studies that provided ranges of exposure levels are included. The solid lines across the bars indicate median values. The numbers at the top represents the number of sampling sites. An asterisk (*) denotes the minimal detectable concentration. From HEI 1996.

EXPOSURE ASSESSMENT

Exposure to ethers can occur by inhalation, ingestion, or skin contact. Inhalation is the most common route, but ingestion of drinking water contaminated with ethers is also a concern. Air concentrations have been primarily measured for MTBE; few data are available for other ethers. Preface Figure 2 contains environmental exposure data for MTBE collected through the early 1990s (HEI 1996). More recent data from Europe confirm that the highest exposures are among workers involved in the manufacture and transport of MTBE and of fuels containing MTBE (Hakkola and Saarinen 1996; Saarinen et al 1998; Vainiotalo et al 1998) and among gas station attendants and that exposure levels have decreased since the introduction of vapor recovery systems (CONCAWE 2000). Overall, the general public is exposed to low concentrations of ethers. The highest concentrations are encountered during short-term exposures, such as refueling, for which median levels are 0.3 to 6 ppm and peaks occasionally exceed 10 ppm (HEI 1996). Commuters in automobiles are exposed to much lower concentrations but for longer periods of time (HEI 1996).

METABOLISM

A number of studies conducted in vitro, in animals, and in humans have investigated the metabolism and disposition of MTBE, but limited information is available for other ethers. These studies have shown that MTBE, whether inhaled or ingested, is taken up in the bloodstream and distributed to body fluids. Within the range of concentrations relevant to human exposure, the level of MTBE in the blood is proportional to the MTBE concentration in the inhaled air (Johanson et al 1995). After uptake, MTBE is exhaled unchanged or metabolized with the proportion between pathways depending on the route of exposure and dose administered. The first step in metabolizing MTBE is dealkylation, which yields formaldehyde and TBA (Preface Figure 3). This reaction is catalyzed by cytochrome P450 (CYP)-dependent enzymes that occur in different forms, or isozymes, with different substrate specificities. For example, a study using rat liver microsomes implicated the isozyme Cyp2e1 in MTBE metabolism (Brady et al 1990). Whether other isozymes are also important in MTBE metabolism, whether differences occur at different substrate concentrations, and whether other ethers are metabolized by different isozymes remains to be determined. In addition, whether individual differences in the types of isozyme involved or isozyme polymorphism contribute to individual susceptibility and sensitivity to oxygenates warrants investigation. Some of these ques-
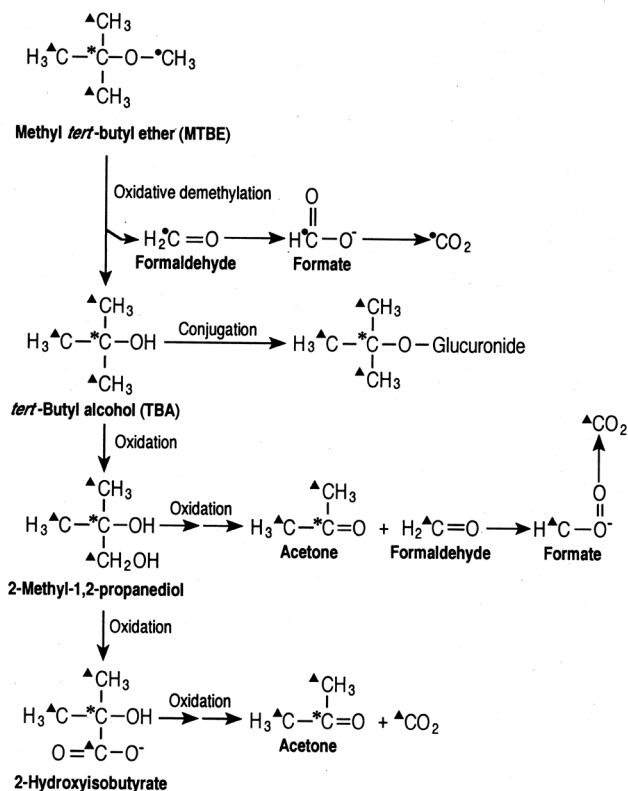
Figure 3. Pathway for MTBE metabolism derived from published studies. The symbols represent the specific carbons that could be labeled and their respective fates: ● = a methyl ether carbon; ∗ = central tertiary carbon of a *tert*-butyl group; ▲ = primary carbons of a *tert*-butyl group. From HEI 1996.

tions have been investigated in the research by Dr Hong and colleagues as described in this Report.

SHORT-TERM HEALTH EFFECTS

To evaluate the systemic, neurotoxic, and irritant effects of ethers, short-term studies have been conducted in laboratory animals exposed to nonlethal doses of MTBE. Several studies noted increases in liver, kidney, and adrenal gland weight in rats exposed for 28 days to 3,000 or 8,000 ppm of MTBE, 4,000 ppm of ETBE by inhalation, 0.09 to 1.75 g/kg MTBE, or 0.5 to 1.0 g/kg TAME by gavage (IIT Research Institute 1991, 1992; Chun et al 1992; Daughtrey and Bird 1995). Neurotoxic effects, primarily in the form of reduced activity, were observed at 800 ppm MTBE. Exposure concentrations in these experiments were much higher, however, than those likely to be encountered by the general public. Therefore, these effects are not expected to occur in humans after brief exposures by inhalation, even if they are repeated over time. Whether ingestion of ethers at sufficient levels through contaminated drinking water

presents a health risk remains to be determined (Dourson and Felter 1997; Stern and Tardiff 1997).

After introduction of oxygenated fuels, some people exposed to MTBE-containing gasoline vapors voiced complaints about headaches, nausea, and sensory irritation. Several community studies investigated the prevalence of symptoms in occupational settings (eg, among service station attendants or taxi drivers) and in the general population with conflicting results (reviewed in HEI 1996). Whereas some studies failed to find a correlation between MTBE exposure and prevalence of symptoms in garage workers, other studies found that people with higher blood levels of MTBE were more likely to report symptoms. However, controlled human exposure studies failed to document significant sensory irritation from MTBE alone (Prah et al 1994; Cain et al 1996; Nihlén et al 1998). Further efforts to identify and characterize sensitive individuals are needed (HEI 1996).

LONG-TERM HEALTH EFFECTS

No epidemiologic studies of the long-term health effects of exposure to MTBE have been conducted. Evidence from animal bioassays demonstrated that long-term, high-level exposure to MTBE via oral or inhalation routes might cause cancer in rodents under certain circumstances. Since these experiments were conducted with high doses that cause acute toxicity, it remains unclear whether these effects are relevant to the human situation (HEI 1996). At present, the EPA is reviewing the research to determine whether MTBE should be classified as a probable human carcinogen. Metabolism of MTBE could contribute to carcinogenic effects, for instance, through formation of TBA and formaldehyde, which is listed as a probable human carcinogen, class B-1 (US EPA 1993).

HEI'S OXYGENATES RESEARCH PROGRAM

The HEI Oxygenates Evaluation Committee identified a number of research needs to address uncertainties about the health effects of oxygenates by themselves and as part of gasoline mixtures (for a complete list, see HEI 1996):

- A comprehensive assessment of personal exposure to oxygenates in public and occupational settings.
- Controlled human exposure studies to evaluate metabolism, symptoms, and neurotoxic effects in potentially sensitive individuals after exposure to MTBE alone and mixed with gasoline.
- Epidemiologic and animal studies to investigate the potential risk of human cancer from exposure to

MTBE alone and in combination with gasoline vapors and vehicle exhaust.

- Comprehensive assessment, including metabolism studies, of other ethers (eg, ETBE, TAME, DIPE) if they are to be used widely.

To address some of these research needs, HEI issued a Request for Applications, RFA 95-1, *Comparative Metabolism and Health Effects of Ethers Added to Gasoline to Increase Oxygen Content*. Three studies on the metabolism of ethers were funded and are published together in this report. The first study, *Human Cytochrome P450 Isozymes in Metabolism and Health Effects of Gasoline Ethers* (Hong et al), defines the roles of various cytochrome isozymes in the metabolism of MTBE, ETBE, and TAME. Dr Hong proposed to study ether metabolism in rat and human liver microsomes and in rat and monkey nasal tissue, and to identify polymorphisms of a particular isozyme, CYP2E1, in a human subpopulation of individuals who reported sensitivity to MTBE exposure. The second study, *Biotransformation of MTBE, ETBE, and TAME After Inhalation or Ingestion in Rats and Humans* (Dekant et al), compares the metabolism of MTBE, ETBE, and TAME as a function of time and concentration and investigates ether metabolism after inhalation and ingestion. The third study, *MTBE Inhaled Alone and in Combination with Gasoline Vapor* (Benson et al), investigates the uptake and metabolism of MTBE in rats after inhalation of MTBE alone or in combination with gasoline vapors. This study mimics human exposure to MTBE and gasoline vapor during refueling, addressing the question of whether MTBE metabolism changes in the presence of other substances at concentrations relevant to the human situation.

Taken together, these three studies provide information on the metabolism of MTBE alone and combined with gasoline evaporative emissions, and on the metabolism of other ethers in rats and humans. The concentrations of MTBE used in the two inhalation studies were selected to represent high ambient levels and the majority of occupational exposures (ie, 4 and 40 ppm). The results were expected to

- characterize possible differences between rats and humans in the biotransformation of ethers and facilitate extrapolation of health effects data from animals to humans,
- provide information on whether exposure to MTBE in combination with gasoline vapor affects the metabolism of MTBE, and
- help determine whether sensitivity to MTBE exposure is related, at least in part, to different forms of the CYP enzyme, which is responsible for its metabolism.

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ABBREVIATIONS AND OTHER TERMS

BTEX	benzene, toluene, ethyl benzene, xylene
CO	carbon monoxide
CYP	cytochrome P450
DIPE	diisopropyl ether
ETBE	ethyl *tert*-butyl ether
MTBE	methyl *tert*-butyl ether
NAAQS	National Ambient Air Quality Standard
RFG	reformulated gasoline
TAME	*tert*-amyl methyl ether
TBA	*tert*-butyl alcohol
US EPA	US Environmental Protection Agency

Human Cytochrome P450 Isozymes in Metabolism and Health Effects of Gasoline Ethers

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ABSTRACT

To reduce the production of carbon monoxide and other pollutants in motor vehicle exhaust, methyl *tert*-butyl ether (MTBE*), ethyl *tert*-butyl ether (ETBE), and *tert*-amyl methyl ether (TAME) are added to gasoline as oxygenates for more complete combustion. Among them, MTBE is the most widely used. The possible adverse effect of MTBE in humans is a public concern, but the human enzymes responsible for metabolism of these gasoline ethers and the causes or factors for increased sensitivity to MTBE in certain individuals are totally unknown. This information is important to understanding the health effects of MTBE in humans and to assessing the human relevance of pharmacokinetics and toxicity data obtained from animals.

In the present study, we demonstrated that human liver is active in metabolizing MTBE to *tert*-butyl alcohol (TBA), a major circulating metabolite and an exposure marker of MTBE. The activity is localized in the microsomal fraction but not in the cytosol. Formation of TBA in human liver microsomes is NADPH-dependent and is significantly inhibited by carbon monoxide, which inhibits cytochrome P450 (CYP) enzymes. These results provide strong evidence that CYP enzymes play a critical role in the metabolism of MTBE in human livers. Human liver is also active in the oxidative metabolism of 2 other gasoline ethers,

ETBE and TAME. We observed a large interindividual variation in metabolizing these gasoline ethers in 15 microsomal samples prepared from normal human livers. The activity level (pmol metabolite/min/mg) ranged from 204 to 2,890 for MTBE; 179 to 3,134 for ETBE; and 271 to 8,532 for TAME. The microsomal activities in metabolizing MTBE, ETBE, and TAME correlated highly with each other (r = 0.91 to 0.96), suggesting that these ethers are metabolized by the same enzyme(s).

Correlation analysis of the ether-metabolizing activities with individual CYP enzyme activities in the human liver microsomes showed that the highest degree of correlation was with CYP isoform 2A6 (CYP2A6)[†] (r = 0.94 for MTBE, 0.95 for ETBE, and 0.90 for TAME), which is constitutively expressed in human livers and known to be polymorphic. CYP2A6 displayed the highest turnover number in metabolizing gasoline ethers among a battery of human CYP enzymes expressed in human B-lymphoblastoid cells. CYP2A6 coexpressed with human CYP reductase by a baculovirus expression system was also more active than CYP isoform 2E1 (CYP2E1) in the metabolism of MTBE, ETBE, and TAME.

Kinetic studies on MTBE metabolism with human liver microsomes (n = 3) exhibited an apparent Michaelis constant (K_m) of 28 to 89 μM and a maximum rate of metabolism (V_{max}) of 215 to 783 pmol/min/mg. Metabolism of MTBE, ETBE, and TAME by human liver microsomes was inhibited by coumarin, a known substrate of human CYP2A6, in a concentration-dependent manner. Monoclonal antibody against human CYP2A6 caused a significant inhibition (75% to 95%) of the metabolism of MTBE, ETBE, and TAME in human liver microsomes. Taken together, these results clearly indicate that, in human liver, CYP2A6 is a major enzyme responsible for metabolism of MTBE, ETBE, and TAME. Although CYP2E1 metabolizes diethyl ether and was previously suggested to be involved

[†] The CYP superfamily components are differentiated in the text as follows: human genes are in captial letters and italics; rodent genes have an initial capital letter and are in italics; human enzymes are in uppercase Roman type; rodent enzymes have an initial capital letter and are in Roman type. A table of cited CYP components is given at the end of this Investigators' Report.

in MTBE metabolism, an antibody against CYP2E1 did not inhibit the metabolism of these ethers in human liver microsomes. Liver microsomes prepared from *Cyp2e1*-null mice lacked 2e1 activity, which was assayed as *N*-nitrosodimethylamine (NDMA) demethylation. However, the levels of ether-metabolizing activity were not significantly different from those of liver microsomes from the sex-matched and age-matched C57BL/6N and 129/Sv mice, which are the parental lineage strains of *Cyp2e1*-null mice and are both *Cyp2e1*$^{+/+}$. These results indicate that CYP2E1 plays a negligible role in the metabolism of MTBE, ETBE, and TAME in human and mouse livers.

After demonstrating that human CYP2A6 is a major enzyme for the metabolism of MTBE and other gasoline ethers, we examined possible sequence variations in the *CYP2A6* gene in 23 individuals who claimed to be sensitive to MTBE exposure. Three novel *CYP2A6* missense variants, Ser29Asn in exon 1, Arg64Cys in exon 2, and Lys476Arg in exon 9 were identified, and the existence of a reported genetic variant, Leu160His (CYP2A6v1), confirmed. The complementary DNA (cDNA) of the wildtype CYP2A6 and these variants, except the Lys476Arg variant, were generated by site-directed mutagenesis and the corresponding proteins were expressed by a baculovirus/Sf9 cell system. In comparison to the wildtype CYP2A6, the Ser29Asn and Arg64Cys variants showed a small trend toward reduced activity in metabolizing MTBE, ETBE, and TAME, whereas the Leu160His variant showed a total loss of ether-metabolizing activity. Whether *CYP2A6* genetic polymorphism plays a role in an individual's metabolic capability and sensitivity to MTBE and other gasoline ethers remains to be studied.

As inhalation constitutes a major route for human exposure to gasoline ethers and some of the reported symptoms attributed to MTBE exposure appear to be related to olfactory sensation, we examined the ether metabolism in the rat olfactory mucosa and compared it with that in the other rat tissues. The metabolic activity of rat olfactory mucosa microsomes was 46-fold higher than that of the liver microsomes in metabolizing MTBE, and 37-fold and 25-fold higher, respectively, in metabolizing ETBE and TAME. No detectable activity was found in the microsomes prepared from the lungs, kidneys, or olfactory bulbs of the brain. The apparent K_m and V_{max} values for metabolism of MTBE, ETBE, and TAME in rat olfactory microsomes were similar, ranging from 87 to 125 µM and 9.8 to 11.7 nmol/min/mg protein, respectively. Our results provide important information on the metabolism of gasoline ethers and are essential for further studies on the relationship between functional *CYP2A6* genetic polymorphism and an individual's sensitivity to MTBE.

INTRODUCTION

GASOLINE ETHERS: HUMAN EXPOSURE AND METABOLISM

The US Clean Air Act Amendments of 1990 require the use of gasoline containing a minimum of 2.7% oxygen (w/v) during the winter in areas that failed to meet the National Ambient Air Quality Standard (NAAQS) for carbon monoxide. Addition of oxygenates to gasoline raises the oxygen content and improves the combustion process, reducing the emission of carbon monoxide.

Ethers added to gasoline as oxygenated additives include MTBE, ETBE, and TAME. Among them, MTBE is the most widely used. MTBE was originally used in the United States in the late 1970s as an octane enhancer of gasoline after the phase-out of lead-containing fuels. The use of MTBE has significantly increased since 1992 in areas with severe air pollution. TAME has a lower vapor pressure than MTBE, which results in lower evaporative emissions, and ETBE can be made from renewable sources. Therefore, these ethers have been proposed to replace MTBE (Health Effects Institute 1996). Currently, approximately 20% of the gasoline sold in the United States contains 2% to 15% MTBE, and the use of MTBE and other ethers as oxygenates may increase over the next decade (Costantini 1993).

Inhalation at petroleum terminals and gas stations constitutes a major route for environmental exposure of MTBE and other gasoline ethers. During refueling, the concentrations of MTBE range from less than 1 to 4 ppm within the personal breathing zone and from 0.01 to 0.1 ppm inside the cars (Clayton Environmental Consultants 1991; Hartle 1993; Lioy et al 1994). Recently, ingestion exposure to MTBE through contaminated well water, resulting from the leakage of underground storage tanks, has also been demonstrated (Squillace et al 1996).

Studies in rats and humans have shown that inhaled MTBE is eliminated primarily through the lungs in exhaled air and through the kidneys in urine (Health Effects Institute 1996). The blood levels of MTBE and TBA, a major circulating metabolite and a suitable marker for MTBE exposure in people, as well as human pharmacokinetics data have been reported (Clayton Environmental Consultants 1991; Prah et al 1994; Johanson et al 1995). In human experimental exposure studies, 2 subjects exposed to 1.39 ppm MTBE for 1 hour showed a rapid rise in blood MTBE concentrations peaking at the end of exposure with a clearance half-time of about 35 minutes. The blood concentration of TBA increased gradually over a couple of hours and maintained the peak level for up to 7 hours after

exposure (Prah et al 1994). Because elimination of TBA in humans and animals has been found to be much slower than elimination of MTBE (Costantini 1993), TBA is believed to be more likely to be involved in the adverse health effects of MTBE (JA Bond, personal communication, 1996). The blood and urine levels of TBA vary considerably among human subjects experimentally exposed to MTBE (Nihlen et al 1994; Cain et al 1996).

TBA is formed by oxidative demethylation of MTBE (Costantini 1993). Deethylation of ETBE also results in the formation of TBA, and demethylation of TAME leads to the formation of *tert*-amyl alcohol (TAA). Previously, Brady and colleagues (1990) reported the metabolism of MTBE by rat liver microsomes in which the involvement of CYP enzymes was implicated. Turini and associates (1998) recently reported that in rat liver microsomes Cyp2b1 appeared to be the major enzyme in oxidation of MTBE and TAME, whereas Cyp2e1 may play a minor role in MTBE demethylation. However, the K_m values of Cyp2b1 for MTBE and ETBE in the Turini report were 5.7 mM and 2.3 mM, respectively, suggesting limited relevance of this finding to ether metabolism in vivo. The metabolism of MTBE and 2 other gasoline ethers in human tissues and the enzymes involved are unknown. This information is important in our understanding of the health effects of MTBE in humans and is critical to assessing the human relevance of pharmacokinetics and toxicity data obtained from animal studies.

HUMAN HEALTH EFFECTS OF MTBE EXPOSURE

The possible adverse effects of MTBE exposure in humans have received increasing attention. In November 1992, shortly after introduction of MTBE into wintertime oxygenated fuel in Fairbanks, Alaska, residents began attributing illnesses to exposure to the oxygenated fuel. The major symptoms reported included headache, nausea or vomiting, burning sensation of the nose or mouth, coughing, dizziness, disorientation, and eye irritation (Middaugh 1992, 1993). Subsequent studies by the Centers for Disease Control and Prevention (1993a,b) and by White and coworkers (1995) showed that in both Alaska and Stamford, Connecticut, an area also participating in the wintertime oxygenated fuel program, persons with higher blood levels of MTBE had more complaints of symptoms. Other reports failed to associate these symptoms with MTBE exposure, however (Mohr et al 1994; Nihlén et al 1994; Prah et al 1994; Cain et al 1996). Although the reportedly sensitive individuals account for only a small percentage of the MTBE-exposed populations and the current information does not clearly implicate MTBE in an increase of symptoms, individuals claiming sensitivity to MTBE may reasonably be considered part of a subpopulation with greater susceptibility.

CYP ENZYMES AND ENVIRONMENTAL TOXICITY

Encoded by different genes, CYP enzymes are a family of hemoproteins that play a key role in metabolism of most environmental chemicals. Depending on the chemicals, the metabolites produced could be more or less toxic than their parent compounds (resulting in metabolic activation or detoxication, respectively). Differences in catalytic activity among different forms of CYP enzymes and their relative distribution in tissues are important factors in determining the toxic action of environmental chemicals (Conney 1967; Guengerich 1991; Nelson et al 1996).

The CYP enzymes are mainly localized in the endoplasmic reticulum (the microsomal fraction in biochemical preparation) of eukaryotic cells. In the reactions catalyzed by CYP enzymes, the enzyme binds the substrate and oxygen while the CYP reductase acts as an electron carrier shuttling electrons from NADPH to the enzyme (Lu and West 1980). In recent years, the role of genetic polymorphism of CYP enzymes in environmental toxicity has received great attention. A single amino acid substitution resulting from missense sequence variations, such as those seen in single nucleotide polymorphism, can have a profound effect on protein structure and function if it occurs in a critical region. For a particular CYP enzyme, substitution of a single amino acid can have a general effect toward any or all of its substrates by affecting the heme binding or reductase binding site. Genetic polymorphism is therefore believed to be an important factor in determining an individual's sensitivity to environmental chemicals through altering the expression level and function of CYP enzymes (Daly et al 1994; Hong and Yang 1997).

SPECIFIC AIMS

Prior to our study, little was known about the metabolism of MTBE and other gasoline ethers in human tissues and the responsible human enzymes. Because the CYP enzymes play a key role in the metabolism of most environmental chemicals, we hypothesized that human CYP enzymes are responsible for the metabolism of MTBE and other gasoline ethers. We further speculated that the increased sensitivity reported for certain exposed individuals could be linked to the difference in their metabolic capability due to genetic polymorphism of the ether-metabolizing CYP enzyme.

Our hypothesis was tested with the following specific aims:

- To characterize metabolism of MTBE and related gasoline ethers in human liver and to identify the major human enzymes involved.
- To investigate the relationship between genetic polymorphism of ether-metabolizing enzymes and sensitivity to MTBE.
- To compare metabolism of MTBE and related ethers in human liver microsomes with metabolism in liver and nasal mucosa microsomes from rats.

In our original proposal, we specifically hypothesized that human CYP2E1 is responsible for the metabolism of MTBE and that the genetic polymorphism of *CYP2E1* might be associated with an individual's sensitivity to MTBE exposure. This hypothesis was modified after our studies demonstrated that CYP2A6 is a major enzyme for the metabolism of MTBE, ETBE, and TAME. Nevertheless, our general working hypothesis on the relationship between the genetic polymorphism of MTBE-metabolizing enzymes and an individual's sensitivity to MTBE exposure did not change.

METHODS AND STUDY DESIGN

CHEMICALS

MTBE, ETBE, TAME, TBA, TAA, and NDMA were purchased from Aldrich Chemical (Milwaukee WI). The purity of these chemicals was greater than 99%, except for TAME, which was 97% pure. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and NADP$^+$ were from Sigma Chemical (St Louis MO). [^{14}C]NDMA (formaldehyde-free, 40 mCi/mmol) was prepared by Stanford Research Institute International (Menlo Park CA), and [^{14}C]formaldehyde (10 mCi/mmol, with a radiochemical purity greater than 95%) was from New England Nuclear (Boston MA). All other chemicals were reagent grade and were obtained from standard suppliers.

ANIMALS

Male Sprague-Dawley rats, C57BL/6N and 129/Sv mice (Taconic Farms, Germantown NY) and female A/J mice (Jackson Laboratory, Bar Harbor ME) were acclimated for 1 week at the animal facility of Rutgers University. They received lab chow (Lab Diet 5012, Richmond Standard, PMI Feeds, St Louis MO) and tap water ad libitum and were maintained in air-conditioned quarters (22° to 25°C)

with 12-hour light-dark cycles. The animals were killed by decapitation; tissues were collected and immediately stored at –80°C. The rat nasal mucosas, including olfactory and respiratory epithelium, were collected according to a procedure previously described (Hong et al 1991).

METABOLISM OF GASOLINE ETHERS

Incubations for the metabolism of MTBE were performed according to a previously described protocol (Brady et al 1990). The incubation mixture (0.4 mL final volume) contained 50 mM Tris-HCl (pH 7.4), 10 mM MgCl$_2$, 150 mM KCl, an NADPH-generating system (0.4 mM NADP$^+$, 10 mM glucose 6-phosphate, 0.2 unit glucose-6-phosphate dehydrogenase), and microsomal or cytosolic proteins or expressed CYP enzymes. The substrate concentrations are specified in the text. Incubations were carried out in sealed headspace vials, and the reaction was initiated by injecting the NADPH-generating system into the solution. After a 30-minute incubation at 37°C, the reaction was terminated with 50 µL each of 25% ZnSO$_4$ and saturated Ba(OH)$_2$. The metabolism rates were linear under our assay conditions. For the blanks, ZnSO$_4$ and Ba(OH)$_2$ were added into the incubation mixture prior to initiating the reaction with the NADPH-generating system. The same incubation and gas chromatography (GC) conditions were used for analyzing the metabolism of ETBE and TAME.

For chemical inhibition, coumarin was dissolved in 2 µL of methanol and added into the incubation mixture prior to initiating the reaction. For immunoinhibition, antibodies against CYP2A6 and CYP2E1 (Gentest, Woburn MA) were preincubated with the human liver microsomes for 10 minutes at 4°C and were incubated with the rest of the reaction mixture for 30 minutes at 37°C.

For kinetic studies, the metabolizing activities were determined at 6 to 7 different substrate concentrations and the determinations were always repeated at least twice to ensure data reproducibility. Kinetic parameters (K_m and V_{max}) were determined by curve fitting to the Michaelis-Menten equation followed by nonlinear regression analysis using Enzymekinetics V1.11 software (Trinity Software, Plymouth NH).

Concentrations of TBA (a metabolite of MTBE and ETBE) and TAA (a metabolite of TAME) were determined by headspace GC (Hong et al 1997). A Perkin-Elmer model 8500 gas chromatograph was used with a Carbopack B/5% Carbowax 20 M stainless steel column (⅛ inch × 6 feet, Supelco, Bellefonte PA) and an HS-101 headspace autoinjector. The carrier gas was helium and the flow rate was 20 mL/min. The injector and flame ionization detector were at 160°C, while the oven temperature was 60°C. Under these

analytical conditions, the retention times (minutes) of these chemicals on headspace GC were MTBE, 5.2; ETBE, 10.3; TAME, 14.6; TBA, 6.77; and TAA, 19.4. Preestablished standard curves were used to quantitate levels of TBA and TAA. To enhance the detection sensitivity in the metabolism study using expressed CYP enzymes and 0.1-mM substrate concentration, a 4 μL aliquot of the reaction mixture after incubation was injected directly into the GC for metabolite analysis. The oven temperature was increased to 86°C for TBA analysis and to 93°C for TAA analysis. The injector and detector temperatures were increased to 240°C.

MICROSOMES AND CYTOSOL PREPARATION FROM HUMAN LIVER

Liver samples from liver cancer patients (37 to 80 years old) were provided by the Affiliated Tissue Retrieval and Distribution Service of the Cancer Institute of New Jersey (New Brunswick NJ). Prior to cryosurgery, these patients signed a consent form permitting the research use of their resected tissues. The tissue collection procedures were preapproved by the Institutional Review Board. The samples were neighboring tissues of the liver tumors and were normal in morphology. They were snap frozen in liquid nitrogen within 30 minutes of surgical removal to assure freshness, transferred to our laboratory in liquid nitrogen, and stored at –80°C prior to use.

Tissues were homogenized in ice-cold Tris-HCl buffer (pH 7.4, containing 1.15% KCl) with an Ultra-Turrax polytron (Janke and Kunkel, Staufen, Germany). Microsomes and cytosolic fractions were prepared by differential centrifugation (Hong and Yang 1985). Microsomes from the infected Sf9 cells were prepared by a brief sonication followed by a centrifugation at 105,000g for 60 minutes.

The protein content was determined by the Lowry method (Lowry et al 1951). Total CYP content was determined as previously described (Patten and Koch 1995). For CYP content determination, microsomal proteins were diluted to 1 mg protein/mL with 0.1M phosphate buffer, and aliquots were poured into two 1-cm optical path cuvettes. After recording the baseline, the sample cuvette was bubbled with carbon monoxide for 20 seconds. The difference in absorption spectrum was measured by scanning from 500 to 400 nm immediately after adding dithionite to the sample cuvette, with the other cuvette as a reference.

HETEROLOGOUSLY EXPRESSED HUMAN CYP ENZYMES

Twelve individual human CYP enzymes expressed in the human AHH-1 TK$^{+/-}$ B-lymphoblastoid cell line were purchased from Gentest. These cells contain very low levels of endogenous CYP enzyme activity, and each cell line was engineered to consistently express a particular human CYP cDNA.

The HepatoScreen kit (Human Biologics, Phoenix AZ) contained 15 human liver microsomal samples prepared from organ donors who died in accidents. The CYP enzyme activity profiles of these human liver microsome samples were based on the following determinations: CYP1A2, 7-ethoxyresorufin O-dealkylation; CYP2A6, coumarin hydroxylation; CYP2B6, *S*-mephenytoin N-demethylation; CYP2C9, tolbutamide hydroxylation; CYP2C19, *S*-mephenytoin hydroxylation; CYP2D6, dextromethorphan O-demethylation; CYP2E1, chlorzoxazone hydroxylation; CYP3A4, dextromethorphan N-demethylation; CYP3A4/5, testosterone oxidation. The human liver microsomes and B-lymphoblastoid cell microsomes were shipped on dry ice and stored at –80°C until use.

Cyp2e1 KNOCKOUT MICE

Cyp2e1-null mice were produced as described elsewhere (Lee et al 1996). In brief, embryonic stem cells of 129/Sv mouse origin were used for gene targeting. After selection, the embryonic stem cells containing an inactivated *Cyp2e1* allele were microinjected into the C57BL/6N blastocysts. Homozygotes of the *Cyp2e1*-null mice were produced by crossing the F1 generation (Lee et al 1996). The breeding pairs of *Cyp2e1*-null mice were transported from the laboratory of Dr Frank Gonzalez at the National Cancer Institute, National Institutes of Health (Bethesda MD), and the *Cyp2e1*-null mouse colony was established at the Nelson Animal Facility at Rutgers University (Piscataway NJ).

NDMA demethylase activity in the liver microsomes was determined with [^{14}C]NDMA at a low concentration (40 μM). This assay is highly specific for CYP2E1 activity (Yang et al 1990). The product, [^{14}C]formaldehyde, was determined by a radiometric method as described previously (Hong et al 1989). Briefly, the reaction mixture contained 50 mM Tris-HCl (pH 7.4), 10 mM MgCl$_2$, 150 mM KCl, an NADPH-generating system (0.4 mM NADP$^+$, 10 mM glucose-6-phosphate, and 0.2 IU glucose-6-phosphate dehydrogenase), 40 μM [^{14}C]NDMA (0.05 μCi), and 50 μg microsomal protein in a total volume of 0.1 mL. The reaction was initiated by addition of the generating system, incubated for 15 minutes at 37°C, and stopped with the addition of 30 μL of 1M NaCH$_3$COOH (sodium acetate, pH 4.5) in ice. The mixture was then incubated at 50°C for 30 minutes after the addition of dimedone. The formaldehyde-methane derivative was extracted into hexane and mixed with scintillation liquid Betafluor (Packard, Meriden CT) for counting.

IDENTIFICATION AND CHARACTERIZATION OF *CYP2A6* VARIANTS

Dr Mohr (coinvestigator on this project) developed a screening questionnaire to determine symptoms due to exposure to gasoline containing MTBE. A roster was developed of members of Oxybusters (a self-help group of individuals in New Jersey who report that they are sensitive to exposure to MTBE), and persons who reported MBTE sensitivity to the New Jersey Department of Environmental Protection or the National Reformulated Gasoline Hotline. Letters were then sent to randomly selected persons asking for their consent to participate in this study and querying them regarding age, medical history, and employment status.

From those who responded, all individuals under 18 years of age, without employment, or with any major medical conditions including heart disease, asthma, major psychiatric diagnoses, chronic fatigue, or multiple chemical sensitivities were excluded from further participation. Of the responders who met the above criteria and agreed to participate, a list of 50 common symptoms, including those that have been attributed to MTBE exposure as well as others, were sent to each participant along with a self-addressed stamped envelope. Each individual was asked to indicate which 5 symptoms they most associate with exposure to MTBE. Their responses were tallied to indicate the 5 most frequent symptoms reported in this group. All individuals who did not report at least 3 of the top 5 symptoms on their form were excluded from further consideration.

The selected subjects who were willing to participate in the study were asked to read and sign a written informed consent to allow a finger stick using a sterile lancet. All blood samples were collected under the supervision of a licensed physician at the Clinical Center of the Environmental and Occupational Health Sciences Institute (Piscataway NJ). The collected blood samples were stored at −20°C prior to analysis. Genomic DNA was isolated from human blood by our established method and serves as a template for polymerase chain reaction (PCR) amplification (Deng et al 1999).

The *CYP2A6* gene is highly homologous (> 95%) to *CYP2A7* and *CYP2A13* genes and to *CYP2A7* pseudogenes. To ensure the specificity of amplification of *CYP2A6* exons, a 2-step nesting PCR method was developed (Fernandez-Salguero et al 1995). Briefly, the complete sequence of the *CYP2A6* gene (7.8 kilobase [kb]) was first amplified by using 2 *CYP2A6*-specific primers (F4, R4), which anneal, respectively, in the 5′ and 3′ untranslated regions (UTR) of the *CYP2A6* gene. The reaction mixture contained 100 ng genomic DNA, 0.2 μM of each primer, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.8 mM magnesium acetate, and 2 U of rTth DNA polymerase (XL-PCR kit, Perkin-Elmer). A hot start protocol was used as instructed by the manufacturer. PCR amplification was performed by 31 cycles of denaturing at 93°C for 1 minute, and then annealing and extending at 66°C for 6 minutes. PCR products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide.

In the second-step PCR, the 7.8-kb PCR product was used as a template, and each of the 9 exons of the *CYP2A6* gene was amplified by nesting PCR using exon-specific primers. All primers were synthesized by Operon (Alameda CA). PCR reactions were performed on a Perkin-Elmer GeneAmp PCR system 2400 (Norwalk CT). The PCR amplification of each exon was carried out in 25 μL containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM dNTPs and 1 unit DNA polymerase (Amplitaq). The reaction started at 94°C for 1 minute, followed by 30 cycles of 94°C for 30 seconds, 58° to 64°C (depending on the annealing temperature of different PCR primers) for 30 seconds, and 72°C for 30 seconds. At the end of last cycle, the reaction was extended to 7 minutes at 72°C. If the DNA sequence needed to be determined, the PCR products were separated by a low melting 3% NuSieve gel electrophoresis and purified with a DNA purification kit (Promega Wizard, Madison WI). Each exon was then directly sequenced using thermosequenase kit (Amersham, Arlington Heights IL).

The pCMV5 plasmid vector containing full-length human *CYP2A6* cDNA was constructed and provided by Dr Chris Patten (Gentest). *CYP2A6* cDNA variants were generated by a site-directed mutagenesis protocol previously described (Jones and Howard 1991). Two pairs of primers were used to generate each mutation. Each pair of primers was designed to amplify the whole circular plasmid, pCMV5 containing *CYP2A6* cDNA (6 kb), and to create the mutation at one end. After the 2 PCR products were mixed and denatured, a nicked plasmid with the desired mutation was formed and selected by transforming the competent bacteria. Each introduced mutation was confirmed by repeated sequencing using four primers to cover the entire cDNA sequence.

EXPRESSION OF HUMAN CYP2A6 PROTEINS BY BACULOVIRUS/SF9 INSECT CELL SYSTEM

For the expression, the full-length cDNA of wild type or variant *CYP2A6* was excised from the pCMV5 vector by *Eco*RI and *Xba*I digestion and purified from the low-melting agarose gel after electrophoresis. The cDNA was then inserted into a transfer vector p₂Bac (In Vitrogen), which was used to transform *Escherichia coli* DH5α for

amplification. The p_2Bac-h2A6 DNA (purified by a Qiagen column) was mixed with AcMNPV DNA (in 3:1 ratio) and cationic liposomes to transfect Sf9 insect cells cultured on 6-cm dishes. After incubation for several days, the recombinant virus containing h2A6 cDNA was identified by visualization of occlusion minus (occ$^-$) plaques under a microscope. The recombinant viruses were amplified in Sf9 cells and their titers determined. Sf9 cells were infected with these viruses to produce the recombinant CYP2A6 proteins. Infection times for producing the baculovirus recombinant CYP2A6 were approximately 72 hours, and hemin was included in the medium as previously described (Patten and Koch 1995).

For coexpression of human CYP2A6 and CYP2E1 and the human CYP reductase, *Spodoptera frugigerda* (Sf9) insect cells were infected simultaneously either with the *CYP2A6* and the reductase recombinant viruses or with the *CYP2E1* and the reductase recombinant viruses. In our later studies, we used a BAC-to-BAC baculovirus expression system developed by Gibco/BRL (Grand Island NY), which uses site-specific transposition in *E. coli* hosts to produce recombinant viral DNA instead of through homologous recombination in insect cells. This method eliminated plaque screening and multiple rounds of viral amplification, and is therefore much faster and easier than the conventional one.

Immunoblotting was used to determine the CYP2A6 content in the membrane fraction of the infected Sf9 cells and was carried out as described (Pan et al 1992). Proteins were separated by sodium lauryl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to a nitrocellulose sheet. The nitrocellulose sheet was then incubated with an anti-CYP2A6 antibody (Gentest) followed by binding of a secondary antibody and immunostained. We used a detection method based on enhanced chemiluminescence, which is at least 10-fold more sensitive than the alkaline phosphatase detection method.

The metabolism of coumarin to 7-hydroxycoumarin was determined as described (Tan et al 1997). A total volume of 0.5 mL reaction mixture contained 50 mM Tris-HCl buffer, pH 7.4, 10 mM MgCl$_2$, 150 mM KCl, 1 mM NADPH and 50 μM coumarin (Sigma Chemical). The reaction was carried out at 37°C for 15 minutes and stopped by adding 60 μL of 15% trichloroacetic acid (TCA) and 1 mL of methylene chloride. The mixture was centrifuged, and 7-hydroxycoumarin in the organic phase was extracted into a 30 mM sodium borate buffer, pH 9.0. Fluorescence was measured in a spectrofluorometer (λ_{ex} = 368 nm, λ_{em} = 453 nm) against a time 0 blank consisting of a complete reaction mixture to which the NADPH regenerating system was added after the addition of TCA.
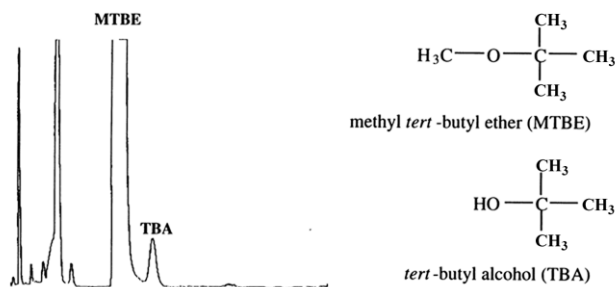
Figure 1. **Headspace gas chromatogram of MTBE metabolism and structures of MTBE and TBA.** Human liver microsomes (600 μg protein) were incubated with 1 mM MTBE at 37°C for 30 minutes. Formation of TBA was determined by headspace GC as described in the Methods and Study Design section.

STATISTICAL METHODS AND DATA ANALYSIS

The reported values are either the mean and standard deviation (SD) or mean and standard error (SE). The quantitative data were analyzed by either analysis of variance followed by the Newman-Keuls test or by *t* test with the Statview 4.5 software (Abacus Concepts, Cary NC). Correlation analysis was performed using the CA-Cricketgraph III version 1.01 software (Computer Associates International, Islandia NY).

RESULTS

METABOLISM OF GASOLINE ETHERS IN HUMAN LIVER

Our established headspace GC method was satisfactory in analyzing the alcohol products formed during the metabolism of gasoline ethers. A representative gas chromatogram of MTBE and TBA is shown in Figure 1. With this method, we first examined a set of human liver samples from liver cancer patients. All of the 8 human liver microsome samples were found to be active in metabolizing MTBE to TBA. The activities ranged from 86 to 175 pmol/min/mg protein, with an average activity of 124 (Table 1). In comparison with the activity in this set of human liver microsomes, the level of MTBE metabolizing activity in rat and mouse liver microsomes is approximately 2-fold higher (Table 1). However, the activity level in rat and mouse is lower than that in the human liver microsomes from accident victims.

In contrast with human liver microsomes, little TBA was formed when the human liver cytosol fractions were incubated with MTBE (Table 1) although a 2.5-fold higher protein concentration was used in the incubation. This

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Table 1. Metabolism of MTBE in Liver Microsomes and Cytosol[a]

Species	Activity (pmol TBA/min/mg protein)	
	Microsomes	Cytosol
Human ($n = 8$)	124 ± 11	1.3 ± 0.5[b]
Rat ($n = 5$)	284 ± 14	ND
Mouse ($n = 4$)	288 ± 29	ND

[a] The formation of TBA was assayed at 1 mM substrate concentration for MTBE-metabolizing activity. The values are the mean ± SE; ND, not detectable; n, number of the samples. Each rat or mouse sample was pooled from 3 animals.

[b] Significantly different from microsomes ($P < 0.05$).

Note: The microsomal MTBE-metabolizing activity was found to be substantially higher (845 pmol TBA/min/mg protein) in human livers from accident victims (see Figure 2).

subcellular localization of the activity in metabolizing MTBE to TBA was also demonstrated in the rat and mouse livers. Because CYP enzymes are mainly localized in the endoplasmic reticulum (the microsomal fraction) in liver cells, this finding suggests that CYP enzymes could be responsible for the catalyzed reaction. CYP-catalyzed reactions are known to require NADPH and are susceptible to carbon monoxide inhibition (Lu and West 1980). To further establish the role of CYP enzymes in the metabolism of MTBE, we selected 3 human liver microsome samples for determining the effects of NADPH and CO on TBA formation. When the incubation was carried out in the absence of an NADPH-generating system, the microsomal activity in metabolizing MTBE to TBA was not detectable. Bubbling the incubation mixture with 95% CO for 3 minutes prior to initiation of the reaction with MTBE caused an
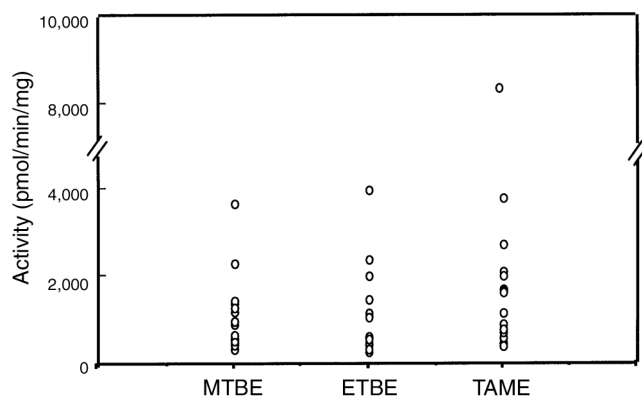
80% reduction in TBA formation (23 ± 8 versus 116 ± 26 pmol/min/mg protein). Together, these results provide strong evidence supporting our hypothesis that CYP enzymes catalyze the metabolism of MTBE in human liver.

The presence of MTBE-metabolizing activity in human liver microsomes was confirmed in a second set of liver samples from normal subjects who died after an accident. All of the liver microsomal samples ($n = 15$) were found to be active in metabolizing MTBE to TBA, confirming our previous finding with human liver samples collected from liver cancer patients. The average level of MTBE metabolizing activity in the second set of samples was much higher than that of the previous samples (845.1 versus 124.9 pmol TBA/min/mg protein), however, which probably reflects the difference between normal and tumor-bearing liver tissues. The human liver microsomal samples were also active in metabolizing ETBE and TAME. A large interindividual variation in metabolizing MTBE, ETBE, and TAME was observed (Figure 2). The activity levels (pmol metabolite/min/mg) ranged from 204 to 2,890 for MTBE, 179 to 3,134 for ETBE, and 271 to 8,532 for TAME. The activities in metabolizing these ethers were highly correlated: MTBE versus ETBE, $r = 0.96$; MTBE versus TAME, $r = 0.92$; and ETBE versus TAME, $r = 0.91$ (Figure 3). These high degrees of correlation suggest that MTBE, ETBE, and TAME are metabolized by the same enzyme(s) in human livers.

Michaelis-Menten kinetics were observed in the metabolism of MTBE with human liver microsomal samples ($n = 3$) when the substrate concentrations ranged from

Figure 2. Interindividual variation in metabolism of gasoline ethers in 15 human liver microsomal samples. The liver microsomal samples were prepared from organ donors who died in accidents. The formation of TBA was assayed for MTBE- and ETBE-metabolizing activities. The formation of TAA was assayed for TAME-metabolizing activity.
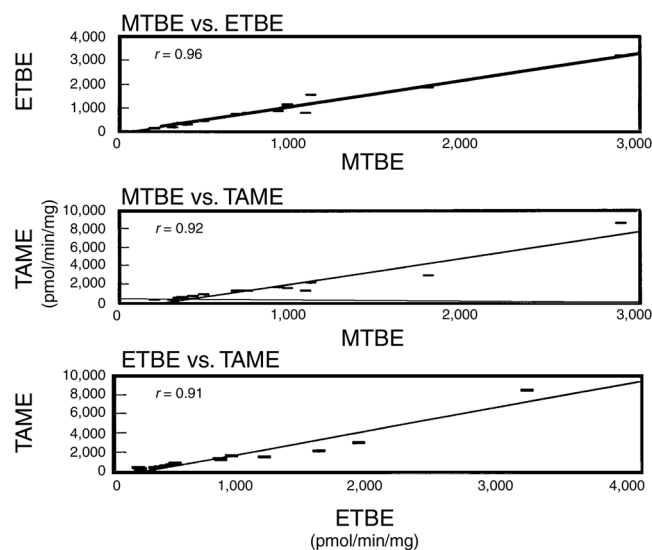
Figure 3. Correlations among metabolizing activities of MTBE, ETBE, and TAME in human liver microsomes. The ether-metabolizing activities shown in Figure 2 were used for correlation analysis.

Table 2. Kinetic Parameters of MTBE Metabolism in Human Liver Microsomes

Sample	K_m (µM)	V_{max} (pmol/min/mg protein)	V_{max}/K_m
HBI 102	89	783	8.8
HBI 107	28	215	7.7
HBI 115	57	501	8.8

Microsomal protein (300 to 450 µg) was incubated with different concentrations of MTBE at 37°C for 30 minutes. The results are the average of 2 separate experiments.

0.01 to 2.5 mM. The apparent K_m values for the metabolism of MTBE ranged from 28 to 89 µM, and the V_{max} values from 215 to 783 pmol/min/mg protein, respectively (Table 2).

Correlation analysis of the ether-metabolizing activities with individual CYP enzyme activities in the human liver microsomes were carried out to help identify the major CYP enzyme(s) responsible for metabolism of MTBE, ETBE, and TAME. The ether-metabolizing activities were highly correlated with the activity of coumarin 7-hydroxylation, a characteristic activity of human CYP2A6. The correlation coefficient was 0.94 for MTBE, 0.95 for ETBE, and 0.90 for TAME (Figure 4). The correlation of these ether-metabolizing activities with the activity of CYP2E1, an enzyme involved in the metabolism of diethyl ether and presumably also for the metabolism of gasoline ethers, was poor (r values from 0.19 to 0.25, Figure 5). As shown in Table 3, the

Table 3. Correlation (r value) of Ether-Metabolizing Activities and Activities of Individual CYP Enzymes in Human Liver Microsomes

Enzymes	MTBE	ETBE	TAME
CYP2A6	0.94	0.95	0.90
CYP2E1	0.25	0.19	0.21
CYP2B6	0.67	0.69	0.87
CYP1A2	0.01	0.01	0
CYP3A4	0.69	0.71	0.87
CYP3A4/5	0.61	0.64	0.70
CYP2C9	0.36	0.28	0.29
CYP2C19	0.18	0.18	0.34
CYP2D6	0	0.01	0

The correlation was analyzed based on the activities (pmol/min/mg protein) of the individual CYP enzymes (provided by Human Biologics) and the metabolizing activities (pmol/min/mg protein) toward the 3 gasoline ethers.

highest degree of correlation was found to be with human CYP2A6. The correlation coefficient values varied for other CYP enzyme activities. The ether-metabolizing activities did not correlate with CYP1A2 or CYP2D6 activities.

ETHER METABOLISM BY HETEROLOGOUSLY EXPRESSED HUMAN CYP ENZYMES

In the presence of coexpressed CYP reductase, both baculovirus-expressed human CYP2A6 and CYP2E1 were

Figure 4. Correlation of CYP2A6 activity with ether-metabolizing activities in human liver microsomes. The ether-metabolizing activities determined in Figure 2 were used for correlation analysis with the activity of coumarin 7-hydroxylation, a characteristic activity of human CYP2A6.

Figure 5. Correlation of CYP2E1 activity with ether-metabolizing activities in human liver microsomes. The ether-metabolizing activities determined in Figure 2 were used for correlation analysis with the activity of chlorzoxazone hydroxylation, a selective activity of human CYP2E1.
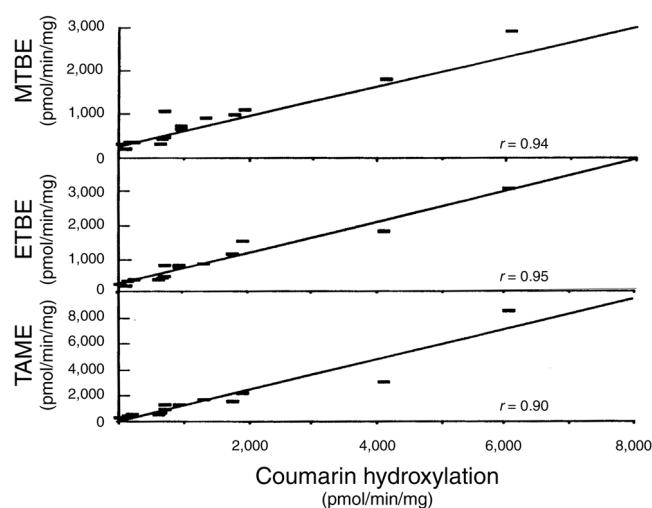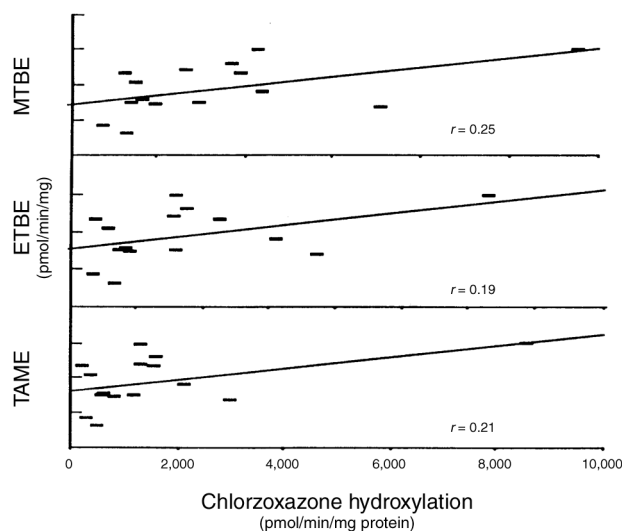
Table 4. Metabolism of MTBE and Other Gasoline Ethers by Human CYP2A6 and CYP2E1 (with Coexpressed CYP Reductase)

	Activity (nmol metabolite/min/nmol CYP)		
	MTBE	ETBE	TAME
CYP2A6	6.1	13.6	37
CYP2E1	0.7	0.8	0.4

Human CYP2A6 and CYP2E1 were individually coexpressed with human CYP reductase via a baculovirus system. The incubation mixture contained 0.04 nmol of CYP2A6 or CYP2E1 and 1 mM substrate. The differences between duplicate incubations or between repeat assays were < 10%.

Table 5. Metabolism of Gasoline Ethers by Heterologously Expressed Human CYP Enzymes[a]

	Activity (pmol metabolite/min/pmol CYP)		
Enzyme	MTBE	ETBE	TAME
CYP2A6	1.45 (0.18)[b]	1.61 (0.29)	2.79 (0.64)
CYP2E1	0.87 (0.12)	0.34 (ND)	0.53 (0.06)
CYP2B6	0.04 (0.03)	0.18 (0.05)	1.05 (0.05)
CYP1A2	ND (ND)	0.13 (—)	— (—)
CYP4A11	0.28 (0.08)	— (0.03)	0.09 (ND)
CYP3A4	ND (ND)	— (ND)	0.32 (0.14)
CYP1A1	ND (ND)	— (—)	0.11 (—)

[a] ND, not detectable; —, not measured. Activities for CYP1B1, CYP2C8, CYP2C9, CYP2C19 and CYP2D6 were not detectable.

