Journal of Toxicology and Environmental Health, Part A

ISSN: 1528-7394 (Print) 1087-2620 (Online) Journal homepage: https://www.tandfonline.com/loi/uteh20

The Metabolic Rate Constants and Specific Activity of Human and Rat Hepatic Cytochrome P-450 2E1 Toward Toluene and Chloroform

John C. Lipscomb , Hugh A. Barton , Rogelio Tornero-Velez , Marina V. Evans , Sattar Alcasey , John E. Snawder & John Laskey

To cite this article: John C. Lipscomb , Hugh A. Barton , Rogelio Tornero-Velez , Marina V. Evans , Sattar Alcasey , John E. Snawder & John Laskey (2004) The Metabolic Rate Constants and Specific Activity of Human and Rat Hepatic Cytochrome P-450 2E1 Toward Toluene and Chloroform, Journal of Toxicology and Environmental Health, Part A, 67:7, 537-553, DOI: 10.1080/15287390490425588

To link to this article: https://doi.org/10.1080/15287390490425588

Published online: 12 Aug 2010.

Journal of Toxicology and Environmental Health, Part A, 67:537–553, 2004

ISSN: 1528–7394 print / 1087–2620 online
DOI: 10.1080/15287390490425588

Taylor & Francis
healthsciences

THE METABOLIC RATE CONSTANTS AND SPECIFIC ACTIVITY OF HUMAN AND RAT HEPATIC CYTOCHROME P-450 2E1 TOWARD TOLUENE AND CHLOROFORM

John C. Lipscomb,[1] Hugh A. Barton,[2] Rogelio Tornero-Velez,[3] Marina V. Evans,[2] Sattar Alcasey,[2] John E. Snawder,[4] John Laskey[5]

[1]U.S. Environmental Protection Agency, Office of Research and Development, National Center for Environmental Assessment, Cincinnati, Ohio, USA
[2]U.S. Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Research Triangle Park, North Carolina, USA
[3]U.S. Environmental Protection Agency, Office of Research and Development, National Exposure Research Laboratory, Research Triangle Park, North Carolina, USA
[4]Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Cincinnati, Ohio, USA
[5]National Caucus and Center on Black Aged Inc.–Senior Environmental Program (NCBA-SEP), Research Triangle Park, North Carolina, USA

Chloroform ($CHCl_3$) is a near-ubiquitous environmental contaminant, a by-product of the disinfection of drinking water sources and a commercially important compound. Standards for safe exposure have been established based on information defining its toxicity, which is mediated by metabolites. The metabolism of $CHCl_3$ is via cytochrome P-450 2E1 (CYP2E1)-mediated oxidation to phosgene, which is known to obey a saturable mechanism. CYP2E1 is a highly conserved form, expressed in all mammalian systems studied, and is responsible for the metabolism of a great many low-molecular-weight (halogenated) compounds. However, the Michaelis–Menten rate constants for $CHCl_3$ oxidation have not been derived in vitro, and the specific activity of CYP2E1 toward $CHCl_3$ has not been reported. In this investigation with microsomal protein (MSP), apparent V_{max} values of 27.6 and 28.3 nmol/h/mg MSP and apparent K_m values of 1 and 0.15 μM in rats and human organ donors, respectively, were demonstrated. The specific activity of CYP2E1 toward $CHCl_3$ in rats and humans was 5.29 and 5.24 pmol/min/pmol

Accepted 24 October 2003.

The views expressed in this article are those of the authors and do not necessarily reflect the views and policies of the U.S. Environmental Protection Agency. This article (NCEA-C-1429) has been reviewed and cleared for public release in accord with the policies of the U.S. EPA, Office of Research and Development, National Center for Environmental Assessment. The mention of trade names does not imply endorsement. The authors are grateful to Cindy Linsky of the North Carolina State University for undertaking critical initial studies and to Dr. Harlal Choudhury for critical comments during manuscript preparation. The authors express their sincere gratitude to organ donors and their families, for without their generous contributions, studies like these would not be possible.

Present address for Sattar Alcasey is Access Business Group, ILL, Ada, MI, USA.