[b] The ether-metabolizing activities determined at 0.1 mM substrate concentration are shown in parentheses.

Differences between duplicate incubations or between 2 separate determinations were < 10%. The incubation was carried out at 37°C for 30 minutes either with 100 pmol of human lymphoblast-expressed enzymes and 1 mM of substrate or with 20 to 50 pmol of the expressed enzymes and 0.1 mM of substrate. The GC detection limit was 0.15 ng for TBA and 0.29 ng for TAA.

Table 6. Effects of Coumarin, a CYP2A6 Substrate, on Metabolism of Gasoline Ethers in Human Liver Microsomes

	Inhibition of Metabolism (%)		
	MTBE	ETBE	TAME
Control	0	0	0
Methanol	21	20	22
Coumarin			
1 µM	42	45	43
10 µM	76	85	76
100 µM	89	99	80

Microsomal proteins (100–150 µg) were incubated with 1 mM substrate in a final volume of 400 µL at 37°C for 30 minutes. Coumarin (dissolved in 2 µL methanol) was added into the incubation mixture prior to initiation of the reaction. Values are the mean of 3 individual samples (HBI 102, 107, and 115).

able to metabolize MTBE, ETBE, and TAME, but CYP2A6 was more active than CYP2E1 in metabolizing all of these ethers (Table 4). We further used a battery of human CYP enzymes expressed in human B-lymphoblastoid cells to determine the capability of each individual CYP form in the metabolism of MTBE, ETBE, and TAME. Among all the available expressed human CYP enzymes, CYP2A6 still showed the highest activities in metabolizing all 3 ethers, and CYP2E1 had the second-highest ether-metabolizing activities (Table 5). No activities were detectable in metabolizing MTBE, ETBE, or TAME by heterologously expressed CYP1B1, CYP2C8, CYP2C9, CYP2C19, or CYP2D6 or in metabolizing MTBE by CYP1A1, CYP1A2, or CYP3A4 (Table 5). CYP2B6 and CYP3A4 showed either low (0.04 pmol TBA/min/pmol CYP) or no activity in metabolizing MTBE (Table 5), even though previous analysis with human liver microsomal samples had revealed some correlation between the ether-metabolizing activities and the activities of these 2 enzymes.

CHEMICAL AND ANTIBODY INHIBITION

Both the correlation analysis with human liver microsomes and the metabolism study with heterologously expressed CYP enzymes suggest that CYP2A6 is a major enzyme for oxidative metabolism of MTBE, ETBE, and TAME. If this is true, addition of a known CYP2A6 substrate such as coumarin in the incubation mixture could be expected to competitively inhibit the metabolism of these ethers. To test this hypothesis, different amounts of coumarin (all dissolved in 2 µL methanol) were added to the incubation mixture prior to initiation of the reaction. The solvent methanol alone caused approximately 20% inhibition of the metabolism of MTBE, ETBE, and TAME by human liver microsomes ($n = 3$). Nevertheless, addition of coumarin clearly inhibited metabolism of these ethers in a concentration-dependent manner (Table 6), suggesting that these gasoline ethers are substrates of human CYP2A6.

We also used an immunoinhibition approach to determine the relative contributions of CYP2A6 and CYP2E1 to metabolism of MTBE, ETBE, and TAME in human liver microsomes ($n = 3$). The maximum effective concentrations of the antibodies were established by prior experiments (data not shown). Under optimal conditions, addition of a monoclonal antibody against human CYP2A6 significantly inhibited (75% to 95%) the metabolism of all 3 ethers in human liver microsomes (Figure 6). In contrast, there was no inhibition by a monoclonal antibody against CYP2E1 (Figure 6) even though the same anti-CYP2E1 antibody inhibited more than 90% of the CYP2E1 activity (assayed as NDMA demethylase) in these human liver microsomes (data not shown).

ETHER METABOLISM IN MOUSE LIVER MICROSOMES LACKING Cyp2e1

An early report suggested that Cyp2e1 may be involved in metabolism of MTBE in rat liver microsomes (Brady et al 1990). Heterologously expressed human CYP2E1 was able to catalyze the oxidative metabolism of these gasoline ethers although its activity was much less than that of human CYP2A6. The activity correlation analysis and antibody inhibition study with human liver microsomes strongly suggest, however, that CYP2E1 is not a major enzyme involved in metabolism of MTBE and other gasoline ethers. We therefore used transgenic *Cyp2e1*-null mice to determine further the role of Cyp2e1 in metabolism of MTBE, ETBE, and TAME. The *Cyp2e1*-null mice were established by gene targeting, and their *Cyp2e1* genes were disrupted (Lee et al 1996).

No NDMA demethylase activity, a highly selective activity of Cyp2e1 in liver microsomes from the *Cyp2e1*-null mice (Table 7), was detectable, confirming a previous report of no *Cyp2e1* mRNA and protein expression in this line of transgenic mice (Lee et al 1996). In contrast, liver
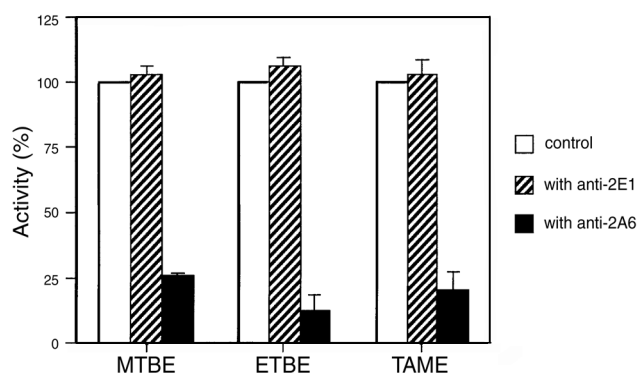
Figure 6. Effects of anti-CYP2A6 and anti-CYP2E1 human antibodies on the metabolism of gasoline ethers in human liver microsomes. The antibodies were preincubated with human liver microsomes for 10 minutes on ice, and the ether-metabolizing activities were determined after incubation in the presence of the antibodies.

microsomes prepared from the C57BL/6N and 129/Sv mice were active in catalyzing the oxidative demethylation of NDMA with a specific activity of approximately 100 pmol/min/mg protein (Table 7). Both the C57BL/6N and 129/Sv mice are parental lineage strains of the *Cyp2e1*-null mice and contain the intact functional *Cyp2e1* alleles (*Cyp2e1*$^{+/+}$). We then compared the female *Cyp2e1*-null mice with their age-matched female C57BL/6N and 129/Sv mice for ether-metabolizing activity in the liver microsomes. As shown in Table 7, the metabolizing activity (pmol/min/mg) in liver microsomes from the *Cyp2e1*-null mice was 540 ± 170 for MTBE, 510 ± 240 for ETBE, and 1114 ± 250 for TAME, when assayed at a 1 mM substrate concentration. These activity levels are not substantially different from those of the C57BL/6N and 129/Sv groups (Table 7). The MTBE-metabolizing activity was the same for the *Cyp2e1*-null mouse group and the control groups when the assay was performed at a lower substrate concentration (0.1 mM). The activity (pmol/min/mg) was 220 ± 60 for *Cyp2e1*-null mice, 250 ± 80 for C57BL/6N mice, and 230 ± 60 for 129/Sv mice (Table 7). Differences between the male and female mice at the same age and between the 2 age groups of the same sex were not significant, although the ether-metabolizing activity in the 8-month-old male mice appeared to be slightly higher than that in the 4-month-old male mice (Table 8).

IDENTIFICATION AND CHARACTERIZATION OF *CYP2A6* VARIANTS

After identifying human CYP2A6 as a major enzyme for metabolism of MTBE and other gasoline ethers, we examined the possible sequence variations in the *CYP2A6* gene in the individuals who claimed to be sensitive to MTBE exposure. The *CYP2A6* gene, a 7.8-kb fragment, was amplified from the blood DNA, and each coding exon was directly sequenced with the exon-specific primers. Figure 7 illustrates how a missense genetic variant was identified. In a total of 23 samples, we identified 3 novel

Table 7. Metabolism of NDMA and Gasoline Ethers in Liver Microsomes from *Cyp2e1*-null Mice and Their Parental Lineage Strains

Strains	Activity (pmol/min/mg protein)			
	NDMA	MTBE	ETBE	TAME
Cyp2e1-null (2e1$^{-/-}$)	ND	540 ± 170 (220 ± 60)	510 ± 240	1114 ± 250
C57Bl/6N (2e1$^{+/+}$)	100 ± 31	670 ± 160 (250 ± 80)	700 ± 120	1010 ± 260
129/Sv (2e1$^{+/+}$)	100 ± 23	740 ± 140 (230 ± 60)	660 ± 140	760 ± 250

Female mice at age of 7 weeks were used. The values are the mean ± SD (n = 4 or 5); ND, not detectable. For NDMA metabolism, microsomal protein (50 μg) was incubated with [^{14}C]NDMA (40 μM) at 37°C for 15 minutes. For ether metabolism, microsomal protein (0.6 mg) was incubated with either 1 mM or 0.1 mM (values in parentheses) substrate at 37°C for 30 minutes.

Table 8. Metabolism of Gasoline Ethers in Liver Microsomes from *Cyp2e1*-null Mice at Different Ages

	Activity (nmol/min/mg protein)		
	MTBE	ETBE	TAME
4 months old			
Male	0.40 ± 0.12	0.45 ± 0.11	0.70 ± 0.32
Female	0.42 ± 0.07	0.33 ± 0.04	0.72 ± 0.08
8 months old			
Male	0.68 ± 0.06	0.68 ± 0.04	1.11 ± 0.14
Female	0.48 ± 0.30	0.40 ± 0.19	0.87 ± 0.47

Microsomal protein (0.6 mg) was incubated with 1 mM substrate at 37°C for 30 minutes. The values are the mean ± SD ($n = 3$).
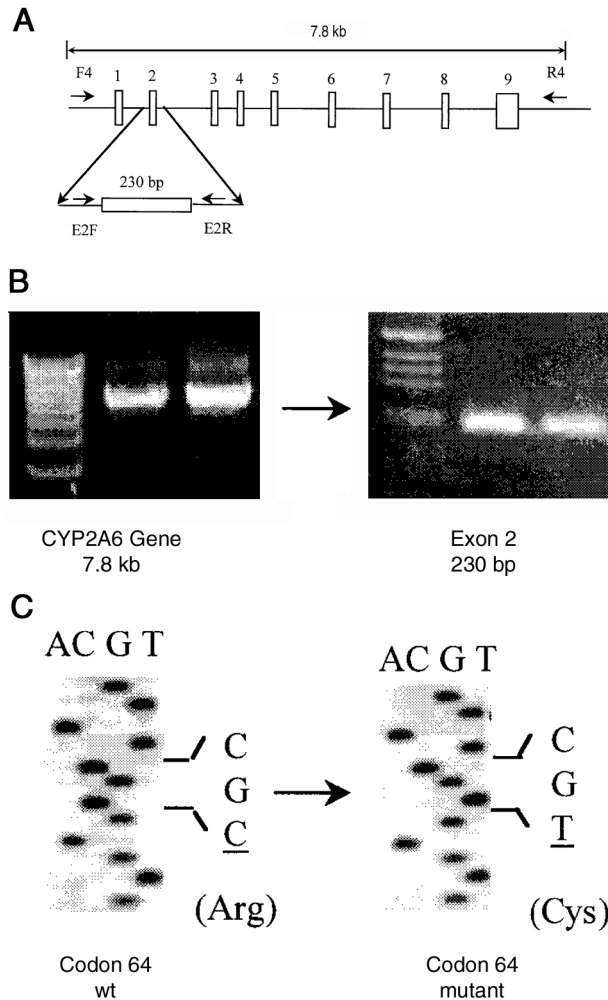
Figure 7. Identification of missense variant (Arg64Cys) in exon 2 of human *CYP2A6* gene. (A) A 2-step PCR amplification strategy, (B) electrophoresis of the amplified PCR products, (C) a single nucleotide variation detected by DNA sequencing. wt = wild type; kb = kilobase; bp = base pairs.

Table 9. Distribution of *CYP2A6* Missense Genetic Variants in Subjects ($n = 23$) Who Claimed to Be Sensitive to MTBE

	Variants			
	Ser29Asn	Arg64Cys	Leu160His	Lys476Arg
Heterozygote	1	1	1	1
Homozygote	1	1	1	1
Allelic Frequency	6.5%	10.9%	2.2%	4.3%

Blood DNA was used for PCR amplification of the *CYP2A6* gene. The variations in the coding exons of the *CYP2A6* gene were determined by DNA sequencing.

CYP2A6 missense variants: Ser29Asn in exon 1, Arg64Cys in exon 2, and Lys476Arg in exon 9. In addition, we confirmed the existence of a previously reported *CYP2A6* polymorphic variant (v1, Leu160His). The distribution of these variants in the test subjects is shown in Table 9.

The cDNA of wildtype CYP2A6 was used to construct the *CYP2A6* variant cDNAs (for Ser29Asn, Arg64Cys, and Leu160His) by site-directed mutagenesis. These cDNAs were then used for protein expression by a baculovirus/Sf9 cell system. Immunoblot analysis using human CYP2A6-specific antibodies demonstrated that the wildtype CYP2A6 and the variant proteins were successfully expressed (Figure 8).

Metabolism of MTBE, ETBE, and TAME was carried out with the expressed CYP2A6 proteins. In comparison with wildtype CYP2A6, the Ser29Asn and Arg64Cys variants
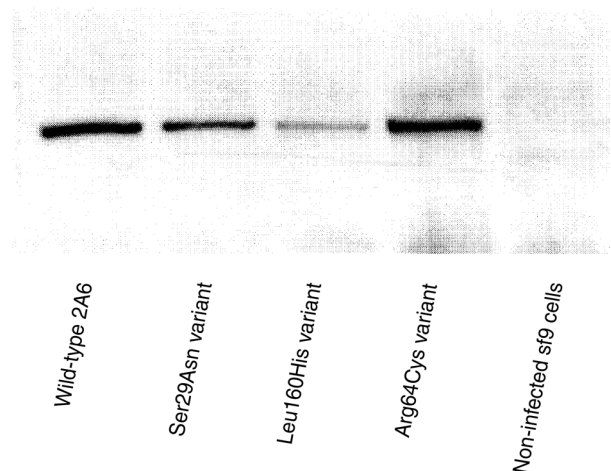
Figure 8. Immunoblot analysis of CYP2A6 proteins expressed in Sf9 cells. CYP2A6 content in the membrane fraction of the Sf9 cells infected with CYP2A6 were determined by immunoblot analysis. For each sample, 20 μg of microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose sheet. The nitrocellulose sheet was then incubated with an anti-CYP2A6 antibody (Gentest) followed by immunostaining and chemiluminescence detection.

Table 10. Metabolism of Gasoline Ethers and Coumarin by Heterologously Expressed Human CYP2A6 Proteins

	Activity (pmol/min/pmol CYP)			
	MTBE	ETBE	TAME	Coumarin
Wildtype	3.5	2.6	3.6	6.8
Variants				
Ser29Asn	2.3	2.1	2.2	6.1
Arg64Cys	2.4	2.0	2.3	3.9
Leu160His (CYP2A6v1)	ND	ND	ND	ND

Values are the average of 2 to 3 separate determinations with the difference < 15%. ND = not detectable.

The final substrate concentration was 0.25 mM. CYP (5 pmol) was used in the incubation except for the Leu160His variant. Since the Leu160His variant has no measurable absorption at 450 nm, its CYP content was quantified by immunoblotting. For the Leu160His variant, the amount of microsomal protein (87 μg) used contained three times more CYP content than the others.

For coumarin 7-hydroxylation assay, 10 μg of microsomal protein was used (300 μg for CYP 2A6v1). Incubation was carried out at 37°C for 15 minutes with 50 μM coumarin.

showed a trend to slightly reduce the activity of metabolizing MTBE, ETBE, and TAME by 20% to 30%. The Leu160His (CYP2A6v1) variant showed a total loss of the ether-metabolizing activities (Table 10). The alterations in the ether-metabolizing activity of the CYP2A6 variant proteins are consistent with the changes in coumarin 7-hydroxylase activity, a characteristic activity of human CYP2A6 (Table 10). The Leu160His variant showed little coumarin 7-hydroxylation activity, even though a 30-fold higher amount of protein was used in the assay (Table 10).

METABOLISM OF GASOLINE ETHERS IN RAT NASAL MUCOSA

Previous studies by other investigators and by us have shown that rat nasal mucosa is highly active in metabolizing several important environmental chemicals (Bond et al 1993; Hadley and Dahl 1983; Hong et al 1991). Because inhalation is the major route for human exposure to MTBE and other gasoline ethers, we determined the ether-metabolizing activities in rat nasal mucosa. A representative chromatogram for the metabolic profile of MTBE in the microsomes prepared from rat liver and olfactory mucosa is shown in Figure 9. Although the metabolic profile is the same, the activity in metabolizing MTBE to TBA is dramatically higher in olfactory mucosa microsomes than in liver microsomes under the same incubation conditions. Further analysis with more samples and using ETBE and TAME as substrates showed that rat olfactory mucosa microsomes exhibit the highest activities in metabolizing all 3 ethers. The microsomal activity level of the olfactory
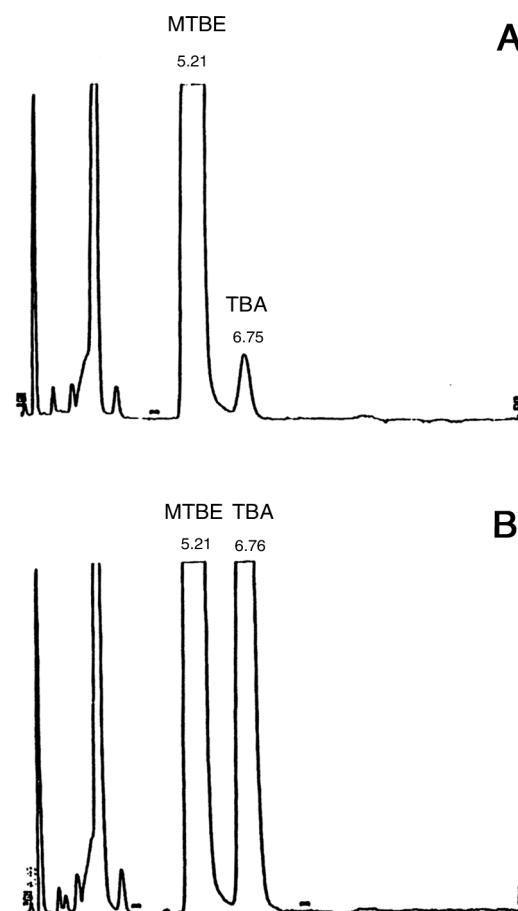
Figure 9. Headspace gas chromatograms of MTBE metabolism in rat liver (A) and olfactory mucosa (B) microsomes. Microsomes (600 μg protein) were incubated with 1 mM MTBE at 37°C for 30 minutes. Formation of TBA was determined by headspace GC. The sensitivity of the detector (absorbance units full scale) was the same for both chromatograms.

mucosa was 46-fold higher than the liver in metabolizing MTBE, and 37-fold and 25-fold higher, respectively, in metabolizing ETBE and TAME (Table 11). The activity level of the olfactory mucosa microsomes was approximately 5-fold to 9-fold higher than that of the respiratory mucosa microsomes, depending on which ether substrate was used. No detectable activities were found in the microsomes prepared from the lungs, kidneys, and olfactory bulbs of these untreated rats (Table 11).

For all the rat tissues examined, no TBA or TAA formation was detected when the cytosolic fractions were used for the incubation with MTBE, ETBE, and TAME (data not shown). When the incubation was carried out in the absence of an NADPH-generating system, the activities in the rat olfactory microsomes in metabolizing MTBE, ETBE, and TAME were undetectable (Table 12). Bubbling of the

Table 11. Metabolism of MTBE, ETBE, and TAME in Microsomes Prepared from Various Rat Tissues

		Activity (nmol/min/mg protein)		
	n	MTBE	ETBE	TAME
Nasal Mucosa				
Olfactory	6	9.3 ± 1.3	8.8 ± 0.6	13.2 ± 0.6
Respiratory	6	1.8 ± 0.3	1.0 ± 0.2	2.1 ± 0.4
Liver	6	0.2 ± 0.01	0.2 ± 0.01	0.5 ± 0.03
Lung	5	ND	ND	ND
Kidney	5	ND	ND	ND
Olfactory Bulb	3	ND	ND	ND

Microsomes were incubated with 1mM substrate in a final volume of 400 μL at 37°C for 30 minutes. The microsomal protein used was 600 μg for liver, lung, kidney, olfactory bulb, and 100 μg for olfactory or respiratory mucosa microsomes.

Values are the mean ± SE; ND, not detectable. Tissues from 3 to 5 rats were pooled to make a single sample; n, number of samples.

Table 12. Effects of NADPH and CO on Metabolism of MTBE and TAME in Rat Olfactory Mucosa Microsomes

	Activity (nmol/min/mg protein)		
	Control	Without NADPH	With CO pretreatment
MTBE	7.1	ND	0.9 (87)
ETBE	9.5	ND	1.2 (88)
TAME	11.4	ND	1.8 (85)

Rat olfactory mucosa microsomes were pooled from 6 samples (each sample was pooled from 5 rats). Microsomes (50 μg) were incubated with 0.25 mM substrate at 37°C for 30 minutes. To determine the effects of NADPH, the NADPH-generating system was omitted from the incubation. The effect of CO was determined by bubbling the microsomal mixture with 95% CO for 3 minutes before the addition of the substrate. The activity values are the average of 2 separate determinations. The percentage of inhibition is shown in parentheses; ND, not detectable.

microsomal incubation mixture with 95% CO for 3 minutes prior to initiation of the reaction caused 85% to 87% reduction in the formation of TBA or TAA (Table 12). These results are consistent with the results of human and mouse livers, providing further evidence to support the role of CYP enzymes in metabolism of these gasoline ethers.

Kinetic studies on metabolism of MTBE, ETBE, and TAME in the rat olfactory mucosa microsomes are summarized in Figure 10. At substrate concentrations ranging from 0.01 to 2.5 mM, Michaelis-Menten kinetics were observed in the formation of TBA from MTBE or ETBE,
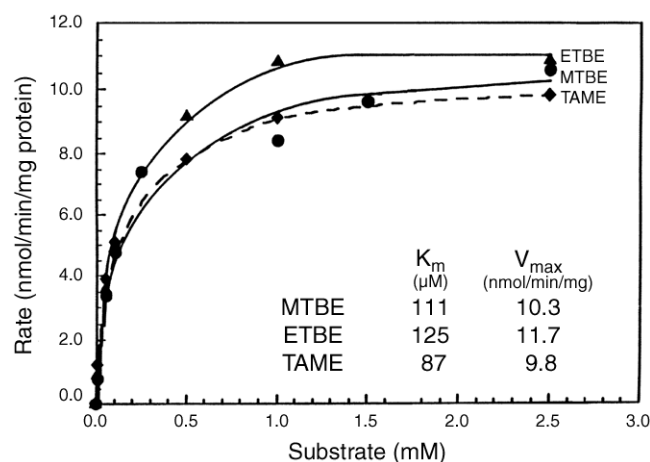
Figure 10. Kinetics of metabolism of MTBE, ETBE, and TAME in rat olfactory mucosa microsomes. Microsomes (50 μg protein) were incubated with the substrates at different concentrations, and the formation of TBA or TAA was determined by headspace GC.

and the formation of TAA from TAME. The apparent K_m and V_{max} values for metabolism of MTBE, ETBE, and TAME were very close, ranging from 87 to 125 μM and from 9.8 to 11.7 nmol/min/mg protein, respectively.

When TAME (0.1 to 0.5 mM) was added into the incubation mixture, a concentration-dependent inhibition was observed in metabolism of MTBE and ETBE (Table 13). On the other hand, metabolism of TAME was also inhibited by the presence of MTBE or ETBE in the incubation mixture. At 0.25 mM of TAME, the presence of MTBE or ETBE in the incubation mixture caused a 24% or 32% decrease (7.0 and 6.2 versus 9.2 nmol/min/mg) in the formation of TAA (Table 13). Metabolism of MTBE, ETBE, and TAME in the olfactory mucosa microsomes was also inhibited to a similar extent by the addition of coumarin. At 50 μM, coumarin caused approximately an 87% inhibition in metabolism of these ethers (data not shown). These results suggest that, as in the human liver, metabolism of MTBE, ETBE, and TAME in the rat olfactory mucosa microsomes are also catalyzed by the same CYP enzyme(s).

DISCUSSION AND CONCLUSIONS

Previously, the pharmacokinetics and toxicity of MTBE were mainly obtained through rodent studies. Prior to our study, metabolism of MTBE and 2 other gasoline ethers in human tissues and the enzymes involved were unknown. This information is important in our understanding of the health effects of MTBE in humans and in assessing the human relevance of the animal data. In the present study, we demonstrate that human liver is active in metabolizing MTBE to TBA. Although some reports indicate that TBA

Table 13. Effects of TAME on Metabolism of MTBE and ETBE in Rat Nasal Mucosa

	Activity (nmol/min/mg protein)			
	MTBE	TAME	ETBE	TAME
TAME in mixture (mM)				
0	6.7		9.2	
0.10	2.8 (58)	4.2	4.8 (48)	3.7
0.25	2.0 (71)	7.0	3.7 (60)	6.2
0.50	1.2 (82)	8.5	2.2 (76)	8.5
TAME alone (mM)				
0.25		9.2		9.2

The microsomes were pooled from 6 samples (each sample was pooled from 5 rats). Microsomal protein (50 μg) was incubated with the substrates in a final volume of 400 μL at 37°C for 30 minutes. The substrate concentrations for MTBE and ETBE were 0.25 mM. Metabolism of MTBE and ETBE was assayed as TBA formation, and metabolism of TAME was assayed as TAA formation. The activity values are the average of duplicate determinations.

The percentage of inhibition is shown in parentheses.

can be further metabolized (Costantini 1993), TBA is not a substrate of alcohol dehydrogenase and has been thought of as a nonmetabolizable alcohol. Although Cederbaum and Cohen (1980) reported metabolism of TBA to formaldehyde by rat liver microsomes, the high value of the apparent K_m for TBA (30 mM) in their findings suggests a limited relevance of this finding to TBA metabolism in vivo. Incubation of rat liver microsomes with 0.1 mM TBA did not result in detectable loss of TBA during the incubation period (Brady et al 1990).

Our study also demonstrates that human liver microsomes are active in metabolizing ETBE and TAME to their alcohol metabolites, TBA and TAA, respectively, and that metabolism of MTBE, ETBE, and TAME is catalyzed by the same CYP enzyme(s). This is consistent with the fact that these 3 ethers are similar in structure and physical-chemical properties. Whereas the exposure, metabolism, and health effects of MTBE have been under extensive investigation, corresponding information for ETBE and TAME is lacking. Therefore, our data could be useful in predicting the pharmacokinetics and health effects of ETBE and TAME from the MTBE studies.

The critical role of CYP enzymes in metabolism of MTBE and 2 other ethers is clearly shown by cellular localization of the ether-metabolizing activities, by NADPH dependence and carbon monoxide inhibition of the ether metabolism in different microsomes, and by metabolism of the ethers by the heterologously expressed CYP enzymes. We further demonstrate that human CYP2A6 is a major

enzyme responsible for oxidative metabolism of MTBE and 2 other gasoline ethers in human liver. This conclusion is based on the results obtained from several complementary approaches:

- the highest level of correlation was obtained with CYP2A6 for the ether-metabolizing activities in human liver microsomes;
- the highest activity of CYP2A6 in metabolizing MTBE, ETBE, and TAME among all the heterologously expressed human CYP enzymes examined;
- a significant inhibition (75% to 95%) of metabolism of all 3 ethers in human liver microsomes by an inhibitory antibody against human CYP2A6; and
- a concentration-dependent inhibition of metabolism of these ethers in human liver microsomes by the CYP2A6 substrate coumarin.

Although each of these approaches has its own limitations, together they provide convincing data in establishing the role of human CYP2A6 in metabolism of MTBE, ETBE, and TAME.

CYP2E1, which is constitutively expressed in liver and other tissues, plays a key role in metabolism of many environmental chemicals, particularly low molecular weight organic molecules (Yang et al 1990, 1992). Because diethyl ether is a good substrate of CYP2E1, and because a partial involvement of Cyp2e1 was implicated in metabolism of MTBE in the rat liver microsomes (Brady et al 1988, 1990), we originally hypothesized that CYP2E1 is responsible for metabolism of MTBE. In the present study, the heterologously expressed CYP2E1 did show activities in metabolizing MTBE, ETBE, and TAME. The poor correlation of the ether-metabolizing activities with the CYP2E1 activity in human liver microsomes, however, and the lack of inhibition of ether metabolism by CYP2E1-specific antibodies strongly suggest that CYP2E1 is not a major enzyme involved in metabolism of MTBE and other gasoline ethers. This conclusion is substantiated by the study with the *Cyp2e1*-null mice, in which the levels of ether-metabolizing activities in the liver microsomes lacking Cyp2e1 were not significantly different from those of the liver microsomes from the wildtype control mice that carried the functional *Cyp2e1* gene.

Like *CYP2E1*, *CYP2A6* is constitutively expressed in various human tissues, with its highest level in the liver, and catalyzes metabolism of many important environmental chemicals and drugs (Aoyama et al 1990; Crespi et al 1990; Yun et al 1991; Camus et al 1993; Pelkonen and Raunio 1995). The existence of functional genetic polymorphism of human *CYP2A6* is strongly indicated by the observation of marked interindividual activity variations in vitro and in vivo as well as the bimodal activity distribution in general

populations (Rautio et al 1992; Iscan et al 1994). Indeed, several human *CYP2A6* variants have been reported, including CYP2A6v1 (Leu160His), CYP2A6v2 (containing multiple missense alterations in exons 3, 6, and 8), and a large deletion variant (Fernandez-Salguero et al 1995; Nunoya et al 1998). The deletion variant is expected to be null in phenotype, and the CYP2A6v1 variant has been reported to lack the catalytic activity for coumarin 7-hydroxylation, a characteristic activity of CYP2A6 (Yamano et al 1990).

In the present study, we identified 3 novel missense *CYP2A6* variants in subjects who claimed to be sensitive to MTBE exposure. Functional analysis of 2 of these 3 genetic variants (Ser29Asn and Arg64Cys) and the Leu160His variant revealed that the Ser29Asn and Arg64Cys variants show a trend to slightly reduced activity in metabolizing MTBE, ETBE, and TAME by 20% to 30%. Whether these changes are significant and biologically relevant remains to be determined. The Leu160His (CYP2A6v1) variant showed a total loss of ether-metabolizing activities. The alterations in ether-metabolizing activity of the CYP2A6 variant proteins are consistent with the changes in coumarin 7-hydroxylase activity. The profound effect of a single amino acid substitution in CYP2A6v1, which is at a position distal to the heme-binding cysteine (codon 439), on CYP2A6 catalytic activity is interesting and the mechanisms involved remain to be studied. The studies with heterologously expressed CYP2A6 variant proteins do not represent the heterozygote situation, but the biochemical characterization does mimic the homozygote situation and may predict the role of the homozygous CYP2A6 variants as part of in vivo metabolism of MTBE and the 2 other ethers.

The presence of individuals who claim to be sensitive to MTBE has raised a public concern on the safety of MTBE. One possible explanation for the reported sensitivity could be related to differences in individual ability to metabolize MTBE. This speculation appears to be consistent with several observations: only a small percentage of exposed people claim to be sensitive to MTBE; blood and urine levels of TBA vary considerably among subjects experimentally exposed to MTBE (Johanson et al 1995; White et al 1995; Cain et al 1996), as do MTBE-metabolizing activities in human liver microsomes (this report); and the catalytic activities of some CYP2A6 genetic variants appear to be significantly altered (this report). The role of genetic polymorphism of *CYP2A6* in the in vivo metabolism of and the susceptibility to MTBE remains to be studied.

In the present study, we demonstrate that rat nasal mucosa has high activity in metabolizing MTBE and 2 other ethers. This finding is consistent with previous reports that rat nasal mucosa is highly active in metabolizing environmental chemicals (Bond 1983; Bond and Li 1983; Hadley and Dahl 1983; Brittebo and Ahlman 1984; Hong et al 1991, 1992). Several CYP enzymes have been demonstrated to be present in rat nasal mucosa (Ding and Coon 1993). At present, the particular CYP forms responsible for metabolism of MTBE, ETBE, and TAME in rat nasal mucosa are not known. Significant inhibition of metabolism of these ethers by coumarin suggests that CYP enzymes responsible for ether metabolism are probably also involved in coumarin metabolism. Preliminary results have shown that baculovirus expressed rat *Cyp2a3*, a predominant CYP form expressed in rat olfactory mucosa and an ortholog of human *CYP2A6* (Liu et al 1996), is active in metabolism of MTBE, ETBE, and TAME (Hong and Ding, unpublished results 1999).

In everyday life, the nasal tissues are exposed to the highest levels of airborne environmental chemicals. Efficient metabolism in situ in the nasal mucosa could be particularly important for inhaled chemicals, such as MTBE, in determining their fate and elicited biological responses (Dahl 1989; Morris 1994). Because inhalation is a major route for human exposure to MTBE and other gasoline ethers, our finding is of interest in consideration of the facts that (1) in situ metabolism of MTBE by olfactory mucosa may play a role in smell sensory irritation related to MTBE exposure and (2) formaldehyde formed from MTBE and TAME, as well as acetaldehyde from ETBE, are reported to be nasal carcinogens in rodents (Swenberg et al 1980). However, the relevance of our finding to the human situation needs to be determined.

IMPLICATIONS

Genetic polymorphism of CYP enzymes is believed to be an important factor in determining an individual's sensitivity to environmental chemicals. We hypothesized that the reported increased sensitivity to MTBE exposure in certain individuals could be due to their altered ability to metabolize MTBE, which may be related to functional genetic polymorphism of MTBE-metabolizing enzymes such as CYP2A6. Demonstration of human CYP2A6 as a major MTBE-metabolizing enzyme and identification of the presence of *CYP2A6* genetic variants with altered MTBE-metabolizing activity are essential steps in testing our hypothesis. If genetic polymorphism of *CYP2A6* is indeed a susceptibility biomarker, it may serve as an objective means of identifying sensitive human subjects and could have great regulatory significance.

Further studies are needed to elucidate the importance of *CYP2A6* genetic polymorphism in MTBE metabolism in vivo and sensitivity to MTBE exposure. One approach is to correlate the *CYP2A6* variant genotypes with the blood levels of MTBE and TBA as well as the biological responses in healthy volunteers subjected to controlled exposure to MTBE. Another approach is to conduct a case-control study to examine whether the allelic frequency distribution of *CYP2A6* genetic polymorphism is significantly different in those individuals self-reported to be sensitive to MTBE when compared with the control population.

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OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

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ABBREVIATIONS AND OTHER TERMS

Ba(OH)$_2$	barium hydroxide
bp	basepair
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CO	carbon monoxide
CYP	cytochrome P450 superfamily
ETBE	ethyl *tert*-butyl ether
GC	gas chromatography
HCl	hydrogen chloride
kb	kilobase
KCl	potassium chloride
K_m	Michaelis constant
MgCl$_2$	magnesium chloride
MTBE	methyl *tert*-butyl ether
NaCH$_3$COOH	sodium acetate
NADP$^+$	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NDMA	*N*-nitrosodimethylamine
PCR	polymerase chain reaction

r	bivariate correlation coefficient
Sf9	*Spodoptera frugigerda*
SDS	sodium dodecyl sulfate
TAA	*tert*-amyl alcohol
TAME	*tert*-amyl methyl ether
TBA	*tert*-butyl alcohol
TCA	trichloroacetic acid
UTR	untranslated regions
V_{max}	maximum rate of metabolism
$ZnSO_4$	zinc sulfate

CYP ELEMENTS CITED IN THIS REPORT

Human Genes	Rodent Genes	Human Proteins	Rodent Proteins
		CYP1A1	
		CYP1A2	
	Cyp2a3		
CYP2A6		CYP2A6	
CYP2A7		CYP2A7	
CYP2A13		CYP2A13	
			Cyp2b1
		CYP2B6	
		CYP2C8	
		CYP2C9	
		CYP2C19	
		CYP2D6	
CYP2E1	*Cyp2e1*	CYP2E1	Cyp2e1
		CYP3A4	
		CYP3A5	
		CYP4A11	

INVESTIGATORS' REPORT

Biotransformation of MTBE, ETBE, and TAME After Inhalation or Ingestion in Rats and Humans

Wolfgang Dekant, Ulrike Bernauer, Elisabeth Rosner, and Alexander Amberg

ABSTRACT

The biotransformation of methyl *tert*-butyl ether (MTBE*), ethyl *tert*-butyl ether (ETBE), and *tert*-amyl methyl ether (TAME) was studied in humans and in rats after inhalation of 4 and 40 ppm of MTBE, ETBE, and TAME, respectively, for 4 hours, and the biotransformation of MTBE and TAME was studied after ingestion exposure in humans to 5 and 15 mg in water. *tert*-Butyl alcohol (TBA), a TBA conjugate, 2-methyl-1,2-propanediol, and 2-hydroxyisobutyrate were found to be metabolites of MTBE and ETBE. *tert*-Amyl alcohol (TAA), free and glucuronidated 2-methyl-2,3-butanediol (a glucuronide of TAA), 2-hydroxy-2-methyl butyrate, and 3-hydroxy-3-methyl butyrate were found to be metabolites of TAME.

After inhalation, MTBE, ETBE, and TAME were rapidly taken up by both rats and humans; after termination of exposure, clearance from blood of the ethers by exhalation and biotransformation to urinary metabolites occurred with half-times of less than 7 hours in rats and humans. Biotransformation of MTBE and ETBE was similar in humans and rats after inhalation exposure. 2-Hydroxyisobutyrate was recovered as a major product in urine. All metabolites of MTBE and ETBE excreted with urine were eliminated with half-times of less than 20 hours. Biotransformation of TAME was qualitatively similar in rats and humans, but the meta-

bolic pathways were different. In humans, 2-methyl-2,3-butanediol, 2-hydroxy-2-methyl butyrate, and 3-hydroxy-3-methyl butyrate were recovered as major urinary products. In rats, however, 2-methyl-2,3-butanediol and its glucuronide were major TAME metabolites recovered in urine.

After ingestion of MTBE and TAME, both compounds were rapidly absorbed from the gastrointestinal tract. Hepatic first-pass metabolism of these ethers was not observed, and a significant part of the administered dose was transferred into blood and cleared by exhalation. Metabolic pathways for MTBE and TAME and kinetics of excretion were identical after ingestion and inhalation exposures.

Results of studies presented here suggest (1) that excretion of MTBE, ETBE, and TAME in rats and humans is rapid, (2) that biotransformation and excretion of MTBE and ETBE are identical in rats, and (3) that biotransformation and excretion of TAME is quantitatively different in rats and humans.

INTRODUCTION

MTBE and several other branched chain alkyl ethers are presently used or are being considered for use as fuel additives to reduce carbon monoxide and hydrocarbon emissions. Exposure of the general population to these ethers may occur at service stations and inside cars. Concentrations of MTBE in the breathing zone of people refueling their cars have been determined to be up to 4 ppm, and concentrations inside cars were determined to be up to 0.1 ppm (Health Effects Institute 1996). MTBE, a current gasoline additive, can contaminate soil and underground water as a result of leaks from underground storage tanks. As part of its National Water Quality Assessment Program (Davidson 1995), the US Geological Survey detected MTBE in 27% of urban wells and in 1.3% of agricultural wells at concentrations of 0.2 mg/L (Reno NV) to 23 mg/L (Denver CO). Although these wells were not used to supply drinking water, these groundwater MTBE levels suggest that oxygenate contamination of drinking water may expose humans to ethers via ingestion. In the future,

TAME, a promising potential additive for gasoline, may similarly contaminate groundwater and result in human exposure through drinking water.

A variety of health effects (such as headache, dizziness, and airway irritation) have been associated with MTBE exposure (ECETOC 1997). Controlled exposure studies generating higher blood levels of MTBE than were expected in the general population have not resulted in reported symptoms or effects on performance in behavioral tests (ECETOC 1997). In animal studies, acute toxicity of MTBE and the other ethers is low, but studies in rats and mice have shown that chronic exposure to very high concentrations of MTBE induced some tumors. Chronic inhalation of MTBE by F344 rats (400, 3,000, and 8,000 ppm, 6 hours/day, 5 days/week) resulted in an increased incidence of nephropathy in both sexes of rats at concentrations of 3,000 and 8,000 ppm and in renal tumors in males at 3,000 ppm (Bird et al 1997). At the highest exposure dose, female mice exhibited an increase in the incidence of liver tumors (Burleigh-Flayer et al 1992). Problems hampering interpretation of this study include decreased survival of high-dose animals and exposures substantially shorter than lifespan. After chronic ingestion of 250 and 1,000 mg/kg MTBE, the incidence of tumors including testicular adenomas in Sprague-Dawley rats increased (Belpoggi et al 1995). The major metabolite of MTBE, TBA, also induced renal tumors in male rats. A mechanism of inducing such tumors in rat kidney by binding MTBE, or more likely the metabolite TBA, to α_{2u}-globulin has been proposed (Takahashi et al 1993), but tumor induction by chemical binding to α_{2u}-globulin is not considered to be relevant to humans (Swenberg 1993). A causative role of α_{2u}-globulin in the renal toxicity of MTBE has been debated, however (Scientific Advisory Board on Toxic Air Pollutants 1995). We have not found any data on toxicity from chronic exposure to the other ethers considered in this proposal. A study on the subchronic toxicity of TAME and ETBE has shown severe central nervous system–depression and increased liver weight after 4 weeks in both sexes of rats exposed to 4,000 ppm for 6 hours/day, 5 days/week, but showed no treatment-related histopathologic findings (White et al 1995).

Acute uptake and disposition of MTBE has been studied in rats and humans (Costantini 1993, Johanson et al 1995b). Inhaled MTBE is rapidly equilibrated in blood and elimination occurs primarily via exhalation of the parent compound and excretion of metabolites in urine. Biotransformation of MTBE in rats appeared to become saturated after high-exposure concentrations; but in the dose range studied in humans (up to 50 ppm), no saturation of the metabolic transformation was observed. The metabolism of MTBE seems to be mediated by cytochrome P450 (CYP) (Brady et al 1990), and several metabolites (TBA, 2-methyl-1,2-propanediol, α-hydroxyisobutyrate, acetone, formaldehyde, and formic acid) were identified in rats (Miller et al 1997). In humans, MTBE is metabolized to TBA in low yields (Johanson et al 1995b). Data on the in vivo biotransformation of other branched chain ethers considered in this proposal are not available. The available data and interpretation of biotransformation of hydrocarbons and structurally related ethers indicate, however, that CYP-mediated oxidations are major pathways in biotransformation of the ethers considered here. Primary alcohols formed by the initial biotransformation may be further oxidized to carboxylic acids or excreted as conjugates (Sipes and Gandolfi 1991).

SPECIFIC AIMS

This study intended to generate comparative data on the biotransformation of ethers added to gasoline in humans and in rats after inhalation exposure and in humans after ingestion exposure. In the first part of the study, the structures of excreted metabolites formed from the ethers in vivo were established as a basis for metabolite quantitation. In the second part, excretion of metabolites of MTBE, ETBE, and TAME was quantified for rats and humans after controlled inhalation exposure. In the third part, biotransformation and kinetics of metabolite excretion were quantified in humans who ingested MTBE and TAME in tap water. The resulting data were used to compare the excretion of metabolites in humans and rats and establish interindividual differences among humans. These data may thus serve as a basis for risk comparisons.

The generated data may also help in the interpretation of the animal toxicity data of the ethers and in the extrapolation of those data to humans. Moreover, with the known structures of metabolites, further studies on the toxicity of the metabolites may be performed for a better characterization of the mechanisms of ether toxicity and the relevance of these mechanisms to humans. The obtained results may also serve as a basis for studying the biotransformation of these ethers when present in gasoline.

Studies of CYP enzymes in the oxidation of MTBE, ETBE, and TAME were also planned in the initial application. After discussion with HEI staff, these studies were not performed because the complicated biotransformation of TAME would have required development of a large number of procedures to quantify the metabolites. Moreover, the metabolism of MTBE, ETBE, and TAME by CYP in vitro has been studied by others (Turini et al 1998; Hong et al 1999a,b) and is addressed in the study by Hong et al in this report. The results obtained by these authors suggest

that several CYP enzymes contribute to oxidation of MTBE, ETBE, and TAME.

METHODS AND STUDY DESIGN

GENERAL STUDY DESIGN

Exposure of human volunteers and of rats by inhalation was performed in a dynamic exposure chamber accommodating 6 human subjects (seated) and 10 rats at 1 time ensuring identical exposure conditions. For each of the ethers, 2 exposure concentrations (4 and 40 ppm) were selected for 4-hour exposures. Toxicokinetics studies after ingestion were restricted to MTBE and TAME and were performed only in humans. Parent ethers and TBA (MTBE and ETBE exposures) and TAA (TAME exposures) were measured in blood samples, and relevant metabolites were measured in urine samples. In addition, volatile metabolites were measured in exhaled breath from 3 human subjects exposed to MTBE by ingestion to obtain complete mass balance.

HUMAN INHALATION EXPOSURE

Three healthy female and three healthy male subjects participated in the inhalation studies (Table 1) (Ertle et al 1972). The subjects were asked to refrain from alcoholic beverages and drugs 2 days before and throughout each experiment. Subjects did not abuse alcohol and were nonsmokers or occasional smokers. Subjects were healthy as judged by medical examination, and clinical blood chemistry confirmed no previous occupational exposure to gasoline ethers. Subjects did not refuel their cars during the 2 days prior to exposure and during the sample collection period.

Each subject was exposed to targeted concentrations of 4 or 40 ppm of each ether (MTBE, ETBE, TAME) for 4 hours in a dynamic exposure chamber (Ertle et al 1972). Ether concentrations in chamber air were measured at different sampling ports in 15-minute intervals by gas chromatography/mass spectrometry (GC/MS). Exposures started at 8 am. A time interval of 4 weeks was kept between exposures. No significant differences in temperature in the chamber, number of air exchanges and relative humidity were observed between the exposures. All human exposures were performed according to guidelines given in the Declaration of Helsinki and under approval of the local institutional review board.

The chamber design is shown in Figure 1. Generation of the chemical and air mix has been described (Ertle et al 1972, Müller et al 1972, 1974, 1975). The chamber had a total volume of 8 m^3, air flow rate was 28 m^3/hour at a temperature of 22°C, and the relative humidity was 50% to 60%. Ether concentrations were identical at different sites in the chamber both during actual exposures and during test runs (chamber tests with identical conditions were performed the day before each human exposure to demonstrate stability). After the exposure, all urine excreted by the subjects was collected for 72 hours at 6-hour intervals,

Table 1. Characteristics and Participation of Human Subjects in Exposure Studies

Subject	Age (year)	Height (cm)	Body weight (kg)	Body fat (%)[a]	Inhalation			Ingestion	
					MTBE	ETBE	TAME	MTBE	TAME
Male									
A	28	175	70	18.5	x		x	x	x
B	28	175	83	24.5	x	x			
C	28	185	75	13.5	x		x		
G	26	176	62	8.5		x	x		x
H	31	176	81	20.5		x			
K	30	179	93	29.5				x	x
M	28	184	80	19.5				x	
Female									
D	38	166	56	29.0	x		x	x	x
E	29	162	61	28.0	x	x		x	x
F	26	165	58	27.5	x	x	x		
I	29	174	60	26.5		x	x		
L	27	180	86	30.5				x	x

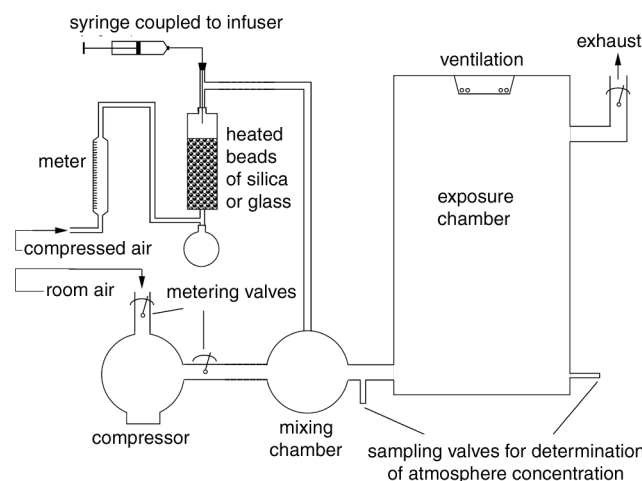[a] Percentage of body fat was measured according to Donoghue (1985).

Figure 1. Inhalation chamber used for exposures to MTBE, ETBE and TAME. The liquid ether is continously added into a stream of air passing over heated glass or silica beads. The vaporized ether is then mixed with room air and introduced into the exposure chamber. The concentration of the ether in the atmosphere in the chamber is determined by taking air samples and quantifying ether content by gas chromatography.

urine volumes were measured, and 2 aliquots (60 mL each) were rapidly frozen directly after each collection and stored at −20°C until sample preparation.

RAT INHALATION EXPOSURE

Male and female F344 rats (Harlan Winkelmann, Borchen, Germany) were kept in cages at constant humidity and temperature (21°C) with a 12-hour light/dark cycle (lights on at 8 am). Before the experiments, the animals were accustomed to the metabolic cages for 3 days, and control urine samples were collected for 12 hours directly before the start of the exposure. All animal experimentation was performed according to the guidelines of the German animal welfare act with permission from the appropriate authorities.

Five male (210 to 240 g) and five female (190 to 220 g) 12-week-old rats were exposed to targeted concentrations of 4 and 40 ppm of each of the ethers (MTBE, ETBE, TAME) in the exposure chamber described for humans. During the exposures, rats were kept in separate Macrolon cages with free access to food and water. After exposure the cages were checked for excreted urine, the animals were transferred to metabolic cages, and urine was collected at 4°C for 72 hours at 6-hour intervals. Blood samples from the tail vein (100 µL) were taken at the end of the exposure. During the exposures, rats did not excrete urine.

ETHER CONCENTRATIONS IN THE EXPOSURE CHAMBER

Samples (50 µL) of chamber air were taken every 15 minutes with a gas-tight syringe. MTBE, ETBE, and TAME in the atmosphere of the exposure chamber were measured by capillary GC/MS. Separation of MTBE and ETBE was performed with a DB624 fused silica column (30 m × 0.25 mm internal diameter [ID], 1.4-µm film) at an oven temperature of 35°C. Injector temperature was 150°C and detector temperature was 200°C; split injection was used with a split ratio of 5:1. During the separation (run time of 5 minutes), the intensity of the major fragment ion in the electron impact mass spectrum of MTBE (mass-to-charge ratio [m/z] 73) or ETBE (m/z 59) was monitored with a dwell time of 80 msec. Separation of TAME was performed with a DB1 fused silica column (30 m × 0.25 mm ID, 1-µm film) at an oven temperature of 35°C. Injector temperature was 150°C and detector temperature was 200°C; split injection was used with a split ratio of 5:1. During the separation (run time of 5 minutes), the intensity (m/z 73) was monitored with a dwell time of 80 msec. Quantitation was based on calibration curves obtained with metered ether concentrations.

HUMAN INGESTION EXPOSURE

[13]C-MTBE was used for the human ingestion exposure to be able to study low-dose metabolite excretion and avoid interference of the high background concentrations of 2-hydroxyisobutyrate present in urine. The analytic procedures were identical to those developed for the inhalation exposures. Methods for determination of ^{13}C-MTBE metabolites were adjusted for the content of ^{13}C in the metabolites with respect to mass spectrometric quantitation. Separation conditions and internal standards were identical as described for inhalation exposures. To study kinetics of excretion and biotransformation of MTBE and TAME, 6 human subjects (3 male and 3 female for each compound; see Table 1) were given 5 and 15 mg of MTBE or TAME in 100 mL of tap water. The subjects consumed the spiked water samples within 30 seconds. Blood samples were taken at 60-minute intervals from hour 0 to hour 4 and at 120-minute intervals from hour 4 to hour 12 after exposure. One additional blood sample was taken 24 hours after exposure. Urine samples were taken at 6-hour intervals for 96 hours after exposure.

ETHERS IN BLOOD

Blood samples (10 mL) from human subjects were collected using heparinized syringes. Blood samples from rats were 100 µL. Part of the blood samples from humans

(0.5 mL) and rats (0.025 mL) was immediately transferred into GC-autosampler vials (2-mL volume for human samples and 0.2-mL volume for rat blood samples). The vials were capped and stored at −20°C until analysis. The remainder of the samples was frozen at −20°C for storage.

For MTBE quantitation, 10 μL of an aqueous solution of ETBE (100 nmol/mL) was added through the septum and the vials were heated to 50°C for 1 hour. MTBE concentrations were quantified by headspace GC/MS by injecting 200 μl of the headspace from the vials using split injection (split ratio of 10:1). Samples were separated using a DB1 coated fused silica column (30 m × 0.25 mm ID, 1-μm film) at 40°C. In addition to monitoring m/z 73 (for MTBE), m/z 59 (the most intensive fragment ion in the electron impact mass spectrum of the internal standard ETBE) was monitored during the separation with dwell times of 80 msec. Quantitation was performed relative to the content of ETBE and referenced to calibration curves with fortified aliquots of blood samples from controls containing 0 to 10 nmol MTBE/mL blood. The method was linear in the range of concentrations used and calibration standards were analyzed with every sample series (usually 20 to 30 samples).

ETBE was quantified using MTBE as internal standard by adding 10 μL of an aqueous solution of MTBE (100 nmol/mL) using conditions as described above for MTBE. Quantitation was performed relative to the content of MTBE and referenced to calibration curves with fortified aliquots of blood samples from controls containing 0 to 20 nmol ETBE/mL blood. The method permitted quantitation of 0.1 nmol MTBE (or ETBE) per 1 mL of blood with a signal-to-noise ratio of 5:1. In the studies with ingestion exposure, a new instrument was used (see Appendix A), which lowered the quantitation limit for MTBE in blood to 0.01 nmol/mL using identical acquisition conditions. Metabolite concentrations in each urine sample collected were determined in duplicate. TAME and TAA in blood were quantified in a single analysis step.

TBA, TAME, AND TAA IN BLOOD

TBA was quantified by GC/MS using deuterated TBA (TBA-d_{10}) as internal standard. To GC-vials (2 mL volume for human samples and 0.2 mL volume for rat blood samples) containing 0.2 mL of human blood or 0.025 mL of rat blood, 5 μL of a TBA-d_{10} solution (1,000 nmol/mL in water) and 160 μL of 1N HCl for human blood or 20 μL of 1N HCl for rat blood samples were added with a microliter syringe through the septum. The vials were then kept at 80°C for 1 hour and 200 μL of the headspace for human blood or 100 μL for rat blood samples were injected into the GC/MS using split injection (split ratio of 10:1). Injector and transfer line temperatures were 220°C. Sam-

ples were separated using a DB1 coated fused silica column (30 m × 0.25 mm ID, 1-μm film) at a temperature of 40°C. The ions m/z 59 (TBA) and m/z 65 (TBA-d_{10}) were monitored during the gas chromatographic separation with dwell times of 80 msec. Quantitation was performed relative to the content of TBA-d_{10} and referenced to calibration curves with fortified aliquots of blood samples from controls containing 0 to 50 nmol/mL TBA. The method was linear in the range of concentrations used and calibration standards were analyzed with every sample series (usually 20 to 30 samples). The method permitted quantitation of 0.2 nmol TBA/mL of blood with a signal-to-noise ratio of 5:1. When identical samples were repeatedly analyzed, deviations of the obtained quantitative results were less than 10%.

For TAME and TAA, 5 μL of an aqueous solution of the internal standard TBA (1,000 nmol/mL) was added through the septum with a microliter syringe, and the vials were then heated to 70°C for 1 hour. TAME and TAA concentrations were quantified by headspace GC/MS by injecting 200 μL of the headspace from the vials using split injection (split ratio of 10:1). Samples were separated using a DB1 coated fused silica column (30 m × 0.25 mm ID, 1-μm film) at a temperature of 40°C. In addition to monitoring m/z 73 (for TAME), m/z 59 (most intensive fragment ion in the electron impact mass spectrum of TAA and the internal standard TBA) were monitored during the separation with dwell times of 80 msec. Quantitation was performed relative to the content of TBA and referenced to calibration curves with fortified aliquots of blood samples from controls containing 0 to 20 nmol TAME and 0 to 20 nmol TAA/mL blood. The method permitted the quantitation of 0.1 nmol TAME/mL of blood with a signal-to-noise ratio of 5:1. In the studies with ingestion exposure, a new instrument was used (see Appendix A), which lowered the quantitation limit for TAME in blood to 0.01 nmol/mL using identical acquisition conditions.

PARENT ETHERS, TBA, AND TAA IN URINE

MTBE, ETBE, and TAME and TBA and TAA in urine samples were quantified by headspace GC/MS as described for blood samples, using 0.5 mL of human urine and 0.2 mL of rat urine. Conjugates of the alcohols in urine were cleaved to TBA by acid treatment.

OTHER URINARY METABOLITES FROM MTBE AND ETBE

To quantify 2-methyl-1,2-propanediol, 50 μL of a solution of the internal standard 1,2-propanediol (1,000 nmol/mL in water) was added to 0.5 mL of human urine or 0.2 mL of rat

urine. All urine samples were then diluted with an equivalent volume of methanol, and 2-methyl-1,2-propanediol was quantified by GC/MS by injecting 1 µL of the obtained samples. Separation was achieved using a fused silica column coated with DB-FFAP (30 m × 0.32 mm ID, 0.25-µm film) with helium as carrier gas (2 mL/min). Samples were separated using a linear temperature program from 50°C to 230°C with a heating rate of 10°C/min. Injector and transfer line temperatures were 280°C. The concentrations of 2-methyl-1,2-propanediol were determined by monitoring m/z 59 and m/z 45 during the gas chromatographic separation with dwell times of 80 msec. Split injection (split ratio of 10:1) was used. Quantitation was performed relative to the content of 1,2-propanediol and referenced to calibration curves with fortified aliquots of blood samples from controls containing 0 to 2,000 nmol/mL 2-methyl-1,2-propanediol. The method was linear in the range of concentrations used, and calibration standards were analyzed with every sample series (usually 20 to 30 samples). The method permitted quantitation of 1 nmol 2-methyl-1,2-propanediol/mL urine with a signal-to-noise ratio of 5:1. When identical samples were repeatedly analyzed, deviations of the obtained quantitative results were less than 15%.

Concentrations of 2-hydroxyisobutyrate in urine were quantified by GC/MS after transformation to the corresponding methyl ester. Urine samples (0.5 mL for humans and 0.2 mL for rats) were mixed with the internal standard 2-hydroxy-2-methyl butyrate (100 µL of a 1,000 nmol/mL solution in water). Samples were then taken to dryness using anhydrous P_2O_5 in an evacuated desiccator. The obtained residues were treated with 500 µL of boron trifluoride in methanol (BF$_3$/methanol; 14% v/v) at 60°C for 30 minutes. Samples were then diluted with 250 µL of water and extracted with 1 mL of chloroform. The chloroform layer was dried over sodium sulfate and 2 µL of the obtained solution was analyzed by GC/MS (splitless injection). Samples were separated on a DB-WAX column (30 m × 0.25 mm ID, 0.25-µm film) using a linear temperature program from 50°C to 230°C with a heating rate of 10°C/min. The intensities of m/z 43, 55, 49, 73, and 89 were monitored during the separation with dwell times of 80 msec. Quantitation was based on the ratio of m/z 59 to 73 (internal standard). Quantitation was performed relative to the content of 2-hydroxy-2-methyl butyrate and referenced to calibration curves with fortified aliquots of urine samples from controls containing 0 to 4,000 nmol/mL 2-hydroxyisobutyrate. The method was linear in the range of concentrations used, and calibration standards were analyzed with every sample series (usually 20 to 30 samples). The method permitted quantitation of 3 nmol 2-hydroxyisobutyrate/mL of urine with a signal-to-noise ratio of 5:1. When identical

samples were repeatedly analyzed, deviations of the obtained quantitative results were less than 18%.

TAME METABOLITES IN URINE

Two methods were used to quantify TAA glucuronides and 2-methyl-2,3-butanediol glucuronides. The first method involved direct analysis of the glucuronides by GC/MS determination of trimethylsilyl derivatives. To quantify glucuronide excretion, 50 µL of a solution of the internal standard glucuronic acid (1,000 nmol/mL) was added to 100 µL of human or rat urine and the mixtures were lyophilized. The residues were treated for 30 minutes with 1 mL of a mixture of hexamethyldisilazane, trimethylchlorosilane, and pyridine (2:1:9, v:v:v) at 80°C in a closed-reaction vial. From the obtained solution, 2 µL was injected into the GC/MS. Separation was performed using a DB1 coated fused silica column (30 m × 0.25 mm ID, 1-µm film). Injector and transfer line temperatures were 310°C. Samples were injected using split injection (split ratio of 10:1), oven temperature was 100°C and increased to 310°C with a rate of 10°C/min. Samples were monitored using m/z 204 and 217. Quantitation was performed relative to the content of glucuronic acid and referenced to calibration curves with fortified aliquots of urine samples from controls containing 0 to 1,000 nmol/mL of the glucuronides, which were isolated from urine of TAME-treated rats by preparative high-performance liquid chromatography (HPLC) (Amberg et al 1999).

This method was not very sensitive and could be used only with samples containing high concentrations of the glucuronides. In addition, the injected mixture resulted in a rapid deterioration of the performance of the mass spectrometer. Therefore, all samples were also analyzed by a simpler and more sensitive method using acid hydrolysis of the glucuronides. Enzymatic hydrolysis was not very effective with reference compounds, however, as glucuronidase did not completely cleave the glucuronides within 24 hours. Under the conditions of the acid hydrolysis, the alcohols formed by the acid hydrolysis were further converted by an acid-catalyzed dehydration to 2-methyl-2-butene (for TAA) and 3-methyl-2-butanone (for 2-methyl-2,3-butanediol). The efficiency of the acid hydrolysis and the dehydration was checked by nuclear magnetic resonance spectroscopy (NMR), monitoring disappearance of the glucuronide signals in urine samples from rats treated with ^{13}C-TAME (Amberg et al 1999).

To quantify the content of free alcohols and glucuronides, TBA was used as an internal standard (TBA is cleaved to 2-methylpropene under acidic conditions). TBA (25 µL from a 1,000-nmol/mL solution in water) and 10M sulfuric acid (60 µL) were added to 200 µL urine in a closed vial. After

1 hour at 90°C, 500 µL of the gas phase from the vial was analyzed by GC/MS. Separation was performed using a DB1 coated fused silica column (30 m × 0.25 mm ID, 1-µm film). Samples were injected using splitless injection, and oven temperature was 40°C. Samples were monitored using *m/z* 56, 70, and 86 by selected ion monitoring. Quantitation was performed relative to the formed 2-methylpropene and referenced to calibration curves with fortified aliquots of urine samples from controls containing 0 to 1,000 nmol/mL of TAA and 2-methyl-2,3-butanediol. The method was linear in the range of concentrations used, and calibration standards were analyzed with every sample series (usually 20 to 30 samples). The method permitted the quantitation of 0.1 nmol of TAA glucuronide and 0.5 nmol 2-methyl-2,3-butanediol glucuronide per mL of urine with a signal-to-noise ratio of 3:1. When identical samples were repeatedly analyzed, deviations of the obtained quantitative results were less than 10%. This method determined the content of free TAA and free 2-methyl-2,3-butanediol and their glucuronides in the samples. Concentrations of the 2 glucuronides were obtained by subtraction of the content of the free alcohols (see below).

To quantify 2-methyl-2,3-butanediol, 25 µL of a solution of the internal standard 1,2-propanediol (1,000 nmol/mL in water) were added to 0.1 mL of human or rat urine. Urine samples were then diluted with 0.9 mL of methanol, and 2-methyl-2,3-butanediol content was quantified by GC/MS by injecting 1 µL of the obtained mixtures. Separation was achieved using a fused silica column coated with DB-FFAP (30 m × 0.32 mm ID, 0.25-µm film) with helium as the carrier gas (2 mL/min). Samples were separated using a linear temperature program from 50°C to 230°C with a heating rate of 10°C/min. Injector and transfer line temperatures were 230°C. The concentration of 2-methyl-2,3-butanediol was determined by monitoring *m/z* 59 and *m/z* 45 during the gas chromatographic separation with dwell times of 80 msec. Split injection (split ratio of 10:1) was used. Quantitation was performed relative to the content of 1,2-propanediol and referenced to calibration curves with fortified aliquots of urine samples from controls containing 0 to 1,000 nmol/mL 2-methyl-2,3-butanediol. The method was linear in the range of concentrations used and calibration standards were analyzed with every sample series (usually 20 to 30 samples) The method permitted the quantitation of 1 nmol 2-methyl-2,3-butanediol/mL urine with a signal-to-noise ratio of 5:1. When identical samples were repeatedly analyzed, deviations of the obtained quantitative results were less than 15%.

Concentrations of 2-hydroxy-2-methyl butyrate and 3-hydroxy-3-methyl butyrate in urine were quantified by GC/MS after transformation to the corresponding methyl esters. Urine samples (0.1 mL for humans and rats) were mixed with 2-hydroxyvaleric acid (internal standard, 25 µL of a 1,000-nmol/mL solution in water). Samples were then taken to dryness using anhydrous P_2O_5 in an evacuated desiccator. The obtained residues were treated with 500 µL of BF_3/methanol (14%) at 60°C for 30 minutes. 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 2 µL of the obtained solutions was analyzed by GC/MS (by splitless injection). Samples were separated on a DB-WAX column (30 m × 0.25 mm ID, 0.25-µm film) using a linear temperature program from 50°C to 230°C with a heating rate of 10°C/min. The intensities of *m/z* 55 and 59 were monitored during the separation with dwell times of 80 msec. Quantitation was performed relative to the content of 2-hydroxyvaleric acid and referenced to calibration curves with fortified aliquots of urine samples from controls containing 0 to 1,000 nmol/mL 2-hydroxy-2-methyl butyrate and 3-hydroxy-3-methyl butyrate. The method was linear in the range of concentrations used and calibration standards were analyzed with every sample series (usually 20 to 30 samples). The method permitted the quantitation of 3 nmol 2-hydroxy-2-methyl butyrate and 3-hydroxy-3-methyl butyrate/mL of urine with a signal-to-noise ratio of 3:1. When identical samples were repeatedly analyzed, deviations of the obtained quantitative results were less than 10%.

BACKGROUND LEVELS OF ETHER METABOLITES IN URINE

Because of considerable and varying concentrations of several ether metabolites in urine samples of human subjects before and 90 hours after exposure, average background concentrations were determined to identify urine samples with increased content of these metabolites. Urine samples from each human subject and from each rat were collected for 6 hours directly before the exposure and 90 to 96 hours after exposure. Mean concentrations of the metabolites in these samples were compared with mean concentrations of metabolites excreted after the exposures and tested for significance (*t* test).

EXHALATION OF MTBE AND TBA

Exhaled breath from the human subjects was collected in air sampling bags (Supelco, Deisenhofen Germany). Portions (300 µL) from the collected air were injected into the GC/MS system, and separation was performed using a fused silica capillary column (DB1, 30 m × 0.25 mm ID, 1-µm film). Samples were injected in the splitless mode and eluted from the column at 35°C with a flow rate of 1 mL/min using helium as the carrier gas. Quantitation of MTBE and TBA in the air samples was done by selected

ion monitoring using m/z 20, 59, and 73. MTBE and TBA concentrations in the samples were referenced to air samples with known concentrations of MTBE and TBA using the peak obtained for neon (m/z 20) as internal standard. The method was linear in response between 1 pmol and 100 pmol MTBE/mL or TBA/mL of air, and calibration samples were run after each analysis series.

STATISTICAL ANALYSES

Statistical analyses of the data were performed using a Student t test in Microsoft Excel spreadsheets. To determine possible sex differences, all data from the male and female animals and from male and female human subjects were compared using a t test. P values of less than 0.05 were considered significant. Half-times were calculated using exponential regression in Excel. The curve-fitting function of the program was used, and curves were stripped based on correlation coefficients. All correlation values (r^2) of greater than 0.95 were considered for separation.

QUALITY CONTROL

The quality policy of the University of Würzburg is to obtain reliable and reproducible data and to ensure maximum protection of all people involved. All animal experiments were performed according to national laws and international guidelines for the humane treatment of laboratory animals. The quality of the data generated was supervised by regular inspections of the experimental protocols, regular maintenance and calibration of all instruments used, and determination of identity and purity of all chemicals involved. All experimental procedures used were described in standard operation procedures. For methods development, results were directly introduced into computer databases and defined criteria for the use of biological samples were developed. All original data are kept on file and samples are stored for reanalysis if required.

All personnel involved in the studies were well educated and properly trained to perform their tasks. Continuing education courses are mandatory and are performed by individuals from this institution and from other institutions.

All laboratory animals used in the study were obtained by certified suppliers. Upon arrival, the animals were inspected by the university veterinary service and housed in the animal facility of the Department of Toxicology. The animal facility is maintained by certified staff trained in animal handling and is regularly inspected by both the university veterinarian and the State of Bavaria veterinarian.

The identity and purity of all chemicals used in the study was checked upon receipt, and lot numbers were recorded. All chemicals were stored according to the specifications of the manufacturer and according to the safety guidelines specified by German law.

All instruments used were calibrated by procedures provided by the manufacturer according to the operation manuals. Records of calibration and regular maintenance were kept on file in computer databases. For complex measurements, internal calibration standards were used and obtained data were referenced to those standards.

RESULTS

METABOLITE STRUCTURES

In order to quantify metabolites excreted after exposures to the different ethers, the structures of these compounds had to be determined, and reference compounds for use as calibration standards had to be synthesized. The methods used and results obtained for this part of the study, which mainly used ^{13}C-labeled ethers, are described in detail in Appendix A. These studies identified all relevant metabolites formed (amounts > 2% of applied dose) from the ethers in rats and in humans.

The studies with MTBE confirmed TBA, 2-methyl-1,2-propanediol, and 2-hydroxyisobutyrate as MTBE metabolites excreted in urine (Miller et al 1997). Based on the confirmed structures of the metabolites, MTBE biotransformation proceeds by oxidation of the methyl group in MTBE to give an intermediate hemiacetal, which decomposes with release of formaldehyde to TBA (Figure 2).

In ETBE-exposed rats, the observed metabolites were identical to those observed in MTBE-treated rats. This observation suggests that TBA is also a major intermediate metabolite of ETBE formed by CYP-mediated oxidation reaction.
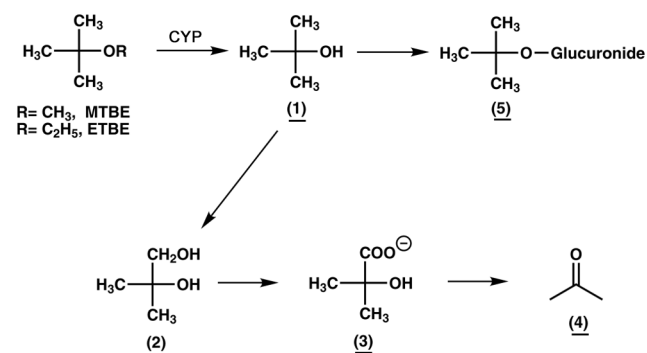

Figure 2. Biotransformation of MTBE, ETBE, and TBA in mammals. Enzymes involved in a transformation are identified along the arrows. Numbers allocated to metabolites excreted in urine are underlined. (1) TBA; (2) 2-methyl-1,2-propanediol; (3) 2-hydroxyisobutyrate; (4) acetone; (5) glucuronide of TBA.

With ETBE, oxidation by CYP seems to occur exclusively at the α-carbon atom of the ethyl ether moiety since metabolites whose formation could be explained by oxidation of the β-carbon (such as *t*-butyl glycol) were not observed. The observation of a preferred oxidation on the α-carbon is in line with observations on the biotransformation of ethyl ether where ether cleavage by α-carbon oxidation is the major pathway of biotransformation (Axelrod 1956). Moreover, aliphatic hydrocarbons such as n-hexane are also preferentially oxidized at the ω-1 atom (Krämer et al 1974). In ETBE biotransformation, the intermediate hemiacetal also decomposes to give TBA and acetaldehyde. The further fate of the aldehydes formed in MTBE and ETBE biotransformation has not been investigated; but they are expected to be rapidly metabolized to formate and acetate, respectively.

Formation of the other MTBE or ETBE metabolites involves further biotransformation of TBA formed in the first step of the metabolic pathway. Conjugation of TBA with activated glucuronic acid results in excretion of the glucuronide conjugate. The two other metabolites (2-methyl-1,2-propanediol and 2-hydroxyisobutyrate) present in urine of TBA-treated animals and also in urine of rats exposed to MTBE and ETBE suggest further oxidative metabolism of the intermediate metabolite TBA. The likely pathway for formation of these metabolites involves oxidation of TBA by CYP to give 2-methyl-1,2-propanediol. TBA is not a substrate for alcohol dehydrogenase, but it is oxidized by rat liver microsomes to formaldehyde and acetone under conditions consistent with an involvement of CYP (Cederbaum and Cohen 1980, Cederbaum et al 1983). CYP-mediated oxidation of a C–H bond in one of the methyl groups of TBA results in excretion of the diol metabolite. Further oxidation of 2-methyl-1,2-propanediol results in 2-hydroxyisobutyrate, which is excreted as a major metabolite of TBA, as well as of MTBE and ETBE (see Figure 2).

The structures of TAME metabolites delineated from experiments in rats and in one human subject suggest a complex biotransformation of TAME (Figure 3). The first step in the biotransformation of TAME is oxidation of the methyl group to give an unstable hemiacetal that decomposes to TAA. The low concentrations of TAA recovered in urine of rats exposed to both TAME and to TAA suggest intensive further metabolism of this alcohol by conjugation and by further oxidation, similar as observed for TBA (Bernauer et al 1998, Cederbaum and Cohen 1980, Johanson et al 1995a). Glucuronidation of TAA seems to be a major step in its biotransformation resulting in excretion of a glucuronide. In addition, TAA is oxidized to several diols in reactions that are likely to involve CYP–catalyzed oxidation of a C–H bond. The major pathway of TAA oxi-
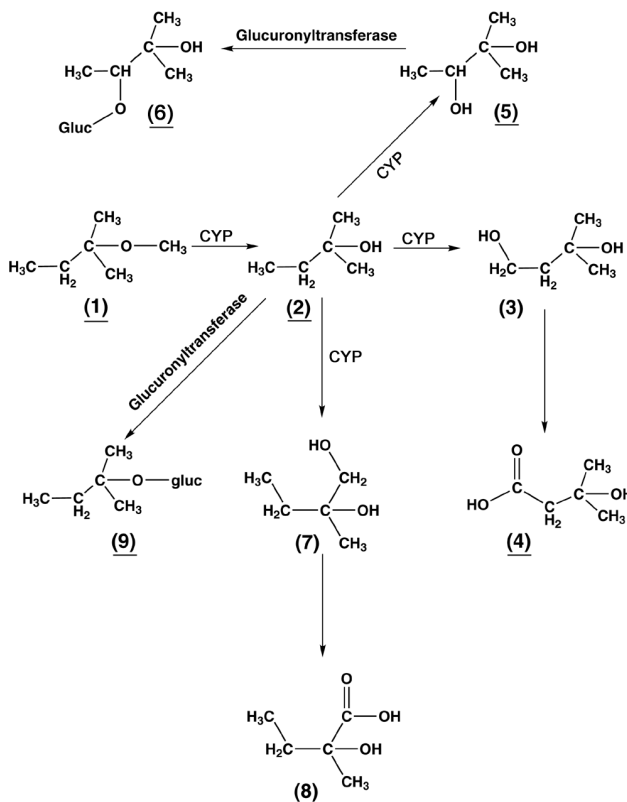
Figure 3. Biotransformation of TAME in mammals. Enzymes involved in a transformation are identified along the arrows. Numbers allocated to metabolites found in urine are underlined. (1) TAME; (2) TAA; (3) 2-methyl-2,4-butanediol; (4) 3-hydroxy-3-methyl butyrate; (5) 2-methyl-2,3-butanediol; (6) glucuronide of 2-methyl-2,3-butanediol, (7) 2-methyl-1,2-butanediol; (8) 2-hydroxy-2-methyl butyrate; (9) glucuronide of TAA.

dation occurs at the C3 atom to give 2-methyl-2,3-butanediol, which, including the glucuronide formed, is the major product of TAME biotransformation excreted in urine of TAME-exposed rats. Oxidation of the carbon atom in 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-methyl butyrate. Oxidation of the methyl group next to the alcohol group in TAA also represents a minor pathway that results in intermediate formation of 2-methyl-1,2-butanediol, which may be further oxidized to give 2-hydroxy-2-methyl butyrate.

Based on the structures of metabolites delineated, methods were developed to quantitate these compounds in urine of human subjects and rats exposed to MTBE, ETBE and TAME in order to obtain complete information on the fate of these ethers in humans and rodents.

MTBE BIOTRANSFORMATION AND KINETICS OF EXCRETION

Human Inhalation Exposure

During the inhalation experiments, the deviations between the targeted concentrations and the actual concentrations of MTBE in the chamber were less than 10% of the targeted values. Actual concentrations were 4.5 ± 0.4 ppm and 38.7 ± 3.2 ppm (mean $\pm$ SD of 16 determinations in 15 minutes intervals over 4 hours). Experimental results on the excretion of MTBE metabolites and half-times in humans after inhalation and ingestion exposure are given in Tables 2 and 3 and in Figures 4 and 5. (Individual data are presented in Appendix B). MTBE was not detected in blood samples from the human subjects taken before exposure. The maximum concentrations of MTBE in blood were determined directly after the end of the inhalation exposure; MTBE concentrations decreased thereafter to reach the limit of detection 8 hours (4 ppm) or 12 hours (40 ppm) after the end of the exposure period. TBA was detected in low concentrations (0.9 ± 0.3 nmol/mL blood) in most of the blood samples taken from the individuals before exposure and in blood samples from unexposed control subjects. Blood samples taken from the human subjects showed statistically significant increases in TBA concentrations from the end of exposure until the 6-hour sampling point after 4 ppm MTBE (Figure 4, panel A) and until the 12-hour sampling point after 40 ppm MTBE (Figure 4, panel B).

In urine samples collected before MTBE exposure and in samples collected from unexposed control subjects, low concentrations of TBA, 2-methyl-1,2-propanediol, and 2-hydroxyisobutyrate were present. In urine samples from exposed individuals, these concentrations were significantly higher than in the preexposure urine samples. After 4 ppm MTBE, only a few urine samples from the human subjects contained significantly increased concentrations of 2-hydroxyisobutyrate (Figure 5, panel A). Statistically significant increases in concentrations of the 3 metabolites were observed at all time points between 0 and 48 hours after the end of exposure to 40-ppm MTBE (see Figure 5, panel B).

Because of much lower background levels, the concentrations of 2-methyl-1,2-propanediol were significantly higher than controls in all samples collected between 0 and 42 hours after exposure to 4 ppm MTBE. TBA (free and conjugated) concentrations were significantly higher than controls in most samples collected between 0 and 30 hours after exposure to 4 ppm MTBE and between 0 and 48 hours after exposure to 40 ppm MTBE (see Figure 5 panels A and B).

No statistically significant differences in amounts of 2-hydroxyisobutyrate excreted or in rates of excretion were seen between males and females. The determined half-times of elimination from urine were also not significantly different after the 4 or 40 ppm MTBE exposure (Table 3). Based on the sum of recovered metabolites, 2-hydroxyisobutyrate represents the major urinary MTBE metabolite,

Table 2. MTBE Exposure: Blood Levels of MTBE and TBA

	Maximum Concentration (µM)	Half-Time (hr)	Maximum Concentration (µM)	Half-Time (hr)
Inhalation	——————— 4 ppm MTBE ———————		——————— 40 ppm MTBE ———————	
Human				
MTBE[a]	1.9 ± 0.4	1.3 ± 0.2	6.7 ± 1.6	1.2 ± 0.2
		2.3 ± 0.3		2.4 ± 0.6
TBA	2.6 ± 0.3[b]	6.5 ± 2.1	21.8 ± 3.7[b]	5.3 ± 2.1
Rat				
MTBE	2.3 ± 1.0	—[c]	5.9 ± 1.8	0.5 ± 0.2
TBA	2.9 ± 0.5[d]	—	36.7 ± 10.8[b]	—
Ingestion	——————— 5 mg MTBE ———————		——————— 15 mg MTBE ———————	
Human				
MTBE[a]	0.10 ± 0.03	0.8 ± 0.1	0.69 ± 0.25	0.7 ± 0.2
		1.8 ± 0.3		1.2 ± 0.3
		8.1 ± 3.0		3.7 ± 0.9
TBA	0.45 ± 0.13	8.1 ± 1.6	1.82 ± 0.63	8.5 ± 2.4

[a] Human elimination of MTBE from blood occurred in 2 phases after inhalation exposure and in 3 phases after ingestion exposure.

[b] Significantly above background ($P < 0.01$).

[c] — = Not determined.

[d] Significantly above background ($P < 0.05$).
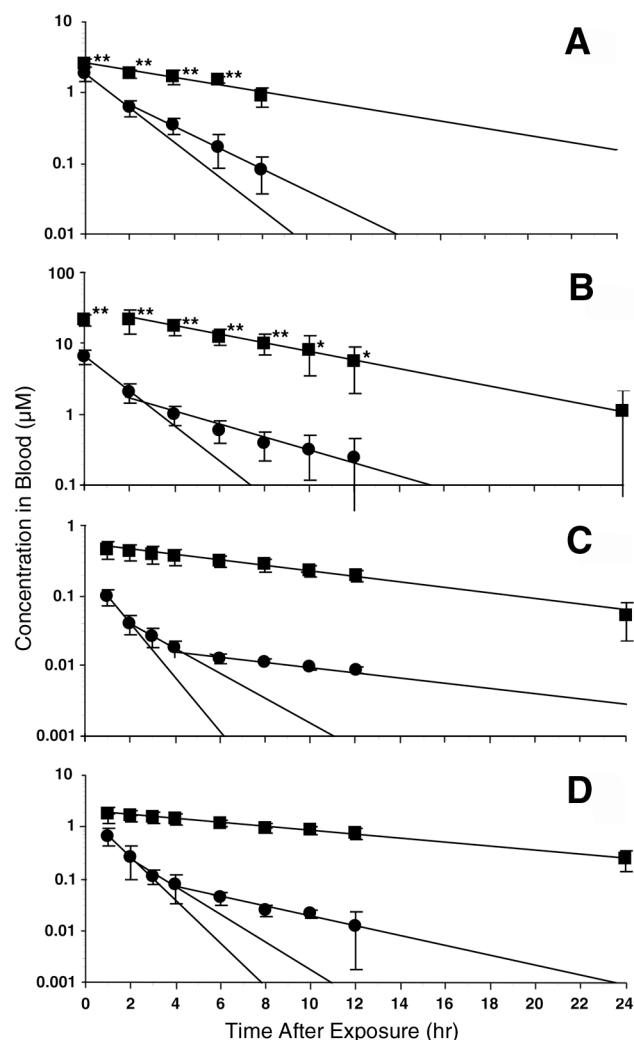
Figure 4. Time-course of MTBE (●) and TBA(■) elimination from blood of 6 human subjects exposed to 4.5 ± 0.4 ppm (panel A) and 38.7 ± 3.2 ppm (panel B) MTBE for 4 hours in a dynamic exposure chamber and to 5 mg (panel C) and 15 mg (panel D) MTBE by ingestion. Statistically significant differences as compared to background TBA concentrations measured in the individuals before and 90 hours after the exposures (** = P < 0.01; * = P < 0.05). Due to the use of ^{13}C-labeled ether for the oral studies, there was no detectable background of metabolites.

whereas TBA and 2-methyl-1,2-propanediol are minor urinary metabolites of MTBE in humans (see Table 3).

Human Ingestion Exposure

Human subjects were exposed to ^{13}C-MTBE in tap water. In these experiments, MTBE labeled with the stable isotope ^{13}C was used to follow the kinetics of MTBE-metabolite excretion without interference from background levels. ^{13}C-MTBE is metabolized similarly to ^{12}C-MTBE, but because of a change in molecular weight by 1 mass unit, metabolites can be measured by MS without interference
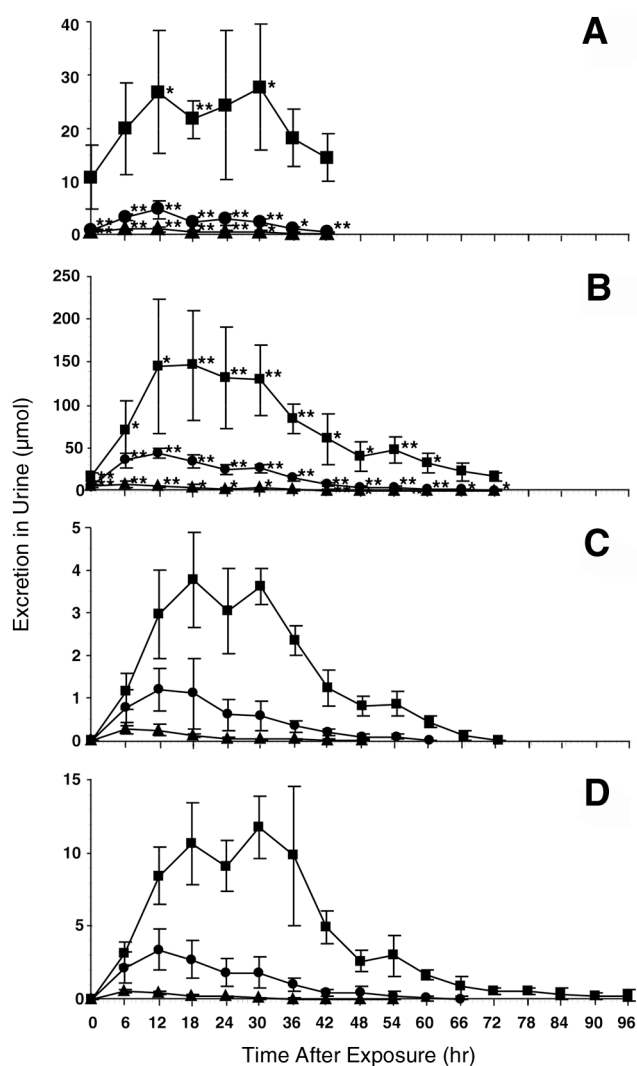
Figure 5. Urinary excretion of 2-hydroxyisobutyrate (■), 2-methyl-1,2-propanediol (●) and TBA (▲) in 6 humans exposed to 4.5 ± 0.4 ppm (panel A) and 38.7 ± 3.2 ppm (panel B) MTBE for 4 hours in a dynamic exposure chamber and to 5 mg (panel C) and 15 mg (panel D) MTBE by ingestion. Numbers (mean ± SD) represent total amount of metabolite excreted in the urine samples collected at 6-hour intervals. Each sample was analyzed in duplicate. Statistically significant differences as compared to background metabolite concentrations were measured in the individuals before and 90 hours after the exposures (** = P < 0.01; * = P < 0.05). Due to the use of ^{13}C-labeled ether for the ingestion exposures, there was no detectable background of metabolites.

from the natural background of ^{12}C compounds. ^{13}C-MTBE was ingested dissolved in local tap water that did not contain detectable concentrations of MTBE. The subjects consumed the spiked water (100 mL) within 20 seconds. Experimental results on the excretion of MTBE metabolites and half-times in humans after ingestion exposure are given in Tables 2 and 3 and in Figures 4 and 5. ^{13}C-MTBE was not detected in blood samples from the human subjects

Table 3. MTBE Exposure: Parent Compound and Metabolites Recovered in Urine

	Total Excreted (μmol)	Background (μmol)	Half-Time (hr)	Total Excreted (μmol)	Background (μmol)	Half-Time (hr)
Inhalation	———————— 4 ppm MTBE ————————			———————— 40 ppm MTBE ————————		
Human						
MTBE	0.3 ± 0.1	ND[a]	5.2 ± 1.0	2.0 ± 1.3	ND	4.3 ± 1.4
TBA	3.4 ± 0.8[b]	0.6 ± 0.4	12.0 ± 3.2	29.9 ± 9.4[b]	0.6 ± 0.4	10.4 ± 1.8
2-Methyl-1,2-propanediol	16.5 ± 3.0[b]	0.2 ± 0.2	7.8 ± 1.8	205.0 ± 15.6[b]	0.3 ± 0.3	9.7 ± 1.5
2-Hydroxyisobutyrate	78.9 ± 27.6	42.7 ± 24.1	10.4 ± 4.4	943.8 ± 332.3[b]	221.5 ± 63.2	17.0 ± 2.5
Rat						
MTBE	ND	ND	—[c]	ND	ND	—
TBA	0.3 ± 0.1[d]	0.2 ± 0.1	5.0 ± 3.6	1.2 ± 0.7[b]	0.3 ± 0.1	4.5 ± 1.2
2-Methyl-1,2-propanediol	0.7 ± 0.3[b]	0.1 ± 0.1	2.9 ± 1.0	2.8 ± 1.0[b]	0.1 ± 0.1	3.4 ± 1.0
2-Hydroxyisobutyrate	1.7 ± 0.3[b]	0.8 ± 0.1	4.9 ± 1.3	11.1 ± 2.8[b]	2.1 ± 0.9	4.4 ± 0.9
Ingestion	———————— 5 mg MTBE ————————			———————— 15 mg MTBE ————————		
Human						
MTBE	0.007 ± 0.002	ND	3.4 ± 0.9	0.10 ± 0.05	ND	5.5 ± 2.0
TBA	0.74 ± 0.22	ND	7.7 ± 2.0	1.6 ± 0.2	ND	8.1 ± 1.4
2-Methyl-1,2-propanediol	5.0 ± 2.4	ND	10.7 ± 2.8	14.2 ± 5.6	ND	9.7 ± 2.3
2-Hydroxyisobutyrate	20.3 ± 3.0	ND	17.4 ± 3.1	67.8 ± 10.6	ND	17.8 ± 3.4

[a] ND = not detected.

[b] Significantly above background ($P < 0.01$).

[c] — = Not determined.

[d] Significantly above background ($P < 0.05$).

taken before exposure. The maximum concentrations of MTBE in blood were determined in the first blood samples taken 1 hour after exposure. MTBE concentrations decreased to reach the limit of detection 12 hours after exposure (see Figure 4, panels C and D). Maximum concentrations of TBA were also determined at the first sampling point, 1 hour after ingestion exposure. TBA concentrations in blood decreased more slowly than MTBE concentrations, and TBA was still present in blood in small but detectable concentrations 24 hours after ingestion. Elimination of MTBE from blood could be separated into 3 phases (see Table 2 and Figure 4, panels C and D). The difference in the kinetics of elimination of MTBE from blood (2 phases after inhalation and 3 phases after ingestion) is most likely due to sampling design (spacing of blood samples). After ingestion, the study design included more closely spaced blood sampling (every hour for 4 hours after ingestion) for a more detailed description of elimination kinetics.

Metabolites observed in urine of humans exposed to MTBE by oral ingestion were identical to those observed after inhalation exposure to MTBE. Based on the amount of 2-hydroxyisobutyrate recovered, this compound also represents the major urinary metabolite of MTBE after ingestion (see Table 3 and Figure 5, panels C and D); again, TBA and 2-methyl-1,2-propanediol were minor urinary metabolites. Kinetics of excretion of the metabolites after oral MTBE exposure were not different from excretion kinetics determined after inhalation exposure. In urine samples from the human subjects, approximately 50% of the administered dose of MTBE was recovered in the form of metabolites (Table 4). The determined half-times for elimination of the metabolites from urine also showed no difference after the 5-mg and 15-mg MTBE exposures (see Table 3) compared to inhalation.

In contrast to MTBE exposures by inhalation, where the extent of exhalation has been described by others (Nihlén 1998b), no data on exhalation of MTBE and TBA after oral MTBE ingestion were available. Therefore, we quantified the exhalation of MTBE and TBA in 3 male individuals exposed to 15 mg of ^{13}C-MTBE. Due to the close spacing of air samples in order to obtain a representative curve for exhalation

Table 4. MTBE Exposure: Calculated Received Dose and Total Recovery of Metabolites[a]

	Received Dose (μmol)	Metabolites Excreted (μmol)	Percentage of Dose	Received Dose (μmol)	Metabolites Excreted (μmol)	Percentage of Dose
Inhalation	———————— 4 ppm MTBE ————————			———————— 40 ppm MTBE ————————		
Human	161	55 ± 9	35 ± 5	1387	958 ± 280	69 ± 20
Rat	3.8	1.6 ± 0.3	42 ± 7	33	13 ± 3	39 ± 9
Ingestion	———————— 5 mg MTBE ————————			———————— 15 mg MTBE ————————		
Human	57	26 ± 5	46 ± 9	170	84 ± 11	49 ± 6

[a] See Amberg et al (2000) for basis for calculations.

(presumed to be rapid), time constraints in sample handling limited the number of individuals who could be studied. Excretion of MTBE by exhalation in the 3 human subjects paralleled the determined blood concentrations and also occurred with 3 half-times (0.25 ± 0.07 hours; 0.64 ± 0.15 hours; 1.74 ± 0.23 hours) identical to those observed for MTBE clearance from blood (Figure 6). The exhalation of TBA was slower, occurred with only a single half-time (6.71 ± 2.17 hours), and also paralleled the clearance of TBA from blood. The amount of exhaled MTBE after oral ingestion was calculated as 26 ± 5 % of dose; TBA exhalation accounted for 6 ± 1 % of dose. Together with the amount of metabolites recovered in urine, the summed excretion of MTBE and TBA accounted for over 80% of the ingested dose of MTBE. In the breath samples taken, only very small concentrations of ^{13}C-acetone were present (< 0.1% of dose), suggesting that acetone is not a major metabolite of MTBE formed in humans after oral MTBE exposure.

Rat Inhalation Exposure

Rats were exposed to the same MTBE concentrations used in the human inhalation studies. The experimental results on metabolite concentrations and excretion are compiled in Tables 2 and 3 and Figure 7. The concentrations of MTBE in blood of rats determined at the end of the

4-hour exposure were similar to those seen in humans after identical exposure concentrations. MTBE was more rapidly cleared from rat blood than from human blood. The determined concentrations of TBA were also of the same order of magnitude both in rats and in humans after exposure to 4-ppm and 40-ppm concentrations of MTBE.

TBA was also detected in low concentrations in blood samples taken from control rats. However, blood samples taken from rats after exposure to 4 ppm and 40 ppm MTBE showed statistically significant increases in TBA concentrations at the end of exposure.

In urine samples collected before MTBE exposure and in samples collected from control rats, TBA, 2-methyl-1,2-propanediol, and 2-hydroxyisobutyrate were present at low concentrations. In samples collected within 24 hours
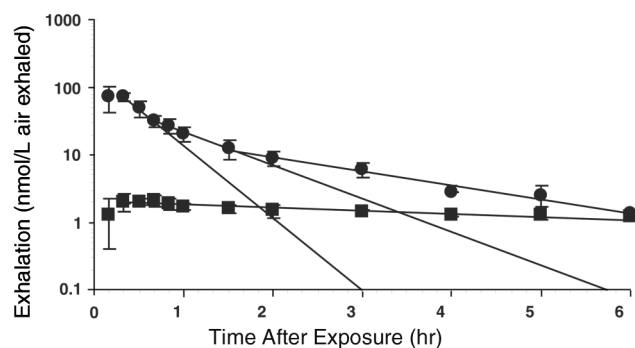
Figure 6. Time course of MTBE (●) and TBA (■) exhalation in 3 male human subjects exposed to 15 mg MTBE by ingestion.
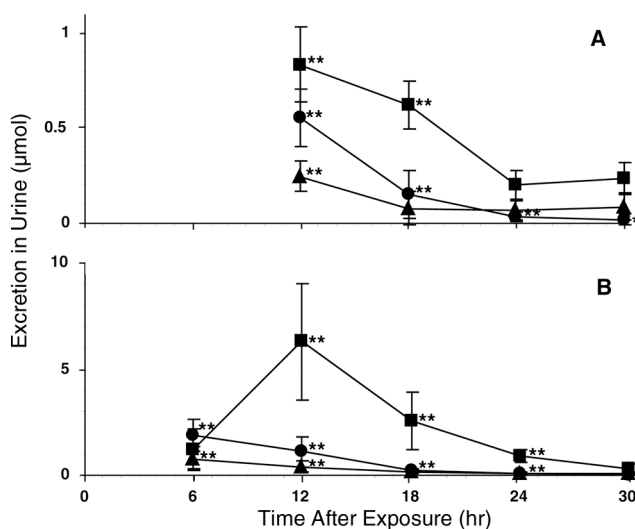
Figure 7. Urinary excretion of 2-hydroxyisobutyrate (■), 2-methyl-1,2-propanediol (●) and TBA (▲) in 10 rats exposed to 4.5 ± 0.4 ppm (panel A) and 38.7 ± 3.2 ppm (panel B) MTBE for 4 hours in a dynamic exposure chamber. Numbers (mean ± SD) indicate total amount of metabolite excreted in the urine samples collected at 6-hour intervals. Each sample was analyzed in duplicate. Significant differences as compared to background levels are indicated (** = $P < 0.01$; * = $P < 0.05$).

of exposing rats to 40 ppm MTBE, concentrations of 2-hydroxyisobutyrate and 2-methyl-1,2-propanediol were significantly higher than in control samples (Figure 7). TBA concentrations were significantly increased above background only at 6 and 12 hours after the end of exposure. After exposure to 4-ppm MTBE, only a few urine samples contained significantly increased concentrations of 2-hydroxyisobutyrate and TBA (Figure 7). Due to much lower background levels, the concentrations of 2-methyl-1, 2-propanediol were significantly higher than preexposure levels in all samples collected between 0 and 24 hours after

exposure to 4-ppm MTBE. Based on the amount of 2-hydroxyisobutyrate, this compound also represents the major urinary metabolite of MTBE (see Table 3) in rats; TBA and 2-methyl-1,2-propanediol were minor urinary metabolites.

ETBE BIOTRANSFORMATION

Human Inhalation Exposure

The study design of ETBE exposures was identical to that of the MTBE exposures except that ingestion studies

Table 5. ETBE Exposure: Blood Levels of ETBE and TBA

	Maximum Concentration (μM)	Half-Time (hr)	Maximum Concentration (μM)	Half-Time (hr)
Inhalation	———————— 4 ppm ETBE ————————		———————— 40 ppm ETBE ————————	
Human				
ETBE[a]	1.3 ± 0.7	1.1 ± 0.2	12.1 ± 4.0	1.1 ± 0.1
				6.2 ± 3.3
TBA	1.8 ± 0.2[b]	8.2 ± 2.2	13.9 ± 2.2[b]	9.8 ± 1.4
Rat				
ETBE	1.0 ± 0.7	0.4 ± 0.0	5.3 ± 1.2	0.8 ± 0.2
TBA	5.7 ± 0.8[b]	—[c]	21.7 ± 4.9[b]	—

[a] Human elimination of ETBE from blood occurred in 2 phases, but the second phase was detectable only after exposure to 40 ppm ETBE. Blood levels for ETBE were below the limit of detection after 4 hours.

[b] Significantly above background ($P < 0.01$).

[c] — = Not determined.

Table 6. ETBE Exposure: Parent Compound and Metabolites Recovered in Urine

	Total Excreted (μmol)	Background (μmol)	Half-Time (hr)	Total Excreted (μmol)	Background (μmol)	Half-Time (hr)
Inhalation	———————— 4 ppm ETBE ————————			———————— 40 ppm ETBE ————————		
Human						
ETBE	0.3 ± 0.1	ND[a]	5.6 ± 1.9	0.9 ± 0.5	ND	3.5 ± 1.9
TBA	5.1 ± 1.3[b]	2.0 ± 0.4	14.6 ± 4.1	22.6 ± 7.5 [b]	2.5 ± 0.5	11.2 ± 2.1
2-Methyl-1,2-propanediol	13.6 ± 4.5[b]	3.5 ± 2.2	10.2 ± 2.0	96.6 ± 23.6[b]	5.8 ± 3.6	12.3 ± 2.3
2-Hydroxyisobutyrate	130.4 ± 21.8[c]	93.6 ± 28.1	20.3 ± 2.7	522.6 ± 149.2[b]	167.4 ± 42.3	28.3 ± 9.1
Rat						
ETBE	ND	ND	—[d]	ND	ND	—
TBA	0.2 ± 0.1	0.2 ± 0.1	—	0.7 ± 0.5[b]	0.2 ± 0.1	4.6 ± 1.4
2-Methyl-1,2-propanediol	0.3 ± 0.1[b]	0.02 ± 0.01	4.0 ± 0.9	1.6 ± 1.1[b]	0.02 ± 0.02	2.6 ± 0.5
2-Hydroxyisobutyrate	2.3 ± 0.5[c]	1.4 ± 0.4	4.7 ± 2.6	10.6 ± 2.2[b]	1.9 ± 0.8	3.0 ± 1.0

[a] ND = not detected.

[b] Significantly above background ($P < 0.01$).

[c] Significantly above background ($P < 0.05$).

[d] — = Not determined.

Table 7. ETBE Exposure: Calculated Received Dose and Total Recovery of Metabolites

	Received Dose (μmol)	Metabolites Excreted (μmol)	Percentage of Dose	Received Dose (μmol)	Metabolites Excreted (μmol)	Percentage of Dose
Inhalation	——————— 4 ppm ETBE ———————			——————— 40 ppm ETBE ———————		
Human	121	50 ± 20	41 ± 17	1092	467 ± 134	43 ± 12
Rat	2.3	1.1 ± 0.7	50 ± 30	21	11 ± 3	53 ± 15

were not performed. Actual ETBE concentrations in the chamber were 4.5 ± 0.6 ppm and 40.6 ± 3.0 ppm (mean ± SD of 16 determinations in 15-minute intervals over 4 hours). Experimental results on the excretion of ETBE metabolites and half-times in humans are given in Tables 5, 6 and 7 and in Figures 8 and 9. ETBE was not detected in blood samples from the subjects taken before exposure. The maximum concentrations of ETBE in blood were determined immediately after the end of the exposure period; ETBE concentrations in blood decreased to reach the limit of detection 4 hours (4 ppm) or 24 hours (40 ppm) after the end of the exposure period. TBA was detected in low concentrations (0.5 ± 0.3 nmol/mL blood) in most of the blood samples taken from the individuals before the exposure. Blood samples taken after exposure to ETBE showed statistically significant increases in TBA concentrations between the end of exposure and the 4-hour blood sampling point after 4 ppm ETBE and at the 24-hour blood sampling point after 40 ppm ETBE (Figure 8).

In the urine samples from exposed individuals, the concentrations of TBA, 2-methyl-1,2-propanediol, and

2-hydroxyisobutyrate were significantly higher than control urine samples. After exposure to 4-ppm ETBE, only a few urine samples from the subjects contained significantly increased concentrations of 2-hydroxyisobutyrate (Figure 9).

Due to much lower background levels, the concentration of 2-methyl-1,2-propanediol was significantly higher than controls in all samples collected between 0 and 36 hours after exposure to 4 ppm ETBE. TBA (free and conjugated) concentrations were significantly elevated above background in most samples collected between 0 and 24 hours after exposure to 4 ppm ETBE and between 0 and 36 hours after exposure to 40 ppm ETBE (see Figure 9). Statistically significant increases in the concentrations of the 3 metabolites were observed at all time points between 0 and 36 hours after the end of exposure to 40 ppm ETBE (see Figure 9).

Judging by the amount of 2-hydroxyisobutyrate recovered, this compound also represents the major urinary metabolite of ETBE (see Table 6); TBA and 2-methyl-1,2-propanediol were minor urinary metabolites of ETBE in humans.
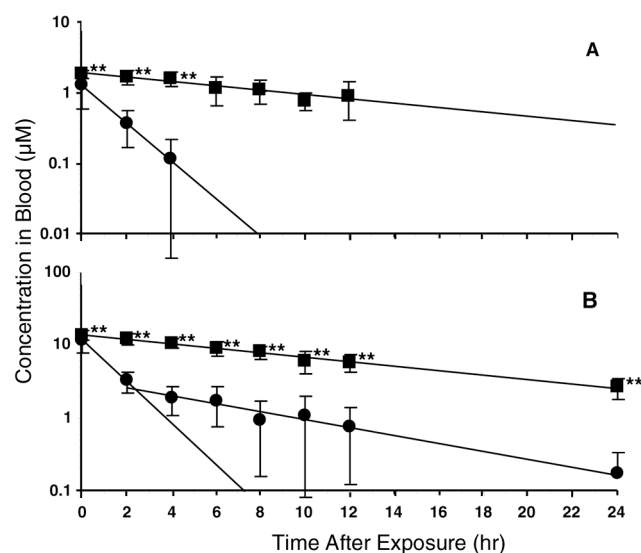
Figure 8. Time-course of ETBE (●) and TBA (■) elimination from blood of 6 human subjects exposed to 4.5 ± 0.6 ppm (panel A) and 40.6 ± 3.0 ppm (panel B) ETBE for 4 hours in a dynamic exposure chamber. Significant differences as compared to background TBA concentrations measured before and 90 hours after exposure (** = $P < 0.01$; * = $P < 0.05$).
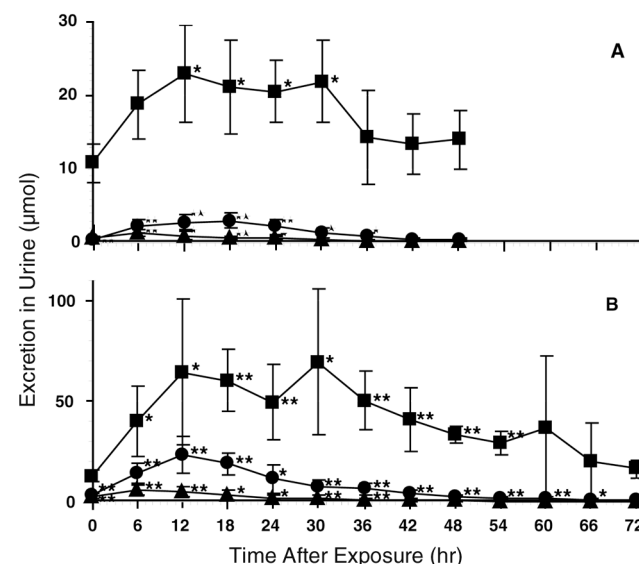
Figure 9. Urinary excretion of 2-hydroxyisobutyrate (■), 2-methyl-1,2-propanediol (●) and TBA (▲) in 6 human subjects exposed to 4.5 ± 0.6 ppm (panel A) and 40.6 ± 3.0 ppm (panel B) ETBE for 4 hours in a dynamic exposure chamber. Numbers (mean ± SD) represent total amount of metabolite excreted in the urine samples collected at 6-hour intervals. Each sample was analyzed in duplicate. Significant differences as compared to background levels are indicated (** = $P < 0.01$; * = $P < 0.05$).
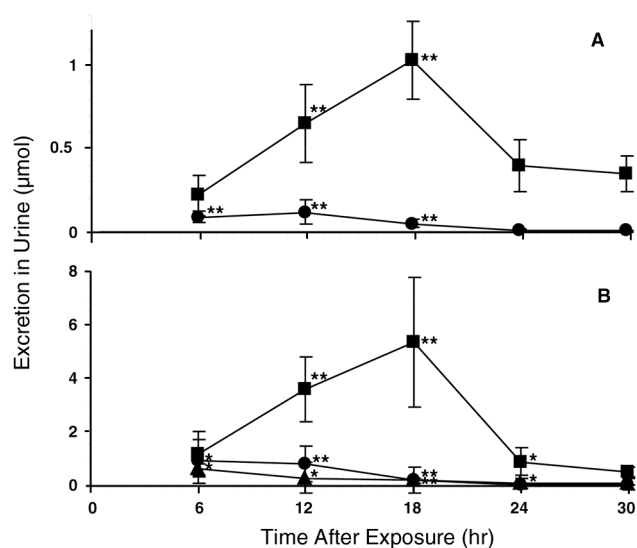
Figure 10. Urinary excretion of 2-hydroxyisobutyrate (■), 2-methyl-1,2-propanediol (●) and TBA (▲) in 10 rats exposed to 4.5 ± 0.6 ppm (panel A) and 40.6 ± 3.0 ppm (panel B) ETBE for 4 hours in a dynamic exposure chamber. Numbers (mean ± SD) represent total amount of metabolite excreted in the urine samples collected at 6-hour intervals. Each sample was analyzed in duplicate. Significant differences as compared to background levels are indicated (** = $P < 0.01$; * = $P < 0.05$).

The rates of excretion and urinary concentrations of 2-hydroxyisobutyrate varied widely among individuals, but no statistically significant differences in these measures were seen between males and females. As with the excretion of 2-hydroxyisobutyrate, the rates of excretion or the total recovery of the other metabolites were not significantly

different between males and females. The determined half-times of excretion with urine were also not significantly different after exposure to 4 ppm and 40 ppm ETBE (see Table 6).

Rat Inhalation Exposure

Rats were exposed to the same ETBE concentrations as used in the human studies. The experimental results on metabolite concentrations and excretion are compiled in Tables 5, 6, and 7 and in Figure 10. The concentrations of ETBE in blood of rats determined after the end of the 4-hour exposure period were lower than those seen in humans after identical exposure concentrations. In contrast to concentrations of ETBE, the determined concentrations of TBA were higher both after 4 ppm and 40 ppm ETBE in rats than in humans subjected to identical exposure concentrations. After exposure to 4 ppm ETBE, the concentrations of 2-hydroxyisobutyrate and 2-methyl-1,2-propanediol in urine samples from exposed rats were significantly increased between 6 and 18 hours after exposure. The concentrations of TBA were not significantly increased (Figure 10). In the urine samples from exposed rats, the concentrations of 2-hydroxyisobutyrate and 2-methyl-1,2-propanediol were significantly increased (as compared to control levels) in urine samples collected within 24 hours after the end of 40-ppm ETBE inhalation (Figure 10). TBA concentrations were significantly above background only between 6 and 18 hours after the end of the exposure. Based on the amount of 2-hydroxyisobutyrate recovered, this compound also represents the major urinary metabolite of ETBE

Table 8. TAME Exposure: Blood Levels of TAME and TAA

	Maximum Concentration (µM)	Half-Time (hr)	Maximum Concentration (µM)	Half-Time (hr)
Inhalation	——————— 4 ppm TAME ———————		————— 40 ppm TAME —————	
Human				
TAME[a]	0.63 ± 0.11	1.4 ± 0.2	4.4 ± 1.7	1.2 ± 0.3
		4.6 ± 1.4		3.5 ± 0.7
TAA	1.02 ± 0.28	5.2 ± 2.0	9.2 ± 1.8	6.9 ± 0.4
Rat				
TAME	1.4 ± 0.8	1.1 ± 0.5	9.6 ± 1.4	0.6 ± 0.1
TAA	1.8 ± 0.2	—[b]	8.1 ± 1.5	—
Ingestion	——————— 5 mg TAME ———————		————— 15 mg TAME —————	
Human				
TAME[a]	0.056 ± 0.031	0.9 ± 0.1	0.140 ± 0.091	1.0 ± 0.3
		1.2 ± 0.2		1.8 ± 0.8
		3.1 ± 0.7		3.0 ± 0.4
TAA	0.42 ± 0.07	4.0 ± 0.9	0.96 ± 0.17	3.7 ± 0.7

[a] Human elimination of TAME from blood occurred in 2 phases after inhalation exposure and in 3 phases after ingestion exposure.

[b] — = Not determined.

(see Table 6) in rats; TBA and 2-methyl-1,2-propanediol were minor urinary metabolites of ETBE.

TAME BIOTRANSFORMATION AND KINETICS OF EXCRETION

Human Inhalation Exposure

During all inhalation experiments, the deviations between the targeted concentrations and the actual concentrations of TAME in the chamber were less than 10% of the targeted values. Average concentrations of TAME in the chamber were 3.8 ± 0.2 ppm and 38.4 ± 1.7 ppm (mean $\pm$ SD of 16 determinations in 15-minute intervals over 4 hours). Experimental results on the excretion of TAME metabolites and half-times in humans are given in Tables 8, 9, and 10 and in Figures 11 and 12. TAME and TAA were

not detected in blood samples from the human subjects taken before exposure. The maximum concentrations of TAME and TAA in blood were determined directly after the end of exposure. TAME concentrations decreased to reach the limit of detection at 12 hours after the end of exposure for both exposures (4-ppm and 40-ppm). Elimination of TAME from blood could be separated into 2 phases with half-times of 1.2 hours and 3.5 hours. Blood samples taken from subjects after exposure to 4 ppm and 40 ppm TAME showed detectable concentrations of TAA for the time between the end of exposure and the 4-hour blood sampling after 4 ppm TAME and between the end of exposure and the 24-hour blood sampling after 40 ppm TAME. Clearance of TAA from blood followed first-order kinetics and was slower than that of TAME.

Table 9. TAME Exposure: Parent Compound and Metabolites Recovered in Urine

	Total Excreted (μmol)	Background (μmol)	Half-Time (hr)	Total Excreted (μmol)	Background (μmol)	Half-Time (hr)
Inhalation	——— 4 ppm TAME ———			——— 40 ppm TAME ———		
Human						
TAME	0.12 ± 0.04	ND[a]	8.1 ± 1.5	1.5 ± 1.1	ND	4.3 ± 1.4
TAA	0.51 ± 0.25	ND	—[b]	5.7 ± 2.0	ND	6.0 ± 1.6
TAA glucuronide	1.8 ± 0.5	ND	8.9 ± 1.7	13.6 ± 2.4	ND	7.0 ± 0.9
2-Methyl-2,3-butanediol	26.8 ± 2.4^c	4.7 ± 1.1	39.8 ± 10.3	261.3 ± 76.3^c	7.9 ± 3.3	31.5 ± 6.1
2-Methyl-2,3-butanediol glucuronide	ND	ND	—	ND	ND	—
2-Hydroxy-2-methyl butyrate	85.7 ± 30.2	56.0 ± 31.4	—	291.8 ± 80.1^c	95.6 ± 53.7	12.3 ± 3.0
3-Hydroxy-3-methyl butyrate	219.3 ± 64.1	192.6 ± 46.9	—	330.9 ± 106.1^d	200.0 ± 68.9	9.8 ± 3.2
Rat						
TAME	ND	ND	—	ND	ND	—
TAA	0.005 ± 0.002	0.005 ± 0.002	—	0.011 ± 0.007^d	0.005 ± 0.002	—
TAA glucuronide	0.082 ± 0.047	ND	6.0 ± 1.6	1.0 ± 0.9	ND	4.1 ± 0.9
2-Methyl-2,3-butanediol	0.71 ± 0.19^c	0.06 ± 0.02	4.7 ± 0.8	4.7 ± 1.1^c	0.1 ± 0.1	4.6 ± 0.9
2-Methyl-2,3-butanediol glucuronide	ND	ND	—	1.7 ± 1.2	ND	4.5 ± 1.4
2-Hydroxy-2-methyl butyrate	0.28 ± 0.08	0.23 ± 0.06	—	0.64 ± 0.12^c	0.26 ± 0.04	4.8 ± 0.6
3-Hydroxy-3-methyl butyrate	1.7 ± 0.8	1.6 ± 1.1	—	2.1 ± 0.9	1.6 ± 0.8	—
Ingestion	——— 5 mg TAME ———			——— 15 mg TAME ———		
Human						
TAME	0.015 ± 0.014	ND	3.4 ± 1.9	0.016 ± 0.024	ND	1.6 ± 0.9
TAA	0.24 ± 0.15	ND	3.5 ± 0.9	0.21 ± 0.13	ND	4.5 ± 3.7
TAA glucuronide	0.31 ± 0.09	ND	7.5 ± 2.0	1.5 ± 0.5	ND	5.6 ± 0.9
2-Methyl-2,3-butanediol	5.4 ± 1.6^c	1.8 ± 1.6	42.5 ± 11.4	22.8 ± 4.4^c	3.6 ± 2.6	37.7 ± 7.1

[a] ND = not detected.

[b] — = Not determined.

[c] Significantly above background ($P < 0.01$).

[d] Significantly above background ($P < 0.05$).

Table 10. TAME Exposure: Calculated Received Doses and Total Recovery of Metabolites

	Received Dose (μmol)	Metabolites Excreted (μmol)	Percentage of Dose	Received Dose (μmol)	Metabolites Excreted (μmol)	Percentage of Dose
Inhalation	——————— 4 ppm TAME ———————			——————— 40 ppm TAME ———————		
Human	102	54 ± 21	53 ± 21	1033	600 ± 186	58 ± 18
Rat	1.9	0.78 ± 0.23	40 ± 12	20	8.1 ± 2.6	42 ± 13
Ingestion	——————— 5 mg TAME ———————			——————— 15 mg TAME ———————		
Human	49	4.2 ± 1.3	9 ± 3	147	21 ± 5	14 ± 3

In urine samples collected from subjects before TAME exposure and in samples collected from control subjects, low concentrations of 2-methyl-2,3-butanediol were present. In addition, high and variable concentrations of 2-hydroxy-2-methyl butyrate and 3-hydroxy-3-methyl butyrate were observed. In urine samples from TAME-exposed individuals, the concentrations of 2-methyl-
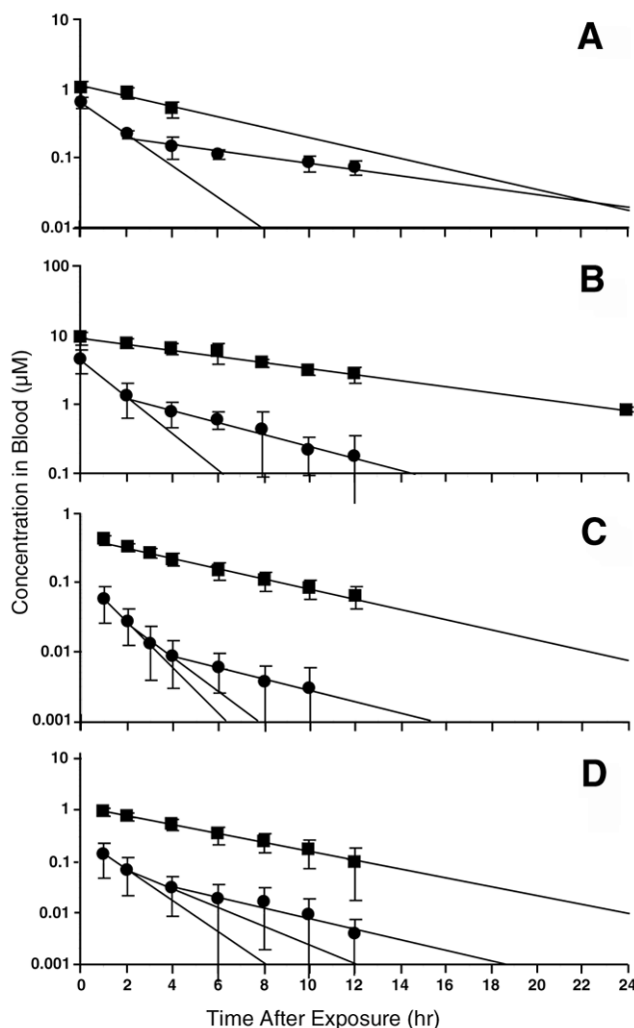

Figure 11. Time course of TAME (●) and TAA (■) elimination from blood of 6 human subjects exposed to 3.8 ± 0.2 ppm (panel A) and 38.4 ± 1.7 ppm (panel B) TAME for 4 hours in a dynamic exposure chamber and to 5 mg (panel C) and 15 mg (panel D) TAME by ingestion.
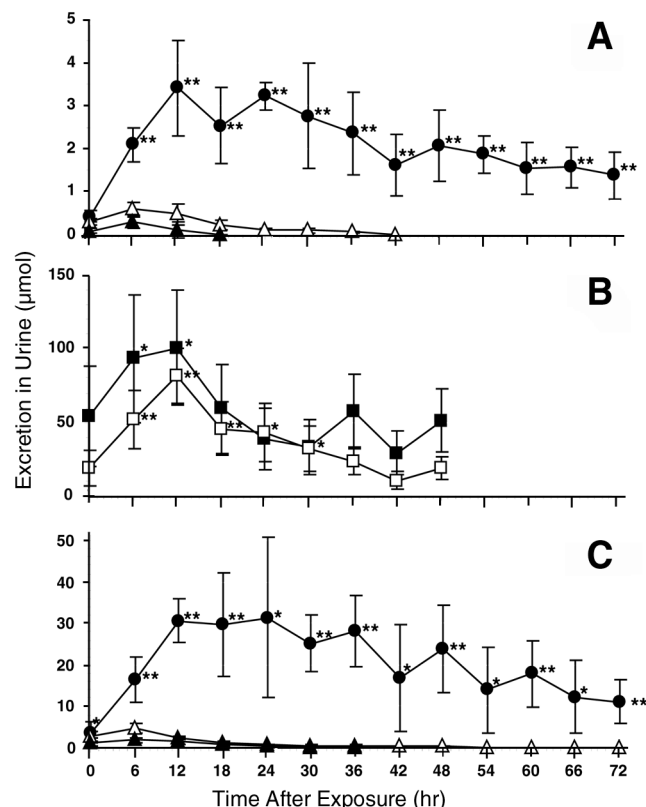
Figure 12. Urinary excretion of 2-methyl-2,3-butanediol (●), TAA glucuronide (△), TAA (▲), 3-hydroxy-3-methyl butyrate (□) and 2-hydroxy-2-methyl butyrate (■) in 6 human subjects exposed to 3.8 ± 0.2 ppm (panel A) and 38.4 ± 1.7 ppm (panels B and C) TAME for 4 hours in a dynamic exposure chamber. Numbers (mean ± SD) represent total amount of metabolite excreted in the urine samples collected at 6-hour intervals. Each sample was analyzed in duplicate. Significant differences as compared to background levels are indicated (** = $P < 0.01$; * = $P < 0.05$). In urine of subjects exposed to 3.8 ± 0.2 ppm TAME, concentrations of 3-hydroxy-3-methyl butyrate and 2-hydroxy-2-methyl butyrate were not significantly elevated.

2,3-butanediol were significantly increased in all urine samples collected until 72 hours after the end of exposure to both 4 ppm and 40 ppm TAME. Elimination of 2-methyl-2,3-butanediol was slow and not complete within the period of observation. After exposure to 4 ppm TAME, none of the urine samples contained significantly increased concentrations of 2-hydroxy-2-methyl butyrate and 3-hydroxy-3-methyl butyrate due to the high and variable background. Statistically significant increases in the concentrations of 2-hydroxy-2-methyl butyrate were observed only between 0 and 30 hours after the end of the 40-ppm exposure. Significantly increased concentrations of 3-hydroxy-3-methyl butyrate were seen only in urine samples taken between 0 and 12 hours after the end of exposure.

Due to the absence of background levels, the concentrations of TAME and TAA could be quantified with higher precision. TAME was detectable in all urine samples from the human subjects collected between 0 and 6 hours after the end of exposure to 4 ppm TAME and between 0 and 12 hours after the end of the exposure to 40 ppm TAME. TAA and its glucuronide were also detected in low concentrations in all urine samples collected between 0 and 36 hours after the end of the 40-ppm exposure. Excretion of these compounds in urine occurred with half-times of less than 10 hours (Figure 12).

Based on the recovered amounts of 2-methyl-2,3-butanediol and 2-hydroxy-2-methyl butyrate, these compounds represent the major excretory metabolites formed from TAME (see Table 9) in humans. In addition, 3-hydroxy-3-methyl butyrate was a major TAME metabolite in urine, whereas free and conjugated TAA and unchanged TAME were only minor excretory products. Large variations were observed in the extent of TAME biotransformation (see Table 10) between the individuals and in the rates of excretion and the urinary concentrations of 2-hydroxy-2-methyl butyrate and 3-hydroxy-3-methyl butyrate. However, there were no statistically significant differences between males and females for the amounts of these acids, of free and conjugated 2-methyl-2,3-butanediol, or of any of the other metabolites excreted or in the rates of excretion of metabolites. The determined half-times of elimination in urine also were not significantly different after the 4-ppm and 40-ppm TAME exposures (Table 10).

Human Ingestion Exposure

The human subjects were also exposed to 5 mg and 15 mg of TAME in tap water. In contrast to the ingestion studies with MTBE, ^{13}C-TAME could not be used due to time-consuming synthetic procedures and expensive
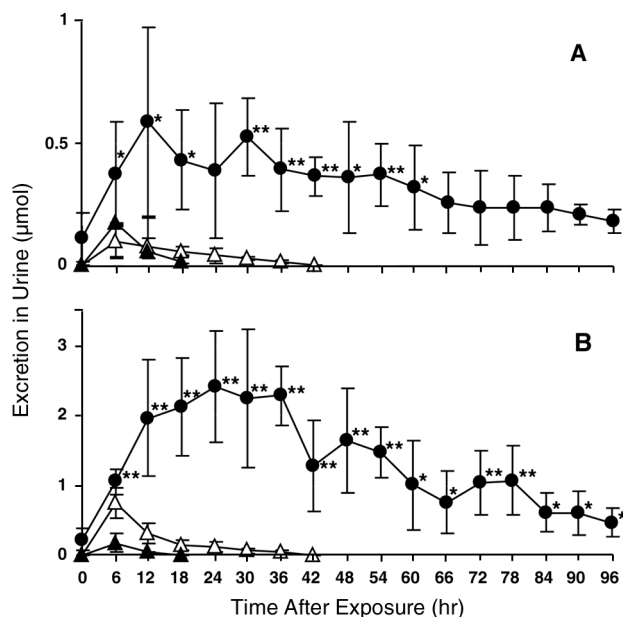
Figure 13. Urinary excretion of 2-methyl-2,3-butanediol (●), TAA glucuronide (△) and TAA (▲) in 6 human subjects exposed to 5 mg TAME (panel A) and 15 mg TAME via ingestion (panel B). Numbers (mean ± SD) represent total amount of metabolite excreted in the urine samples collected at 6-hour intervals. Each sample was analyzed in duplicate. Significant differences as compared to background levels are indicated (** = $P < 0.01$; * = $P < 0.05$).

starting materials. TAME was dissolved in local tap water, which did not contain detectable concentrations of TAME. The subjects consumed the spiked water samples (100 mL) within 20 seconds. Experimental results on the excretion of TAME metabolites and half-times in humans are given in Tables 9 and 10 and in Figures 11 and 13. TAME and TAA were not detected in blood samples from the subjects taken before the exposure. The maximum concentrations of TAME and TAA in blood were determined at the first blood sampling, 1 hour after ingestion. TAME concentrations decreased to reach the limit of detection 12 hours after exposure. Elimination of TAME from blood could be separated into 3 phases (Table 8). Blood samples taken from the human subjects showed detectable concentrations of TAA for the time period between the 1-hour blood sampling and the 12-hour blood sampling. Clearance of TAA from blood followed first-order kinetics and was slower than that of TAME.

As with the inhalation exposures, background concentrations of several metabolites of TAME were present. In the urine samples from TAME-exposed individuals, the concentrations of 2-methyl-2,3-butanediol were significantly increased in all urine samples collected until 96 hours after ingestion of 15 mg TAME. Concentrations of 2-methyl-2,3-butanediol in all urine samples collected up to

60 hours after ingestion of 5 mg TAME were significantly higher than in control samples. No significant increases were observed in the concentrations of 2-hydroxy-2-methyl butyrate and of 3-hydroxy-3-methyl butyrate due to the high and variable background.

TAME was detectable in all urine samples from the subjects collected 6 hours after the ingestion of 5 mg and 15 mg TAME. TAA glucuronide was also detected in all urine samples collected up to 42 hours after ingestion; and free TAA was detected up to 18 hours after TAME ingestion in low concentrations. Excretion of these compounds in urine occurred with half-times of less than 10 hours.

Based on calculations of recovery, 2-methyl-2,3-butanediol represented the major excretory metabolite TAME (see Table 9) in humans after oral ingestion. However, 3 hydroxy-3-methyl butyrate and 2-hydroxy-2-methyl butyrate—which were expected to be formed as major TAME-metabolites in urine based on the results of the inhalation studies with TAME—were not significantly elevated above the high background. Free and conjugated TAA and unchanged TAME were only minor excretory products. No significant differences were observed in the amounts of 2-methyl-2,3-butanediol or the other metabolites excreted, or in the rates of excretion of metabolites, between male and female subjects in the ingestion study. The determined half-times of elimination with urine were not significantly different after ingestion of 5 mg and 15 mg TAME, and only a small part of the dose given was recovered in the form of TAME metabolites (see Table 9).

Rat Inhalation Exposure

Rats were exposed to the same TAME concentrations as used in the human inhalation studies. The experimental results on metabolite concentrations and excretion are compiled in Tables 8 and 9 and in Figure 14. The blood concentrations of TAME in rats determined at the end of the 4-hour exposure period were twice as high as those seen in humans after identical exposure concentrations. The concentrations of TAA were not different between rats and humans after either the 4-ppm or 40-ppm TAME exposures.

In urine samples of rats collected before TAME exposure and in samples collected from control rats, low concentrations of 2-hydroxy-2-methyl butyrate, 3-hydroxy-3-methyl butyrate, 2-methyl-2,3-butanediol, and TAA were present. No significant increases in concentrations of 2-hydroxy-2-methyl butyrate were observed in rat urine samples collected after the 4-ppm exposure, but concentrations were significantly increased in a few urine samples collected within 18 hours after the end of the 40-ppm exposure (Figure 14). Also, no significant increases above background excretion rates were observed in the concentration
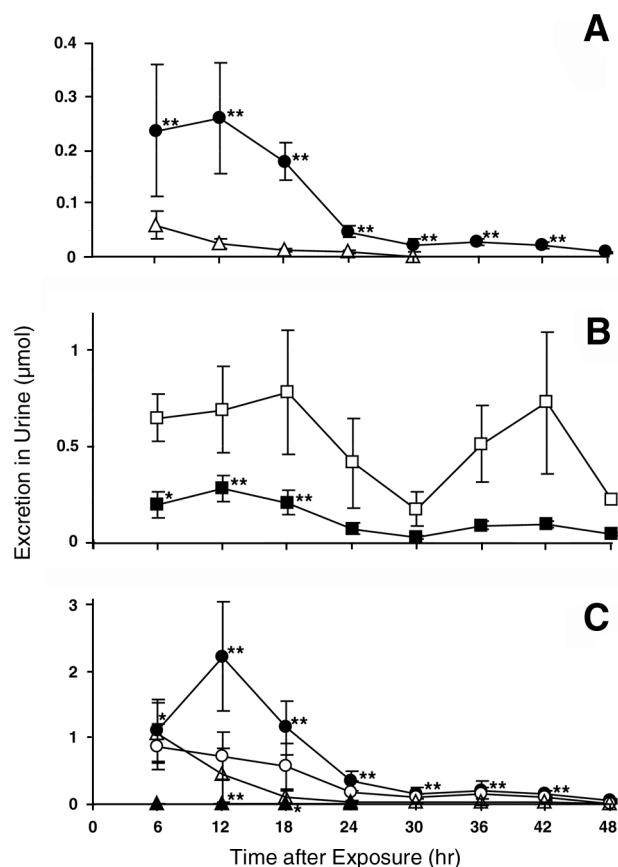

Figure 14. Urinary excretion of 2-hydroxy-2-methyl butyrate (■), 3-hydroxy-3-methyl butyrate (□), TAA glucuronide (△), TAA (▲), 2-methyl-2,3-butanediol (●) and its glucuronide (○) in 10 rats exposed to 3.8 ± 0.2 ppm (panel A) and 38.4 ± 1.7 ppm (panels B and C) TAME for 4 hours in a dynamic exposure chamber. Numbers (mean ± SD) represent total amount of metabolite excreted in the urine samples collected at 6-hour intervals. Each sample was analyzed in duplicate. Significant differences as compared to background levels are indicated (** = $P < 0.01$; * = $P < 0.05$). In urine of rats exposed to 3.8 ± 0.2 ppm TAME, concentrations of metabolites other than 2-methyl-2,3-butanediol (●) and TAA glucuronide (△) were not significantly elevated.

of 3-hydroxy-3-methyl butyrate in any urine samples collected from TAME-exposed rats. Due to much lower background levels, the concentrations of 2-methyl-2,3-butanediol were significantly higher than controls in all samples collected between 0 and 42 hours after both 4-ppm and 40-ppm TAME exposures. The glucuronide of 2-methyl-2,3-butanediol was not detectable in urine samples after 4-ppm exposure, but it was detected in all urine samples collected up to 48 hours after the end of the 40-ppm exposure. The glucuronide of TAA was detected in all urine samples collected up to 24 hours after the end of 4-ppm and 40-ppm exposures. The other metabolites quantified after the 40-ppm exposure were rapidly excreted, and their concentrations in urine samples were below the limit of detection after 24 hours; the presence of

these minor metabolites was not detected in urine samples collected from rats after exposure to 4 ppm TAME.

Based on the amounts of 2-methyl-2,3-butanediol and its glucuronide, these compounds and the TAA glucuronide were the major urinary metabolites of TAME in rat urine (Table 7). Assuming identical retention of TAME after inhalation exposure, humans excreted a significantly higher proportion of the retained TAME as metabolites in urine than rats ($P < 0.03$) (see Table 9).

DISCUSSION

BIOTRANSFORMATION AND KINETICS OF EXCRETION

Humans

To permit a direct comparison of ether disposition and biotransformation, human subjects were exposed to MTBE, ETBE, and TAME by inhalation under identical conditions. To permit further comparison of ether disposition and biotransformation after inhalation and ingestion exposure, human subjects were also exposed to MTBE and TAME by ingestion. The higher dose (15 mg) in the MTBE and TAME ingestion studies was calculated to give body burdens of the ethers identical to the calculated received doses after inhalation of 4 ppm for 4 hours in order to permit delineation of possible differences in ether disposition.

MTBE and ETBE   Results of the inhalation exposures suggest complex toxicokinetics of the ethers in humans. Elimination of MTBE and ETBE from blood occurred in several phases, with the first and second phases after ingestion representing redistribution of the ethers from blood into slowly perfused compartments. The final phase of elimination represents clearance of MTBE and ETBE by exhalation and by biotransformation to TBA and other metabolites. Clearance of MTBE and ETBE from human blood occurred with half-times between 2 and 4 hours, well in agreement with the results of other studies (Nihlén et al 1998a,b, Prah et al 1994). The clearance of TBA from blood after MTBE and ETBE exposures can be described by first-order kinetics; the rate constant is likely to be determined by further biotransformation of TBA since only a minor part of the TBA formed in the human organism from MTBE or ETBE is exhaled or excreted with urine (Nihlén et al 1998a,b).

The blood levels of MTBE and ETBE observed in humans in this study were in good agreement with those seen by Nihlén and colleagues (1998a,b), who exposed humans to similar MTBE and ETBE concentrations (25 ppm for 2 hours). These authors reported MTBE blood concentrations of 12.5 µmol/L and ETBE concentrations of

10 µmol/L at the end of exposure. In contrast to our study, however, where the human subjects were exposed at rest, subjects in the Nihlén study performed light work, which resulted in higher blood levels due to increased respiration and increased blood flow through the lung. Similar blood levels of MTBE were also seen in other studies exposing humans by inhalation, after correcting for exposure levels and exposure times (Cain et al 1996, Pekari et al 1996, Prah et al 1994). With the exception of MTBE, for which a non-linear increase in blood levels occurred between the 4-ppm and 40-ppm ingestion exposures, ether concentrations in blood measured directly after inhalation or ingestion were linearly related to exposure concentrations. The cause for the nonlinearity observed with MTBE in the inhalation studies is not known: saturation or induction of biotransformation is unlikely since a linear relationship was observed between the blood concentrations of TBA and in total metabolites recovered in urine.

Ingestion of MTBE also resulted in measurable blood levels of MTBE and TBA. The concentrations of these compounds in blood of all human subjects depended on the administered dose. The maximum blood levels for MTBE were lower after ingestion than after inhalation. The lower maximum blood levels reported were probably due to differences in sampling frequency, however: the first blood samples in the inhalation studies were taken at the end of the inhalation period, whereas after ingestion, the first blood samples were taken 1 hour after administration. The data on MTBE exhalation collected after ingestion suggest that MTBE is rapidly absorbed from the gastrointestinal tract and maximum blood levels are achieved within 10 to 20 minutes. Since concentration in blood parallels the concentration in exhaled air, extrapolation from the measured 1-hour blood levels using MTBE half-times determined in blood suggest that maximum blood levels after inhalation of 4 ppm for 4 hours and ingestion of 15 mg MTBE are both in the range of 2 µM. These observations indicate that most of the orally administered MTBE at low and environmentally relevant doses is absorbed in the upper gastrointestinal tract, thus bypassing the liver. Absence of a first-pass effect after ingestion of MTBE is also indicated by the extent of recovery of the applied dose in exhaled air, which accounted for approximately 30% of the MTBE dose.

Elimination of MTBE and ETBE from an organism occurs by both exhalation and excretion of water-soluble metabolites formed in the organism. Between 35 and 69% of the calculated inhaled doses of MTBE or ETBE were recovered as metabolites in urine; the remaining MTBE or ETBE taken up by inhalation is presumed to be exhaled. Exhalation of the parent ethers and TBA was not quantified

after inhalation exposure. However, several other studies using inhalation of similar concentrations of MTBE or ETBE have reported that exhalation is a significant pathway of elimination, supporting the assumption that the unaccounted portion of the MTBE and ETBE taken up was exhaled. In 3 studies in humans after MTBE inhalation, uptake of inhaled MTBE and elimination of MTBE and TBA were quantified. These authors recovered between 40% and 60% of the MTBE dose in exhaled air (Nihlén et al 1986; Johanson et al 1995a; Buckley et al 1997). In rats exposed to 400 ppm MTBE for 6 hours, more than 60% of the received dose was recovered in urine (Miller et al 1997). Similar observations were also made after ETBE inhalation. Nihlén and colleagues (1998a) recovered 50% of the ETBE taken up by their human subjects in exhaled air. In our study, approximately 50% of the MTBE or ETBE taken up by inhalation was recovered in the form of metabolites excreted with urine, complementing the data obtained by Nihlén and colleagues (1998a,b).

To obtain information on MTBE exhalation and complete mass balance, exhalation of MTBE after ingestion was quantified in a limited study in human subjects exposed to 15 mg MTBE. The results of the kinetics of elimination of MTBE and TBA with breath indicated that the portion of the dose not eliminated as urinary metabolites was exhaled and resulted in a recovery of greater than 80% of the administered dose. As expected, the half-times determined for MTBE exhalation were identical to those of MTBE clearance from blood.

A major part of the received doses of MTBE and ETBE was eliminated as metabolites in urine. The 3 known metabolites of MTBE and ETBE excreted with urine as well as unchanged MTBE or ETBE were quantified. Due to some water solubility, a minor part of the MTBE or ETBE dose was excreted with urine in humans, confirming previous results (Nihlén et al 1998 a,b).

A major problem in quantitation of urinary metabolites formed from MTBE and ETBE is the high and varying background levels of 2-hydroxyisobutyrate determined in human subjects before exposure or in apparently unexposed control subjects. 2-Hydroxyisobutyrate has been found as a urinary organic acid in humans and is formed endogenously (Liebich and Forst 1984); the relatively high rates of excretion are unlikely to be related to environmental exposure to chemicals such as TBA or isoprene, which are also metabolized to 2-hydroxyisobutyrate (Bernauer et al 1998, Henderson et al 1993). The high and varying background levels hindered exact quantitation of the metabolism of MTBE and ETBE after the low-dose inhalation and may have resulted in underestimation of the extent of MTBE or ETBE biotransfor-

mation. After the 40-ppm exposure in humans, however, 2-hydroxyisobutyrate was confirmed to be the major metabolite of MTBE or ETBE excreted with urine. In humans, 2-methyl-1,2-propanediol was a minor MTBE and ETBE metabolite, and the presence of this compound in urine samples collected before the exposure and in control individuals was likely caused by oxidation of TBA. Background concentrations of TBA were detected in all blood samples and all urine samples, suggesting exposure to TBA from sources other than MTBE or ETBE. For instance, TBA or t-butyl esters are used in food processing and flavoring (CIREP 1989). After ingestion of MTBE, the extent of biotransformation of MTBE to water-soluble metabolites excreted with urine also reached approximately 50% of the administered dose.

When comparing blood levels and the extent of biotransformation of MTBE and ETBE after inhalation, consistently lower blood levels for the parent ether and for TBA, and also for the summarized excretion of urinary metabolites, were seen with ETBE as compared to MTBE. This was likely due to a less efficient uptake and a lower retention of ETBE than of MTBE. Biotransformation of the initially formed TBA from both MTBE and ETBE was identical, resulting in identical profiles of urinary metabolites and identical rates of excretion of these metabolites. None of the exposures performed indicated a gender difference in the disposition of MTBE or ETBE in humans.

The urinary excretion rates of MTBE metabolites after ingestion were identical to the excretion rates after inhalation. With ^{13}C-MTBE, where background levels of metabolites were very low due to the use of isotope-labeled ether, the relative proportions of excreted metabolites were identical to those seen after ^{12}C-MTBE inhalation with 2-hydroxyisobutytrate as major metabolite and with 2-methyl-1,2-propanediol and TBA as minor metabolites.

TAME The TAME biotransformation and kinetics of excretion were even more complex than those of MTBE and ETBE due to the occurrence of many interacting steps. After inhalation exposure, the determined blood levels of TAME and TAA were lower than those obtained with MTBE and ETBE or TBA formed from MTBE and ETBE. This difference may again be due to a lower retention of TAME in the human lung compared to the other ethers, or may be due to a more rapid biotransformation. As with MTBE and ETBE, biotransformation to water-soluble metabolites represents a major pathway of TAME clearance from the organism. Again, problems with high background levels for 1 of the metabolites limits some of the quantitative conclusions drawn from the study. The observed data suggest, however, that TAME biotransforma-

tion is not saturated in humans in the dose range studied and that TAME disposition and biotransformation do not differ between ingestion and inhalation exposure. As seen with MTBE and ETBE, a minor part of the TAME dose is excreted with urine in humans.

The low recovery of TAME metabolites after ingestion is likely to be mainly due to the high background levels of the major metabolites 3-hydroxy-3-methyl butyrate and 2-hydroxy-2-methyl butyrate. No significant differences in the rates of excretion of these compounds were observed in TAME-dosed individuals: the large and varying amounts of these acids in urine of unexposed individuals, and the small contribution of TAME biotransformation to the urinary concentrations of these acids, prevented exact quantitation. The other minor urinary metabolites of TAME could be quantified because background levels were either low or absent altogether.

Rats

MTBE and ETBE   After 4-hour inhalation exposures to 4 ppm and 40 ppm MTBE or ETBE, blood levels of MTBE or ETBE determined in humans and rats immediately after the end of the exposure period did not differ significantly. In general, the time course of elimination of MTBE or ETBE and all metabolites quantified shows that rats excreted these compounds more rapidly than humans. In rats, MTBE cleared from blood with a half-time of 30 minutes, a result in agreement with other studies. The half-time of MTBE elimination from blood was also found to be 30 minutes in rats (Miller et al 1997) and between 2 and 4 hours in humans (Nihlén et al 1998b, Prah et al 1994). As in other studies, no sex differences in the apparent elimination half-times of these compounds were found.

In rat urine, the ether concentrations were already below the limit of detection in the first available samples (6 hours after the end of exposure). The rate of excretion of MTBE or ETBE metabolites in rat urine was slower compared to exhaled breath. All MTBE and ETBE metabolites were eliminated with apparent half-times of elimination of less than 5 hours; in humans, the rate of urinary elimination of MTBE or ETBE was considerably slower. MTBE or ETBE biotransformation in rats and in humans are similar when the relative amounts of metabolites and their concentrations recovered in urine are compared to the doses received (see Tables 2 through 7).

TAME   Immediately after 4-hour inhalation exposures to 4 ppm and 40 ppm TAME, significantly lower blood levels of TAME were obtained in humans than in rats. In general, the time course of elimination of TAME and all metabolites

quantified in this study shows that rats excrete TAME and its metabolites more rapidly than humans. As seen with the other ethers, no sex differences in the apparent half-times of elimination of these compounds were found, and clearance of TAME from blood in rats was more rapid than from blood in humans.

In humans and rats, the known metabolites of TAME and unchanged TAME excreted with urine were quantified. Quantitative results on excretion of the different metabolites suggest major differences in biotransformation of TAME between rats and humans. In rats, TAME is mainly excreted as 2-methyl-2,3-butanediol and its glucuronide; further oxidation of TAA to other products has minor importance because of the more rapid elimination and glucuronide formation. In humans, 2-methyl-2,3-butanediol is eliminated more slowly than in rats. In addition, TAA seems to be more efficiently oxidized to 2-hydroxy-2-methyl butyrate and 3-hydroxy-3-methyl butyrate in humans; this reaction occurs only to a minor extent in rats. These differences are likely to be due to substrate specificities of the enzymes involved in formation of these compounds from TAME and TAA (CYP and glucuronyl transferases). Rates of urinary excretion of TAME metabolites were slower than elimination through exhalation in both species. However, all TAME metabolites quantified in this study were eliminated in rats with apparent half-times of elimination of less than 6 hours; in humans, the urinary elimination of the metabolites formed from TAME was considerably slower.

In addition to qualitative differences, the extent of TAME biotransformation in humans is significantly higher ($P < 0.05$, t test) than in rats when the amounts of metabolites and the relative concentrations recovered in urine are compared to the doses received (Table 9). Between 40% (in rats) and almost 60% (in humans) of the calculated doses of TAME received by inhalation were recovered as metabolites in urine. The rest of the TAME taken up by inhalation is probably exhaled. Exhalation of unchanged TAME was not determined in this study; because of the volatility of TAME and based on studies with the structurally similar MTBE and ETBE, however, it has to be assumed that the unaccounted portion of the received TAME dose is exhaled unchanged both by rats and by humans after the end of the inhalation period. The biotransformation of TAME in both rats and humans seems not to be saturated in the concentration range studied since the percentage of dose recovered as metabolites was identical after both exposure concentrations.

In addition, individuals differed in the extent of TAME biotransformation. These differences were probably due to individual differences in the CYP profile (Hong et al

1999a; Hong et al in this Report). For instance, differences of more than 10 fold have been found in the capacity of human liver microsomes to oxidize TAME to TAA (Hong et al 1999a; Hong et al in this Report). Moreover, further biotransformation of TAME metabolites, which is complex and dose-dependent, is indicated by the limited data available. Future studies identifying the enzymes involved in TAA biotransformation may address this problem.

IMPLICATIONS FOR INTERPRETATION OF TOXICITY DATA

The results presented here provide further details on the biotransformation of MTBE, ETBE, and TAME after low and environmentally relevant doses, and these results may improve the basis for risk assessment of environmental exposures to these ethers. In general, the data suggest that the rat may be a suitable animal model for studying toxic effects of MTBE and ETBE and for extrapolation of these effects to humans because disposition of MTBE and ETBE was identical in both species.

One of the major issues in the risk assessment process for MTBE is the question of which metabolite (or parent compound) is responsible for the diverse toxic effects seen after long-term MTBE administration in animals and the related issue of dose-dependence of these effects. The role of biotransformation reactions and the metabolites formed in toxic effects observed after repeated administration of MTBE, ETBE, and TBA remains unclear. Both the parent ethers, TBA or other metabolites, and probably 2-hydroxy-isobutyrate (due to the large quantities formed and its slow elimination), may be responsible for toxicity. Both MTBE and TBA induce nephropathy in rats and were found to increase the incidence of renal tumors in male rats (Burleigh-Flayer et al 1992, Lindamood et al 1992, Takahashi et al 1993). α_{2u}-Globulin accumulation has also been observed with ETBE (Medinsky et al 1999). It has been suggested that the α_{2u}-globulin nephropathy syndrome may be involved in the formation of renal tumors in male rats after exposure to MTBE and TBA (Prescott-Mathews et al 1997). α_{2u}-Globulin nephropathy involves an impaired degradation of the male rat–specific protein α_{2u}-globulin induced by xenobiotics bound to this protein. The impaired degradation in the renal lysosomes results in accumulation of modified α_{2u}-globulin in the renal tubular epithelial cells, lysosomal rupture, cell death, and cell proliferation. The induced cell proliferation is suggested to be a major contributor to tumor formation. Tumors are seen only in male rats because of the sex-specific and species-specific biosynthesis of α_{2u}-globulin (Swenberg et al 1989). The chemical responsible for a possible binding to α_{2u}-globulin in MTBE effects is not well defined (Prescott-

Mathews et al 1997, Takahashi et al 1993). In vitro studies suggest that unchanged MTBE may be responsible for binding (Poet and Borghoff 1997), but a role for TBA formed as a metabolite has not been ruled out. Furthermore, MTBE is only a weak inducer of α_{2u}-globulin accumulation, and other mechanisms may contribute to the renal toxicity of this ether.

Whereas ETBE has caused similar α_{2u}-globulin accumulation in the kidney as with MTBE, ETBE exposures of rats have also induced pronounced testicular lesions after 13 weeks of exposure (Medinsky et al 1999), an effect not seen with MTBE. The role of biotransformation in the testicular lesions induced by ETBE is unclear. Because of identical pathways and extent of biotransformation to major metabolites for both MTBE and ETBE, a contribution of biotransformation to the induction of testicular lesions by ETBE seems unlikely. However, methoxyacetic acids are known testicular toxins implicated in testicular toxicity of glycol ethers, and *tert*-butoxyacetic acid could be formed by further oxidation of the α-carbon of the ethyl group in ETBE. A minor pathway (or a pathway operative only after exposure to very high concentrations, as used in the toxicity study, or after prolonged exposure) of ETBE biotransformation may result in 2-*tert*-butoxyacetic acid, which is a structural analog to the known testicular toxicant methoxyacetic acid (Sumner et al 1992, Moslen et al 1995, Welsch 1995). In the study described in this report, ETBE biotransformation was investigated only after single exposures to relatively low concentrations. Possibly low affinity pathways for ETBE oxidation that are operative only at very high exposure doses oxidize ETBE to 2-*tert*-butoxyglycol or 2-*tert*-butoxyacetate, or prolonged exposure to ETBE may result in induction of a CYP enzyme catalyzing formation of 2-*tert*-butoxyglycol.

The structures of metabolites of MTBE and ETBE described in this report and the delineated mechanisms of formation do not suggest the formation of reactive intermediates in MTBE and ETBE biotransformation. Moreover, the major metabolites of MTBE and ETBE identified are endogenously formed in relatively large amounts, suggesting the presence of tolerable exposures. Tolerable exposures would be MTBE or ETBE concentrations that do not significantly increase the blood levels or levels of excretion of 2-hydroxyisobutyrate. Thus, based on the results of biotransformation studies, thresholds for MTBE or ETBE exposures without toxic effects may be defined.

The differences in biotransformation of TAME and kinetics of metabolite excretion between humans and rats suggest that these two species may respond differently to potentially toxic effects of TAME. Because only limited data are available on toxic effects of TAME after long-term

inhalation exposure in rats, however, conclusions must be drawn with caution. The potential of TAME to induce acute and chronic toxicity in rats is low; the role of biotransformation in the observed changes in relative organ weights is unknown. Since the formation of reactive metabolites during TAME biotransformation is also not suggested by the structures of metabolites and their mechanisms of formation, covalent binding to macromolecules has to be regarded as unlikely and thresholds are likely for TAME toxicity. Moreover, the major TAME metabolites found in humans are also formed endogenously (Liebich and Forst 1984), and TAME exposures in low concentrations—as expected in the environment—are unlikely to result in a significant increase in the human body burden of these compounds. Thus, under realistic exposure conditions, toxic effects of TAME in humans are unlikely to occur.

BIOMARKERS OF ETHER EXPOSURE

The studies on biotransformation may also help to define useful biomarkers of exposure to MTBE, ETBE, and TAME. Because of likely exposures from sources other than gasoline, determination of TBA in blood or urine does not represent a useful biomarker of exposure of humans to MTBE or ETBE. Unfortunately, 2-hydroxyisobutyrate, the major MTBE and ETBE metabolite excreted in urine, is formed endogenously in large concentrations, also preventing the use of this compound as a biomarker. Significant increases in the excretion of 2-hydroxyisobutyrate are expected to occur only after exposures to high concentrations of MTBE or ETBE that are unlikely for the general population. Due to a much lower background level, 2-methyl-2,3-butanediol concentrations in urine or blood may be useful biomarkers of exposure to the low levels of TAME that are expected from environmental exposures. Based on the present results, the concentration of parent ethers in blood may be the most useful biomarker of exposure to MTBE, ETBE, and TAME. These measurements are simple to perform, but their utility may be hampered by lack of sensitivity at environmentally relevant exposure conditions and the rapid clearance of the ethers from blood.

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APPENDIX A. Structure Elucidation of Ether Metabolites (Formed from MTBE, ETBE, and TAME)

In this series of experiments, the structures of ether metabolites formed in rats and humans were elucidated by labeling ethers with ^{13}C and using ^{13}C-NMR and GC/MS for structure determination. ^{13}C-labeled ethers (more than 99% ^{13}C in a single carbon atom) were used to identify metabolites excreted in urine to avoid the expensive synthesis of ^{14}C-labeled material and to be able to detect nonvolatile metabolites. ^{13}C is a stable isotope that permits sufficiently sensitive metabolite analysis in urine by ^{13}C-NMR and also identifies unanticipated metabolites and their structures. Moreover, the synthetic procedures yielded 2-^{13}C-TBA and ^{13}C-TAA, which were used in metabolism studies (see main report) to better define the fate of these first intermediary metabolites of the ethers in vivo in rats.

For metabolite identification, 2 male and 2 female rats were exposed to ^{12}C-ether or ^{13}C-labeled ether by inhalation. To confirm that metabolic pathways in humans are identical, one human volunteer was exposed to ^{13}C-TAME by inhalation and to ^{13}C-TBA by ingestion. To obtain large amounts of the metabolites, rats were also exposed to ^{13}C-TBA and ^{13}C-TAA by ingestion.

PROCEDURES AND SYNTHESIS

Instrumental Analysis

Liquid secondary ion MS (LSIMS) was performed using a triple quadrupole mass spectrometer (model TSQ 700, Finnigan, San Jose CA) with electrospray ionization. Samples were introduced at a flow rate of 0.5 mL in water adjusted to pH 3 by adding formic acid. LC/MS and LC–MS/MS spectra were obtained by automatically switching between positive-ion and negative-ion modes in alternating scans. Electrospray voltage was 4.5 kV and 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 because of the low ultraviolet absorption of the metabolites. Urine samples were separated on a steel column (25 cm × 4 mm ID) filled with Partisil ODS-III (Whatmann, Germany) using gradient elution.

A linear gradient from 100% water (acidified with formic acid to pH 3) to 50% water/acetonitril in 25 minutes at a flow rate of 1 mL/min was used for separation.

^{13}C-NMR spectra were recorded with a 250-MHz spectrometer (Bruker, Rheinstetten, Germany) or a 600-MHz spectrometer (Avance 600, Bruker). Usually, 2,000 scans were acquired for Fourier transformation (Bernauer et al 1998). GC/MS was performed with a Fisons MD 800 mass spectrometer coupled to a GC 8000 series gas chromatograph and equipped with an AS 800 autosampler and an electron impact source (Fisons Instruments, Mainz, Germany). Some samples from later parts of the study were also analyzed with electron impact ionization using a 5970 mass spectrometer coupled to a 5890 gas chromatograph (Hewlett-Packard) or a 5973 mass spectrometer coupled to a 6890 chromatograph (Hewlett-Packard). Both instruments were equipped with a CTC Combi-PAL autoinjector with capability for headspace injection (Hewlett-Packard). All GC columns were obtained from J&W Scientific (Folsom CA).

Chemicals

MTBE (99.8+% purity), TAME (97+% purity), TBA (99.5+% purity), TAA (99+% purity), 2-hydroxyisobutyrate (98+% purity), TBA-d_{10} (99+% purity), 2-hydroxy-2-methyl butyrate (98+% purity), 3-hydroxy-3-methyl-2-butanone, 1,2-propanediol (99.5+% purity), and 2-hydroxyvaleric acid (98+% purity) were obtained from Aldrich Chemical Company (Deisenhofen, Germany). ETBE (99+% purity), 3-hydroxy-3-methyl butyrate (98+% purity), and 2-methyl-2,4-butanediol (98+% purity) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals were obtained from commercial suppliers in the highest purity available.

Sample Analysis

Urine samples obtained from animals exposed to ^{13}C-labeled ethers and control urine samples (720 µL) were introduced into an NMR tube (5 mm ID) and 80 µL D_2O was added (Bernauer et al 1998). These samples were immediately analyzed by NMR. Some of the urine samples were treated with β-glucuronidase (Sigma, G7846, lot 72H6836) for 30 minutes at 37°C or sulfatase (Sigma, S9751, lot 621-07881) for 48 hours at 37°C (Henderson et al 1993). Aliquots of the treated urine were also analyzed by NMR. For acid treatment, urine samples were acidified to pH 2 with concentrated hydrochloric acid and incubated for 1 hour at 37°C.

Metabolite structures were confirmed by GC/MS. A portion (0.5 mL) of the urine sample was introduced into a 1.5-mL vial, acidified with 2N HCl to pH 4, and kept at 80°C for 30 minutes. The headspace (100 µL) 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 ethyl acetate, and 1 µL of the ethyl acetate layer was injected into the GC. To identify acidic metabolites, 0.5 mL of the urine samples was taken to dryness by lyophilization and the obtained residues were treated with 500 µL of BF$_3$/methanol (14%) at 60°C for 30 minutes. 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 obtained solution was injected into the gas chromatograph. Separation was performed on a fused silica column (30 m × 0.25 mm ID; 0.25-µm film) coated with DB-WAX. For analysis, a temperature gradient from 35°C to 230°C with a heating rate of 10°C/minute was applied. Electron impact mass spectra (70 eV) were recorded and metabolite peaks identified by comparing chromatograms of urine samples from treated rats with those of untreated controls.

Exposure of One Human Subject to ^{13}C-TBA and ^{13}C-TAME

^{13}C-TBA (5 mg/kg) was administered in a gel capsule to a single human subject (age 44 years, body weight 80 kg). Urine was collected at 12-hour intervals for 48 hours and analyzed by ^{13}C-NMR. To study human biotransformation of ^{13}C-TAME, a 2-L gas sampling bag (Linde, Giessen, 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 subject inhaled the contents of the bag for 4 minutes and exhaled into the bag to ensure maximum uptake of ^{13}C-TAME. After the end of the exposure, urine samples were taken at 6-hour intervals for 48 hours.

Exposure of Rats to ^{13}C-Labeled Ethers

The chamber for inhalation exposure of rodents is shown in Figure A.1; more details are described in the literature (Loizou et al 1994, Urban and Dekant 1994). The animals were introduced into the chamber at 10 AM, and the calculated amounts of ^{12}C-ethers or ^{13}C-labeled ethers were introduced with a microliter syringe. The concentration of ether in the chamber was monitored every 10 minutes by an automatic gas-sampling valve. An aliquot (100 µL) of the gas phase was introduced into a capillary GC (HP 5970) and separated with a DB1 coated fused silica column (DB1, J&W Scientific; 40 m × 0.18 mm ID, 0.4-µm film). Ether concentrations in the air were quantified by flame ionization detection (FID). After the end of the 6-hour exposure period, the animals were transferred to metabolic cages and urine was collected on ice at 24-hour intervals over 48 hours. Two male and two female rats were individually exposed to both the ^{13}C-ether and the ^{12}C-ether.
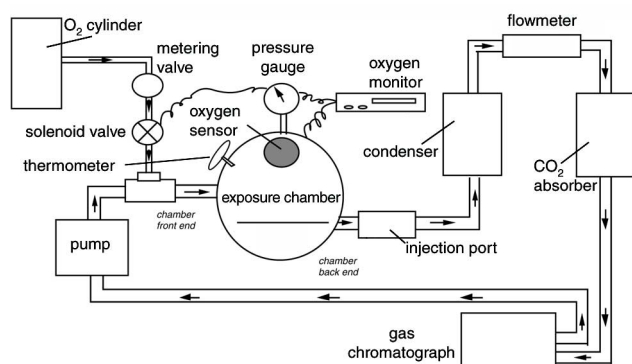
Figure A.1. Closed exposure system for rodents. The volatile xenobiotic is injected into the system to generate an atmosphere with a specific concentration, which then is cycled through the system. Carbon dioxide exhaled by the animals is trapped, and oxygen concentrations are monitored and automatically adjusted. Samples of the atmosphere are automatically analyzed by gas chromatography to determine the concentration of xenobiotic over time in the chamber.

Synthesis of 2-^{13}C-TBA

An ethereal solution of 60 mmol of CH_3I (Merck, Darmstadt, Germany) was slowly added to equimolar amounts of magnesium turnings (1.5 g) covered by 5.0 mL diethyl ether. The Grignard reaction was initiated by adding traces of iodine (Nicholson and Wilson 1987, Aldrich 1998) (Figure A.2). The mixture was then stirred for 30 minutes at flux; an ethereal solution of 50 mmol of 2-^{13}C-acetone (lot P-7787, Cambridge Isotope Laboratories, Andover MA) was added within 30 minutes; and the mixture was kept at flux for 2 hours. After cooling, hydrolysis was performed with 50 mL of an ice-cold, saturated NH_4Cl solution. The layers were separated and the aqueous layer was extracted 5 times with 10 mL of diethyl ether. The ethereal layers were combined and dried over Na_2CO_3. After evaporation of the solvent, the residue was distilled to yield 2-^{13}C-TBA (42% yield, 97% GC/FID purity).

The structure of the reaction product was confirmed by ^{1}H-NMR, ^{13}C-NMR, and MS. For 2-^{13}C-TBA, the ^{1}H-NMR (250 MHz, D_2O) profile was δ 1.25, 9H [d; J_{HC} = 4 Hz;

Figure A.2. Synthetic routes to ^{13}C-MTBE and ^{13}C-ETBE from ^{13}C-acetone. 1 = Acetone; 2 = methylmagnesium iodide; 3 = TBA; 4 = methanol; 5 = ethanol; 6 = MTBE; 7 = ETBE.

(CH_3)$_3$COH]. The ^{13}C-NMR (63 MHz, D_2O) profile was as follows: δ 32.4 [d; J_{CC} = 39.1 Hz; (CH_3)$_3$COH]; 72.6 [s; d, with 0.5% of a satellite doublet; J_{CC} = 39 Hz; (CH_3)$_3$COH]. The MS (70 eV) profile was: m/z 60 [100% M$^+$-CH_3]; 58 [40%]; 44 [31%]; 42 [40%].

Synthesis of ^{13}C-MTBE and ^{13}C-ETBE from 2-^{13}C-TBA

A solution of 20 mmol of methanol (for the synthesis of MTBE) or ethanol (for the synthesis of ETBE) was heated to 70°C; next, 10 mmol of 2-^{13}C-TBA was added with a syringe. With increasing temperature, an azeotropic mixture of MTBE (or ETBE), methanol (or ethanol), and TBA was distilled off. Methanol (or ethanol) was removed from the reaction mixture by boiling over sodium for 1 hour. Further purification was performed by slow distillation with dry-ice cooling on a Büchi-GKR-51 Kugelrohr apparatus (Zurich, Switzerland) to yield 26% 2-^{13}C-MTBE or 21% 2-^{13}C-ETBE. The GC/FID purities of both reaction products were greater than 95%.

The structures of the reaction products were confirmed by ^{1}H-NMR, ^{13}C-NMR (Cederbaum and Cohen 1980), and MS. For 2-^{13}C-MTBE, the ^{1}H-NMR (250 MHz, D_2O) profile was as follows: δ 1.22, 9H [d; J_{HC} = 4 Hz; (CH_3)$_3$COCH$_3$]; 3.24, 3H [d; J_{HC} = 4 Hz; (CH_3)$_3$COCH$_3$]. The ^{13}C-NMR (63 MHz, D_2O) profile was as follows: δ 28.8 [d; J_{CC} = 40 Hz; (CH_3)$_3$COCH$_3$]; 51.6 [s; (CH_3)$_3$COCH$_3$]; 77.9 [s; d, with 0.5% of a satellite doublet; J_{CC} = 40 Hz; (CH_3)$_2$COCH$_3$]. The MS (70 eV) profile was: m/z 74 [100% M$^+$-CH_3]; 58 [59%]; 56 [21%]; 44 [50%]; 42 [38%].

For 2-^{13}C-ETBE, the ^{1}H-NMR (250 MHz, D_2O) profile was as follows: δ 1.15, 4H [t; J = 7 Hz; (CH_3)$_3$COCH$_2$CH$_3$]; 1.23, 9H [d; J_{HC} = 4 Hz; (CH_3)$_3$COCH$_2$CH$_3$]; 3.54, 2H [q; J = 7 Hz; J_{HC} = 2 Hz; (CH_3)$_3$COCH$_2$CH$_3$]. The ^{13}C-NMR (63 MHz, D_2O) profile was as follows: δ 10.0 [s; (CH_3)$_3$COCH$_2$CH$_3$]; 29.3 [d; J_{CC} = 39 Hz; (CH_3)$_3$COCH$_2$CH$_3$]; 60.2 [s; (CH_3)$_3$COCH$_2$CH$_3$]; 77.7 [s; d, with 0.5% of a satellite doublet; J_{CC} = 40 Hz; (CH_3)$_3$COCH$_2$CH$_3$]. The MS (70 eV) profile was: m/z 88 [94% M$^+$-CH_3]; 60 [100%]; 58 [88%]; 56 [12%]; 44 [23%]; 42 [45%]; 41 [16%].

Synthesis of 2-Methyl-1,2-propanediol

2-Methyl-1,2-propanediol was prepared by reduction of the ethyl ester of 2-hydroxyisobutyrate. NaBH$_4$ (4 mmol) was dissolved in 12 mL isopropanol followed by addition of 10 mmol of 2-hydroxybutyric acid ethyl ester at room temperature. The mixture was then stirred overnight. To dissolve the precipitated solid, 2N HCl was added to the reaction mixture. After extraction with ethyl ether, the organic layers were combined and dried over K_2CO_3. After
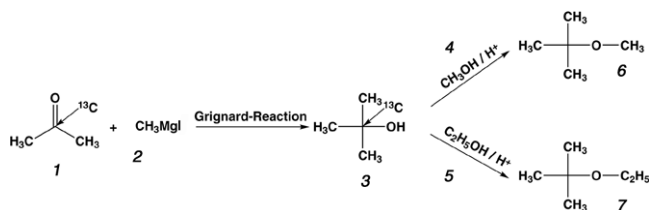
removal of the solvent, 2-methyl-1,2-propanediol was isolated by fractional distillation under reduced pressure.

Characterization of 2-methyl-1,2-propanediol was accomplished under the following conditions. The ^{1}H-NMR (250 MHz, D$_2$O) profile was as follows: δ 1.19, 6H [s; (C$\underline{H}_3$)$_2$C(OH)CH$_2$(OH)]; δ 3.42, 2H [s; (CH$_3$)$_2$C(OH)C$\underline{H}_2$(OH)]. The ^{13}C-NMR (63 MHz, D$_2$O) profile was as follows: δ 27.4 [s; ($\underline{C}$H$_3$)$_2$C(OH)CH$_2$(OH)]; δ 72.6 [s; (CH$_3$)$_2$C(OH)$\underline{C}$H$_2$(OH)]; δ 74.2 [s; (CH$_3$)$_2$$\underline{C}$(OH)CH$_2$ (OH)]. MS (70 eV): m/z 75 [8%, M$^+$-CH$_3$]; 59 [70%]; 57 [28%]; 55 [12%]; 43 [100%]; 42 [22%]; 41 [33%].

Synthesis of 2-^{13}C-TAA

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 (see Figure A.2). The Grignard reaction was initiated by addition of traces of iodine (Kropf 1975, Hünig et al 1979). The mixture was stirred for 30 minutes at reflux; a solution of 50 mmol of 2-^{13}C-acetone (lot P-7787, Cambridge Isotope Laboratories, Andover MA) in diethyl ether was added over 30 minutes; and the mixture was kept at reflux for 2 hours. After cooling, hydrolysis was performed with 30 mL of an ice-cold, saturated NH$_4$Cl solution. The layers were separated, and the aqueous layer was extracted 5 times with 10 mL of diethyl ether. The ether layers were combined and dried over K$_2$CO$_3$. After evaporation of the solvent, the residue was distilled to yield 2-^{13}C-TAA (61% yield, 98% GC/FID purity).

The structure of the reaction product was confirmed by ^{1}H-NMR, ^{13}C-NMR, and MS. For 2-^{13}C-TAA, the ^{1}H-NMR (250 MHz, D$_2$O) profile was as follows: δ 0.89, 3H [t; J = 8 Hz; J_{HC} = 4 Hz; C$\underline{H}_3$CH$_2$C(CH$_3$)$_2$OH]; 1.19, 6H [s; J_{HC} = 4 Hz; CH$_3$CH$_2$C(C$\underline{H}_3$)$_2$OH]; 1.52, 2H [q; J = 8 Hz; J_{HC} = 4 Hz; CH$_3$C$\underline{H}_2$C(CH$_3$)$_2$ OH. The ^{13}C-NMR (63 MHz, D$_2$O) profile was as follows: δ 10.7 [s; $\underline{C}$H$_3$CH$_2$C(CH$_3$)$_2$OH]; 30.0 [d; J_{CC} = 40 Hz; CH$_3$CH$_2$C($\underline{C}$H$_3$)$_2$OH]; 38.1 [d; J_{CC} = 39 Hz; CH$_3$$\underline{C}H_2$C(CH$_3$)$_2$OH]; 75.0 [s; d, with 0.5% of a satellite doublet; J_{CC} = 39 Hz; CH$_3$CH$_2$$\underline{C}$(CH$_3$)$_2$OH]. The MS (70 eV) profile was: m/z 74 [85% M$^+$-CH$_3$]; 60 [100% M$^+$-C$_2$H$_5$]; 56 [68%]; 44 [33%]; 43 [17%]; 42 [19%]; 41 [6%]; 40 [12%].

Synthesis of 2-^{13}C-TAME from 2-^{13}C-TAA

A mixture of methanol (60 mmol) and 10% H$_2$SO$_4$ (6 mL) was heated to 65°C; next, 20 mmol of 2-^{13}C-TAA was added with a syringe (Meerwein 1975). By increasing the temperature of the mixture to 100°C, an azeotropic mixture of 2-^{13}C-TAME, methanol, 2-methyl-2-butene, 2-methyl-1-butene, and TAA was distilled off. Methanol was removed from the reaction mixture by extraction with water. Further purification was performed by slow distilla-

tion 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 greater than 98%.

The structure of the reaction product was confirmed by ^{1}H-NMR, ^{13}C-NMR, and MS. For 2-^{13}C-TAME, the ^{1}H-NMR (250 MHz, D$_2$O) profile was as follows: δ 0.85, 3H [t; J = 8 Hz; J_{HC} = 4 Hz; C$\underline{H}_3$CH$_2$C(CH$_3$)$_2$OCH$_3$]; 1.17, 6H [s; J_{HC} = 4 Hz; CH$_3$CH$_2$C(C$\underline{H}_3$)$_2$OCH$_3$]; 1.55, 2H [q; J = 8 Hz, J_{HC} = 4 Hz; CH$_3$C$\underline{H}_2$C(CH$_3$)$_2$OCH$_3$]; 3.20, 3H [s; J_{HC} = 4 Hz; CH$_3$CH$_2$C(CH$_3$)$_2$OC$\underline{H}_3$]. The ^{13}C-NMR (63 MHz, D$_2$O) profile was as follows: δ 10.4 [s; $\underline{C}$H$_3$CH$_2$C(CH$_3$)$_2$OCH$_3$]; 26.5 [d; J_{CC} = 36 Hz; CH$_3$CH$_2$C($\underline{C}$H$_3$)$_2$OCH$_3$]; 33.9 [d; J_{CC} = 38 Hz; CH$_3$$\underline{C}H_2$C(CH$_3$)$_2OCH_3$]; 51.2 [s; CH$_3CH_2$C(CH$_3$)$_2O\underline{C}H_3$]; 80.1 [s; d, with 0.5% of a satellite doublet; J_{CC} = 40 Hz; CH$_3$CH$_2$$\underline{C}$(CH$_3$)$_2OCH_3$]. The MS (70 eV) profile was: m/z 88 [75% M$^+$-CH$_3$]; 74 [100% M$^+$-C$_2$H$_5$]; 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 (Hünig et al 1979). LiAlH$_4$ (30 mmol, 1,120 mg) was dissolved in 15 mL diethyl ether followed by the slow addition of 12 mmol of 3-hydroxy-3-methyl-2-butanone in 10 mL diethyl ether at room temperature. The mixture was then stirred for 2 hours at reflux. After cooling 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$_2$CO$_3$. After removal of the solvent, 2-methyl-2,3-butanediol was isolated by fractional distillation under reduced pressure.

The structure of 2-methyl-2,3-butanediol was confirmed by ^{1}H-NMR, ^{13}C-NMR, and MS. The ^{1}H-NMR (250 MHz, D$_2$O) profile was as follows: δ 1.15, 3H [d; J = 6 Hz; C$\underline{H}_3$CH(OH)C(CH$_3$)$_2$OH]; 1.17, 9H [s; CH$_3$CH(OH)C(C$\underline{H}_3$)$_2$OH]; 3.63, 1H [q; J = 6 Hz; CH$_3$C$\underline{H}$(OH)C(CH$_3$)$_2$OH]. The ^{13}C-NMR (63 MHz, D$_2$O) profile was as follows: δ 19.3 [s; $\underline{C}$H$_3$CH(OH)C(CH$_3$)$_2$OH]; 25.9 [s; CH$_3$CH(OH)C($\underline{C}$H$_3$)$_2$OH]; 27.0 [s; CH$_3$CH(OH)C($\underline{C}$H$_3$)$_2$OH]; 76.4 [s; CH$_3$CH(OH)$\underline{C}$(CH$_3$)$_2$OH]; 76.7 [s; CH$_3$$\underline{C}$H(OH)C(CH$_3$)$_2$OH]. The MS (70 eV) profile was: m/z 89 [9% M$^+$-CH$_3$]; 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 diethyl ether followed by slow addition of 12 mmol of 2-hydroxy-2-methylbutyric acid in 10 mL diethyl ether at room temperature. The mixture was then stirred for 2 hours at reflux. After cooling 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 removing the solvent, 2-methyl-1,2-butanediol was isolated by fractional distillation under reduced pressure.

The structure of 2-methyl-1,2-butanediol was confirmed by ^{1}H-NMR, ^{13}C-NMR, and MS. The ^{1}H-NMR (250 MHz, D_2O) profile was as follows: δ 0.89, 3H [t; $J = 8$ Hz; C$\underline{H}_3$CH$_2$C(CH$_3$)(OH)CH$_2$OH]; 1.13, 3H [s; CH$_3$CH$_2$C(C$\underline{H}_3$)(OH)CH$_2$OH]; 1.52, 2H [q; $J = 8$ Hz; CH$_3$C$\underline{H}_2$C(CH$_3$)(OH)CH$_2$OH]; 3.43, 2H [s; CH$_3$CH$_2$C(CH$_3$)(OH)C$\underline{H}_2$OH]. The ^{13}C-NMR (63 MHz, D_2O) profile was as follows: δ 9.9 s; $\underline{C}$H$_3$CH$_2$C(CH$_3$)(OH)CH$_2$OH]; 24.5 [s; CH$_3$CH$_2$C($\underline{C}$H$_3$)(OH)CH$_2$OH]; 32.8 [s; CH$_3$$\underline{C}H_2$C(CH$_3$)(OH)CH$_2$OH]; 70.8 [s; CH$_3CH_2$C(CH$_3$)(OH)$\underline{C}H_2$OH]; 76.4 [s; CH$_3CH_2$$\underline{C}$(CH$_3$)(OH)CH$_2$OH]. The MS (70 eV) profile was: m/z 89 [41% M$^+$-CH$_3$]; 75 [89%]; 73 [98%]; 71 [54%]; 58 [42%]; 57 [94%]; 55 [83%]; 53 [30%]; 45 [73%]; 42 [100%]; 41 [79%].

CHARACTERIZATION OF METABOLITES

The use of ^{13}C-labeled ethers permitted detection and identification of metabolites by ^{13}C-NMR. Moreover, comparison of the mass spectra of urine constituents from animals treated with ^{12}C-compounds or ^{13}C-labeled compounds also permitted identification of peaks representing metabolites due to the difference of 1 mass unit in selected fragments of ^{13}C-containing metabolites and identification of metabolites of the ^{13}C-ethers, which were also formed endogenously (eg, acetone).

Biotransformation of MTBE and ETBE

Two male and two female rats were individually exposed by inhalation for 6 hours to 2,000 ppm (initial concentration) of ^{12}C- and ^{13}C-MTBE or ^{12}C- and ^{13}C-ETBE. Monitoring of the chamber air concentrations of MTBE and ETBE indicated a continuous decrease of the air concentrations of ethers due to the uptake of ethers by the rats and metabolism to less volatile metabolites. Six hours after the end of the exposure, ether concentrations in the chamber were below 400 ppm, indicating intensive metabolism of the ethers by the rats. Analysis of the gas phase of the chambers did not indicate formation of volatile or exhaled ether metabolites detectable by FID. Urine samples were collected at 24-hour intervals for 48 hours and analyzed by GC/MS and ^{13}C-NMR (^{13}C-ethers only). Typical NMR spectra for urine samples are shown in Figure A.3.

The NMR spectra showed several resonances that were also present in the urine samples from control animals (see Figure A.3). The structures of those endogenous products were assigned by comparison with literature data and ref-
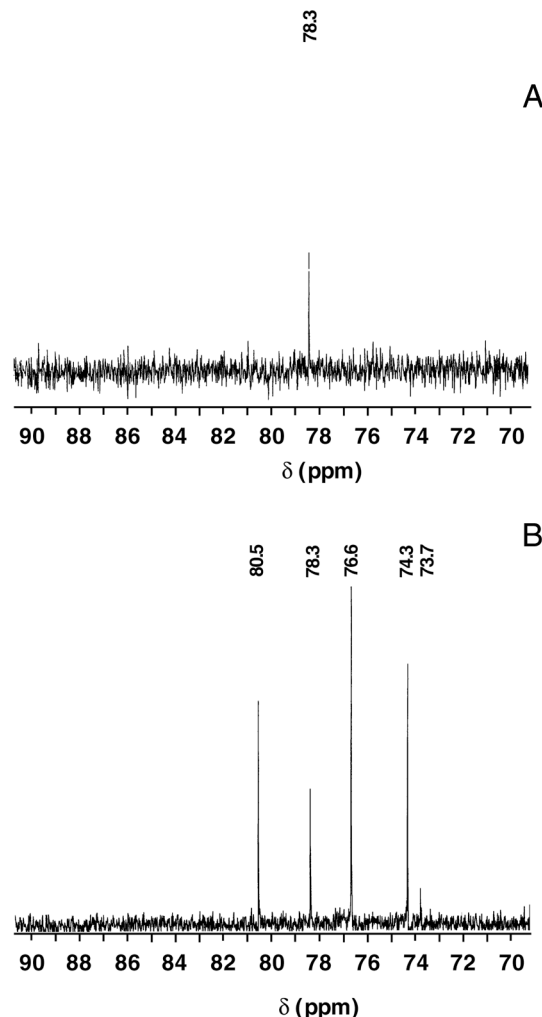

Figure A.3. ^{13}C-NMR spectra (63 MHz). (A) Urine sample from an untreated female rat serving as control; (B) urine sample taken 24 hours after a female rat was exposed to 2,000 ppm ^{13}C-MTBE for 6 hours. Fourier transformation utilized 2,000 scans. Only the shift range between 70 and 90 ppm is shown. Structural assignments were: δ 73.7, unknown; δ 74.3, 2-methyl-1,2-propanediol; δ 76.6, 2-hydroxyisobutyrate; δ 80.5, TBA glucuronide.

erence spectra (Fukuoka et al 1987, Nicholson and Wilson 1987). In addition, the range of chemical shifts between 70 and 90 ppm, where only a single resonance was observed in the ^{13}C-NMR spectra of control urine (δ 78.3), showed several signals indicative of MTBE metabolites (δ 73.7, 74.3, 76.6, 80.5). The structures of these metabolites were revealed by a combination of ^{13}C-NMR and GC/MS. The NMR signal at δ 73.7 most likely represents a conjugate of TBA since this signal disappeared after treating the urine with glucuronidase/sulfatase at 37°C with a concomitant increase in intensity of the signal for TBA. Moreover, the signal at δ 73.7 also disappeared and the TBA signal increased when the urine samples were treated with acid
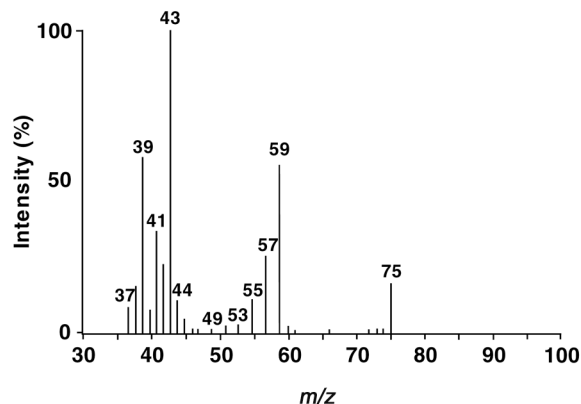
Figure A.4. Mass spectrum of 2-methyl-1,2-propanediol in urine collected for 24 hours after the end of exposure of a male rat to ^{12}C-MTBE (2,000 ppm) for 6 hours. A compound with a similar mass spectrum with several fragments shifted by one mass unit was observed in the urine of rats exposed to ^{13}C-MTBE.

to cleave acid labile conjugates. The exact nature of this conjugate could not be identified.

Calculations to predict the structure based on the chemical shift of the signal at δ 74.3 suggest that this metabolite may represent 2-methyl-1,2-propanediol. The ^{13}C-NMR spectrum of synthetic 2-methyl-1,2-propanediol also showed a resonance at δ 74.3 attributed to the C2 atom. The presence of 2-methyl-1,2-propanediol is also suggested by the MS results of urine analysis for MTBE-treated rats (Figure A.4). The mass spectrum of the ^{12}C-MTBE metabolite in urine and that of the synthetic reference were identical, confirming 2-methyl-1,2-propanediol as a urinary metabolite of MTBE. Moreover, in the mass spectrum of the peak representing 2-methyl-1,2-propanediol after separation of the urine samples of rats treated with ^{13}C-MTBE, several signals were shifted by 1 mass unit, further supporting the assigned structure (*m/z* 76, 60, 58, 56, 44, 42, 40).

The NMR signal δ 76.6 exhibited an identical chemical shift as the C2 atom of hydroxyisobutyrate and was greatly increased when authentic hydroxyisobutyrate was added to the urine sample. This observation confirms hydroxyisobutyrate as a metabolite of ^{13}C-MTBE excreted with the urine. The structure of 2-hydroxyisobutyrate was also confirmed by mass spectrometry (data not shown).

The NMR signal at δ 80.5 also represents a major metabolite of MTBE in rat urine. Based on initial data, the chemical shift of the signal indicates the presence of an electron-withdrawing group next to the C atom carrying the ^{13}C label but no major structural change in the molecule. The signal at δ 80.5 was decreased in intensity by 80% (relative to the urea signal at δ 165.5) when the urine sample was treated for a prolonged time (48 hours at 37°C) with glucuronidase/sulfatase, with a parallel increase in the intensity of the signal

representing TBA. Moreover, the signal at δ 80.5 disappeared, and the TBA resonance was increased in intensity relative to the urea signal when urine samples were treated with acid to cleave acid labile conjugates such as glucuronides. In summary, these data suggest that the metabolite may represent a conjugate of TBA. Studies using refined correlation (COSY) NMR suggest that this metabolite, in contrast to data published (Bernauer et al 1998), represents TBA glucuronide.

Comparison of the NMR spectra recorded from urine samples of treated male and female rats did not indicate major differences between the individual animals; it also did not indicate sex differences in structures of metabolites formed or in relative signal intensities when the spectra were recorded under identical acquisition conditions. Identical metabolites were also present, albeit at lower concentrations, in urine samples of both male and female rats treated with ^{13}C-MTBE collected between 24 and 48 hours after exposure. In addition to structure elucidation of metabolites by ^{13}C-NMR, all urine samples obtained in these studies were also analyzed by headspace GC/MS. In addition to the metabolites suggested by NMR analysis in urine of rats treated with ^{13}C-MTBE, the peak representing TBA was shifted by 1 mass unit and the peak representing acetone showed fragments representing both ^{12}C-acetone and ^{13}C-acetone (Figure A.5), indicating the formation of ^{13}C-acetone as a metabolite of ^{13}C-MTBE. The concentrations of ^{13}C-acetone present in urine were, however, too low to result in a signal in the ^{13}C-NMR spectra.

The chemical shifts of the observed signals in urine from ^{13}C-ETBE-exposed rats were identical to the signals observed in urine of animals exposed to ^{13}C-MTBE. Therefore, the metabolites formed were identical with those resulting from MTBE and TBA metabolism (δ 72.6, TBA;
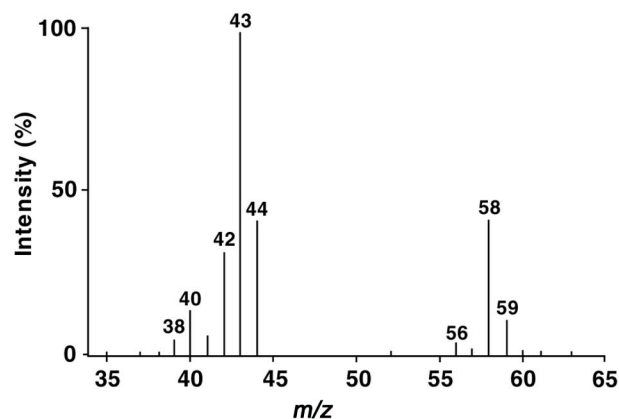

Figure A.5. Mass spectrum of ^{13}C-acetone in urine of male rat exposed to ^{13}C-MTBE. The major fragments were shifted by one mass unit, indicating the presence of [13]C.

δ 74.3, 2-methyl-1,2-propanediol; δ 76.6, 2-hydroxyisobutyrate; δ 80.5, TBA glucuronide). This indicates that the major ETBE-metabolites, as well as the major MTBE-metabolites, result from TBA formed in the first metabolic step of ether biotransformation. Again, no significant differences in signal intensities were observed in urine samples of male and female rats exposed to ^{13}C-ETBE. In urine samples collected between 24 and 48 hours after exposure, only the signal at δ 76.6 ppm was present, indicating that 2-hydroxyisobutyrate is excreted slowly. Neither high-resolution NMR nor GC/MS analysis of urine samples from ETBE-exposed rats indicated the presence of ETBE metabolites formed by oxidation of the β-carbon of the ethyl group in ETBE (eg, *tert*-butyl glycol or *tert*-butoxyacetic acid). Small amounts of ^{13}C-acetone were also present in urine samples of rats exposed to ^{13}C-ETBE as indicated by GC/MS headspace analysis.

Biotransformation of TAME

Two male and two female rats were individually exposed by inhalation to 2,000 ppm (initial concentration) of ^{12}C-TAME or ^{13}C-TAME. Analysis of the chamber air by GC did not reveal the formation of volatile and exhaled metabolites of TAME detectable by the FID used. Urine samples were collected and analyzed by GC/MS, LC–MS/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 A.6. The

Figure A.6. ^{13}C-NMR spectrum (151 MHz, 2,000 scans) of a 24-hour urine sample from a male rat exposed to 2,000 ppm of 2-^{13}C-TAME for 6 hours. Structural assignments for the NMR signals were: δ 72.8, 3-hydroxy-3-methyl butyrate; δ 74.9, TAA; δ 76.4, 2-methyl-2,3-butanediol; δ 76.7, glucuronide of 2-methyl-2,3-butanediol; δ 79.8, 2-hydroxy-2-methyl butyrate; δ 83.1, glucuronide of TAA.

^{1}H-decoupled ^{13}C-NMR spectrum showed several resonances that were also present in urine samples from control animals. 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 a single resonance was observed in the ^{13}C-NMR spectra (δ 78.3). Urine from ^{13}C-TAME–exposed animals showed several signals in the chemical shift range indicating 3 major and at least 3 minor metabolites.

The NMR signal at δ 72.8 was identical in chemical shift to the C2 atom (which carries the ^{13}C label) of 3-hydroxy-3-methyl butyrate. The presence of this compound in the urine of both [12]C- and ^{13}C-TAME–treated rats was confirmed by GC/MS. The GS separation of the urine samples 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-methyl butyrate (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-methyl butyrate 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 the C2 atom in TAA. This compound was also identified as a minor TAME metabolite by GC/MS (data not shown).

The signal at δ 76.4 represents a major TAME-metabolite in the rat. The C2 atom of synthetic 2-methyl-2,3-butanediol also showed 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 A.7). 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. 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 from urine of ^{13}C-TAME–treated rats at δ 76.7 was identified as a glucuronide of 2-methyl-2,3-butanediol based on the following observations: (1) Electrospray MS/MS of the metabolite isolated from the urine of ^{13}C-TAME–exposed rats by HPLC showed signals at *m/z* 326 and 342 (Figure A.8), and (2) The metabolite from ^{12}C-TAME had signals at *m/z* 325 and 341, indicating the addition of two sodium or one sodium and one potassium atom, respectively, to the molecular ion of 2-methyl-2,3-butanediol glucuronide (see Figure A.8). The ion at *m/z* 326 produced a prominent sec-
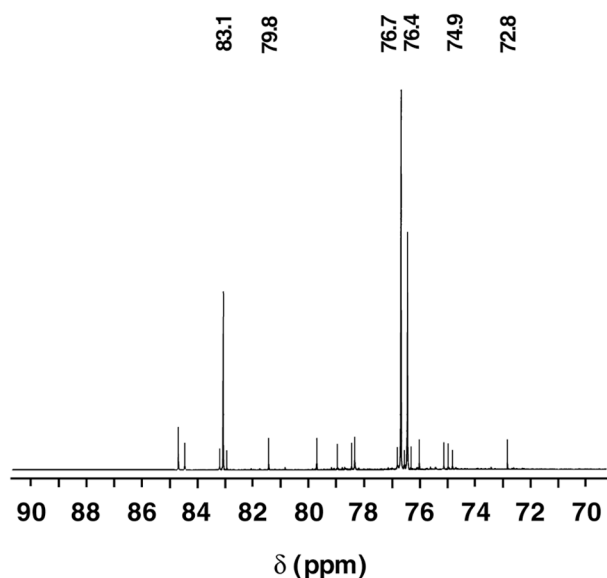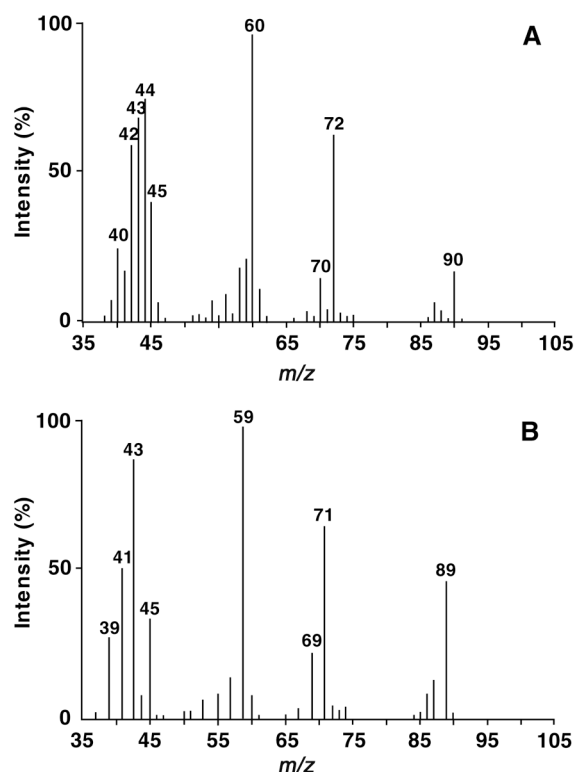
Figure A.7. Mass spectra of 2-methyl-2,3-butanediol in male rat urine. The samples were collected for 24 hours after the end of the animal's exposure to **(A)** 2-^{13}C-TAME (2,000 ppm) for 6 hours and **(B)** ^{12}C-TAME (2,000 ppm) for 6 hours.

Figure A.8. Electrospray mass spectrum (A) and further fragmentation (*m/z* 326) (B) of a glucuronide of 2-methyl-2,3-butanediol with ^{13}C-resonance at δ 76.7 and a collision energy of 30 mV. Spectra were recorded after direct loop injection of the metabolite isolated by preparative HPLC.

ondary ion at *m/z* 150 and several minor fragments indicating the consecutive loss of water and/or ketene. COSY-NMR spectra (Figure A.9) of the isolated peak showed a set of resonances typical for glucuronic acids and their derivatives (Aldrich 1998) (δ 3 to 5 ppm) and the presence of the 2-methyl-2,3-butanediol moiety containing [13]C (δ 1 to 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.

The third intensive 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 sample. This observation suggests that the compound also represents a glucuronide. This assumption is supported by electrospray mass spectra (Figure A.10) and COSY-NMR spectra (Figure A.11) of the isolated compound. The mass spectra 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 TAA. Moreover, the NMR spectra also were
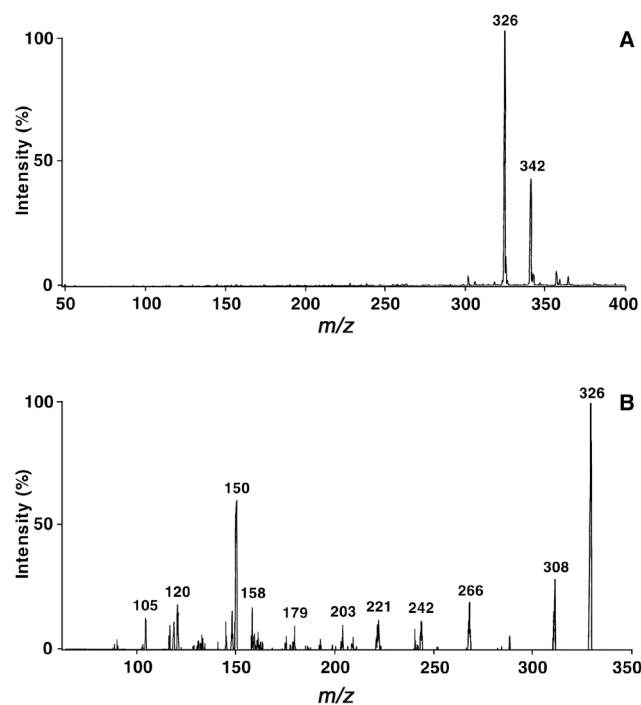

Figure A.9. COSY-NMR spectra of a glucuronide of 2-methyl-2,3-butanediol with ^{13}C-resonance at δ 76.7. For recording the ^{1}H-NMR spectra, the metabolite was isolated by preparative HPLC and dissolved in D[2]O.

Figure A.10. Electrospray mass spectrum (A) and further fragmentation (*m/z* 310) (B) of a glucuronide of TAA with ^{13}C resonance at δ 83.1 and collision energy of 30 mV. Spectra were recorded after direct loop injection of the metabolite isolated by preparative HPLC.
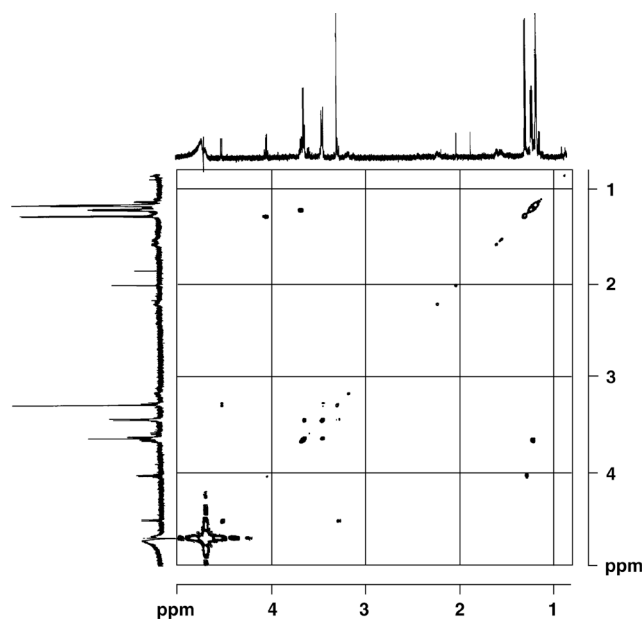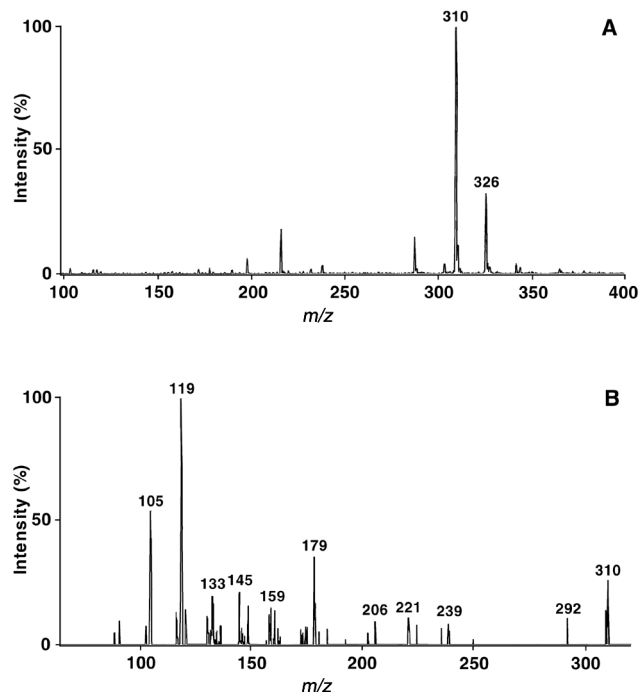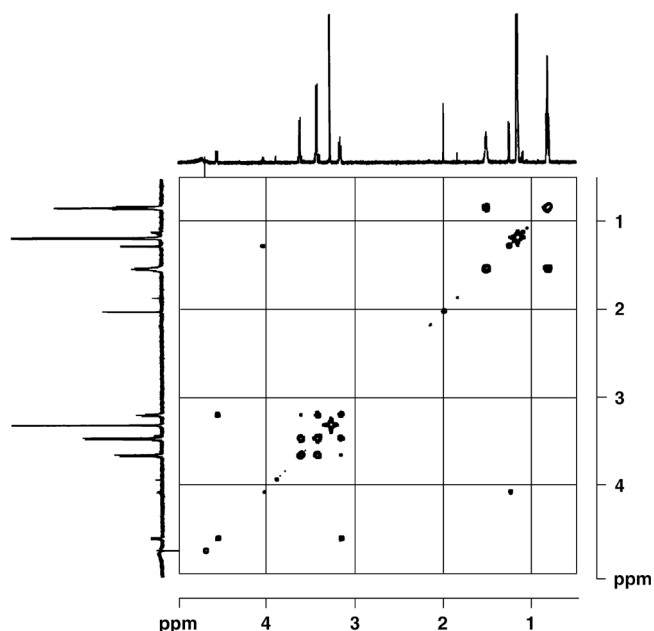
consistent with the presence of the glucuronic acid moiety (δ 3 to 5) and the TAA moiety in the molecule.

Of the other minor metabolites present, the compound giving the signal at δ 79.8 was identified as 2-hydroxy-2-methyl butyrate 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 urine samples from male and female rats exposed to ^{13}C-TAME. Identical metabolites were also present in rat urine samples collected between 24 and 48 hours after ^{13}C-TAME exposure. Relative concentrations of these metabolites were different, however, in the samples between 24 and 48 hours than those in the 0-to-24 hour sampling period, indicating differences in excretion kinetics of the individual metabolites (data not shown).

To confirm that metabolism of TAME is identical in humans and rats, a single human subject 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 liquid) was added to a 2-L gas sampling bag filled with 2 L of pure oxygen. The subject inhaled the TAME-containing oxygen for 4 minutes and exhaled into the sampling bag. This procedure ensured that most of the TAME present in the sampling bag was taken up by the subject. No major discomfort or symptoms of toxicity were associated with procedure. Urine of the subject was collected in 6-hour fractions for 48 hours and analyzed
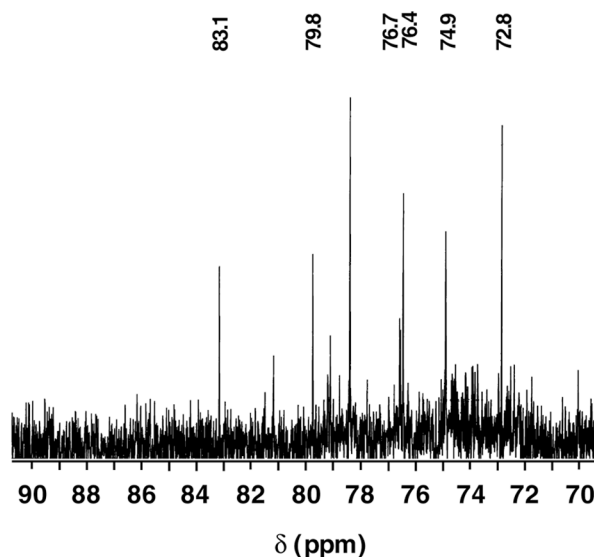

Figure A.11. COSY-NMR spectra of a glucuronide of TAA with ^{13}C resonance at δ 83.1. For recording of ^{1}H-NMR spectra, the metabolite was isolated by preparative HPLC and dissolved in D_2O.

Figure A.12. ^{13}C-NMR spectrum (151 MHz, 32,000 scans) of 6-hour urine sample from a male human subject exposed to 27,000 ppm of TAME (in a 2-L gas sampling bag) for 4 minutes. Structural assignments for the NMR signals were: δ 72.8, 3-hydroxy-3-methyl butyrate; δ 74.9, TAA; δ 76.4, 2-methyl-2,3-butanediol; δ 76.7, glucuronide of 2-methyl-2,3-butanediol; δ 79.8, 2-hydroxy-2-methyl butyrate; δ 83.1, glucuronide of TAA.

by ^{13}C-NMR. The presence of identical metabolites of TAME as observed in rats was indicated by the recorded ^{13}C-NMR spectra (Figure A.12). In contrast to concentrations found in rat urine, free ^{13}C-TAA 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 similar concentrations in the human urine samples (based on relative signal intensities in ^{13}C-NMR). Metabolites of ^{13}C-TAME were also detected in human urine samples collected up to 48 hours after TAME inhalation.

Biotransformation of TBA and TAA

Studies of the metabolism of ^{13}C-TBA and ^{13}C-TAA were included to confirm the structures of metabolites downstream from the formation of TBA and to identify whether metabolic reactions of MTBE at sites other than the methyl ether moiety may occur. Moreover, ^{13}C-TBA and ^{13}C-TAA were available in sufficient amounts from synthesis of the ^{13}C-labeled ethers. Male rats (n = 3/experiment) were treated with either ^{12}C-TBA and ^{13}C-TBA or ^{13}C-TAA and ^{12}C-TAA by gavage (250 mg/kg in corn oil). Urine samples were collected at 24-hour intervals for 48 hours and analyzed by GC/MS or ^{13}C-NMR.

A typical NMR spectrum of a urine sample from a TBA-treated rat is shown in Figure A.13. Again, at chemical shifts between 70 and 90 ppm, where only a single resonance (δ 78.3) of an endogenous compound was present, several signals indicative of TBA metabolites (δ 72.6, 73.7, 74.3, 76.6, 80.4) were observed. The chemical shifts of these signals
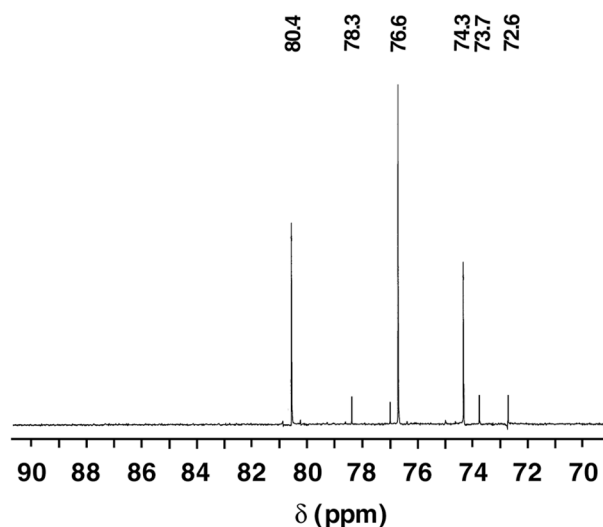
were identical to those seen in urine after ^{13}C-MTBE exposure. Based on these observations and MS data identical to those obtained with MTBE, we identified TBA, TBA glucuronide, 2-hydroxyisobutyrate, and 2-methyl-1,2-propanediol as urinary metabolites of TBA. Also, in the inhalation MTBE exposure, 2-hydroxyisobutyrate was the most prominent metabolite based on signal intensities after acquiring spectral data under identical conditions. Headspace analysis by GC/MS of urine samples also indicated small amounts of ^{13}C-acetone in urine samples of ^{13}C-TBA–treated animals.

In the urine of a single human subject dosed with ^{13}C-TBA (5 mg/kg), the ^{13}C-NMR spectra recorded (Figure A.14) showed the presence of TBA, 2-hydroxyisobutyrate and 2-methyl-1,2-propanediol. These metabolites were present in all urine samples analyzed. In contrast to rat urine samples in which TBA glucuronide was observed as a major excretory product after TBA administration, this conjugate was present only in traces in the human urine samples analyzed.

The ^{13}C-NMR spectra of the urine samples (Figure A.15) collected after treatment of rats with ^{13}C-TAA was similar to those obtained from rats treated with ^{13}C-TAME, suggesting that all the metabolites formed from ^{13}C-TAA are identical to those formed from ^{13}C-TAME. Based on relative signal intensities in ^{13}C-NMR, we identified that TAA glucuronide, 2-methyl-2,3-butanediol, and its glucuronide are also major metabolites of ^{13}C-TAA excreted in urine; free TAA, 2-hydroxy-2-methyl butyrate, and 3-hydroxy-3-methyl butyrate were identified by ^{13}C-NMR as minor metabolites. In urine samples taken 48 hours after exposure, only 2-

Figure A.13. ^{13}C-NMR spectrum (63 MHz) of a 24-hour urine sample from a male rat administered ^{13}C-TBA (250 mg/kg) by gavage. Fourier transformation utilized 2,000 scans. Structural assignments were: δ 72.6, TBA; δ 73.7, unknown; δ 74.3, 2-methyl-1,2-propanediol; δ 76.6, 2-hydroxyisobutyrate; δ 80.4, TBA glucuronide.

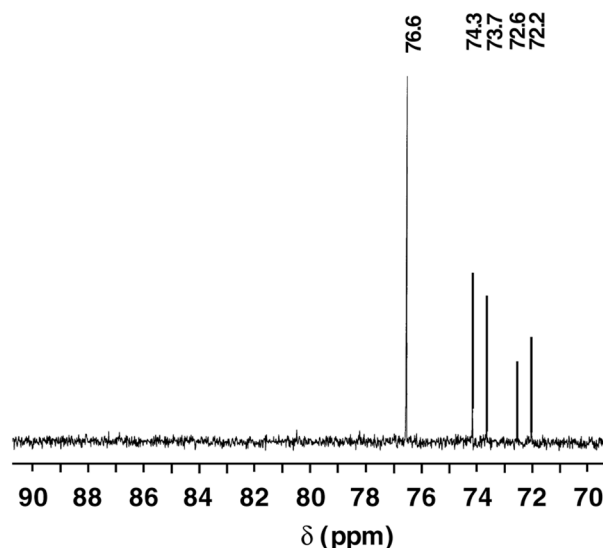**Figure A.14. ^{13}C-NMR (63 MHz) of a 24-hour urine sample from a male subject who ingested 5 mg/kg ^{13}C-TBA in tap water.** Structural assignments were: δ 72.6, TBA; δ 73.7, unknown; δ 74.3, 2-methyl-1,2-propanediol; δ 76.6, 2-hydroxyisobutyrate.
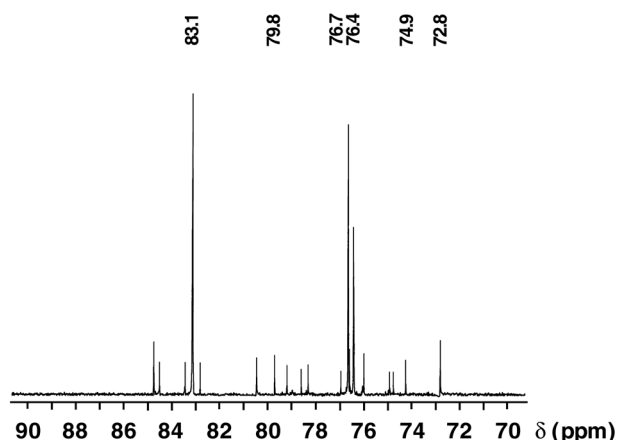
Figure A.15: **^{13}C-NMR spectrum (63 MHz, 2,000 scans) of a 24-hour urine sample from a male F344 rat treated orally with 250 mg/kg 2-^{13}C-TAA.** The following structural assignments for the NMR signals were made: δ 72.8, 3-hydroxy-3-methyl butyrate; δ 74.9, TAA; δ 76.4, 2-methyl-2,3-butanediol; δ 76.7, glucuronide of 2-methyl-2,3-butanediol; δ 79.8, 2-hydroxy-2-methyl butyrate; δ 83.1, glucuronide of TAA.

methyl-2,3-butanediol and its glucuronide were present, suggesting rapid elimination of the TAA glucuronide.

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APPENDIX B. Individual Human Blood Levels and Metabolite Excretion After Exposures to MTBE, ETBE, and TAME

Table B.1. MTBE and TBA Blood Concentrations in Humans Exposed to MTBE for 4 Hours

	Inhalation of 4 ppm MTBE				Inhalation of 40 ppm MTBE			
	MTBE		TBA[a]		MTBE		TBA[a]	
Subject	Max. Conc. (µM)	Half-Time (hr)	Max. Conc. (µM)	Half-Time (hr)	Max. Conc. (µM)	Half-Time[b] (hr)	Max. Conc. (µM)	Half-Time (hr)
A	1.2 ± 0.1	1.3 2.0	3.1 ± 0.4	4.2	9.3 ± 0.1	1.1 1.6	20.6 ± 1.6	7.6
B	2.0 ± 0.2	1.4 2.6	2.5 ± 0.0	9.8	4.5 ± 0.8	1.3 2.5	23.3 ± 0.5	4.8
C	1.8 ± 0.2	1.6 2.6	2.2 ± 0.3	5.6	6.2 ± 0.5	1.3 1.8	28.8 ± 1.6	3.9
D	2.5 ± 0.1	1.2 2.1	2.5 ± 0.2	8.9	5.9 ± 0.0	1.0 2.4	22.0 ± 5.5	2.9
E	1.6 ± 0.0	1.0 2.3	3.0 ± 0.5	5.3	6.2 ± 0.4	1.0 3.4	16.6 ± 4.3	4.1
F	2.2 ± 0.2	1.2 1.9	2.4 ± 0.1	4.9	8.1 ± 0.2	1.5 2.6	19.8 ± 0.6	8.7
Mean	1.9 ± 0.4	1.3 ± 0.2 2.3 ± 0.3	2.6 ± 0.3[c]	6.5 ± 2.1	6.7 ± 1.6	1.2 ± 0.2 2.4 ± 0.6	21.8 ± 3.7[c]	5.3 ± 2.1

[a] Background concentrations of TBA were 0.9 ± 0.3 µM in the 4-ppm and the 40-ppm studies.

[b] Elimination of MTBE from blood occurred in two phases; half-times for both phases were calculated.

[c] Statistically significant above background ($P < 0.01$).

Table B.2. Received Doses of MTBE in Humans and Amount of Metabolites Recovered in Urine[a]

	4 ppm MTBE[b]		40 ppm MTBE[b]	
Subject	Total Excreted Metabolites (µmol)	Percentage of Received Dose	Total Excreted Metabolites (µmol)	Percentage of Received Dose
A	62	39	820	59
B	44	27	844	61
C	62	38	1,295	93
D	48	30	868	63
E	67	42	1,360	98
F	50	31	563	41
Mean	55 ± 9	35 ± 5	958 ± 280	69 ± 20

[a] Received doses were calculated as 161 µmol (4.5 ± 0.4 ppm) and 1,387 µmol (38.7 ± 3.2 ppm) based on an alveolar ventilation rate of 9 L/min and a retention of 0.4. Urine samples were collected in 6-hour intervals for 72 hours. Numbers are the mean of 2 determinations of each metabolite in the sample. The results of repeated measurement of the same sample showed deviations of less than 10% in the analyte measured. Numbers were corrected for metabolite excretion in unexposed individuals.

[b] All values are corrected for background.

Table B.3. ETBE and TBA Blood Concentrations in Humans Exposed to ETBE for 4 Hours[a]

| | 4 ppm ETBE | | | | 40 ppm ETBE | | | |
| | ETBE | | TBA | | ETBE | | TBA[a] | |
Subject	Max. Conc. (μM)	Half-Time (hr)	Max. Conc. (μM)	Half-Time (hr)	Max. Conc. (μM)	Half-Time[b] (hr)	Max. Conc. (μM)	Half-Time (hr)
B	1.6 ± 0.1	1.0	2.0 ± 0.3	6.2	17.5 ± 5.1	0.9 3.2	15.0 ± 0.2	9.9
G	2.6 ± 0.1	1.0	2.0 ± 0.2	7.5	12.0 ± 0.2	1.2 3.7	10.7 ± 1.8	10.1
H	0.8 ± 0.1	1.2	1.6 ± 0.2	9.5	16.4 ± 1.1	1.1 7.5	13.7 ± 1.1	10.5
E	1.2 ± 0.2	0.8	1.6 ± 0.1	5.3	9.8 ± 0.1	1.0 11.1	12.2 ± 1.4	7.2
F	0.4 ± 0.1	1.2	1.9 ± 0.7	9.8	7.2 ± 0.7	0.9 3.6	15.4 ± 0.5	11.5
I	1.4 ± 0.6	1.5	2.0 ± 0.2	10.7	9.8 ± 0.6	1.3 8.4	16.4 ± 2.7	9.6
Mean	1.3 ± 0.7	1.1 ± 0.2	1.8 ± 0.2[c]	8.2 ± 2.2	12.1 ± 4.0	1.1 ± 0.1 6.2 ± 3.3	13.9 ± 2.2[c]	9.8 ± 1.4

[a] Background concentrations of TBA were 0.9 ± 0.2 μM in the 4 ppm study and 0.5 ± 0.3 μM in the 40 ppm study.

[b] Elimination of ETBE from blood occurred in two phases; half-times for both phases could be determined after exposure to 40.6 ± 3.0 ppm. After exposure to 4.5 ± 0.6 ppm ETBE, blood levels were below the detection threshold.

[c] Significantly above background ($P < 0.01$).

Table B.4. Received Doses of ETBE in Humans and Amount of Metabolites Recovered in Urine[a]

| | 4 ppm ETBE | | 40 ppm ETBE | |
Subject	Total Excreted Metabolites (μmol)	Percentage of Received Dose	Total Excreted Metabolites (μmol)	Percentage of Received Dose
B	35	29	643	59
G	74	61	380	35
H	28	23	270	25
E	67	55	556	51
F	65	53	521	48
I	33	28	432	40
Mean	50 ± 20	41 ± 17	467 ± 134	43 ± 12

[a] Received doses were calculated as 121 μmol (4.6 ± 0.6 ppm) and 1092 μmol (40.6 ± 3.0 ppm) based on an alveolar ventilation rate of 9 L/ min and a retention of 0.3 in humans. Urine samples were collected at 6-hour intervals for 72 hours. Numbers are the mean of 2 determinations of each metabolite in the sample. The results of repeated measurement of the same sample showed deviations of less than 10% in the analyte measured. Numbers were corrected for metabolite excretion in unexposed individuals.

Table B.5. TAME and TAA Blood Concentrations in Humans Exposed to TAME for 4 Hours

Subject	4 ppm TAME				40 ppm TAME			
	TAME		TAA		TAME		TAA	
	Max. Conc. (μM)	Half-Time[a] (hr)	Max. Conc. (μM)	Half-Time (hr)	Max. Conc. (μM)	Half-Time[a] (hr)	Max. Conc. (μM)	Half-Time (hr)
A	0.58 ± 0.02	1.3 3.4	0.73 ± 0.10	4.8	3.4 ± 0.0	1.1 3.2	8.4 ± 1.0	7.3
C	0.57 ± 0.03	1.5 6.2	0.70 ± 0.02	5.6	5.6 ± 0.0	1.7 4.0	7.5 ± 0.4	7.4
G	0.83 ± 0.11	1.2 3.9	1.33 ± 0.42	2.5	7.2 ± 0.1	0.9 2.2	8.2 ± 0.0	7.0
D	0.51 ± 0.06	1.3 5.8	0.90 ± 0.08	8.6	2.9 ± 0.0	1.2 4.1	7.9 ± 0.1	6.9
F	0.63 ± 0.08	1.6 2.8	1.18 ± 0.13	4.3	4.2 ± 0.2	1.0 4.0	10.9 ± 0.4	6.6
I	0.66 ± 0.02	1.3 5.3	1.27 ± 0.13	5.5	3.1 ± 0.2	1.0 3.6	12.0 ± 0.2	6.2
Mean	0.63 ± 0.11	1.4 ± 0.2 4.6 ± 1.4	1.02 ± 0.28	5.2 ± 2.0	4.4 ± 1.7	1.2 ± 0.3 3.5 ± 0.7	9.2 ± 1.8	6.9 ± 0.4

[a] Elimination of TAME from blood occured in two phases; half-times for both phases could be determined after exposure to 38.4 ± 1.7 ppm.

Table B.6. Received Doses of TAME in Humans and Amount of Metabolites Recovered in Urine[a]

Subject	4 ppm TAME		40 ppm TAME	
	Total Excreted Metabolites[b] (μmol)	Percentage of Received Dose	Total Excreted Metabolites (μmol)	Percentage of Received Dose
A	29	29	593	57
C	77	75	394	38
G	41	40	500	48
D	44	43	513	50
F	51	50	675	65
I	84	82	927	90
Mean	54 ± 21	53 ± 21	600 ± 186	58 ± 18

[a] Received doses were calculated as 102 μmol (3.8 ± 0.2 ppm) and 1,033 μmol (38.4 ± 1.7 ppm) based on an alveolar ventilation rate of 9 L/min and a retention of 0.3. Urine samples were collected at 6 hour intervals for 72 hours. Numbers are the mean of 2 determinations of each metabolite in the sample. The results of repeated measurement of the same sample showed deviations of less than 10% in the analyte measured. Numbers were corrected for metabolite excretion in unexposed individuals.

[b] Without 3-hydroxy-3-methyl butyrate.

Table B.7. MTBE and TBA Blood Concentrations in Humans 1 Hour After Ingestion of MTBE

	5 mg MTBE				15 mg MTBE			
	MTBE		TBA		MTBE		TBA	
Subject	Max. Conc. (µM)	Half-Time[a] (hr)	Max. Conc. (µM)	Half-Time (hr)	Max. Conc. (µM)	Half-Time[a] (hr)	Max. Conc. (µM)	Half-Time (hr)
A	0.10 ± 0.01	0.9 1.7 7.1	0.42 ± 0.00	8.4	0.58 ± 0.03	0.8 1.3 3.5	1.33 ± 0.03	10.1
K	0.06 ± 0.00	0.9 1.9 12.8	0.40 ± 0.02	7.2	0.38 ± 0.02	0.6 1.4 3.6	1.39 ± 0.06	8.2
M	0.14 ± 0.01	0.9 1.4 4.8	0.26 ± 0.02	11.0	1.05 ± 0.02	1.2 1.1 2.2	1.48 ± 0.05	11.8
D	0.09 ± 0.02	0.8 2.2 6.3	0.52 ± 0.03	7.6	0.89 ± 0.23	0.6 1.0 3.8	2.97 ± 0.00	6.2
E	0.10 ± 0.01	0.6 1.4 10.3	0.64 ± 0.03	6.3	0.69 ± 0.03	0.6 0.9 4.7	2.08 ± 0.02	5.5
L	0.09 ± 0.00	0.7 1.9 7.0	0.47 ± 0.00	8.4	0.52 ± 0.01	0.5 1.7 4.4	1.64 ± 0.11	9.1
Mean	0.10 ± 0.03	0.8 ± 0.1 1.8 ± 0.3 8.1 ± 3.0	0.45 ± 0.13	8.1 ± 1.6	0.69 ± 0.25	0.7 ± 0.2 1.2 ± 0.3 3.7 ± 0.9	1.82 ± 0.63	8.5 ± 2.4

[a] Elimination of MTBE from blood occurred in three phases. Half-times for the three phases were calculated.

Table B.8. Metabolites Recovered in Human Urine After Ingestion of MTBE[a]

	5 mg MTBE		15 mg MTBE	
Subject	Total Excreted Metabolites (µmol)	Percentage of Received Dose	Total Excreted Metabolites (µmol)	Percentage of Received Dose
A	26	46	81	48
K	30	52	87	51
M	17	29	64	38
D	29	51	97	57
E	31	54	88	52
L	24	42	85	50
Mean	26 ± 5	46 ± 9	84 ± 11	49 ± 6

[a] Ingested doses were 170 µmol (15 mg MTBE) and 57 µmol (5 mg MTBE). Urine samples were collected in 6 hour intervals for 96 hours. Numbers are the mean of 2 determinations of each metabolite in the urine sample. The results of repeated measurement of the same sample showed deviations of less than 10% in the analyte measured.

Table B.9. TAME and TAA Blood Concentrations in Humans 1 Hour After Ingestion of TAME

Subject	5 mg TAME				15 mg TAME			
	TAME		TAA		TAME		TAA	
	Max. Conc. (μM)	Half-Time[a] (hr)	Max. Conc. (μM)	Half-Time (hr)	Max. Conc. (μM)	Half-Time[a] (hr)	Max. Conc. (μM)	Half-Time (hr)
A	0.078 ± 0.000	0.9 1.4 2.7	0.38 ± 0.01	5.2	0.134 ± 0.007	1.5 3.0 3.5	0.88 ± 0.03	4.0
G	0.108 ± 0.002	0.9 1.3 3.6	0.43 ± 0.01	4.4	0.315 ± 0.046	1.1 1.4 2.7	0.94 ± 0.04	4.2
K	0.036 ± 0.005	0.8 1.0 1.8	0.33 ± 0.03	4.0	0.137 ± 0.005	0.6 1.2 2.8	0.75 ± 0.04	3.6
D	0.031 ± 0.002	1.1 1.4 3.6	0.51 ± 0.01	3.3	0.059 ± 0.000	1.0 2.3 3.3	0.94 ± 0.00	4.4
E	0.051 ± 0.001	0.9 0.9 3.2	0.48 ± 0.01	2.6	0.116 ± 0.004	0.8 1.7 2.5	1.27 ± 0.02	2.3
L	0.034 ± 0.008	0.9 1.1 3.5	0.39 ± 0.01	4.3	0.079 ± 0.005	1.1 1.0 3.1	0.98 ± 0.01	3.6
Mean	0.056 ± 0.031	0.9 ± 0.1 1.2 ± 0.2 3.1 ± 0.7	0.42 ± 0.07	4.0 ± 0.9	0.140 ± 0.091	1.0 ± 0.3 1.8 ± 0.8 3.0 ± 0.4	0.96 ± 0.17	3.7 ± 0.7

[a] Elimination of TAME from blood occurred in three phases. Half-times for the three phases were calculated.

Table B.10. Amount of Metabolites Recovered in Human Urine After Ingestion of TAME[a]

Subject	5 mg TAME		15 mg TAME	
	Total Excreted Metabolites (μmol)	Percentage of Received Dose	Total Excreted Metabolites (μmol)	Percentage of Received Dose
A	5.5	11	19	13
G	3.0	6	17	12
K	5.3	11	25	17
D	5.1	10	28	19
E	3.4	7	19	13
L	2.7	6	17	12
Mean	4.2 ± 1.3	9 ± 3	21 ± 5	14 ± 3

[a] Ingested doses were 49 μmol (5 mg TAME) and 147 μmol (15 mg TAME). Urine samples were collected in 6 hour intervals for 96 hours. Numbers are the mean of 2 determinations of each metabolite in the urine sample. The results of repeated measurement of the same sample showed deviations of less than 10% in the analyte measured. Numbers were corrected for metabolite excretion in unexposed individuals.

ABOUT THE AUTHORS

Wolfgang Dekant is the principal investigator of the research project. He is professor of toxicology in the Department of Toxicology and Pharmacology at the University of Würzburg, Germany. He received his diploma in chemistry and a doctoral degree in biochemistry from the University of Würzburg and spent one year as postdoctoral fellow at the University of Rochester, New York. His research interests focus on biotransformation of toxic agents and the use of mechanistic toxicology and biomarkers in risk assessment.

Alexander Amberg worked on the project as doctoral student (Dr rer nat) and was responsible for methods development and data collection. He received a diploma in food chemistry from the University of Würzburg and submitted his thesis work in the summer of 2000.

Ulrike Bernauer directed the inhalation exposures. She received a doctoral degree in Biochemical Toxicology from the University of Würzburg and is currently a research associate at the Bundesinstitut für gesundheitlichen Verbraucherschutz in Berlin, Germany. Her research interests focus on the role of enzymatic transformations in the toxicity of air pollutants.

Elisabeth Rosner directed the ingestion exposures. She earned her doctoral degree in biochemical toxicology from the University of Würzburg and is currently a staff toxicologist at a contract laboratory, RCC, in Basel, Switzerland.

OTHER PUBLICATIONS RESULTING FROM THIS RESEARCH

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ABBREVIATIONS AND OTHER TERMS

BF$_3$/methanol	boron trifluoride in methanol
COSY-NMR	correlation NMR
CYP	cytochrome P450
D$_2$O	deuterated water
ETBE	ethyl *tert*-butyl ether
FID	flame ionization detection
GC/MS	gas chromatography/mass spectrometry
HCl	hydrochloric acid
HPLC	high-performance liquid chromatography
ID	internal diameter
LC–MS	liquid chromatography–mass spectrometry
LSIMS	liquid secondary ion mass spectrometry
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MTBE	methyl *tert*-butyl ether
m/z	mass-to-charge ratio
NMR	nuclear magnetic resonance spectroscopy
TAME	*tert*-amyl methyl ether
TAA	*tert*-amyl alcohol
TBA	*tert*-butyl alcohol
TBA-d_{10}	deuterated TBA

MTBE Inhaled Alone and in Combination with Gasoline Vapor: Uptake, Distribution, Metabolism, and Excretion in Rats

Janet M Benson, Edward B Barr, and Jennifer R Krone

ABSTRACT

The purpose of these studies was to extend previous evaluation of methyl *tert*-butyl ether (MTBE)* tissue distribution, metabolism, and excretion in rats to include concentrations more relevant to human exposure (4 and 40 ppm) and to determine the effects of coinhalation of the volatile fraction of unleaded gasoline on the tissue distribution, metabolism, and excretion of MTBE. Groups of male F344 rats were exposed nose-only for 4 hours to 4, 40, or 400 ppm ^{14}C-MTBE or to 20 or 200 ppm of the light fraction of unleaded gasoline (LFG) containing 4 or 40 ppm ^{14}C-MTBE, respectively. To evaluate the effects of repeated inhalation of LFG on MTBE tissue distribution, metabolism, and excretion, rats were exposed for 4 hours on each of 7 consecutive days to 20 or 200 ppm LFG with MTBE (4 or 40 ppm) followed on the eighth day by a similar exposure to LFG containing ^{14}C-MTBE. Subgroups of rats were evaluated for respiratory parameters, initial body burdens, rates and routes of excretion, and tissue distribution and elimination. The concentrations of MTBE and its chief metabolite, *tert*-butyl alcohol (TBA), were measured in blood and kidney immediately after exposure, and the major urinary metabolites—2-hydroxyisobutyric acid (IBA) and 2-methyl-1,2-propanediol (2MePD)—were measured in urine. Inhalation of MTBE alone or as a component of LFG had no concentration-dependent effect on respiratory

minute volume. The initial body burdens of MTBE equivalents achieved after 4 hours of exposure to MTBE did not increase linearly with exposure concentration.

MTBE equivalents rapidly distributed to all tissues examined, with the largest percentages distributed to liver. The observed initial body burden did not increase linearly between 4 and 400 ppm. At 400 ppm, elimination half-times of MTBE equivalents from liver increased and from lung, kidney, and testes decreased compared with the two smaller doses. Furthermore, at 400 ppm the elimination half-time for volatile organic compounds (VOCs) in breath was significantly shorter and the percentage of the initial body burden of MTBE equivalents eliminated as VOCs in breath increased significantly. These changes probably reflect a saturation of blood with MTBE at 400 ppm and strongly suggest that the uptake and fate of MTBE are notably different at exposure concentrations above and below 400 ppm.

Single and repeated coexposure to 20 and 200 ppm LFG with MTBE had opposite effects on the total body burden of MTBE equivalents present at the end of exposures compared with those achieved after 4 and 40 ppm MTBE exposures: 20 ppm LFG increased and 200 ppm LFG significantly decreased the burdens of MTBE equivalents present. The effects of coexposure to LFG on blood levels of MTBE equivalents paralleled the effects on body burden. These differences in overall uptake of MTBE equivalents cannot be attributed to alterations of minute volume. The reason for the increase in overall uptake after 20-ppm LFG exposure is not clear. Decreased MTBE absorption (uptake) after single and repeated coexposure to 200 ppm LFG may be due to a decrease in solubility of MTBE in blood caused by inhalation of other hydrocarbons. Investigations on the blood/air partition coefficient of MTBE in the absence and presence of LFG would be needed to confirm this hypothesis.

Single and repeated coexposure to either 20 or 200 ppm LFG significantly decreased the percentage of the initial body burden from MTBE equivalents in tissues, including liver, kidney, and testes, immediately and 72 hours after

exposure compared with those occurring after inhalation of 4 ppm or 40 ppm MTBE. The shift in tissue distribution after single exposure cannot be completely explained by changes in tissue elimination rates for MTBE equivalents because elimination rates were faster only among rats repeatedly exposed to 200 ppm LFG. However, coexposure yielded faster elimination of MTBE equivalents in urine (repeated exposures only) and as VOCs and CO_2.

Analysis of cytochrome P450 monooxygenase in liver and nasal mucosa obtained from rats repeatedly exposed to 200 ppm LFG showed no induction of activity compared to activity in these tissues in unexposed rats (J-Y Hong, personal communication, 1999). This finding suggests that induction of cytochrome P450 monooxygenases may not have contributed to the changes in tissue distribution and elimination of MTBE equivalents observed with repeated inhalation of LFG in our studies (although additional studies would be needed to confirm this).

In conclusion, these findings indicate that the uptake and fate of inhaled MTBE are altered upon increasing exposure levels from 4 to 400 ppm and suggest that toxic effects observed previously upon repeated inhalation of 400 ppm or greater may not necessarily be linearly extrapolated to lower concentrations. Furthermore, coexposure to LFG, whether single or repeated, decreases tissue burdens of MTBE equivalents and enhances the rate of elimination of MTBE and its metabolites, thereby possibly reducing the toxic effects of MTBE compared with its effects when inhaled alone.

INTRODUCTION

MTBE is a commonly used gasoline additive originally used as an octane enhancer but now used to increase the oxygen content of fuel in order to reduce CO and other harmful components of engine exhaust. In 1993, over 24 billion pounds of MTBE was manufactured in the United States. Inhalation is a major route of exposure for individuals involved in production, transport, and distribution of MTBE or MTBE-containing fuel. Levels of occupational exposure are generally less than 10 ppm. The general public is exposed by inhalation to much lower MTBE concentrations (Figure 1). Exposure to MTBE-contaminated drinking water is an increasing concern in many areas of the United States.

The possible health effects of MTBE have been widely studied (see reviews in Costantini 1993; Health Effects Institute 1996; National Science and Technology Council 1997). Despite public claims of headache, eye, nose and throat irritation, nausea and vomiting, and dizziness after
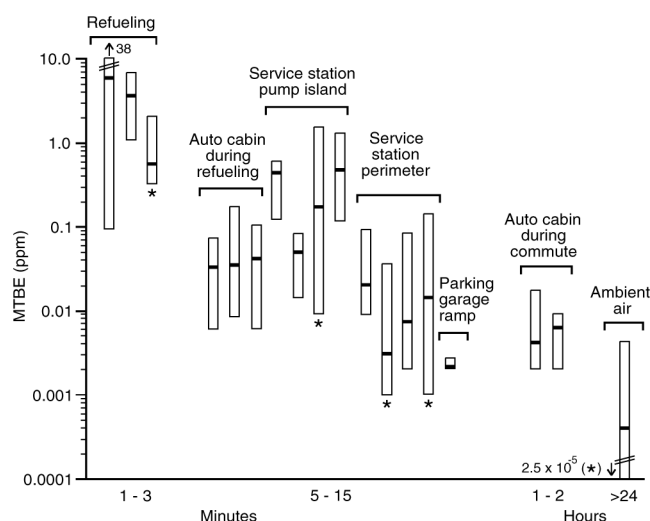

Figure 1. Concentrations of MTBE in air encountered by general population. The solid lines across the bars indicate median values. An asterisk (*) denotes the minimal detectable concentration. Modified from Health Effects Institute (1996).

exposure to MTBE-containing fuels, these symptoms were generally not reported among humans in controlled laboratory experiments (Prah et al 1994; Johanson et al 1995; Nihlén et al 1998). Repeated inhalation of high levels of MTBE by rats has not resulted in persistent or cumulative neurotoxic effects (Daughtrey et al 1997). Further, repeated inhalation of MTBE by rats, mice, and rabbits has not been associated with reproductive or developmental effects (except in the presence of maternal toxicity) (Bevan et al 1997a,b).

Results of rodent bioassay studies suggest that MTBE is carcinogenic. An increased incidence of lymphomas and leukemias (combined) and Leydig cell adenomas occurred in rats ingesting 250 mg MTBE/kg/day for 2 years (Belpoggi et al 1997). Leydig cell adenomas (rats), renal tubular adenomas and carcinomas (rats), and hepatocellular adenomas and carcinomas (mice) increased significantly in F344 rats and CD-1 mice exposed to up to 8,000 ppm MTBE for 2 years (Bird et al 1997). Although the validity of these results has been debated, they raise concern regarding adverse health consequences for people chronically exposed to MTBE.

Knowledge of the uptake, distribution, fate, and metabolism of MTBE is important to understand the results of toxicity studies in animals and humans and to extrapolate the results obtained in animals to humans. Inhaled MTBE in humans is rapidly absorbed into the blood stream and then rapidly eliminated after termination of exposure (Cain et al 1996; Buckley et al 1997; Nihlén et al 1998). Net respiratory uptake of MTBE is relatively low (32% to 42%), however, because it has a relatively low blood–to–alveolar-air partition

coefficient (Nihlén et al 1995, 1998). Therefore, much of the inhaled MTBE is exhaled unchanged. In humans, MTBE is rapidly metabolized to TBA. Although the rate of TBA elimination from blood is somewhat slower than that of MTBE, TBA is excreted in exhaled air along with MTBE. In addition, TBA can undergo conjugation to form a glucuronide or be metabolized to 2MePD. The latter compound undergoes further metabolism to form a sulfate conjugate IBA (Dekant et al in this Report). MTBE and TBA have not been identified as urinary metabolites (Cain et al 1996; Buckley et al 1997; Nihlén et al 1998), but conjugated TBA, 2MePD, and IBA have been identified in urine of humans exposed to MTBE (Dekant et al in this Research Report). Metabolic pathways for MTBE and TBA are shown in Figure 2.

The pharmacokinetics of inhaled MTBE in rats has been summarized by Miller and coworkers (1997), who evaluated the effects of exposure concentration and repeated exposure on uptake, metabolism and excretion. The rats were exposed nose-only to 400 or 8,000 ppm ^{14}C-labeled MTBE once for 6 hours or to 400 ppm ^{14}C-MTBE for 6 hours/day for 8 days. Single exposure to 400 or 8,000 ppm for 6 hours led to an apparent steady-state concentration of MTBE in blood after 2 hours. Peak plasma levels were 10 μg/mL and 493 μg/mL for the 400-ppm and 8,000-ppm exposure groups, respectively. After repeated inhalation of 400 ppm, the peak plasma level decreased to 9 mg/mL on the eighth day, and the area under the curve of plasma concentration plotted against time suggested that repeated MTBE inhalation induced its own metabolism.
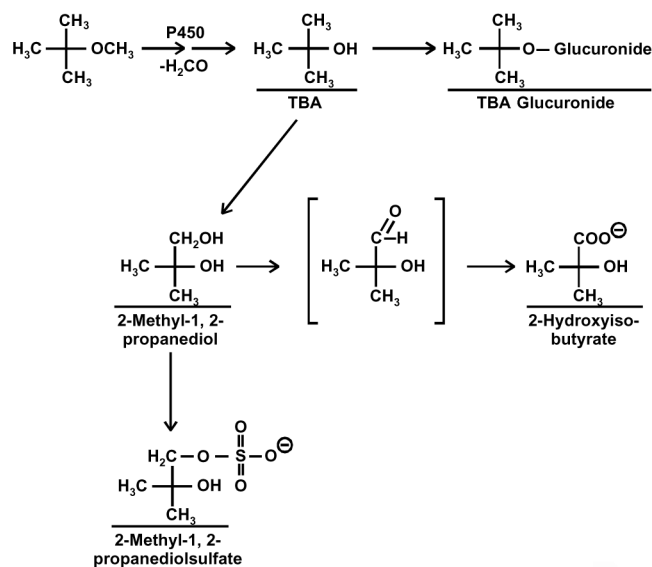

Figure 2. Metabolic pathways of MTBE and its major metabolite, TBA.

After a single exposure to 400 ppm MTBE, 65% of the inhaled dose was excreted in urine (Miller et al 1997). Repeated exposure increased the percentage of the dose excreted in urine to approximately 72%, further suggesting increased metabolism. In contrast, after inhalation of 8,000 ppm the fraction of the inhaled dose excreted in urine was decreased and the fraction of unmetabolized MTBE exhaled in air increased, suggesting saturation of metabolism at high exposure concentrations. For all exposure groups, approximately 80% of the radioactivity expired into the air was exhaled during the first 3 hours after dosing; 90% had been exhaled by 6 hours after dosing. Although MTBE and TBA were present in the exhaled air, most of the exhaled radioactivity was associated with MTBE. Metabolites found in urine were 2MePD (approximately 14% of the radioactivity excreted in urine) and IBA (70% of the radioactivity excreted in urine).

Dekant and coworkers in this Research Report compared the metabolism and excretion of MTBE in rats and humans exposed to identical concentrations of MTBE. Humans (3 males and 3 females) and rats (5 males and 5 females) were exposed to 4 and 40 ppm MTBE for 4 hours in a dynamic exposure system. In humans, urine samples were collected for 72 hours, and blood samples were obtained over 48 hours. At the end of the exposure, MTBE concentrations in blood of rats and humans were 5.9 ± 1.8 μM and 6.7 ± 1.6 μM, respectively. MTBE concentrations in blood after a 4-ppm exposure were also comparable (2.3 ± 1.0 μM in rats and 1.9 ± 0.4 μM in humans). MTBE was rapidly cleared from blood, with a half-time of 2.6 hours in humans and 0.5 hour in rats. Concentrations of TBA in blood of rats and humans after the 4-ppm and 40-ppm exposures were also comparable. IBA was the major excretory product in urine; TBA and 2MePD were minor metabolites. Elimination halftimes for the MTBE metabolites were 10 to 17 hours for humans and 3.4 to 4.5 hours for rats. Results of these studies indicated elimination of MTBE was slower for humans than rats but that the metabolites produced by both species were the same.

A physiologically based pharmacokinetic model for MTBE in male F344 rats has been developed by Borghoff and coworkers (1996).

Most people are not exposed to high concentrations of MTBE, nor are they generally exposed to MTBE alone but as a component of gasoline vapors. The purpose of studies in this report was to evaluate MTBE uptake and fate in rats at concentrations more relevant to human exposure and to determine the effects of coinhalation of the volatile fraction of unleaded gasoline on the uptake and fate of MTBE.

Specific objectives of this study were to:

1. Determine the linearity of uptake, distribution, metabolism, and excretion of ^{14}C-labeled MTBE in F344/N rats inhaling 4, 40, or 400 ppm for 4 hours.

2. Determine uptake, distribution, metabolism, and excretion of MTBE inhaled as a component of LFG. For these studies, F344/Crl BN rats were exposed for 4 hours to 20-ppm and 200-ppm LFG which contained 20% MTBE (by weight) and was tagged with ^{14}C-MTBE at a known specific activity.

3. Determine the effect of repeated inhalation of unleaded gasoline vapor containing MTBE on uptake, distribution, metabolism, and excretion of subsequently inhaled MTBE. For these studies, F334/Crl BN rats were exposed 4 hours/day for 7 consecutive days to 20 or 200 ppm LFG containing 20% MTBE. On the eighth day the uptake and fate of 20% by weight of ^{14}C-MTBE inhaled as a component of LFG was determined.

METHODS AND STUDY DESIGN

CHEMICALS

Uniformly labeled ^{14}C-MTBE (specific activity of 2.0 mCi/mmol; 98% radiochemically pure and chemically pure, Appendix A) was custom synthesized by Wizard Laboratories (Davis CA). The ^{14}C-MTBE was provided in sealed ampules containing 1 mCi ^{14}C-MTBE each and stored at –80°C until used.

The LFG was prepared by Chevron Research and Technology Company (Richmond CA) and was obtained through the American Petroleum Institute. The fraction was prepared by distilling unleaded gasoline at 130°C and condensing the components volatilized at this temperature. The chemical composition of the LFG is provided in Appendix A. The LFG was received in two 1-gallon metal cans and stored at about 8°C.

The MTBE and MTBE metabolite analytic standards, TBA, 2MePD, and IBA, were purchased from Aldrich Chemical Company (St Louis MO). Ultima Gold XR liquid scintillation cocktail was purchased from Packard Instrument (Meriden CT). Deuterated MTBE and TBA used as internal standards for quantitation of MTBE and TBA in blood, kidney, and urine were purchased from Cambridge Isotope Laboratories (Andover MA).

ANIMALS

Purchase, Housing, and Care

Male F344/N rats (10 to 12 weeks old) were purchased from Harlan Sprague-Dawley (Indianapolis IN) for exposure to 4, 40, and 400 ppm MTBE. Male F344/Crl BN rats (10 to 12 weeks old) were purchased from Charles River Laboratories (Wilmington MA) for all other exposures because pulmonary infections were identified in F344/N rats supplied by Harlan Laboratories for other studies at our Institute. No infections were identified in animals used in these studies. The rats were quarantined for 2 weeks before exposure and were 12 to 14 weeks old at the time of exposure. The rats were housed in polycarbonate cages with hardwood chip bedding and filter caps. Animal rooms were maintained at 21 ± 1°C with a relative humidity of 20% to 60%. There was a 12-hour light-dark cycle with light beginning at 0600. Food (Harlan Teklad certified rodent diet [W], Harlan Teklad, Madison WI) and water were provided ad libitum.

Randomization and Identification

Any rats that appeared sick at the time of randomization were excluded from the study. Within 7 days before exposures started, animals were randomly assigned to groups by weight and identified by ear tag. Animal assignments and body weights are provided in Appendix B.

Conditioning for Nose-Only Exposure

Before being exposed the rats were conditioned twice to the nose-only tubes. On the first occasion, the rats were placed in the exposure tubes for 0.5 hour. The second conditioning period took place for 2 hours on a separate day.

INHALATION EXPOSURE

Generator Solutions

Generator solutions were prepared immediately before each exposure according to the calculations described in Appendix C. For the coexposures, MTBE was added to the light fraction of the gasoline mixture. MTBE is present in whole unleaded gasoline at a concentration of about 14% by weight. Because of its relatively low boiling point, however, MTBE is enriched in the light boiling fraction and constitutes about 20% of the light boiling fraction of the gasoline. Because the effects of inhalation of the light boiling fraction on MTBE uptake, distribution, metabolism, and excretion were being evaluated in these studies, MTBE was added to the LFG in these studies to a final concentration of 20% by weight (see Appendix C).

Preliminary studies indicated that addition of 2 mCi ^{14}C-MTBE to the total amount of unlabeled MTBE or LFG/MTBE for each 4-hour exposure would result in deposition of approximately 1 μCi ^{14}C activity in each rat. The specific activities of the MTBE used for each exposure were determined by quantitating the ^{14}C activity per micromole of MTBE in each generator solution.

Exposure System

The exposure system consisted of a syringe pump, gastight Hamilton syringe, J tube (glass for MTBE exposures; stainless steel for LFG exposures), and a 96-port brass nose-only exposure chamber. The entire system was enclosed within a glove box. Liquid MTBE and LFG were metered at an appropriate rate into the J tube, and the vapors were carried by a stream of N_2 into the chamber and mixed with chamber supply air. The flow rate through the chamber was 20 to 30 L/min. Oxygen concentration and chamber temperature were monitored throughout the exposure.

Characterization of Test Atmospheres

For MTBE exposures, the total concentration of MTBE vapor in the chambers was monitored using a Miran 1A infrared analyzer (Wilks Enterprises, South Norwalk CT) with a monitoring wavelength of 3.4 µm (C-H stretch, suitable for quantitating total hydrocarbon content) and a gas chromatograph (GC) (GOW MAC Instrument, Bridgewater NJ). The GC was equipped with a stainless steel column packed with 1.5% OV101 100/120 Chromosorb (Alltech Associates, Deerfield IL) and operated at a temperature of 200°C. Both instruments were calibrated daily. Five point calibrations were designed to bracket the target exposure concentration for that day. The infrared analyzer was calibrated by injecting appropriate volumes of MTBE into the calibration loop and then, after stabilization, recording the output voltage. For calibrating the GC, 1-mL volumes of vapor were transferred from the calibration loop to the GC with a gas-tight syringe, and the peak areas were recorded.

For the LFG exposures, the total concentration of hydrocarbon vapor in the chambers was monitored using an infrared analyzer with a monitoring wavelength of 3.4 µm. The analyzer was calibrated daily (5 point calibrations) with known concentrations of fuel vapors injected into the calibration loop. Periodically, the profile of gasoline components was determined by GC with flame ionization detection (HP 5890, Hewlett-Packard, Palo Alto CA). The chromatographic conditions were supplied by the American Petroleum Institute through the courtesy of Dr Gary Hoffman, Huntington Life Sciences. A Petrocol DH 50.2 (50 m × 0.2 mm; 0.5 mm film thickness) column (Supelco, Sigma-Aldrich, Highland IL) was used to resolve the hydrocarbon peaks. Column temperature conditions were as follows: Initial temperature 35°C (12 minutes); ramp to 70°C at 10°C/min; ramp to 200°C at 15°C/min, and hold at 200°C for 1.83 minutes. The column was calibrated on exposure days using several hydrocarbon standards with retention times ranging from 5.6 minutes (butane) to 17.8 minutes (MTBE).

EXPERIMENTAL DESIGN

The study protocol is provided in Appendix D. The uptake, distribution, metabolism, and excretion of inhaled MTBE in F344 rats were evaluated under 3 exposure conditions.

1. Single 4-hour inhalation exposure to 4, 40, or 400 ppm ^{14}C-MTBE.

2. Single 4-hour inhalation coexposure to 20 or 200 ppm LFG (total hydrocarbon) containing 4 or 40 ppm ^{14}C-MTBE, respectively (20% by weight).

3. Repeat 4-hour/day inhalation coexposure to 20 or 200 ppm LFG (total hydrocarbon) containing 4 or 40 ppm MTBE, respectively, for 7 consecutive days. On the eighth day, the LFG mixture contained ^{14}C-MTBE at a known specific activity.

For each exposure, the rats were divided into 4 analysis subgroups (Table 1).

Table 1. Distribution of Endpoint Measures for Each Exposure

Analysis Subgroup	Endpoint Measures	Number of Animals	Time Point[a]
A	Amount of MTBE inhaled and absorbed	5	Immediately after 4-hr exposure
B	Initial body burden of MTBE	5	Immediately after 4-hr exposure
C	Pathways of excretion, identification and quantitation of major metabolites in urine	5	72 hr after end of 4-hr exposure
D	Uptake, distribution, metabolism, elimination	33[a]	0.5, 1, 2, and 4 hr of exposure; 2, 4, 8, 12, 24, 48, and 72 hr after exposure

[a] Three rats were killed at each time point.

Subgroup A: Amount of MTBE Inhaled and Effect of Test-Atmosphere Inhalation on Respiratory Parameters

Respiratory measurements (frequency and tidal volume) were made on 5 rats by plethysmography (Medinsky et al 1985) to estimate the amount of vapors inhaled. Immediately after the exposure, these rats were killed using an overdose of pentobarbital, and the entire carcass was frozen in liquid N_2. Thawed, depelted carcasses were digested in tetraethylammonium hydroxide (TEAH). Subsequent quantitation of ^{14}C activity was used to determine the amount of MTBE equivalents present at the end of exposure. Because we did not measure the amount of MTBE or metabolites exhaled during the exposures, accurate determination of absorption could not be made.

Subgroup B: Body Burden of MTBE Equivalents at End of 4-Hour Exposure

After the first MTBE exposure (40 ppm), we determined that Subgroup A rats could not be used to estimate the initial body burdens (that is, the body burden of MTBE equivalents at the end of the 4-hour exposure) for the remaining exposed rats. This was because the shape of plethysmograph tubes and the configuration of rats within the tubes were different from those of the remaining rats exposed within more conical restraint tubes. The blunt shape with associated dead space caused an underestimation of MTBE deposition in the remaining rats. Therefore, a separate subgroup of rats was included in all subsequent exposures for determination of the initial body burdens. This subgroup was also killed within minutes after termination of exposures, frozen in liquid N_2, and stored at –80°C until processed for analysis.

The initial body burdens obtained using these rats were generally lower than the values obtained by taking the sum of MTBE equivalents (in micromoles) excreted and retained at 72 hours by the Subgroup C rats. Therefore, only initial body burden data obtained from the Subgroup C rats are reported.

Subgroup C: Excretion Pathways, Identification of Major Metabolites, and Confirmation of Initial Body Burden

Five rats were placed in glass metabolism cages after a 4-hour exposure for collection of urine and feces and for measurement of radioactive VOCs, MTBE and TBA, and $^{14}CO_2$ in exhaled air. The time between removal from the inhalation chamber and start of expired air collection was approximately 10 minutes. Vessels used to collect urine and feces were kept in ice. Charcoal traps were used for the collection of MTBE and TBA in exhaled air. A dimethylformamide (DMF)-containing impinger was included after the charcoal traps for collecting TBA and acetone. A second impinger containing 2N potassium hydroxide (KOH) was used for collection of exhaled $^{14}CO_2$. Samples were collected periodically over 72 hours. After the 72-hour collections, the rats were killed by intraperitoneal injection of an overdose of pentobarbital. Blood was collected by cardiac puncture; liver, kidneys, lungs, heart, brain, perirenal fat, and gonads were removed and weighed. The tissues and carcasses were immediately frozen in liquid N_2 and stored at –80°C pending analysis. The animal cages were also rinsed, and the washings were stored at –20°C pending analysis.

The sum of MTBE equivalents in micromoles excreted and remaining in the tissues of rats were also used to estimate the initial burden of MTBE in the rats at the end of the 4-hour exposure. The values obtained from Subgroup C rats were used to calculate the percentage of initial body burden in excretion and tissues.

Subgroup D: Uptake, Distribution, Metabolism, and Tissue Elimination

This subgroup consisted of 33 rats. At various time points during and after exposure (Table 1), groups of 3 rats were anesthetized using pentobarbital and bled by cardiac puncture. Blood, lung, liver, kidney, heart, perirenal fat, brain, testes, and carcass were taken for measurement of total ^{14}C activity and for metabolite identification and measurement (kidney). Weighed portions of blood, liver, lung, kidney (left), and testes (left) were saved separately for potential subsequent quantitation of MTBE and TBA.

PROCESSING SAMPLES FOR RADIOANALYSIS

Tissues

Weighed samples of tissue and whole carcasses (without pelt) were digested using a 35% solution of TEAH (Sachem, Austin TX, 1 mL/g tissue). The weight of each digest was recorded. Weighed aliquots of digest were mixed with Ultima Gold XR scintillation cocktail for quantitation of ^{14}C activity. Aliquot counts for each sample were corrected for the total weight of the corresponding sample digest.

Urine: Total ^{14}C Activity

The specimens were collected into preweighed vials and reweighed after collection to determine the total amount of urine obtained. Aliquots (100 µL) of the samples were weighed into liquid scintillation vials. Liquid scintillation fluid (15 mL) was added to each vial before counting. Aliquot counts for each sample were corrected for the total weight of the urine sample collected.

Feces: Total ^{14}C Activity

Each fecal sample was diluted (1:1) with 1% Triton X100 and homogenized. The total combined weight of each sample was determined and recorded. Aliquots (200 mg) of each homogenate were weighed into tared vials, and 15 mL of scintillation cocktail was added. Aliquot counts were corrected for the total weight of each sample.

Charcoal and DMF: Total ^{14}C Activity

The charcoal in each trap was stored at room temperature in sealed, 1-pint canning jars pending analysis. The charcoal contained in each trap was weighed. Triplicate aliquots (2 g) were extracted by sonicating for 15 minutes with 10 mL DMF. The extractions were carried out at room temperature. The sonicates were centrifuged at 10°C for 30 minutes at 1,700 rpm to remove charcoal particles. Each extract was transferred to scintillation vials for quantitation of radioactivity. If the third extract contained significant amounts of radioactivity, 2 additional extracts were performed. For each sample, total ^{14}C activity was the sum of activity from each extraction performed. Extrapolations were performed if the fifth extract still contained measurable activity.

Volumes of DMF in each bubbler from the trap were measured and recorded. Aliquots (1 mL) were transferred to scintillation vials. Scintillation cocktail (Ultima Gold) was added, and ^{14}C activity was counted and corrected for the total volume of DMF in each sample.

CO$_2$ Traps

Immediately after each collection, the total volume of 2N KOH in each bubbler from the trap was measured and recorded. Aliquots (1 mL) were mixed with scintillation cocktail and counted for ^{14}C activity. The activity was corrected for the total volume of KOH in each bubbler sample.

Cage Washes

The total weight of each cage wash was determined and recorded. Aliquots (1 mL) of each were weighed into scintillation vials, mixed with scintillation cocktail, and counted for ^{14}C activity.

Radioanalysis

Radioactivity in the samples was quantitated by analysis in a liquid scintillation analyzer (Tri-Carb model 2500TR, Packard Instrument Company, Meriden CT). Quench correction was performed by the automatic instrument software. Samples were counted for 30 minutes. Minimum detectable activities for each sample type were calculated using the method of Altschuler and Pasternack (1963). The limit of detection was approximately 60 gross counts; limits of quantitation were approximately 140 gross counts. Limits of quantitation for each tissue type are provided in Appendix E.

Quantitation of MTBE and TBA in Blood and Kidney

MTBE and TBA concentrations in blood and kidney were quantitated by headspace analysis using gas chromatography/mass spectrometry (GC/MS). The equipment consisted of a data station (HP-UX), a mass selective detector (HP 5970), and a GC (HP 5890) (Hewlett-Packard).

MTBE and TBA were resolved using a Rtx-1 column (30 m × 0.32 mm ID; Restek Corporation, Bellfonte PA). The helium carrier gas flow was 2.0 mL/min. The initial column temperature of 40°C was held for 1 minute; the temperature was then ramped at 40°C/min for a final temperature of 200°C. The final temperature was held for 2 minutes. The injection and detector temperatures were 220°C. The total run time was 7.5 minutes.

The MS was operated in the Sim acquisition mode with a 3-minute solvent delay. The mass-to-charge ratio (m/z) was monitored for the following ions: 59.00, 65.00, 73.00, and 76.00. The dwell time per ion was 50 milliseconds.

Blood Analyses The MS was calibrated daily over 1 of 3 working concentration ranges, depending on the samples being analyzed: 0.1 to 5 nmol/0.5 g blood; 5 to 70 nmol/0.5 g blood; and 70 to 1,000 nmol/0.5 g blood. For the calibrations, known quantities of MTBE and TBA were added to 0.5 g control blood in 2-mL crimp-top vials. Deuterated TBA (TBA-d_{10}) and MTBE (MTBE-d_3) were added: 25 nmol of each. The vials were capped and stored in liquid N$_2$ until used.

Thawed standards were heated to 90°C for 30 minutes. Headspace (100 μL) was injected onto the GC column. Each standard was run at least 3 times for each calibration. The ratio of the ion peak areas (59/65 nmol/0.5 g blood for TBA and 73/76 nmol/0.5 g blood for MTBE) was plotted against the corresponding TBA or MTBE concentration to obtain daily standard curves. The correlation coefficient (r^2) of the standard curve had to exceed 0.95 for sample analyses to proceed.

Blood samples for exposed rats were prepared in the same manner as the blood standards. Blood (0.5 g) was weighed into a 2-mL crimp-top glass vial, and TBA-d_{10} and MTBE-d_3 (25 nmol) were then added. The vials were stored in liquid N$_2$ or heated immediately to 90°C for 30 minutes for headspace analysis.

Kidney Analyses For kidney samples, 2 calibration curves were generated: 0.1 to 250 nmol and 5.0 to 2,500 nmol. The

quantity of internal standard added to the samples was determined by the expected quantity of metabolites in the sample as indicated by the radioactivity content of the sample. If the expected quantity of metabolites was less than or equal to 100 nmol, 25 nmol of internal standard was added. If the expected quantity of metabolites was greater than 100 nmol, 1,000 nmol of internal standard was added. The peak area ratios were assumed to be equal to the ratios of the analytes and the internal standards [that is, (25 nmol MTBE)/(25 nmol MTBE-d_3) = 1.0]. This assumption was tested and documented. The slope for the low-range calibration was 0.04, and the slope for the high-range calibration was 0.001.

The tissue was prepared by freeze-fracturing 250 to 300 mg of tissue in a Bessman tissue pulverizer (Biospec Products, Bartlesville OK). The pulverized tissue was quickly transferred to a 20-mL headspace vial containing 2 mL 0.5 M succinct acid solution. Internal standard (5 µL of 25.0 nmol or 1,000 nmol) was added to each vial; the vials were sealed and stored at −80°C pending analysis. Sample analysis procedures were the same as those used for blood.

Identification and Quantitation of 2MePD and IBA in Urine

Metabolites in urine were resolved using high-performance liquid chromatography (HPLC; model 1050, Hewlett-Packard). The chromatographic procedure was a modification of that reported by Miller and colleagues (1997). An aliquot of each urine sample (100 µL) containing a known amount of ^{14}C activity was injected onto a C_{18} reversed-phase column (Pinnacle ODS, 150 × 4.6 mm, 5 µm; Restek Corporation, Bellfonte PA). The mobile phase was 0.05 M potassium phosphate buffer (pH 7) containing 2% methanol. The mobile phase was run isocratically at a flow rate of 1 mL/min. The column eluant was collected (0.2-minute fractions) using a fraction collector. The ^{14}C activity in each fraction was determined by ligand scintillation counting for 5 minutes.

Two peaks of radioactivity were detected in urine. The metabolites in each peak were identified using liquid chromatography–tandem mass spectrometry (LC–MS/MS). The material to be analyzed was obtained by collecting fractions from 5 runs of a single urine sample. Aliquots (100 µL) of each fraction were counted for ^{14}C activity to identify fractions containing metabolites. Fractions containing each metabolite were pooled for identification of metabolites.

The LC–MS/MS parameters were optimized for identification of the 2 likely urinary metabolites, 2MePD and IBA, using known standards. Aliquots (100 µL) of each pooled metabolite sample were injected onto an HPLC (model 10ADVP, Shimadzu Company, Kyoto, Japan) equipped with a Hypersil BDS column (50 × 2.1 mm, 3 µm;

MetaChem Technologies, Torrence CA). The metabolites were eluted using a mobile phase of 99% methanol/1% of a 100-mM ammonium acetate buffer at a flow rate of 200 µL/min. The eluant was directed to an electrospray MS (API 365, PE Biosystems, Foster City CA) that was monitored for negative ion pairs (103.0/56.8 for IBA and 91.2/72.6 for 2MeDP).

The first peak eluting from the reversed-phase column was positively identified by LC–MS/MS as IBA; the second was identified as 2MePD. Spectra of collected fractions were compared with those of authentic standards. The total activity in each peak in each sample was summed and corrected for the volume of urine collected. The nanomoles of each metabolite were calculated based on the specific activity of the ^{14}C-MTBE for the corresponding exposure. Mass spectra of the isolated metabolites and standards are provided in Appendix H.

DATA ANALYSIS AND STATISTICAL EVALUATION

Data Analysis and Statistical Comparisons

Group mean values, standard deviations, and standard errors were calculated for all parameters measured.

Differences between matched pairs of data (that is, baseline minute volumes versus minute volumes in the same group of animals measured during the exposure) were compared using a paired two-tailed t test (GraphPad Software, San Diego CA).

Comparisons of endpoints were made among: (1) groups exposed to 4, 40, and 400 ppm MTBE (effect of exposure concentration); (2) groups exposed once or repeatedly to 4 ppm MTBE and 20 ppm LFG (effect of coexposure to a low concentration of LFG); and (3) groups exposed once or repeatedly to 40 ppm MTBE and 200 ppm LFG (effect of coexposure to a higher concentration of LFG). These comparisons were evaluated using a one-way analysis of variance with a Tukey-Kramer postevaluation test to adjust for multiple comparisons (GraphPad Software). The significance level was $P \leq 0.05$.

Modeling of Elimination of MTBE Equivalents

The total amount of MTBE equivalents present in the rats at the end of exposure (initial body burden) was estimated by measuring the ^{14}C activity in groups of 5 rats killed immediately after exposure (Subgroup B) and by taking the sum of the micromoles of MTBE equivalents excreted and retained by the Subgroup C rats. Values from the Subgroup C rats were used to calculate a mean initial body burden for each exposure group. Except for blood, which was not removed from the rats in toto, the amounts of MTBE equivalents in tissues from each rat were normalized

to the mean initial body burden for that exposure group. Mean percentage of initial body burden was calculated for each group at the time of death so that the extent of tissue uptake, distribution, and elimination could be directly compared among exposure groups.

For evaluation of rates of elimination of MTBE equivalents from tissues and excreta, the elimination data for each exposure group were fit with a 2-component negative exponential equation, a single-component negative equation, and a single-component negative exponential equation incorporating a constant for a nonclearing component (SAS/STAT User's Guide, 1990, Chapter 29, NLIN Procedure). Models were chosen for goodness of fit (correlation coefficient) and statistical significance of the fit parameters ($P < 0.05$). Elimination from blood and in excreta were best fit using a single-component negative exponential equation (single-compartment model), the same model used by Miller and associates (1997) and Amberg and coworkers (1999) to describe elimination of MTBE and/or TBA from blood and urine. Elimination from tissues was best described using a single-component negative exponential incorporating a constant, describing a nonclearing

component, possibly resulting from incorporation of ^{14}C derived from the initial demethylation of MTBE into the single-carbon synthetic pool. Differences in elimination parameters as a function of MTBE exposure concentration and as a function of coexposure to LFG and MTBE were evaluated using Wald statistics (Gallant 1975).

RESULTS

EXPOSURE ATMOSPHERE

Results of exposures conducted with 4, 40, and 400 ppm MTBE and LFG containing MTBE are summarized in Table 2. Achieved MTBE exposure concentrations were within 5% of target by infrared analysis and within 11% of target by GC analysis. LFG exposure concentrations were achieved within 10% of target for all but the 20-ppm single LFG exposure, for which the achieved concentration was 34% higher than target. GC analyses of the hydrocarbon composition of the exposure atmosphere for the 200 ppm LFG single exposure remained relatively constant throughout the 4 hours of exposure (Appendix C).

Table 2. Exposure Summary and Achieved MTBE Body Burdens After ^{14}C-MTBE Alone and in Combination with LFG

Exposure Group[a]	MTBE or LFG Achieved ppm[b]		[14]C Specific Activity (µCi/µmol)	MTBE Initial Body Burden[c]	
	Infrared	GC		µmol	µmol/kg
4 ppm MTBE	4.21 ± 0.47 (11)	3.82 ± 0.88 (11)	2.0	0.87 ± 0.09	4.29 ± 0.41
40 ppm MTBE	39.4 ± 12.5 (15)	42.7 ± 5.55 (11)	0.082	23.1 ± 1.45	74.2 ± 4.58
400 ppm MTBE	412 ± 11.3 (12)	444 ± 38.4 (12)	0.006	298 ± 10.9	1217 ± 34
20 ppm Single LFG	26.8 ± 19.3 (36)	NA[d]	1.02	1.91 ± 0.07	8.26 ± 0.32[e]
20 ppm Repeat LFG	21.0 ± 1.9[f] (18)	NA[d]	0.704	1.50 ± 0.06	6.92 ± 0.21[e]
200 ppm Single LFG	220 ± 52.8 (10)	NA[g]	0.157	11.2 ± 0.89	40.9 ± 3.21[h]
200 ppm Repeat LFG	173.6 ± 38.9[i] (36)	NA[f]	0.131	9.51 ± 0.53	49.2 ± 2.05[h]

a Note that 20 ppm LFG contains 4 ppm MTBE and 200 ppm LFG contains 40 ppm MTBE.

[b] Results are the mean ± SD; number of animals for each value is given in parentheses.

[c] Results are the mean ± SE of 5 animals.

[d] GC analysis was not performed because the concentration of LFG components other than MTBE was below the limit of detection.

[e] Body burden was significantly greater than that occurring when 4 ppm MTBE was inhaled alone. One-way analysis of variance.

[f] The daily mean ± SE of the seven 20-ppm LFG exposures before exposure to 20 ppm LFG containing 4 ppm ^{14}C-MTBE was 19.6 ± 0.63 ($n = 7$).

[g] No usable chromatograms were obtained for the 200-ppm exposure due to instrument malfunction.

[h] Body burden was significantly less than when 40 ppm MTBE was inhaled alone ($P \leq 0.05$).

[i] The daily mean ± SE of the seven 200-ppm LFG exposures before exposure to 200 ppm LFG containing 40 ppm ^{14}C-MTBE was 194.8 ± 7.84 ($n = 7$).

MTBE EQUIVALENTS IN RATS AT END OF EXPOSURE

The initial body burdens of MTBE equivalents at the end of the 4-hour exposures are compared in Table 2. The observed initial body burden did not increase linearly between 4 and 400 ppm. Additional exposure concentrations would need to be evaluated in order to describe accurately the relationship between exposure concentration and body burden at exposure concentrations at or below 400 ppm. Single and repeated inhalation of 20 ppm LFG significantly increased the concentration of MTBE equivalents in the rats at the end of exposure, whereas single and repeated inhalation of 200 ppm LFG significantly decreased the body burden of MTBE equivalents achieved at the end of exposure.

RESPIRATORY PARAMETERS

Baseline minute volumes ranged from 252 to 297 mL/min and were not significantly different among the 7 exposure groups (Table 3). The effect of MTBE concentration or LFG concentration or exposure duration on minute volumes showed no consistent trend when compared to baseline values measured within the same exposure group. When comparing the effect of MTBE exposure concentration on minute volume measured during exposure, the group mean minute volume of rats inhaling 400 ppm MTBE was significantly greater than the group mean minute volumes of rats inhaling 4 and 40 ppm MTBE. Group mean minute volumes of rats coexposed once or repeatedly to 20 or 200 ppm LFG and MTBE were not significantly different from those of rats exposed to 4 or 40 ppm MTBE, respectively. Individual animal data are provided in Appendix F.

UPTAKE AND ELIMINATION OF MTBE EQUIVALENTS IN BLOOD

Uptake and elimination of MTBE equivalents (^{14}C activity derived from MTBE and its metabolites) in blood as a function of MTBE exposure concentration are presented in Figure 3. The effects of single and repeated coexposure to 20 ppm and 200 ppm LFG on the concentrations of MTBE equivalents in blood during and after exposure are shown in Figures 4 and 5, respectively. In all cases, MTBE equivalents were rapidly absorbed into the blood with peak concentrations occurring prior to completion of the 4-hour exposure period. Peak blood concentrations in rats exposed to MTBE alone increased with exposure concentration. Although peak concentrations of MTBE equivalents in rats exposed to 4 ppm MTBE alone or to 20 ppm LFG were similar (7 to 9 µM), peak concentrations of MTBE equivalents in rats exposed to 200 ppm LFG (once or repeatedly) were less than half that achieved in rats exposed to 40 ppm MTBE alone.
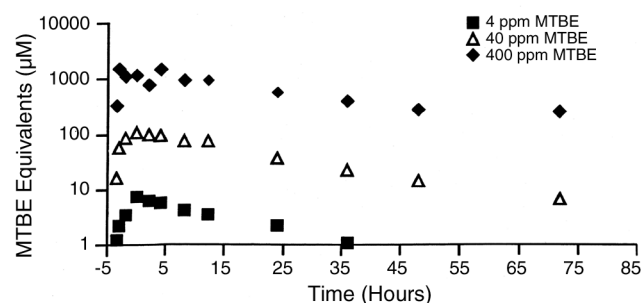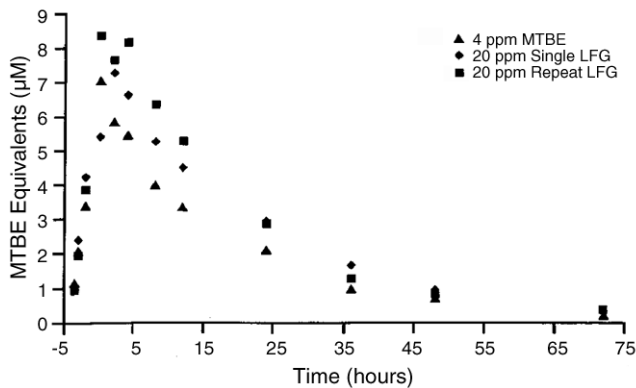

Figure 3. Blood uptake and elimination of MTBE equivalents during and after single exposure to MTBE. Data points represent the means of 3 animals.

Table 3. Effect of Exposure on Respiratory Parameters

Parameter	4-ppm MTBE Exposure[a]			40-ppm MTBE Exposure[a]			400 ppm MTBE
	MTBE	Single LFG 20 ppm	Repeat LFG 20 ppm	MTBE	Single LFG 200 ppm	Repeat LFG 200 ppm	
Baseline minute volume (mL/min)	297 ± 18.9	261 ± 23.0	292 ± 21.6	261 ± 17.2	252 ± 20.2	294 ± 10.0	292 ± 15.6
Exposure minute volume (mL/min)	246 ± 14.4[b]	259 ± 17.0	275 ± 20.6[b]	238 ± 11.5	286 ± 19.3[b]	278 ± 13.1	314 ± 10.7[c]
Number of animals	4	4	5	4	4	5	5

[a] Results represent the mean ± SE.

[b] Mean significantly different than baseline minute volume for same group. Two-tailed t test for matched samples, $P \leq 0.05$.

[c] Mean significantly different from minute volume of the 4 ppm and 40 ppm MTBE rats. Analysis of variance corrected with Tukey-Kramer multiple comparisons test, $P \leq 0.05$.

The half-time for elimination of MTBE equivalents from blood of rats exposed to 400 ppm MTBE was significantly longer than the elimination half-times for rats exposed to either 4 or 40 ppm MTBE (Table 4). Both single and repeated coexposure to 200 ppm LFG yielded a significantly lower elimination half-time of MTBE equivalents compared with that occurring in rats inhaling 40 ppm MTBE alone. The effects of coexposure to 20 ppm LFG were less clear, with single coexposure having a lower elimination half-time and repeated exposure having no apparent effect.

UPTAKE AND ELIMINATION OF MTBE EQUIVALENTS IN SELECTED TISSUES

MTBE equivalents distributed to all tissues examined. Within each MTBE exposure group, concentrations of MTBE equivalents (nmol/g tissue) in all the tissues examined were similar (Figure 6). Immediately and 72 hours after exposure, liver contained the greatest percentage of the initial MTBE burden (Figures 7A and B). Generally, rats inhaling 4 ppm MTBE accumulated and retained a

Figure 4. Blood uptake and elimination of MTBE equivalents during and after coexposure to 20 ppm LFG. Data points represent the means of 3 animals.
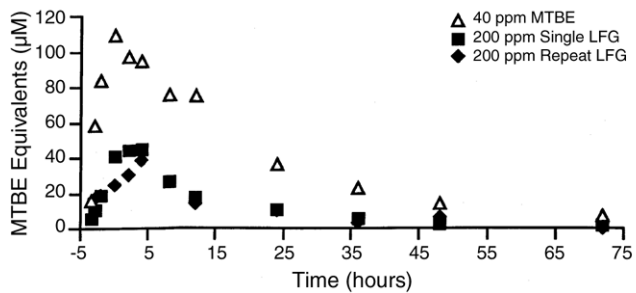
Figure 5. Blood uptake and elimination of MTBE equivalents during and after coexposure to 200 ppm LFG. Data points represent the means of 3 animals.

Table 4. Elimination of MTBE Equivalents from Blood[a]

Exposure Group	Elimination Half-Time [hr (95% CI)]	
4 ppm MTBE	14.7	(13.3–16.1)
40 ppm MTBE	16.5	(13.6–21.0)
400 ppm MTBE	30.1[b,c]	(26.6–38.5)
20 ppm Single LFG	31.5[b]	(23.1–46.2)
20 ppm Repeat LFG	15.7	(14.7–16.9)
200 ppm Single LFG	10.2[c]	(9.24–11.4)
200 ppm Repeat LFG	12.2[c]	(11.7–12.6)

[a] Data were fit using a single-component negative exponential function, $y = ae^{-bx}$, where a = the y intercept (initial blood burden at the end of the 4-hour exposure) and b = the elimination parameter. The elimination half-time (hr) = 0.693/b.

[b] Value significantly different from the half-time for 4 ppm MTBE.

[c] Value significantly different from the half-time for 40 ppm MTBE.

larger percentage of the initial MTBE body burden in liver, lung, brain, testes, and heart than did rats inhaling 40 or 400 ppm MTBE. The exception to this pattern was the kidney.

Generally, single and repeated coexposure to gasoline vapor reduced the percentages of initial MTBE body burden present in tissues immediately and 72 hours after the ^{14}C-labeled LFG coexposure compared with the corresponding percentages present in these tissues from rats inhaling ^{14}C-MTBE alone (Figures 8 and 9). The lower percentage of the initial MTBE body burden after LFG coexposure cannot be attributed to a shift in the distribution of MTBE equivalents to fat (Table 5). Individual animal data are provided in Appendix G.
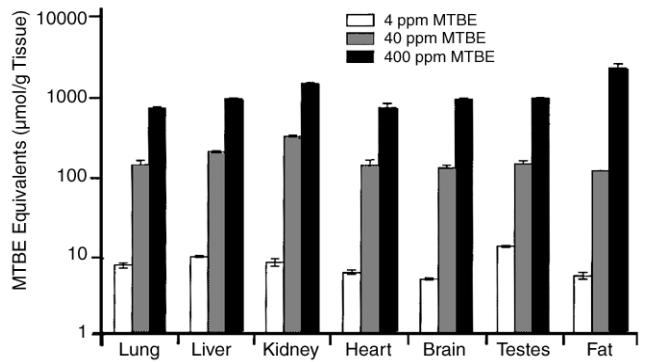
Figure 6. Selected tissue distribution of MTBE equivalents (Mean ± SE) after exposure to MTBE. Data points represent the means of 5 animals.
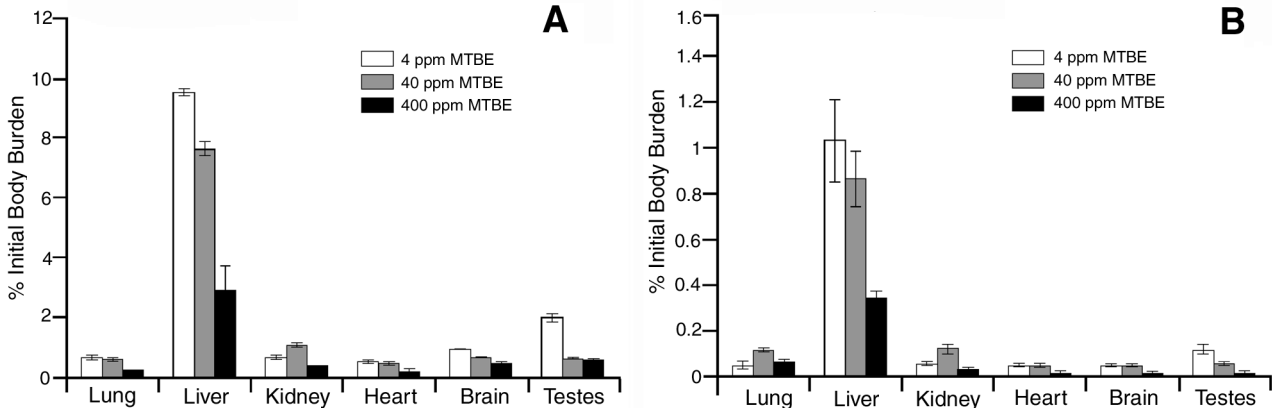
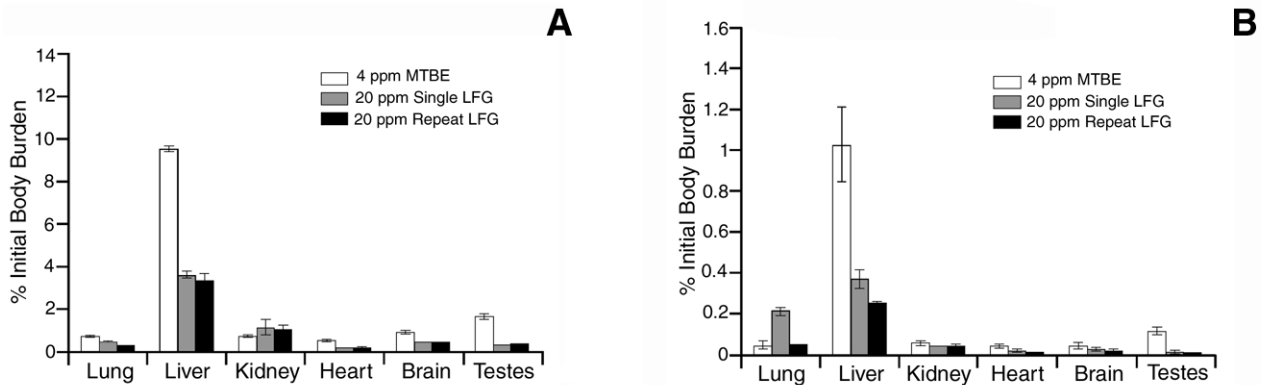**Figure 7. Selected tissue distribution of MTBE equivalents (mean ± SE) after exposure to MTBE. (A)** At end of exposure (n = 5). Mean values after 40 ppm (kidney, brain, testes) or 400 ppm (all organs presented) differed significantly from mean values for these organs after 4 ppm. Mean values after 40 ppm in liver, kidney and heart differed significantly from mean values for these organs after 400 ppm. **(B)** At 72 hours after end of exposure (n = 7 to 8). Mean values after 40 ppm (lung, kidney, testes) or 400 ppm (liver, heart, brain, testes) differed significantly from mean values for these organs after 4 ppm. Mean values after 40 ppm differed significantly from the mean values after 400 ppm for liver, kidney and testes.

Figure 8. Selected tissue distribution of MTBE equivalents (mean ± SE) after coexposure to 20 ppm LFG. (A) At end of exposure (n = 5). Mean values for lung, liver, heart, brain, and testes after single or repeat coexposures differed significantly from the values for these organs after 4 ppm MTBE alone. Mean value for lung after single coexposure differed significantly from this value for lung after repeat coexposure. **(B)** At 72 hours after end of exposure (n = 7 to 8). Mean values after single coexposure (lung, liver, heart, testes) or repeat coexposure (liver, heart, testes) differed significantly from values for these organs after 4 ppm MTBE alone. Mean values for single coexposure (lung, liver) also differed significantly from values for these organs after repeat coexposure.
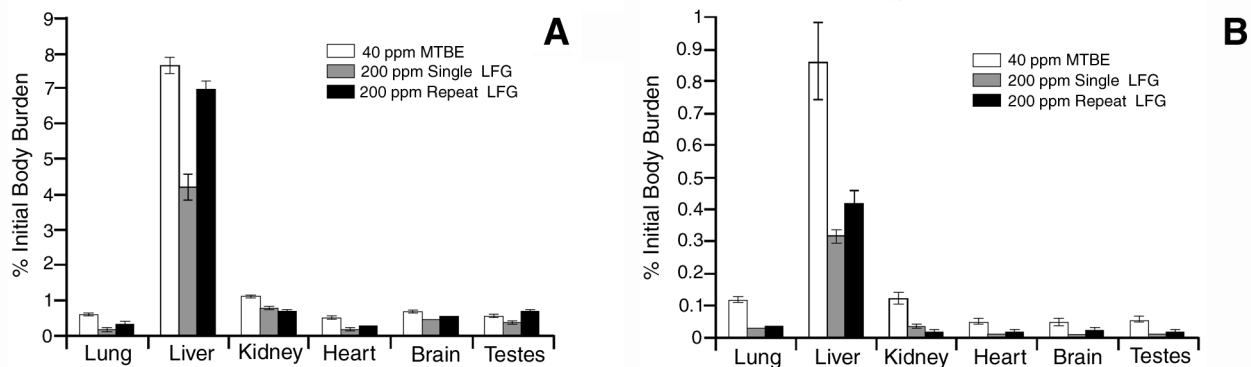

Figure 9. Selected tissue distribution of MTBE equivalents (mean ± SE) after coexposure to 200 ppm LFG. (A) At end of exposure (n = 5). Mean values for lung, liver, kidney, heart and brain after single or repeat coexposures and for testes after single exposure differed significantly from the values for these organs after 40 ppm MTBE alone. Mean value for liver, heart, brain and testes after single coexposure differed significantly for these organs after repeat coexposure. **(B)** At 72 hours after end of exposure (n = 3). Mean values after single coexposure (lung, liver, kidney, heart) or repeat coexposure (lung, liver, heart, testes) differed significantly from values for these organs after 40 ppm MTBE alone.

Elimination half-times were generally less than 12 hours for liver and less than 24 hours for lung, kidney, and testes (Table 6). In all cases, a small fraction (less than 1%) of the initial tissue burden did not appear to undergo elimination. This retained fraction most likely represents incorporation of [14]C (from formaldehyde generated from the initial demethylation reaction to TBA; Figure 2) into the single-carbon synthetic pool, a consequence of using uniformly ^{14}C-labeled MTBE in these experiments.

Rates of tissue elimination of MTBE equivalents were affected by MTBE exposure concentration in a tissue-dependent manner. Elimination of MTBE equivalents from liver was significantly slower among rats exposed to 40 and 400 ppm MTBE compared with elimination from liver in rats exposed to 4 ppm MTBE (Table 6). In contrast, elimination of MTBE equivalents from lung was significantly faster among rats exposed to 400 ppm MTBE than rats exposed to 4 ppm MTBE. Similarly, the rates of elimination of MTBE equivalents from kidney and testes were significantly faster for rats exposed to 400 ppm MTBE than for rats exposed to either 4 or 40 ppm MTBE.

Effects of single and repeated exposure to LFG on elimination of MTBE equivalents from tissues depended on the concentration and duration of LFG exposure as well as tissue type. Elimination half-time of MTBE equivalents from liver and kidney were significantly longer among rats exposed once and repeatedly to 20 ppm LFG containing 4 ppm MTBE than among rats exposed to 4 ppm MTBE alone. Single coexposure to 200 ppm LFG had no effect on the elimination half-time of MTBE equivalents from any tissue compared with the half-time for tissues from rats exposed to 40 ppm MTBE alone. In contrast, elimination half-times of

MTBE equivalents from lung, liver, kidney, and testes among rats repeatedly exposed to 200 ppm LFG were significantly faster than among rats exposed once to 40 ppm MTBE.

Table 5. Distribution of MTBE Equivalents in Fat

	% IBB in Fat	
Exposure Group	Immediately After Exposure[a]	72 hr After Exposure[b]
4 ppm MTBE	8.84 ± 1.03	0.24 ± 0.05
40 ppm MTBE	9.49 ± 0.12	0.49 ± 0.09
400 ppm MTBE	11.9 ± 1.67	0.21 ± 0.06
20 ppm Single LFG	7.2 ± 0.61	1.05 ± 0.88[c]
20 ppm Repeat LFG	5.48 ± 0.34[c]	0.76 ± 0.17[c]
200 ppm Single LFG	7.67 ± 1.76	0.24 ± 0.04[d]
200 ppm Repeat LFG	6.85 ± 0.57	0.18 ± 0.01[d]

[a] Results are the mean ± SE of 3 animals.

[b] Results are the mean ± SE of 5 to 8 animals.

[c] Mean significantly different from 4 ppm MTBE.

[d] Mean significantly different from 40 ppm MTBE.

Table 6. Elimination of MTBE Equivalents from Liver, Lung, Kidney, and Testes

Exposure Group	Elimination Half-Time [hr (95% CI)][a]		IBB Retained at 72 hr [% (95%CI)]
Liver			
4 ppm MTBE	4.71	(4.03–5.63)	1.67 (1.37–1.96)
40 ppm MTBE	10.3[b]	(8.56–12.8)	0.96 (0.60–1.31)
400 ppm MTBE	9.36[b]	(7.70–12.2)	0.41 (0.33–0.51)
20 ppm Single LFG	9.24[b]	(7.45–12.2)	0.64 (0.48–0.80)
20 ppm Repeat LFG	14.1[b]	(11.2–19.2)	0.53 (0.24–0.82)
200 ppm Single LFG	9.49	(7.14–13.9)	0.52 (0.39–0.64)
200 ppm Repeat LFG	5.87[c]	(4.91–7.37)	0.14 (0.48–0.62)
Lung			
4 ppm MTBE	12.1	(9.49–16.9)	0.19 (0.14–0.23)
40 ppm MTBE	15.2	(1.11–23.9)	0.10 (0.04–0.14)
400 ppm MTBE	8.24[b]	(6.02–13.1)	0.08 (0.07–0.09)
20 ppm Single LFG	16.3	(11.4–28.9)	0.30 (0.23–0.38)
20 ppm Repeat LFG	11.4	(10.2–12.8)	0.12 (0.11–0.13)
200 ppm Single LFG	18.7	(15.8–23.1)	0.02 (0.01–0.04)
200 ppm Repeat LFG	11.4[b]	(9.9–13.1)	0.05 (0.04–0.06)
Kidney			
4 ppm MTBE	9.36	(7.96–11.6)	0.08 (0.06–0.10)
40 ppm MTBE	11.6	(9.24–15.1)	0.12 (0.08–0.16)
400 ppm MTBE	8.56[c]	(7.79–9.49)	0.05 (0.04–0.05)
20 ppm Single LFG	16.1[b]	(12.2–23.1)	0.04 (0.001–0.08)
20 ppm Repeat LFG	14.1[b]	(12.2–16.9)	0.04 (0.01–0.07)
200 ppm Single LFG	12.8	(11.2–15.1)	0.04 (0.02–0.05)
200 ppm Repeat LFG	7.00[c]	(6.41–7.70)	0.03 (0.03–0.03)
Testes			
4 ppm MTBE	13.2	(10.5–18.7)	0.12 (0.05–0.19)
40 ppm MTBE	11.4	(7.79–21.0)	0.05 (0.02–0.08)
400 ppm MTBE	4.55[b,c]	(4.10–5.1)	0.03 (0.03–0.04)
20 ppm Single LFG	10.6	(9.36–12.6)	0.02 (0.02–0.04)
20 ppm Repeat LFG	12.6	(11.0–14.4)	0.03 (0.02–0.05)
200 ppm Single LFG	10.5	(9.49–11.7)	0.02 (0.02–0.02)
200 ppm Repeat LFG	6.30[c]	(5.63–7.07)	0.04 (0.03–0.04)

[a] Elimination data were fit using a single-component negative exponential function incorporating a constant, $y = a + be^{-cx}$, where a = percentage of tissue burden not clearing, b = the y intercept (initial tissue burden at the end of the 4-hour exposure), and c = the elimination parameter. The elimination half-time (hr) = 0.693/c.

[b] Value significantly different from the half-time for 4 ppm MTBE.

[c] Value significantly different from the half-time for 40 ppm MTBE.

EXCRETION PATHWAYS

In all cases, MTBE and/or its metabolites were excreted chiefly in urine and to a lesser extent in exhaled breath (VOCs and CO_2) and feces. An increase in exposure concentration from 4 to 400 ppm resulted in a significant decrease in the percentage of metabolites excreted in urine and a significant increase in the percentage of MTBE equivalents exhaled (Figure 10A).

Single coexposure to 20 ppm LFG resulted in a significant shift from urinary excretion of metabolite to excretion of VOCs in breath (Figure 10B). This shift was reversed to a certain extent upon repeated exposure to 20 ppm LFG. Repeated exposure also resulted in a significant increase in the amount of MTBE equivalents exhaled as CO_2. Single and repeated coexposure to 200 ppm LFG resulted in a significantly lower percentage of MTBE metabolites excreted in urine and increased percentage of MTBE equivalents exhaled in breath as VOCs and CO_2 (Figure 10C).

Rats exposed to 400 ppm exhaled the largest percentage of their initial burden as VOCs and the smallest percentage as CO_2.

RELATIVE RATES OF EXCRETION OF MTBE EQUIVALENTS

Urine

The majority of urinary excretion of MBTE and its metabolites occurred 36 to 48 hours after exposure. The rate of excretion of MTBE equivalents in urine was unaffected by MTBE exposure concentration or single coexposure to LFG (Table 7). Repeated coexposure to 20 ppm and 200 ppm LFG, however, yielded a significantly greater excretion rate compared with that occurring after inhalation of 4 and 40 ppm MTBE, respectively.

VOCs in Breath

The majority of VOCs was excreted in breath within the first 12 hours after exposure. Exposure to 400 ppm MTBE and coexposure to LFG led to significantly higher rates of excretion of VOCs in breath compared with exposure to 4 or 40 ppm MTBE (Table 7). Single and repeated inhalation of 20 and 200 ppm LFG led to higher rates of excretion of VOCs compared with inhalation of 4 and 40 ppm MTBE, respectively (Table 7).

MTBE-Derived CO_2

The majority of MTBE-derived CO_2 was excreted within the first 12 hours after exposure. Repeated coexposure of rats to 20 ppm LFG generated a significantly higher rate of excretion of metabolized MTBE as CO_2 compared with that observed for rats inhaling 4 ppm MTBE (Table 7). Both

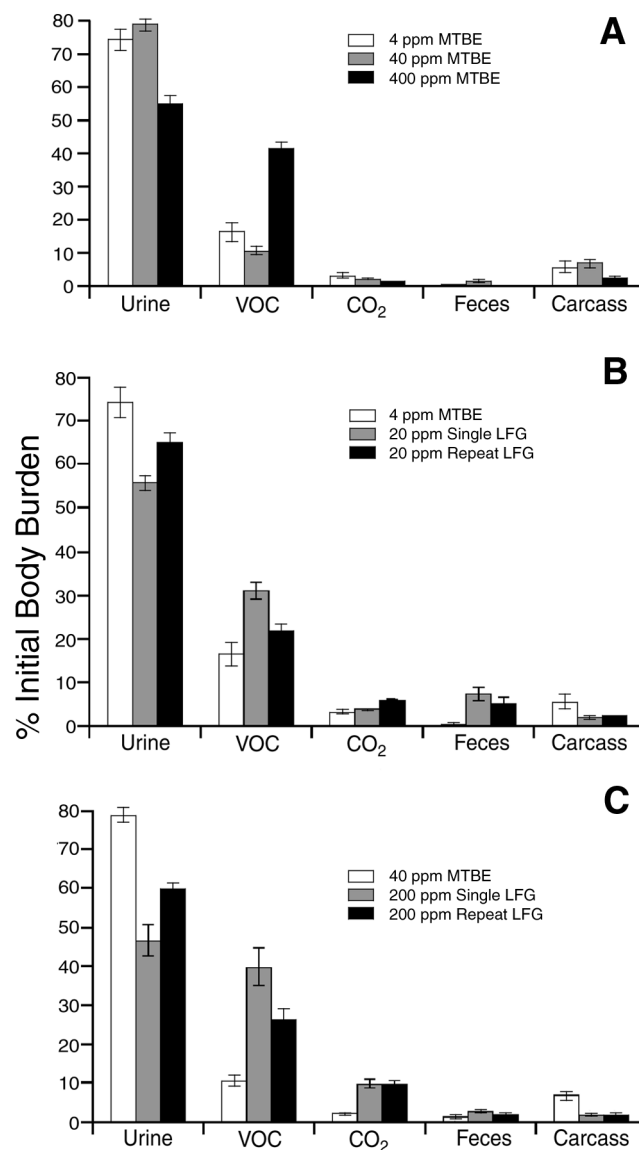single and repeated inhalation of 200 ppm MTBE yielded a significantly greater rate of excretion of MTBE as CO_2.

Figure 10. Relative percentage of MTBE equivalents in exhaled breath, urine, and feces. **(A)** Inhalation of 4, 40 or 400 ppm MTBE. Mean percentages after 400 ppm (urine, VOC, carcass) and after 40 ppm (feces) differed significantly from values after 4 ppm via these routes. **(B)** Single and repeat coexposures to 20 ppm LFG. Mean percentages after single (VOC, feces) and repeat (urine, CO_2, feces) coexposures differed significantly from elimination via these routes after 4 ppm MTBE. Mean percentages after repeat coexposure to 20 ppm LFG for urine and CO_2 differed significantly from percentages for these routes after single coexposure to 20 ppm LFG. **(C)** Single and repeat coexposures to 200 ppm LFG. Mean percentages in urine, VOC, CO_2 and carcass after single or repeat coexposure to 200 ppm LFG differed significantly from elimination via these routes after 40 ppm MTBE. Mean percentages after repeat coexposure to 200 ppm LFG for urine and VOC differed significantly from percentages for these routes after single coexposure to 200 ppm LFG.

Table 7. Elimination of MTBE and/or Its Metabolites in Urine and Exhaled Air

	Elimination Half-Time [hr (95% CI)][a]		
Exposure Group	Urine	VOC	CO$_2$
4 ppm MTBE	13.9 (12.4–15.8)	5.17 (4.56–5.97)	19.2 (18.2–21.0)
40 ppm MTBE	12.8 (11.9–13.9)	10.2 (8.77–11.9)	27.7[b] (24.8–31.5)
400 ppm MTBE	12.4 (11.4–13.3)	2.15[b,c] (1.97–3.55)	19.2[c] (18.2–20.3)
20 ppm Single LFG	12.4 (12.2–12.8)	4.08[b] (3.77–4.41)	13.3[b] (12.8–13.9)
20 ppm Repeat LFG	11.0[b] (10.7–11.4)	1.42[b] (1.34–1.51)	11.2[b] (10.5–12.2)
200 ppm Single LFG	12.8 (11.9–13.9)	6.19[c] (5.73–6.66)	13.3[c] (12.8–13.9)
200 ppm Repeat LFG	10.8[c] (9.9–11.9)	3.51[c] (2.91–4.44)	16.5[c] (15.8–16.9)

[a] Elimination data were fit using a single-component negative exponential function $y = e^{-ax}$, where a = the elimination parameter (hours^{-1}). The elimination half-time (hr) = 0.693/a.

[b] Significantly different from the half-time for 4 ppm MTBE alone.

[c] Significantly different from the half-time for 40 ppm MTBE alone.

BLOOD, KIDNEY AND URINE ANALYSES

MTBE and TBA in Blood

Concentrations of MTBE and TBA in whole blood were determined by headspace GC/MS. Only TBA was detected in blood of rats exposed to 4 ppm MTBE (Figures 11A and 12A). The concentrations were highly variable and detectable only after 2 hours of exposure until 2 hours after the end of exposure.

With MTBE exposures of 40 and 400 ppm, MTBE was detected in blood within 30 minutes, and TBA was first detected within 1 hour of exposure (Figures 11B and 11C).
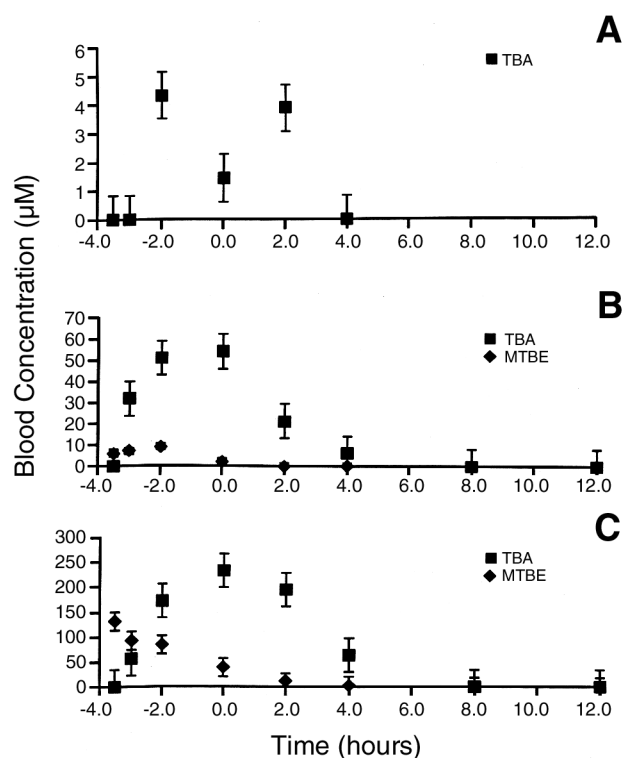
Figure 11. Concentrations of MTBE and TBA in blood after exposure to 4 ppm MTBE (A), 40 ppm MTBE (B), and 400 ppm MTBE (C). Results are expressed as mean ± SE (*n* = 3).
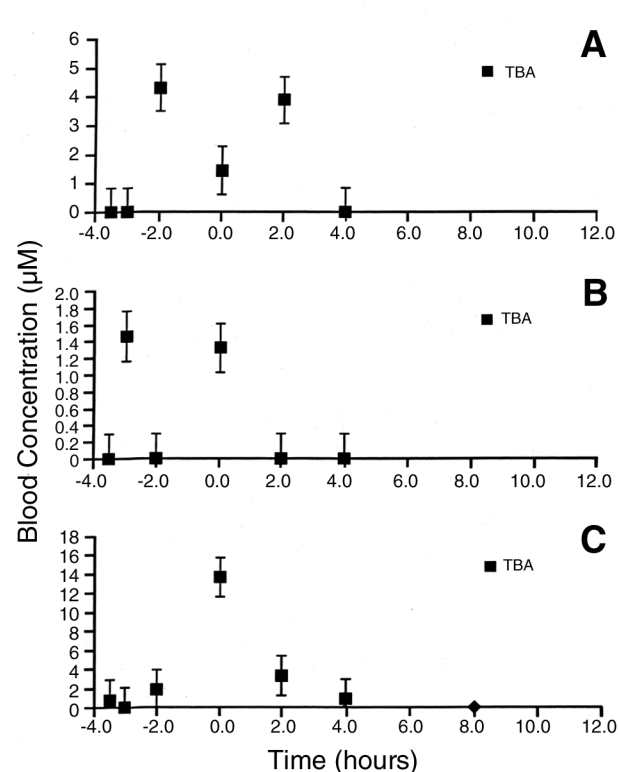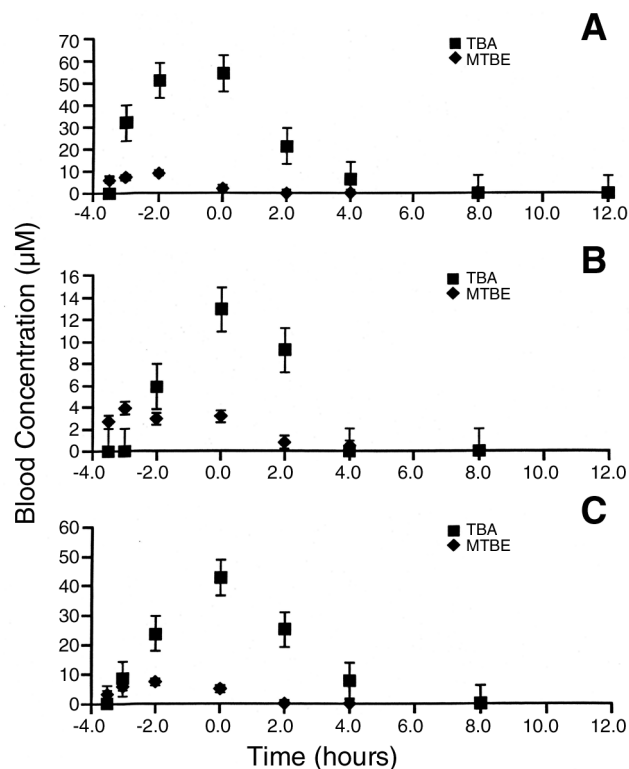

Figure 12. Concentrations of MTBE and TBA in blood after single exposure to 4 ppm MTBE (A), single coexposure to 20 ppm LFG (B), and repeat coexposure to 20 ppm LFG (C). Results are expressed as mean ± SE (*n* = 3). MTBE levels were below the limit of quantitation.

By 2 hours of exposure, concentrations of TBA greatly exceeded those of MTBE; elimination of TBA was slower than that of MTBE.

With single coexposure to 20 ppm LFG, only TBA was detected in blood (Figure 12B). Concentrations of TBA in blood during this exposure were quite variable, and no TBA was detected after the exposure ended. Repeated coexposure to LFG greatly increased the concentration of TBA in blood (Figure 12C), with detection beginning after only 30 minutes of exposure and levels remaining above the measurable limit through 4 hours after exposure.

The apparent rate at which TBA concentrations increased in blood and the concentrations achieved were notably lower in rats coexposed to 200 ppm LFG compared with rats inhaling 40 ppm MTBE alone (Figures 13A and B). Single coexposure to 200 ppm LFG also appeared to reduce the rate of elimination of MTBE from the blood (Figure 13B). Repeated coexposure to 200 ppm LFG appeared to have little effect on the maximum levels of MTBE and TBA in blood but elicited a slower rate of increase in TBA blood concentrations during the exposure (Figure 13C).

In all cases neither MTBE nor TBA were detected in blood by 8 hours after exposure. Lack of sufficient data points precluded actual estimation of elimination half-times for MTBE and TBA from blood.

MTBE and TBA in Kidney

Concentrations of MTBE and TBA in kidney were measured only in rats killed immediately after the 4-hour exposure. Although MTBE was not detected in blood of rats exposed to 4 ppm MTBE or to 20 ppm LFG, MTBE rather than TBA comprised 99% to 100% of the head-
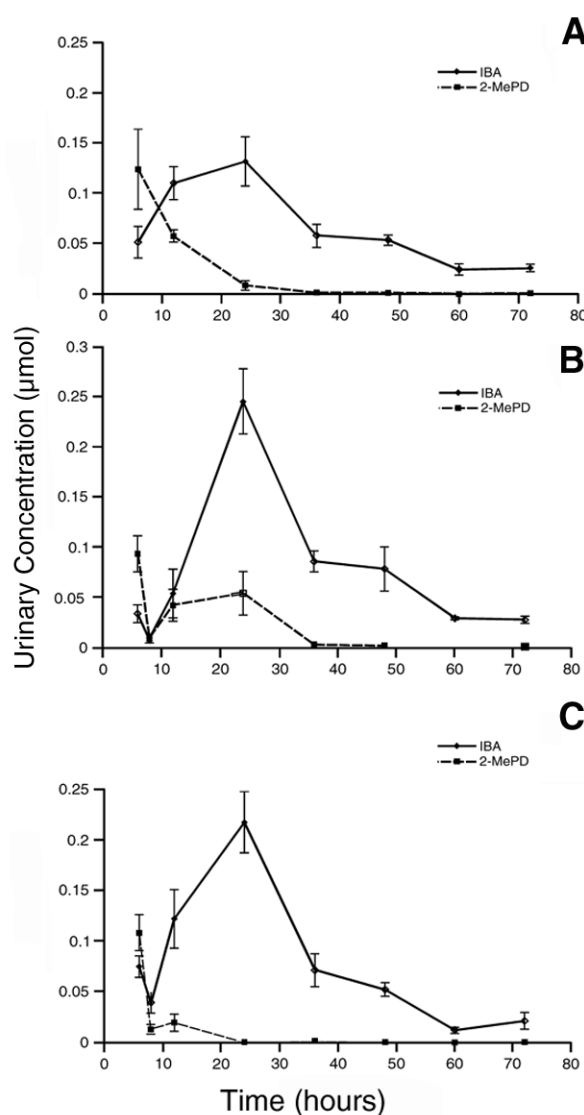

Figure 13. Concentrations of MTBE and TBA in blood after single exposure to 40 ppm MTBE (A), single coexposure to 200 ppm LFG (B), and repeat coexposure to 200 ppm LFG (C). Results are expressed as mean ± SE ($n = 3$).

space quantitated by GC/MS of kidney tissue (Table 8). MTBE was also present in greater concentrations than TBA in kidneys of rats in the remaining exposure groups. The nonvolatile metabolites, IBA and 2MePD, and tissue-bound metabolites would not have been detected using this procedure.

Table 8. Concentrations of MTBE and TBA in Kidney[a]

Exposure Group	nmol/g Kidney			MTBE (% of Total)
	MTBE	TBA	Sum	
4 ppm MTBE	21.1 ± 1.66	2.13 ± 0.68	21.3	91
40 ppm MTBE	78.7 ± 2.32	43.7 ± 3.52	122.4	64
400 ppm MTBE	174 ± 3.32	175 ± 8.77	349	50
20 ppm Single LFG	71.5 ± 17.0	< LOQ[b]	71.5	100
20 ppm Repeat LFG	40 ± 3.40	< LOQ[b]	40	100
200 ppm Single LFG	91.1 ± 5.38	22.9 ± 0.50	114	80
200 ppm Repeat LFG	70.4 ± 7.38	23.6 ± 1.09	94	75

[a] Results are the mean ± SE of 3 animals.

[b] Value is below the limit of quantitation (LOQ) of the method.

IBA and 2MePD in Urine

The radioactivity in urine was separable into 2 peaks. The first peak essentially eluted in the void volume of the column and was identified by LC–MS/MS as IBA. The second peak eluting shortly thereafter was identified by LC–MS/MS as 2MePD. Recovery of radioactivity from the column was essentially complete, indicating no retention of metabolites on the column. TBA or its conjugates were not identified in urine.

The excretion of IBA and 2MePD in urine of rats is shown in Figures 14 through 16. Both 2MePD and IBA were present in urine at 6 hours after the end of exposure

to MTBE alone and to LFG with MTBE. At that time, the concentrations of 2MePD were consistently higher than that of IBA among all exposure groups. Concentrations of 2MePD decreased rapidly thereafter, except in rats repeatedly exposed to 200 ppm LFG, when the concentrations peaked 12 hours after exposure (Figure 16C). The concentrations of IBA peaked at 24 hours after exposure, except in rats repeatedly exposed to 200 ppm LFG, for which IBA concentrations peaked 12 hours after exposure (Figure 16C). In all cases, IBA was detectable in urine 72 hours after exposure, whereas 2MePD was not. IBA was the primary metabolite excreted in urine, comprising 70% to 80% of the total urinary excretion (data not shown).
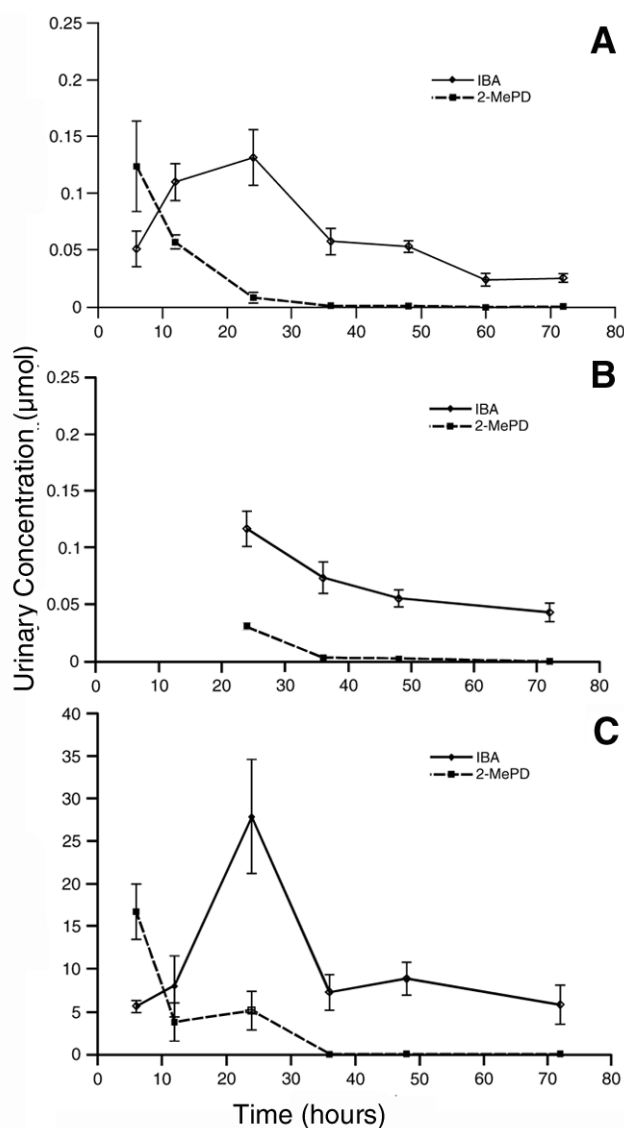
Figure 14. Excretion of IBA and 2MePD in urine after exposure to 4 ppm MTBE (A), 40 ppm MTBE (B), and 400 ppm MTBE (C). Results are expressed as mean ± SE ($n = 4$ or 5). Samples from the 6-hour and 12-hour collections for 40 ppm MTBE were inadvertently lost.
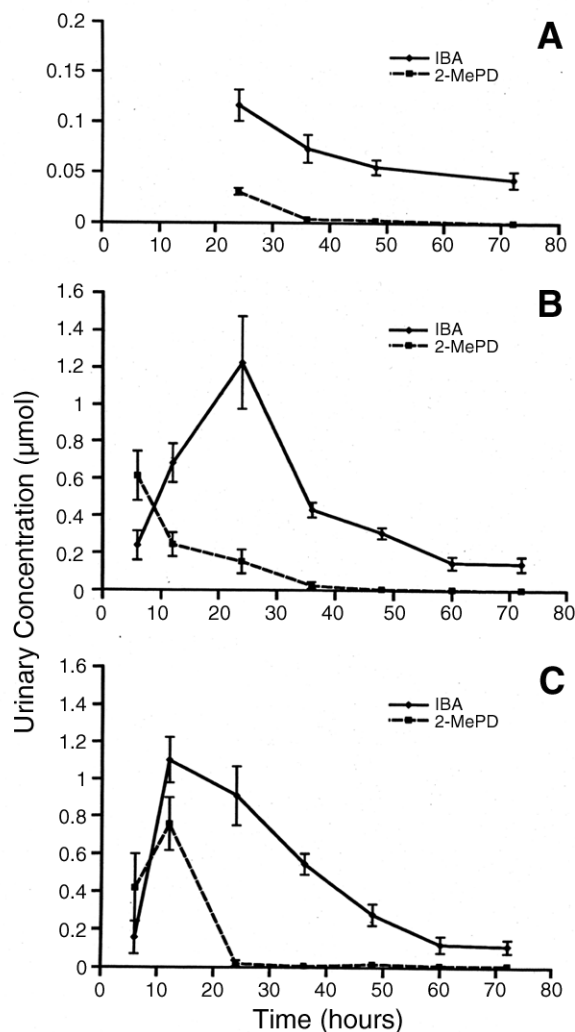

Figure 15. Excretion of IBA and 2MePD in urine after single exposure to 4 ppm MTBE (A), single coexposure to 20 ppm LFG (B), and repeat coexposure to 20 LFG (C). Results are expressed as mean ± SE ($n = 4$ or 5).

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Figure 16. Excretion of IBA and 2MePD in urine after single exposure to 40 ppm MTBE (A), single coexposure to 200 ppm LFG (B), and repeat coexposure to 200 LFG (C). Results are expressed as mean ± SE (n = 4 or 5).

DISCUSSION AND CONCLUSIONS

The overall objectives of this study were to evaluate the uptake and fate (including disposition, elimination, and metabolism) of MTBE in rats inhaling 4, 40, and 400 ppm once for 4 hours and to determine the effects of single and repeated coexposure to LFG with MTBE. Our studies extend previous work in that the uptake and fate of MTBE were evaluated over a lower concentration range, which is more relevant to human environmental and occupational exposure. Our studies are also unique because the effects of coexposure to gasoline vapor on the uptake and fate of MTBE were evaluated. These assessments are important

because people are exposed by inhalation to MTBE in combination with the highly volatile fraction of gasoline.

Inhalation of MTBE alone or as a component of LFG had no concentration-dependent effect on respiratory minute volume. This is consistent with humans who inhaled 5 to 50 ppm MTBE for 2 hours and also had no significant changes in minute volume (Nihlén et al 1998).

The body burdens of MTBE equivalents achieved after 4 hours of exposure to MTBE did not increase linearly with exposure concentration. This finding contrasts with the work of Savolainen and coworkers (1985), who reported linear uptake of inhaled MTBE over a range of 50 to 300 ppm in humans. Body burdens achieved in rats inhaling MTBE in this study were also much lower (0.87 versus 3.3 µmol for the 4 ppm 4-hour exposure; 23 versus 38 µmol for the 40-ppm 4-hour exposure) than calculated dose estimates reported by Dekant et al (in this Research Report) as per the European Centre for Ecotoxicology and Toxicology of Chemicals (1997). The body burden achieved after 4 hours of exposure to 400 ppm MTBE was also lower (298 versus approximately 330 µmol) than expected based on use of the same respiratory parameters (0.169 L/min and 0.5 retention fraction) (Miller et al 1997, Amberg et al 1999, Dekant et al in this Research Report). Our achieved burdens were also low compared with values that would be predicted using the minute volumes measured during our exposures (0.240 to 0.314 L/min).

The reason for the differences between the measured and predicted calculated burdens is not clear. The differences might also be due in part to differences between the predicted retention fractions (0.5 used by Dekant et al in this Research Report, and Miller et al 1997) and actual retained fractions. Nihlén and coworkers (1998) and Riihimaki and associates (1996) reported a net respiratory uptake of 30% to 40% in humans and indicated that this relatively low rate of absorption is due to the relatively low blood to air partition coefficient for MTBE. These data suggest that the 0.5 retained fraction might actually overestimate retention of inhaled MTBE. On the other hand, the measured body burdens were probably underestimated to a small degree because of loss of ^{14}C in exhaled breath between the end of exposure and placement of rats in the metabolism cages. Based on the determined pathways of excretion, however, this would have the least impact at 4 ppm MTBE where the disparity between measured and achieved burdens is the greatest.

Uptake of MTBE equivalents into blood was rapid. The concentrations of MTBE equivalents in blood (µM MTBE equivalents) was consistently higher than the corresponding concentrations of MTBE and TBA in blood because the former case measured ^{14}C activity derived

from MTBE, TBA, and their metabolites, not the concentration of individual components. The half-times for elimination of MTBE equivalents from blood was on the order of 10 to 20 times longer than the half-time for elimination of either MTBE or TBA, again because the measured radioactivity was derived from all metabolites. The half-times for elimination of MTBE equivalents from blood of rats exposed to 4 and 40 ppm MTBE were similar to those for elimination of MTBE equivalents in urine (primarily contributed by IBA and 2MePD) and as $^{14}CO_2$.

The concentrations of MTBE and TBA in blood immediately after exposure to 4 and 40 ppm MTBE in these studies correlate well with the values reported in the section by Dekant et al. TBA concentrations determined in these studies were slightly higher (53.9 versus 36.7 µM for 40 ppm exposure; 4.4 versus 2.9 µM for 4 ppm exposure), while concentrations of MTBE were slightly lower (2.3 versus 5.9 µM for 40 ppm exposure; none detected versus 2.3 µM for 4 ppm exposure). Too few data points were available to calculate elimination half-times for MTBE and TBA, but our data confirm earlier findings that TBA is eliminated from blood more slowly than is MTBE (Miller et al 1997, Dekant et al in this Research Report).

MTBE equivalents rapidly distributed to all tissues examined with the largest percentages of the initial body burden of MTBE equivalents distributed to liver. The observed initial body burden did not increase linearly, between 4 and 400 ppm. At 400 ppm, elimination half-times of MTBE equivalents from liver increased and from lung, kidney, and testes decreased compared with the two smaller doses. Furthermore, at 400 ppm the elimination half-time for VOCs in breath was significantly shorter and the percentage of the initial body burden of MTBE equivalents eliminated as VOCs in breath increased significantly. These changes probably reflect a saturation of blood with MTBE at 400 ppm and strongly suggest that the uptake and fate of MTBE are notably different at exposure concentrations above and below 400 ppm. A similar effect has been observed for isobutene inhaled by rats at concentrations between 400 and 4,000 ppm (Henderson et al 1993).

Urine was the major route of excretion of MTBE metabolites. MTBE equivalents at 74%, 79% and 55% of the initial body burden were excreted in urine of rats exposed to 4, 40, and 400 ppm MTBE, respectively. By comparison, Dekant and coworkers reported that approximately 40% of the initial dose of rats inhaling 4 and 40 ppm MTBE was excreted in urine. This discrepancy may be due to the fact that the initial doses in the Dekant studies were calculated, not measured, values. The percentage of urinary excretion in our studies for rats exposed to 400 ppm is more consistent with the value of 64% reported by Miller and colleagues (1997)

for rats inhaling 400 ppm MTBE for 6 hours, although this value might be an underestimate due to possibly inaccurate values for retention of radioactivity in carcass.

The amounts of metabolites excreted in urine of rats inhaling 4, 40, and 400 ppm MTBE were 0.66, 18.3, and 163 µmol, respectively. By comparison, Dekant and coworkers reported total urinary excretion of 2.7 and 15.1 µmol for rats inhaling 4 and 40 ppm MTBE. We identified the two major urinary metabolites, IBA and 2MePD. In addition to these metabolites, Dekant and coworkers identified and quantitated TBA in urine of rats inhaling both 4 and 40 ppm MTBE, using GC/MS. Recovery of radioactivity from the high-performance liquid chromatography column used in our study was excellent, but TBA may have coeluted with the IBA or 2MePD peaks. IBA and 2MePD quantitated by HPLC accounted for 0.61 of the 0.66 µmol total metabolites excreted in urine. Because of the loss of samples from early time points, a similar calculation cannot be made for the 40 ppm samples.

Single and repeated coexposure to 20 and 200 ppm LFG had opposite effects on the total body burden of MTBE equivalents present at the end of exposures compared with those achieved after 4 and 40 ppm MTBE exposures. Significantly higher burdens of MTBE equivalents were measured with 20 ppm LFG and lower burdens with 200 ppm MTBE. The effects of coexposure to LFG on blood levels of MTBE equivalents paralleled the effects on body burden. These differences in overall uptake of MTBE equivalents cannot be attributed to alterations of minute volume by the different LFG exposures. The reason for the greater overall uptake after 20 ppm LFG exposure is not clear. Decreased MTBE absorption (uptake) after single and repeated coexposure to 200 ppm LFG may be due to a decrease in solubility of MTBE in blood caused by the inhalation of other hydrocarbons. Investigations on the blood/air partition coefficient of MTBE in the absence and presence of LFG would be needed to confirm this hypothesis.

Single and repeated coexposure to either 20 or 200 ppm of the volatile fraction of gasoline yielded significantly lower percentages of the initial MTBE equivalents body burden in tissues, including liver, kidney, and testes, immediately after and 72 hours after exposure compared with those occurring after inhalation of 4 ppm or 40 ppm MTBE. The shift in tissue distribution after single exposure cannot be completely explained by changes in tissue elimination rates for MTBE equivalents, because elimination rates were increased only among rats repeatedly exposed to 200 ppm LFG. However, coexposure led to more rapid elimination of MTBE equivalents in urine (repeated exposures only) and as VOCs and CO_2.

MTBE is a substrate for cytochrome P450-mediated dealkylation. Metabolic activities for MTBE metabolism are 46 times faster in nasal mucosa than in liver; no metabolic activity has been associated with lung or kidney (Hong et al 1997). Repeated MTBE inhalation or TBA injection did not alter cytochrome P450 monooxygenase activities (Savolainen et al 1985; Turini et al 1998). Furthermore, analysis of cytochrome P450 monooxygenase in liver and nasal mucosa obtained from rats repeatedly exposed to 200 ppm LFG showed no induction of activity compared to unexposed rats (J-Y Hong, personal communication, 1999). Taken together, these findings suggest that induction of cytochrome P450 monooxygenases may not have contributed to the changes in tissue distribution and elimination of MTBE equivalents observed with repeated inhalation of LFG in our studies (although additional studies would be needed to confirm this).

In conclusion, our results indicate that the uptake and fate of inhaled MTBE are altered upon increasing exposure levels from 40 to 400 ppm, suggesting that toxic effects observed previously upon repeated inhalation of concentrations of 400 ppm or greater may not necessarily be linearly extrapolated to estimate effects at lower concentrations. Furthermore, single or repeated coexposure to LFG yields lower tissue burdens of MTBE equivalents and increases the rate of elimination of MTBE and its metabolites, thereby possibly reducing the toxic effects of MTBE compared with its effects when inhaled alone.

ACKNOWLEDGMENTS

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APPENDICES AVAILABLE ON REQUEST

The following appendices are available by contacting the Health Effects Institute at 955 Massachusetts Avenue, Cambridge MA 02139, USA, by phone (+1 617-876-6700), fax (+1 617-876-6709), or e-mail (pubs@healtheffects.org). Please give the full title of report and the titles of the appendices you wish to request.

A. Certificates of Analysis for ^{14}C-Labeled MTBE and Chemical Composition of Unleaded Gasoline

B. Animal Assignments and Body Weights

C. Calculation of Composition of Generator Solutions

D. Study Protocol FY96-035, Toxicokinetics of Methyl tert-Butyl Ether Inhaled Alone and in Combination with Gasoline Vapor

E. Limits of Detection and Quantitation for Radiochemical and GC/MS Analyses

F. Individual Animal Respiratory Parameters

G. Individual Animal Tissue Distribution of MTBE Equivalents

H. Mass Spectral Confirmation of the Identity of Urinary Metabolites

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Edward B Barr received his MSEE from the University of New Mexico. He has extensive experience in the development of inhalation systems for exposure of animals to vapors and particles. His interests are the development of innovative methods for generating biological and chemical aerosols.

Jennifer L Krone received her PhD in analytical chemistry from Arizona State University. Her research interests are in applying mass spectrometry to biological research. She is presently with Proteometrics in New York, New York.

ABBREVIATIONS AND OTHER TERMS

CI	confidence interval
CO_2	carbon dioxide
DMF	dimethylformamide
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
HPLC	high-performance liquid chromatography
IBA	2-hydroxyisobutyric acid
IBB	initial body burden
KOH	potassium hydroxide
LC–MS/MS	liquid chromatography–tandem mass spectrometry
LFG	light fraction of unleaded gasoline
m/z	mass-to-charge ratio
2MePD	2-methyl-1,2-propanediol
MTBE	methyl *tert*-butyl ether
MTBE-d_3	deuterated MTBE
r^2	bivariate coefficient of determination
R^2	multivariate coefficient of determination
TBA	*tert*-butyl alcohol
TBA-d_{10}	deuterated TBA
TEAH	tetraethylammonium hydroxide
VOCs	volatile organic compounds

COMMENTARY
Health Review Committee

INTRODUCTION

Widespread introduction of oxygenates such as methyl *tert*-butyl ether (MTBE*) to reduce emissions of carbon monoxide and other pollutants has increased the potential for human exposure to such chemicals and possible related health effects. To better understand the potential health effects of the gasoline additives MTBE, ethyl *tert*-butyl ether (ETBE) and *tert*-amyl methyl ether (TAME), detailed knowledge of their metabolism in humans is essential. A number of studies conducted in vitro, in animals, and in humans have investigated the uptake, metabolism and excretion of inhaled MTBE, but limited information is available for other ethers, such as ETBE and TAME. In addition, potential exposure of humans through ingestion of water contaminated due to gasoline spills or leaky underground storage tanks has become an important issue (see Brown 1997; Stern and Tardiff 1997). Comparative data between rats and humans, obtained through inhalation as well as ingestion of low doses of ethers, are needed to better understand the human health risks associated with exposure to ethers in gasoline in the environment. Information is also needed on the effect on the uptake and metabolism of MTBE of simultaneously inhaling other substances that are present in gasoline.

The first step in metabolizing MTBE is dealkylation, which yields formaldehyde and *tert*-butyl alcohol (TBA) (see Preface Figure 3). This reaction is catalyzed by cytochrome P450 (CYP)[†] dependent enzymes, which occur in different forms, or isozymes, and which have different metabolizing activity for different compounds. A study using rat liver microsomes implicated the isozyme Cyp2e1 in MTBE metabolism (Brady et al 1990). Whether other isozymes are also important remains to be determined. Considerable interest has focused on whether the presence or absence of certain isozymes contributes to differences in ether metabolism among individuals and whether this, in turn, is linked to differences in sensitivity to ether exposure.

In 1996, HEI funded 3 studies under RFA 95-1, *Comparative Metabolism and Health Effects of Ethers Added to Gasoline to Increase Oxygen Content*.[‡] Jun-Yan Hong and colleagues at the University of Medicine and Dentistry of New Jersey and the Robert Wood Johnson Medical School responded with a proposal to determine the metabolic pathways of MTBE, ETBE, and TAME in human liver microsomes and to identify the major CYP isozyme(s) responsible for their metabolism. Once the primary isozyme responsible for metabolizing MTBE had been identified, the investigators planned to test the hypothesis that genetic polymorphism may be linked to differences in sensitivity to MTBE among humans. The results were expected to provide information on whether sensitivity to MTBE exposure is related, at least in part, to different forms of the CYP enzymes.

Wolfgang Dekant and colleagues at the University of Würzburg proposed to expose rats and humans by inhalation to MTBE, ETBE, and TAME at relatively low doses (4 and 40 ppm) and to extend data on MTBE metabolism to include the ethers ETBE and TAME, which had not been studied extensively. In this study, metabolites would be identified and measured in blood and urine of rats and humans. In addition to inhalation, humans were exposed by ingestion to equivalent doses of MTBE or TAME (5 and 15 mg) in order to address the possibility of exposure to ethers via contaminated drinking water.

Janet Benson and colleagues at Lovelace Respiratory Research Institute proposed to expose rats through inhalation to low and intermediate doses of MTBE (4, 40 and 400 ppm) to study uptake, distribution, metabolism, and excretion of MTBE and its metabolites. In addition, the investigators proposed to expose the animals to a mixture of MTBE and gasoline vapors because human exposure to MTBE occurs predominantly as a component of gasoline. They hypothesized that coexposure of MTBE with gasoline

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*A list of abbreviations and other terms appears at the end of each Investigators' Report.

This document has not been reviewed by public or private institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views of these parties, and no endorsement by them should be inferred.

[†] The CYP superfamily components are differentiated in the text as follows: human genes are in captial letters and italics; rodent genes have an initial capital letter and are in italics; human enzymes are in uppercase Roman type; rodent enzymes have an initial capital letter and are in Roman type. A table of cited CYP components is given at the end of this Investigators' Report.

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vapors might change the uptake and metabolism of MTBE in the body. They chose gasoline vapor concentrations of 20 and 200 ppm, containing 20% MTBE by weight, to achieve levels of 4 and 40 ppm MTBE.

TECHNICAL EVALUATION OF HONG REPORT

The specific objectives of the proposed study by Hong et al were:

1. To characterize the metabolism of MTBE and related ethers in human liver microsomes and to establish the role of human CYP2E1 in the metabolism of MTBE.

2. To investigate the relationship between *CYP2E1* polymorphism and sensitivity to MTBE. Genotype distribution frequency of *CYP2E1* was to be determined in subjects reported to be sensitive to MTBE exposure.

3. To compare the metabolism of MTBE and related ethers in human liver microsomes and nasal mucosa microsomes from rats and monkeys.

SUMMARY OF RESULTS

Hong and colleagues have provided compelling experimental data confirming that metabolism of MTBE, ETBE, and TAME by liver homogenates depends upon CYP enzymes. First, they showed that metabolizing activity is located in the microsomal fraction, not in the cytosol. They also showed that the metabolism depended upon the presence of NADPH, an electron carrier for CYP, and was inhibited when carbon monoxide, a potent inhibitor of CYP enzymes, was added to the reaction mixture. In spite of a large interindividual variation in ether metabolism observed across normal human liver samples, microsomal activities of each ether correlated strongly within individual samples. Metabolic activity of human liver microsomes was compared among ethers, and a correlation coefficient was calculated. The correlation coefficients for MTBE versus ETBE, MTBE versus TAME, and ETBE versus TAME metabolism ranged from $r = 0.91$ to $r = 0.96$, suggesting that these ethers are metabolized by the same enzyme(s).

Hong and coworkers subsequently used human lymphoblastoid cell lines, engineered to each express DNA of a particular human CYP gene, to study the involvement of particular CYP isozymes on ether metabolism. Of twelve isozymes evaluated, CYP2A6 showed the highest ether metabolizing activity in vitro (ie, formation of TBA from MTBE and ETBE or TAA from TAME) in human liver microsomes, whereas CYP2E1 was shown to have the second highest ether-metabolizing activity. Further evidence for the involvement of CYP2A6 was provided when addition of monoclonal antibody against human CYP2A6 caused 75% to 95% inhibition of ether metabolism in human liver microsomes, whereas addition of antibody against CYP2E1 had no effect. Further, liver microsomes from *Cyp2e1*-deficient mice, which lack Cyp2e1 activity, showed no reduction in ether metabolism when compared with enzyme activity in liver microsomes from normal Cyp2e1 wild-type mice. These results were interpreted as suggesting a major role for the CYP2A6 isozyme as opposed to the CYP2E1 isozyme in ether metabolism under the in vitro conditions of high ether substrate concentrations utilized in these studies.

After demonstrating the importance of CYP2A6, Hong examined possible sequence variations in the *CYP2A6* gene in 23 individuals who claimed to be sensitive to MTBE. One previously reported genetic variant of *CYP2A6* was confirmed and three novel variants were identified. To test for the ether metabolizing activity of the variants of *CYP2A6*, complementary DNA of the wild type and three of the variants was generated and the corresponding proteins were expressed in a baculovirus Sf9 cell system to test for their metabolic activity. Two variants were shown to have reduced activity in metabolizing MTBE, ETBE, and TAME and one showed a total loss of activity. No data were presented for metabolic activity of the fourth variant.

Finally, the ether metabolizing capacity of rat nasal mucosa microsomes was demonstrated to be higher than in liver microsomes. No detectable activities were found in microsomes prepared from lungs, kidneys, and olfactory bulbs of the brain. Ether metabolism in liver microsomes from monkeys and from rats exposed to a mixture of MTBE and gasoline was not reported.

DISCUSSION

The HEI Health Review Committee concluded that the experiments were conducted in a careful manner and that the experiments provided solid evidence for participation of CYP enzymes in dealkylation of the ethers used in gasoline. The use of several in vitro tests, different tissues, and mice lacking the *Cyp2e1* gene to investigate ether metabolism was considered a major strength of this study. These studies were complemented by identification of isozyme polymorphisms in a small group of human subjects who had reported sensitivity to MTBE exposure. In general, the results of this study have furthered our understanding of the role of CYP enzymes in metabolism of ethers added to gasoline.

The HEI Health Review Committee identified some issues that were important for interpretation of the results. Based on the information provided in Hong's report, the HEI Health

Review Committee thought that the relative importance of CYP2A6 in metabolism of ethers had not been fully established. The main reason to interpret these results cautiously was that the concentration of MTBE in these in vitro studies was high (1 mM and 100 μM) and therefore was likely to be well beyond the range of concentrations encountered in human exposures, where blood levels up to 35 μM have been reported (Moolenaar et al 1994; White et al 1995a). Thus, the relative role of CYP2A6 in MTBE metabolism under conditions of ambient exposures remains unclear. Second, appreciable activity of CYP2E1 was noted in those studies, and at lower substrate concentrations the contribution of CYP2E1 may have been more pronounced than at the higher concentrations used in this study.

The involvement of several isozymes in ether metabolism has been indicated in multiple animal studies. For instance, Brady and coworkers (1990) found that Cyp2e1 was involved in MTBE metabolism. Turini and colleagues (1998) found that Cyp2b1 had the highest activity in metabolizing MTBE and ETBE and that Cyp2e1 had appreciable activity in metabolizing MTBE but not ETBE; Cyp1a1 and Cyp2c1 were only slightly active in this study. Pharmacokinetic modeling of metabolism of MTBE and TAME performed by Borghoff and colleagues has confirmed the hypothesis that more than one cytochrome would be involved. Pharmacokinetic modeling was unsuccessful when only one isozyme was assumed to be responsible for ether metabolism; therefore, two saturable pathways for ether metabolism were described. The first pathway involved a high capacity/low affinity enzyme, which the investigators thought represented Cyp2a6, and the second pathway involved a low capacity/high affinity enzyme, which they thought represented Cyp2e1 (Borghoff et al 1996; Collins et al 1999; Collins et al, personal communication, 2000). Together, these studies support the involvement of more than one isozyme in the metabolism of MTBE, ETBE, and TAME.

The HEI Health Review Committee thought that the authors' effort to collect data from human tissue aids in extrapolating from animal to human data. In several experiments, human liver tissue was used to measure the activity of several isozymes on ether metabolism. The Committee noted, however, that the use of tissue from liver cancer patients in the first assay (see Hong Table 1) was somewhat problematic. Although the investigators took care to only use healthy liver tissue from these patients, it is difficult to assume that these samples reflected the metabolic capacity of normal liver. In a subsequent experiment (see Hong Figure 2) microsomes obtained from the liver of accident victims had a substantially higher specific activity than in liver microsomes from cancer patients. The results

obtained with microsomes from liver cancer patients (Hong Table 1) should therefore be interpreted with caution. In addition, due to the low number of samples (n = 2 in some experiments), statistical comparisons were not always possible (see, for example, Hong Table 10).

The potential utility of *Cyp2e1*-deficient mice as a model remains unclear. Since gene-disrupted mice have a life-long deficit in the gene that has been knocked out, compensatory mechanisms are likely to have altered that particular system during development (for example, altered patterns of CYP expression). The consequences of those compensatory mechanisms for the system studied remain subject to debate.

In terms of the interspecies and intraspecies comparison of CYP activity in liver and nasal microsomes, more research needs to be performed. The investigators originally intended to study monkey liver and nasal tissues in addition to rat tissues. Since nasal metabolism could be a major route of elimination for inhaled material, and since activity in rat nasal tissue appeared to be much higher than in liver tissue, such a study would have been very interesting. Whether metabolism of ethers in nasal mucosa in humans is a major pathway of elimination also remains to be determined.

The detection of three new polymorphisms is potentially very interesting. However, the selection of a population subgroup based on self-reported symptoms, and the lack of an adequate control group, prevent any conclusion about the prevalence or importance of these isozymes in the general population. In addition, not nearly as many people were recruited for this study as had been projected in the study proposal: of 300 proposed subjects, only 23 subjects were recruited. No causal relationship between the occurrence of these reduced-activity polymorphisms and sensitivity to MTBE exposure can be assumed from these data.

The HEI Health Review Committee recommends further experiments with sensitive and nonsensitive people to establish the occurrence of polymorphisms for several CYP enzymes possibly involved in metabolism of ethers added to gasoline (ie, for CYP2A6, for CYP2E1, and possibly for other isozymes as well). After those data become available, the question of whether low-activity isozymes could be responsible for increased sensitivity to ethers might be addressed. The HEI Health Review Committee therefore thought it disappointing that the original aim, to study CYP2E1 in detail, was abandoned in favor of focusing almost exclusively on CYP2A6. The Review Committee thought that a more extensive comparison of the two isozymes, both in their metabolic activity and in the occurrence of polymorphisms, would have contributed to

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better understanding of their respective roles in vivo in metabolism of ethers added to gasoline and human sensitivity to MTBE.

CONCLUSIONS

The HEI Health Review Committee identified three major conclusions from this research:

1. CYP enzymes are important in the metabolism of ethers added to gasoline in rats and humans.

2. Human hepatic CYP2A6 and CYP2E1 appear to be involved in metabolism of ethers added to gasoline, but the relative contribution of these isozymes at ambient exposure levels has not been ascertained.

3. Specific activities for CYP enzymes in metabolizing ethers added to gasoline are roughly comparable in rat and human liver tissue. Further, the enzymatic activity for ether metabolism in rat nasal mucosa is substantially higher than in rat liver. Whether comparable enzymes and activities for metabolism of ethers added to gasoline exist in the nasal mucosa of humans remains unknown.

TECHNICAL EVALUATION OF DEKANT REPORT

The specific objectives of the proposed study by Dekant et al were:

1. To establish the structures of metabolites formed from the ethers MTBE, ETBE and TAME in rats and humans.

2. To quantify the time- and concentration-dependent excretion of ether metabolites after inhalation of 4 and 40 ppm MTBE, ETBE and TAME in rats and humans.

3. To quantify the time- and concentration-dependent excretion of ether metabolites after ingestion of 5 and 15 mg of MTBE and TAME in humans.

SUMMARY OF METHODS

Metabolite Characterization

To identify metabolites in urine, four rats were exposed to MTBE, ETBE, or TAME through inhalation. Compounds labeled with carbon-13 were synthesized to allow tracking of metabolites originating from the ether exposure (as opposed to endogenous sources). Metabolites were identified and quantified using nuclear magnetic resonance spectroscopy (NMR) and gas chromatography/mass spectrometry (GC/MS). Two male and two female rats were

exposed to 2,000 ppm ^{13}C-labeled MTBE, ETBE and TAME for 6 hours, and urine samples were collected at 24 and 48 hours after the end of exposure. To confirm the metabolic pathways, the ^{13}C-labeled metabolites TBA and TAA were administered orally (250 mg/kg in corn oil) and urine samples were collected after 24 and 48 hours. To determine the metabolic pathway for TAME in humans, 5 mg/kg ^{13}C-TBA was administered to one subject and urine samples were collected every 12 hours for 48 hours. In addition, the subject inhaled air from a 2-L bag containing 27,000 ppm ^{13}C-TAME for 4 minutes, and urine samples were collected every 6 hours for 48 hours thereafter. (^{13}C is a stable isotope and is not considered a health hazard.)

Inhalation Exposure in Rats and Humans

Six humans (3 males, 3 females) and 10 rats (5 males, 5 females) were exposed simultaneously in an exposure chamber to 4 or 40 ppm of MTBE, ETBE or TAME for 4 hours. In humans, blood samples were collected every 2 hours for 12 hours and at 24 hours after the end of exposure. In rats, one blood sample was collected at the end of exposure. Urine was collected every 6 hours until 72 hours after the end of exposure in humans and rats. The rats were transferred to metabolic cages for this purpose. Air samples were taken from the exposure chamber every 15 minutes to confirm the exposure concentrations. Ethers and metabolites were measured using GC/MS.

Oral Exposure in Humans

Six humans (3 males, 3 females) consumed 5 or 15 mg of MTBE or TAME in 100 mL tap water. Blood samples were collected every hour for 4 hours, then every 2 hours until 12 hours, and at 24 hours after exposure. Urine was collected every 6 hours until 96 hours after the end of exposure. After ingestion of 15 mg MTBE, exhaled breath was collected every 10 minutes for the first hour, every 30 minutes for the second hour, and every hour thereafter until 6 hours after the end of exposure.

Data Analysis

The maximum concentration of a compound was compared to its background level using a Student t test. Datasets for male and female rats or humans were also compared using a t test. Half-lives were calculated using exponential regression and curve fitting. No formal comparisons were made among data sets for the three ethers or between rat and human data.

SUMMARY OF RESULTS

After MTBE and ETBE exposure, 4 metabolites were identified: TBA, a TBA conjugate, 2-methyl-1,2-pro-panediol, and 2-hydroxyisobutyrate. After exposure to TAME, 6 metabolites were identified: free and glucu-ronidated 2-methyl-2,3-butanediol, TAA, a glucuronide of TAA, 2-hydroxy-2-methyl butyrate, and 3-hydroxy-3-methyl butyrate. After inhalation, all ethers were rapidly taken up by both rats and humans and subsequently cleared from blood by exhalation of the ether parent com-pounds and biotransformation to urinary metabolites. The biotransformation of MTBE and ETBE followed identical pathways and was similar for rats and humans, except that ether metabolism in rats was more rapid. 2-Hydroxyisobu-tyrate was the major metabolite in urine. The biotransfor-mation of TAME followed slightly different pathways in humans and rats: in humans 2-methyl-2,3-butanediol, 2-hydroxy-2-methyl butyrate, and 3-hydroxy-3-methyl butyrate were major metabolites in urine whereas in rats 2-methyl-2,3-butanediol and its glucuronide were major uri-nary metabolites. In rats, the concentrations of ethers in blood at the end of exposure were similar to those in humans for MTBE, lower for ETBE, and twice as high for TAME. After ingestion of MTBE and TAME in humans, the metabolic pathways were identical to the pathways fol-lowing inhalation exposure of these compounds. A hepatic first pass effect (ie, liver metabolism after absorp-tion through the gut before a compound enters the general circulation) was not observed.

DISCUSSION

Overall, Dekant and colleagues are commended for their efforts in presenting a detailed characterization and quan-tification of ether metabolites in both rodents and human subjects. The primary study objectives were achieved in terms of identifying ether metabolites and identifying met-abolic pathways after inhalation and ingestion in humans and rats. These data will contribute to the toxicokinetic database used for human health risk assessment of expo-sure to ethers, both after environmental exposure during refueling and after exposure to ethers in contaminated drinking water supplies.

Humans and rats were exposed simultaneously in a con-trolled environment chamber. This study design ensured that data from rat and human exposures were largely com-patible, except that the microenvironment in the rat cages may have been slightly different from the general atmo-sphere in the exposure chamber. In order to limit the pos-sibility of background levels of ethers or major metabolites, the subjects were asked to abstain from alcohol and drug use and not to refuel their car two days

before the experimental sessions. Air samples were taken throughout the exposure period and ether concentrations were close to the target values of 4 and 40 ppm. The report does not clarify where the samples were taken inside the chamber, whether a gradient existed throughout the chamber, or whether the ether concentration reached the target values inside the rat chambers. Variations in ether concentration within the exposure chamber may have con-tributed to individual differences in ether uptake or differ-ences in exposure levels between humans and rats. On the other hand, the results obtained in this study are consis-tent and strengthen the confidence in the exposure methods used.

Concerning the study design, composition of the human exposure groups was not optimal: some individuals partic-ipated in one exposure, and others participated in as many as four exposures. The large individual differences in the rate of ether metabolism could be ascribed to differences in the levels of metabolizing enzymes, in particular CYP (see Hong et al, this report). Therefore, exposing the same indi-viduals, for instance, to MTBE or TAME by both ingestion and inhalation would have been preferable. Three individ-uals were exposed to MTBE or TAME by ingestion only, but for these individuals no comparative data from inhala-tion exposure are available. A more careful assignment of subjects to the different exposure conditions would have strengthened the comparisons and statistical analysis of these data.

Dekant and coworkers have developed sophisticated methods to identify and quantify individual metabolites and used these methods to detect compounds with low detection limits. Their data confirm the existing knowl-edge about the metabolic pathways of MTBE and ETBE and extend the knowledge about the metabolism of TAME. Compounds were detected consistently at low levels in blood and urine after exposure to 4 ppm of MTBE, ETBE or TAME. In terms of quantifying metabolite compounds, however, the investigators identified a problem when high background levels of metabolites were detected, mostly for 2-hydroxyisobutyrate and TBA. As a result, levels of these metabolites in urine after exposure to 4 ppm of MTBE, ETBE or TAME were not significantly different from con-trol levels due to high background levels. At the higher exposure concentration of 40 ppm, these problems were less prominent. Consequently, these metabolites which occur endogenously do not appear to be suitable as biom-arkers of ether exposure.

Concerning the half-lives calculated for several com-pounds, it was not clear how the investigators determined that the metabolic pathway occurred in one, two or three phases, each with its own half-life of ether biotransforma-

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tion. Dekant hypothesizes that the sampling times may have influenced the outcome in this respect. For example, the blood sampling schedule after ingestion contained more time points in the first hour after exposure than the equivalent sampling schedule after inhalation. This closer sampling scheme resulted in calculation of three half-lives after ingestion exposure to MTBE compared to two half-lives after inhalation exposure. The authors conclude that MTBE metabolism probably is no different after ingestion than after inhalation and that the relevance of finding different half-lives is therefore questionable. In general, this study found half-lives for MTBE and ETBE metabolism similar to those reported in other studies (Prah et al 1994; Buckley et al 1997; Nihlén et al 1998a,c). The reported blood levels are also similar to those found previously (Prah et al 1994; Cain et al 1996; Buckley et al 1997; Nihlén et al 1998a; Vainiotalo et al 1998).

In conclusion, this study has provided valuable data on the metabolism of MTBE, ETBE and TAME after inhalation and ingestion. The metabolic pathways for MTBE and ETBE were confirmed, and the pathway for TAME was described. ETBE metabolism was similar to MTBE metabolism, whereas TAME metabolism followed a different pathway with the formation of several more metabolites. The metabolism was similar for rats and humans except that the rate of metabolism was faster in rats. Most importantly, the data for ingestion and inhalation of MTBE and TAME in humans were similar, indicating that inhalation data may be used in addition to ingestion data to model environmental exposure to ethers in water as part of the human health risk assessment.

TECHNICAL EVALUATION OF BENSON REPORT

The specific objectives of the proposed study by Benson et al were:

1. To determine the uptake, metabolism, and excretion of MTBE in rats inhaling 4, 40, or 400 ppm for 4 hours.
2. To determine the uptake, metabolism, and excretion of MTBE during and after single or repeat inhalation of the light fraction of gasoline (LFG) containing MTBE.

Male rats were exposed nose-only for 4 hours to ^{14}C-MTBE alone (4, 40 or 400 ppm) or to LFG containing ^{14}C-MTBE (20 ppm or 200 ppm LFG containing 4 and 40 ppm MTBE, respectively). A separate group was exposed to the same concentrations of LFG containing unlabeled MTBE for 7 days and to LFG containing ^{14}C-MTBE on day 8.

The MTBE used in these experiments was labeled with ^{14}C, which allowed tracking of MTBE and its metabolites in tissues, blood, urine, feces, and exhaled air at several time points during and after exposure. Tissues examined were liver, kidneys, lungs, heart, brain, perirenal fat, and gonads. Because composition of the ^{14}C-labeled material was not determined, recovery of [14]C (expressed in *MTBE equivalents*) reflects both the parent compound and all of its metabolites (whether known or unknown). The total of MTBE equivalents in all body compartments at the end of the exposure was calculated as the *initial body burden*.

Benson and colleagues also directly measured levels of MTBE and the metabolite TBA in blood and the metabolites 2-hydroxyisobutyrate and 2-methyl-1,2-propanediol in urine, using GC/MS and liquid chromatography, respectively. Samples were obtained at 0.5, 1, 2, and 4 hours during exposure and at 2, 4, 8, 12, 24, 48, and 72 hours after exposure. To determine possible changes in breathing, which would affect uptake, ventilation rates were monitored during the 4 hours of exposure.

SUMMARY OF KEY RESULTS

Benson did not find any changes in ventilation rates after exposure to MTBE alone or to LFG containing MTBE. MTBE uptake, measured as initial body burden at the end of the exposure period, was not linear in the 4 to 400 ppm range. At 400 ppm, uptake mechanisms may have started to saturate and as a result more MTBE was directly exhaled. At the end of exposure, MTBE equivalents in tissues were detected predominantly in liver although the level per gram tissue was the same as in other tissues. Exposure to 4 ppm MTBE in 20 ppm LFG was not associated with major changes in MTBE uptake compared to inhalation of 4 ppm MTBE alone. In contrast, inhalation of 40 ppm MTBE in 200 ppm LFG led to a significantly lower uptake of MTBE compared to inhalation of 40 ppm MTBE alone. Similarly, the rate of elimination of MTBE equivalents from blood, lung, liver, kidney, and testes increased, possibly due to induction of metabolizing enzymes.

The major route of excretion of MTBE equivalents was urine. To a lesser extent, MTBE and TBA were excreted as volatile organic compounds and CO_2 in breath; very little was excreted in feces. Inhaling 400 ppm MTBE alone or MTBE in 20 or 200 ppm LFG led to a larger amount of MTBE equivalents excreted in air as opposed to urine. The metabolite TBA was consistently present at higher levels than MTBE in blood and was eliminated from blood more slowly. No MTBE or TBA was detected at 2 and 8 hours after exposure, respectively. Peak levels were detected during exposure for MTBE and at the end of exposure for TBA. 2-Hydroxyisobutyrate was the primary metabolite

detected in urine. In general, 2-hydroxyisobutyrate levels in urine peaked around 24 hours after exposure and returned to baseline levels by 72 hours after exposure. Levels of the metabolite 2-methyl-1,2-propanediol were highest at the first sampling timepoint, 6 hours after exposure, and returned to baseline by 24 hours after exposure.

DISCUSSION

The HEI Review Committee members commend Benson and colleagues for their thorough approach and carefully conducted experiments as well as for the detailed data collection. They have presented interesting findings on MTBE metabolism in rats at lower doses of MTBE than used in previous toxicokinetic studies and unique data on changes in MTBE uptake with gasoline coexposure. These results may have implications for human health issues related to exposure to MTBE in the environment (for instance, during refueling). The Health Review Committee identified some issues that may be addressed in future studies. These issues are outlined below.

In this study, rats were exposed to radiolabeled MTBE with all carbon atoms labeled uniformly. Groups of rats were killed at several timepoints during and after inhalation, and tissue samples were taken for analysis. The method of measurement, scintillation counting, counted all molecules with a radioactive ^{14}C atom incorporated, including the parent compound ^{14}C-MTBE and all its metabolites. This method provided valuable information on the time course of MTBE and metabolite elimination from the body, including the relative distribution of MTBE equivalents over body compartments, such as lung and other tissues, blood, and urine. Another benefit of this method was that only compounds that originated from MTBE were measured and compounds that may have been present endogenously were excluded. Compounds that are present endogenously in detectable quantities may interfere with detection of the same compound due to inhalation (see, for instance, Dekant et al in this report).

A disadvantage of the method, however, is that it did not distinguish between MTBE and individual metabolites. To provide additional time course data for MTBE and TBA, Benson also measured MTBE and TBA levels directly in blood. At the level closest to human exposure levels (ie, 4 ppm MTBE), however, MTBE levels in blood were below the detection limit and a time course of metabolism could not be determined. This lack of low-dose data limits the usefulness of the study for extrapolation from rats to humans for human health risk assessment of MTBE exposure at low doses.

When calculating MTBE uptake into the body at the end of the exposure period, the fraction of MTBE that was eliminated *during* the 4-hour exposure should ideally have been taken into account (see Nihlén et al 1998a). In this investigation, Benson did not correct for the elimination of MTBE during the exposure period, and it remains unclear to what extent such correction would have affected the results. Compared to other studies, Benson found a lower initial body burden and lower levels of MTBE and TBA in blood (see for example Dekant et al in this report). These differences could be due to differences in instrument sensitivity, measurement techniques, or exposure conditions.

Benson has presented extensive data on the distribution of MTBE equivalents in blood, urine, several tissues, and breath. In addition, the present study assessed three metabolites, TBA, 2-hydroxyisobutyrate and 2-methyl-1,2-propanediol and determined their levels in blood or urine. The time courses of the metabolite levels logically followed the metabolic pathway, in which MTBE is converted to TBA, which is converted to 2-methyl-1,2-propanediol, which is subsequently converted to 2-hydroxyisobutyrate (see Preface Figure 3): TBA levels peaked first, followed by 2-methyl-1,2-propanediol, and finally 2-hydroxyisobutyrate. Because some metabolite levels were detected in blood and others in urine, what fraction of MTBE converted to these and other metabolites is not clear. Which metabolites may have been present in other tissue compartments (besides blood or urine) is unknown. The formation of conjugates such as glucuronides (reported by Amberg et al 1999 and others) was not investigated in this study. Some information on metabolites other than 2-hydroxyisobutyrate and 2-methyl-1,2-propanediol is presented in the study by Dekant et al in this report.

The finding that gasoline coexposure reduced MTBE uptake and increased elimination rates from blood and urine is interesting: it implies that the health effects of MTBE exposure during refueling and other activities may be less than previously thought. At the same time, this finding is puzzling because a mechanism for such an effect has not been proposed or identified. Possibly, simultaneous inhalation of volatile components in gasoline could alter the solubility of MTBE in blood. Whether this is indeed the case, and which compounds in gasoline might be involved, could be the subject of future investigations.

CONCLUSIONS

The Health Review Committee agrees with the conclusions of Benson et al that

1. MTBE uptake is not linear between 4 and 400 ppm, suggesting that extrapolation from high to low doses

for human risk assessment should also be nonlinear, and

2. single and repeat coexposure with gasoline reduces MTBE uptake and increases its elimination from blood and urine, thereby possibly reducing the toxic effects associated with inhalation of MTBE in a gasoline mixture (such as occurs during refueling).

GENERAL DISCUSSION

HEALTH EFFECTS OF ETHERS AND METABOLITES

When considering possible health effects of MTBE, it is important to recognize the different ways in which people may be exposed: inhalation in the workplace while handling or transporting pure MTBE or MTBE-containing gasoline; inhalation at service stations while refueling vehicles with gasoline containing MTBE; or ingestion of or dermal contact with drinking water contaminated with MTBE. The possible health consequences of exposure to MTBE (see Preface) may range from acute to chronic, depending on the exposure route, concentration and duration, and sensitivity of individuals. At the time when the studies described in this report started, information was lacking on the health effects of MTBE and its metabolites as well as on the acute or chronic health effects of exposure to other ethers (such as ETBE and TAME) and their metabolites. Whereas the metabolite formaldehyde has been classified as a probable human carcinogen (US EPA 1993), the carcinogenic potential of most other metabolites has not been assessed. Furthermore, whether differences in metabolism contributed to the observed sensitivity to the effects of MTBE in people was unclear. The studies described in this report have furthered our knowledge on some of these issues.

EXPOSURE CONCENTRATIONS

MTBE inhalation exposure concentrations used in the Benson and Dekant studies were 4, 40 (both studies), and 400 ppm (Benson only). Benson had originally proposed to use 3, 30 and 300 ppm for 6 hours, and Dekant had proposed to use 5 and 40 ppm for 4 hours. To yield comparable data, the final exposure concentrations and duration for the two studies, 4 and 40 ppm for 4 hours, were decided at an investigator's workshop held on August 1, 1996. The 4 and 40 ppm concentrations are closer to human environmental exposure levels (see Lioy et al 1994) and much lower than concentrations up to 8,000 ppm used in earlier pharmacokinetic and toxicology studies in animals (Savolainen et al 1985; Miller et al 1997). Similarly,

animal studies using ETBE and TAME have used concentrations up to 5,000 ppm (Sun and Beskitt 1995; White et al 1995b; Dorman et al 1997; Medinsky et al 1999). Preface Figure 2 illustrates the human environmental exposure levels, which range from parts per billion to low parts per million. Human controlled exposure studies have used concentrations of 1.1 to 50 ppm for MTBE (Prah et al 1994; Johansen et al 1995; Cain et al 1996; Nihlén et al 1998a,c; Fiedler et al 2000; Pierce et al, personal communication 2001) and ETBE (Nihlén et al 1998b; Nihlén and Johanson 1999), which are closer to environmental exposures than the concentrations used in animal studies.

The exposure concentrations for gasoline vapor in the Benson study were 20 and 200 ppm of gasoline with a 20% MTBE content of 4 and 40 ppm, respectively, to reflect the two lowest concentrations in the experiments with MTBE alone. These concentrations are within the range of human exposure observed during short-term handling of fuel in the workplace (such as refineries, marketing terminals and service stations). Commentary Table 1 contains data collected in the US and Europe on environmental exposure to gasoline vapors that may contain up to 14% MTBE. Gasoline vapor exposures, measured using personal samplers over an 8-hour workday, are highest during occupational exposure and vary from an average of 5.6 ppm for a truck driver to a maximum of 409 ppm. Exposure levels have decreased since the introduction of vapor recovery systems (see Conservation of Clean Air and Water in Western Europe [CONCAWE] 2000). Levels of short-term exposure during refueling at gas stations vary from an average of 1.8 ppm to a maximum of 190 ppm. The 20 and 200 ppm gasoline vapor concentrations used in the Benson study, which were selected to match the 4 and 40 ppm MTBE exposures, fall well within this range.

MTBE BODY BURDEN AND BLOOD LEVELS

One of the questions that has been addressed by the Dekant and Benson studies is how much of the inhaled or ingested MTBE is actually taken into the body, based on blood levels of MTBE at the end of exposure. Their data are in good agreement with those collected in earlier studies. Commentary Table 2 contains a compilation of experimental data on MTBE metabolism in humans and rats. We calculated a received dose, which takes into account the exposure concentration, the exposure duration, the ventilation rate, and a retention factor. The calculated received dose is intended to approximate the initial body burden of MTBE exposure, the amount of MTBE retained in the body, which may be determined from the actual levels of MTBE in blood, tissues, urine, and exhaled breath. The calculated received dose and initial body burden numbers

Commentary Table 1: Environmental Exposure to Gasoline Vapors

Exposure Condition	Total Hydrocarbon (ppm)			Duration	Source
	Mean	SE	Maximum		
Manufacturing & Distribution					
Refinery (Maintenance Workers)	_6.5_ [a]		_119_	8 hr	CONCAWE 2000
Truck Driver (Top Loading)	_28.3_		_244_	8 hr	CONCAWE 2000
Truck Driver (Bottom Loading, with Vapor Recovery)	_5.6_		_32.7_	8 hr	CONCAWE 2000
Truck Driver (Bottom Loading with Vapor Recovery)	_20.1_		_182_	1 hr	CONCAWE 2000
Refinery (Personal Sampler)	13.7	49.5	60 [b]	< 7 hr	Enterline and Viren 1985
Refinery (Area)	15.2	103.3	50 [b]		Enterline and Viren 1985
Marketing Terminal (Personal Sampler)	71.5	182.4	340 [b]	< 7 hr	Enterline and Viren 1985
Marketing Terminal (Area)	13.4	37.8	46.7 [b]		Enterline and Viren 1985
Truck Driver (Top Loading)	_159_		_409_	1 hr	Hakkola and Saarinen 1996
Terminal	1.4		30.1	8 hr TWA [c]	Halder et al 1986
Bulk Handling	15		226	8 hr TWA	Phillips and Jones 1978
Truck Loading (without Vapor Recovery)	130	14.2		< 1 hr	Smith et al 1993
Truck Loading (with Vapor Recovery)	17	2.8		< 1 hr	Smith et al 1993
Truck Driver (without Vapor Recovery)	14	1.5		8 hr TWA	Smith et al 1993
Truck Driver (with Vapor Recovery)	9	1.6		8 hr TWA	Smith et al 1993
Service Stations					
Self Service Refueling (Unleaded) [d]	35.5		190	1–2 min	API 1993
Self Service Refueling (Premium) [d]	27.5		95	1–2 min	API 1993
Service Station (Stage II, Breathing Zone) [e]	2.3	4.5	13	4 hr	API 1994
Service Station Attendant (Summer, Stage II) [e]	_1.8_		_14_	15 min	API 1995
Service Station Attendant (Summer, Stage II) [e]	_2.5_		_26_	8 hr	API 1995
Service Station Attendant (Winter, Stage II) [e]	_1.9_		_17_	15 min	API 1995
Service Station Attendant (Winter, Stage II) [e]	_1.6_		_4.3_	8 hr	API 1995
Service Station Attendant (no Vapor Recovery)	5.5		22.4	8 hr	CONCAWE 2000
Self Service Refueling (Stage I) [e]	_25_		_153_	~ 1min	Hakkola and Saarinen 2000
Self Service Refueling (Stage II) [e]	_5.2_		_37_	~ 1min	Hakkola and Saarinen 2000
Service Station Area	1.0		32.5	8 hr TWA	Halder et al 1986
Self Service Refueling	_6.8_		_46_	< 5 min	Kearney and Dunham 1986
Service Station Attendant	114				McDermott and Vos 1979
Service Station Area (Summer)	0.15		2.3		PACE 1987
Service Station Area (Winter)	0.46		5.4		PACE 1989
Service Station Attendant	3.1	2.9		8 hr TWA	Wen et al 1984

Gasoline may contain up to 14% MTBE. Gasoline exposure is expressed as the concentration of total hydrocarbon.

[a] Numbers in italics were converted from mg/m^3 to ppm using the molecular weight for n-hexane (86.17).

[b] 95th percentile.

[c] TWA = Time-weighted average.

[d] Stage II vapor recovery system at 2 of 5 stations.

[e] Stage I or II vapor recovery systems.

Commentary Table 2. Exposure conditions, calculated received dose, and peak MTBE blood levels in humans and rats as reported in the literature[a]

	N	MTBE Exposure		Calculated Received Dose		Average MTBE Peak Blood Levels (μg/L)
		Concentration (ppm or mg)	Duration (hr)	Absolute [IBB] by Bodyweight (mg)	(mg/kg)	
Humans						
Cain et al 1996	4	1.7 ppm	1	1.3	0.02	17.2
Buckley et al 1997	1M	1.4 ppm	1	0.7 [1.5]	0.01	8.2
	1F	1.4 ppm	1	0.9 [1.5]	0.01	14.1
Dekant et al 2001	6	4 ppm	4	14	0.21	167
	6	40 ppm	4	120.4	1.79	590
	6	5 mg	oral	5	0.07	8.8
	6	15 mg	oral	15	0.2	61
Fiedler et al 2000	31	1.1 ppm	0.25	0.2	0.003	1.23
	31	1.7 ppm	0.25	0.3	0.004	1.75
Nihlén et al 1998a	10	5 ppm	2	19.4	0.25	114
	10	25 ppm	2	97.2	1.25	572
	10	50 ppm	2	194.4	2.49	1,144
Pierce et al 2001[b]	5	2.5 ppm	2	11.8	0.17	54.9
Rats						
Benson et al 2001	3	4 ppm	4	0.4 [0.1]	1.7	ND[c]
	3	40 ppm	4	3.4 [2.0]	13	800
	3	400 ppm	4	46.5 [26.3]	155	11,463
Dekant et al 2001	10	4 ppm	4	0.3	1.5	202
	10	40 ppm	4	2.8	12.8	519
Miller et al 1997	8	400 ppm	6	44.7	240	14,500
	8	8,000 ppm	6	890.8	4,789	534,500
	8	40 mg/kg	intravenous	8.3	40	10,000
	8	40 mg/kg	gavage	8	40	14,000
	8	400 mg/kg	gavage	78.4	400	119,000

[a] The received dose was calculated by the following formula: exposure concentration × conversion factor × exposure duration × ventilation rate × retention factor. The ventilation rate for humans was 9 L/min except in the Johansen (22.5 L/min) and Pierce (11.7 L/min) studies, where subjects were exercising. The ventilation rate in rats was 0.169 L/min except in the Benson study, where ventilation rates were measured to be 0.24–0.31 L/min. The retention factor was 0.4 in humans and 0.5 in rats (see Dekant et al in this report). The retention factor used by Pierce was 0.63. The number in square brackets next to the calculated received dose is the reported initial body burden.

[b] Pierce CH, Morgan MS, Dills RL, Chung HS, Kalman DA. 2001. Unpublished data, Department of Environmental Health, University of Washington.

[c] ND = not detected.

usually exclude the amount that was exhaled during exposure, which may circulate in the exposure atmosphere and complicate the calculations (see Nihlén et al 1998a).

The initial body burden calculated in the human studies varies from 0.2 to 195 mg MTBE per person. The lowest values were obtained during short-term exposures to low concentrations (1.1 ppm for 15 minutes; Fiedler et al 2000) and the highest value was obtained during longer exposure to higher concentrations (50 ppm for 2 hours; Nihlén et al 1998a). The only reported initial body burden (Buckley and associates 1997) closely resembles the calculated received dose (initial body burden of 1.5 mg versus received dose of 1.1 mg per person). The peak MTBE blood levels reported vary from 1 to 1,144 µg/L blood. The relationship between calculated received dose and average peak MTBE blood levels is shown in Commentary Figure 1A. With some small variation at the lower doses, received dose correlates strongly with blood level (r^2 = 0.98). Most studies reported a rapid rise in MTBE blood levels, which peaked at the end of the exposure period and declined rapidly afterward.

The received doses calculated in the rat inhalation studies (Miller et al 1997; Benson et al, this report; Dekant et al, this report) are similar among the 4, 40 and 400 ppm inhalation exposures (Commentary Table 1). Calculations of the received dose were based on a 0.169 L/minute ventilation rate and a 0.5 retention factor. The actual reported initial body burdens as measured by Benson were lower, however (0.1 versus 0.4, 2.0 versus 3.4, and 26.3 versus 46.5 mg for 4, 40 and 400 ppm, respectively). Benson reported a higher ventilation rate (0.24 to 0.31 L/min), which tended to increase the calculated received dose. In addition, the actual retention factor may be lower than the 0.5 used in the calculations. When comparing the calculated received dose with the average peak MTBE blood levels in rats, there is a strong correlation among the inhalation studies up to the 400 ppm dose (Commentary Figure 1B, r^2 = 0.98). The peak MTBE blood levels after gavage and intravenous exposure to MTBE in rats are relatively high, however, compared to the blood levels after inhalation. This difference could be partly attributed to the fact that MTBE is exhaled during inhalation exposure and a bolus injection may lead to faster and more efficient uptake.

ELIMINATION OF MTBE

Regarding MTBE elimination from blood in humans, several studies have reported between one and three phases with varying elimination rates. The first phase is very rapid, with an MTBE half-life of 1 to 5 minutes (Buckley et al 1997; Nihlén et al 1998a). In the second
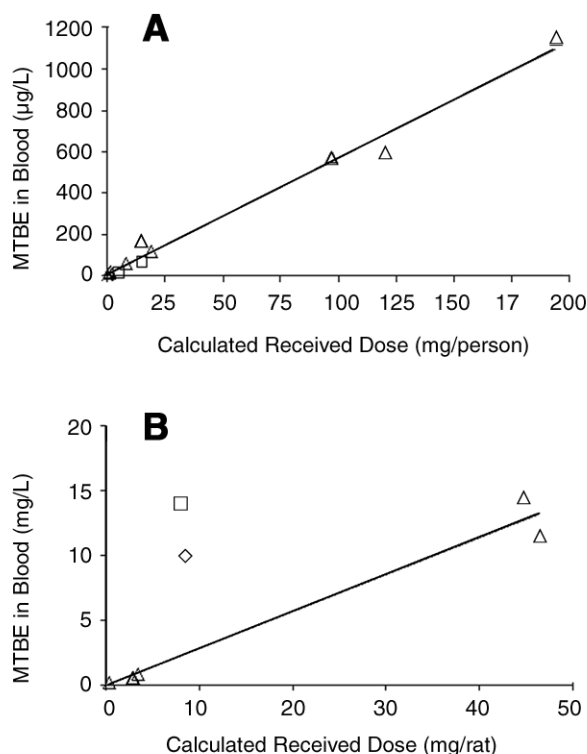
Commentary Figure 1. Correlation between calculated received dose and average peak MTBE levels in blood in humans and rats. (A) Human data from Commentary Table 2. △ = Inhalation, □ = ingestion. The linear regression line is based on inhalation and ingestion data. (B) Rat data from Commentary Table 2. △ = Inhalation, □ = gavage, ◇ = intravenous injection. Note that the highest inhalation dose of the Miller study is not included in this graph. The linear regression line is based on the inhalation data shown in the graph.

phase the MTBE half-life varies from 15 minutes to 1.3 hours (Nihlén et al 1998a; Dekant et al, this report). In the third elimination phase much longer half-lives of 8 to 30 hours have been reported (Buckley et al 1997; Nihlén et al 1998a; Dekant et al, this report). The differences in reported elimination rates may reflect differences in the exposure conditions and in blood sampling procedures. For instance, when relatively few samples are obtained, fitting a pharmacokinetic model to the data becomes more difficult. When comparing elimination rates between exposure routes, the study by Dekant reported shorter half-lives after ingestion compared to inhalation in humans. The half-lives reported for elimination of MTBE from blood in rats are very similar between studies and range from 10 to 30 minutes (Miller et al 1997; Benson et al, this report; Dekant et al, this report), suggesting that the disposition of MTBE in rats is much faster than in humans. Because the elimination of MTBE is so rapid in rats, the frequency of sampling in rats may not have been adequate to record a rapid first phase.

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In general, these data suggest good correlation between inhalation exposure and corresponding peak blood levels in humans and in rats. This similarity indicates that MTBE blood levels can provide a reasonable biomarker for MTBE exposure levels, but only for at most a few hours after exposure. In addition, sensitive assays are needed to accurately measure the low MTBE blood levels after environmental exposure in the parts per billion or low parts per million range. For instance, MTBE blood levels after the 4-ppm exposure in rats were below the limit of detection in the Benson study but were detected in the Dekant study in which a more sensitive assay was used.

MTBE METABOLISM AND BIOMARKERS

Previously, the metabolic pathway for MTBE and other ethers was shown to involve CYP enzymes in the conversion to formaldehyde and TBA, which is then converted to 2-methyl-1,2-propanediol and 2-hydroxyisobutyrate. The study by Hong et al has demonstrated that CYP2A6, in addition to CYP2E1, is involved in the metabolism of MTBE as well as ETBE and TAME. The study by Dekant et al has confirmed the metabolic pathways, further characterized the metabolites formed from MTBE and ETBE, and established the metabolic pathway for TAME. The metabolic pathway for ETBE was shown to be virtually identical to the pathway for MTBE, which considerably expands the limited knowledge of ETBE metabolism. TBA is a candidate for use as a biomarker of MTBE exposure due to a longer half-life than MTBE (8 hours, Dekant et al this report) and the fact that it reaches blood levels comparable to MTBE blood levels. However, the fact that TBA is formed in the body as a breakdown product of other alcohols impairs its suitability as a biomarker for ethers added to gasoline. Metabolites formed exclusively from MTBE metabolism would be better candidates. The study by Dekant has identified several metabolites that could be used as biomarkers and established an analytic method to detect them at low levels. Now that these analytic tools have been established, further toxicokinetic studies are recommended.

One of the important contributions of the Dekant study is the demonstration that metabolic pathways of MTBE and TAME are very similar after inhalation and ingestion. This has important implications for use of the much more extensive data on toxicity of inhaled MTBE for the risk assessment of exposure to MTBE in water. Dekant used 5 and 15 mg per person (in 100 ml, which is equivalent to 50 and 150 mg/L). These doses and exposures are well above what is expected to occur through ingestion of contaminated drinking water. Average environmental exposure levels have been reported at 0.25 ppb (0.34 ng/L) with a

maximum of 2 ppb (2.7 ng/L) from atmospheric deposition and an average of 0.36 ppb (0.49 ng/L) with a maximum 64 ppb (86.5 ng/L) as a worst case from environmental contamination due to gasoline spills (Stern and Tardiff 1997). The current drinking water advisory issued by EPA is 20 to 40 μg/L, a level recommended to avoid unpleasant taste and odor.

MTBE is usually inhaled as a mixture with gasoline vapors during refueling. This exposure mechanism raises an important issue addressed by the Benson study of whether exposure to gasoline containing MTBE would alter the uptake, distribution and elimination of MTBE. Benson showed that gasoline coexposure reduced the uptake and increased the elimination rate for MTBE in some cases. The results were somewhat inconsistent, however: elimination rates increased after a single exposure to 20 ppm LFG containing 4 ppm MTBE but not after repeated coexposure to the same dose. In contrast, both single and repeated coexposure to the 200-ppm dose of LFG containing 40 ppm MTBE increased the elimination rate significantly compared to the equivalent MTBE dose. These results imply that coexposure may reduce the possibility of observing acute health effects of MTBE, although these results need to be confirmed.

SUSCEPTIBLE POPULATIONS

Several reports have suggested that a subgroup of the population may be more sensitive to exposure to MTBE-containing gasoline (Moolenaar et al 1994; White et al 1995a). Some studies have exposed human subjects in a controlled setting while measuring symptoms such as eye and nose irritation. Under these controlled conditions, reporting of symptoms was not significantly increased during exposure to MTBE (Prah et al 1994; Cain et al 1996; Nihlén et al 1998c), even though subjects should be able to detect exposure to MTBE due to its odor. A recent study used gasoline coexposure to mask the smell of MTBE, and subjects were unable to distinguish between the gasoline vapors with or without MTBE (Fiedler et al 2000). Nonetheless, self-reported sensitive subjects reported more symptoms when exposed to gasoline vapor containing 15% MTBE compared to 11% MTBE or no MTBE although a clear dose-response relationship could not be established.

The study by Hong in this report approached the question of why some people may react differently when exposed to MTBE-containing gasoline. The hypothesis tested was that liver enzymes responsible for elimination of MTBE from the body may be polymorphic and may function at a lower catalytic rate in sensitive people compared to the population at large. Hong focused on one par-

ticular liver enzyme, CYP2A6, and identified three variants (at least one of which had significantly reduced activity in metabolizing ethers). Hong then determined that the variants were present in a low frequency (one in 23) in blood samples from self-reported sensitive people. Much larger studies will be required to define the distribution of these isozymes in the general population and to ascertain whether any of the CYP2A6 variants substantively contribute to sensitivity to MTBE. Polymorphisms in other enzymes involved in the metabolism of ethers should also be considered.

RECOMMENDATIONS

The studies reported here have furthered our knowledge on the metabolism of ethers added to gasoline and, in particular, have helped us to understand the similarities between metabolism of inhaled and ingested MTBE as well as the effects on uptake and metabolism of MTBE coexposed with gasoline.

Investigations are recommended to extend the toxicokinetic data for these ethers after inhalation and ingestion. What are the fate and potential health effects of metabolites, including their potential to cause cancer? Is formaldehyde the major metabolite with carcinogenic potential (see Kerns et al 1983; Cirvello et al 1995; International Agency for Research on Cancer 1995)? What is the role of tissues other than liver in metabolizing ethers? What are the possible effects of gasoline coexposure on ether uptake, distribution and excretion in humans? Conversely, what is the effect of MTBE exposure on the uptake, distribution and elimination of other potentially more harmful gasoline components (such as benzene)?

Further research is also necessary before the extent and validity of polymorphisms as indicators of individual susceptibility can be demonstrated. What is the relative importance of various CYP isozymes for ether metabolism in vivo? What is the frequency of enzyme polymorphisms relevant to the metabolism of ethers added to gasoline in the general population? Do the resulting phenotype differences make some individuals more susceptible to potential health effects of ether exposure? What is the metabolic basis for differential responses to exposure to ethers?

Although many questions remain about toxicity of MTBE metabolites and sensitivity of certain individuals, the data presented in this report suggest that previous data on ether metabolism after inhalation can be valuable for risk assessment of both inhalation and ingestion exposures. In addition, findings that coexposure with gasoline decreases MTBE uptake suggest that the health effects of exposure to these oxygenates are not likely to be greater,

and may be somewhat less, than those previously reported.

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ABBREVIATIONS AND OTHER TERMS

CYP	cytochrome P450
ETBE	ethyl *tert*-butyl ether
GC/MS	gas chromatography/mass spectrometry
LFG	light fraction of gasoline
MTBE	methyl *tert*-butyl ether
NMR	nuclear magnetic resonance spectroscopy
r	bivariate correlation coefficient
r^2	bivariate coefficient of determination
TAME	*tert*-amyl methyl ether
TBA	*tert*-butyl alcohol
TWA	time-weighted average

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