Address correspondence to John C. Lipscomb, U.S. Environmental Protection Agency, Office of Research and Development, National Center for Environmental Assessment, Cincinnati, OH 45268, USA. E-mail: lipscomb.john@epa.gov

CYP2E1, respectively. Toluene metabolism to benzyl alcohol (BA), another CYP2E1-dependent reaction, was also highly dependent on CYP2E1 content in humans, and was more efficient than was $CHCl_3$ metabolism. The specific activity of human CYP2E1 toward toluene metabolism in human MSP was 23 pmol/min/pmol CYP2E1. These results demonstrate that differences in CYP2E1 content of MSP among individuals and between species are largely responsible for observed differences in toluene and $CHCl_3$ metabolism in vitro.

Cytochrome P-450 2E1 (CYP2E1) is a member of the family of membrane-bound "microsomal" enzymes in mammals, which is (1) constitutively expressed at different levels between species and amongst humans, accounting for approximately 10% of spectrally determined P-450 content (Davis et al., 2002; Shimada et al., 1994, 1999; Snawder & Lipscomb, 2000), (2) responsible for the metabolism of a great many low-molecular-weight halocarbons, and (3) the form responsible for metabolism of low concentrations of solvents. For these reasons, CYP2E1 phenotypic and genotypic variations have been evaluated as a biomarker for exposure to volatile organic compounds (Lucas et al., 2001). While several polymorphisms in the CYP2E1 gene are known, studies evaluating the quantitative impact of CYP2E1 genetic polymorphisms on metabolic activity continue, with few conclusive results to date.

Toluene is used (1) as a solvent for paints, lacquers, and adhesives, (2) as a component of gasoline, and (3) in numerous manufacturing processes. Toluene is a groundwater contaminant whose conversion to benzyl alcohol (BA) is catalyzed by CYP2E1 at low concentrations (Wang & Nakajima, 1991). Liver and kidney toxicity (weight changes) have been used to quantify human health risk to toluene following oral exposure (NTP, 1989). Foo et al. (1990) demonstrated significant reduction in performance on six out of eight neurological tests following occupational toluene exposure, and diminished performance showed a negative correlation with exposure. Low to moderate toluene exposure also produces tiredness, confusion, weakness, drunken-type actions, memory loss, nausea, loss of appetite, and loss of hearing and color vision. These symptoms usually disappear when exposure is stopped. High levels of toluene may produce nephrotoxic effects (ATSDR, 2001). Liver and kidney weight changes were identified as the most sensitive (critical) effect for setting the oral noncancer reference dose (RfD), and neurological effects were identified as the critical effect in setting inhalation noncancer reference concentration (RfC) (U.S. EPA, 2003).

Chloroform ($CHCl_3$) is a problematic drinking-water contaminant and by-product of the disinfection of drinking water with chlorine (Richardson, 1998; Rook, 1974). $CHCl_3$ is carcinogenic to rats, and assessment of its risk involves nonlinear concentration extrapolation because of the formation of reactive metabolites via CYP2E1 and their detoxication by normal cellular systems, including glutathione (Brown et al., 1974; Sipes et al., 1977). Cytotoxicity is evident following exposure to $CHCl_3$ and appears to be dependent on the formation of reactive metabolites (phosgene) from $CHCl_3$ and the interaction of phosgene with cellular components. Phosgene is scavenged and cytotoxicity

is reduced with glutathione supplementation. When the rate of formation of reactive metabolites exceeds the rate of detoxication and/or repair, cytotoxicity occurs, predisposing the tissue to a carcinogenic response (U.S. EPA, 2001). $CHCl_3$ metabolism has been assessed through various experimental designs, in several mammalian species, and with differing degrees of specificity. Studies with rodent liver preparations have indicated that CYP2E1 is the enzyme most responsible for $CHCl_3$ metabolism at low concentrations (Brady et al., 1989; Testai et al., 1996). Although other enzymes can contribute to $CHCl_3$ metabolism at higher concentrations (Testai et al., 1996), these concentrations are unlikely to be found in tissues of humans exposed to $CHCl_3$ through drinking water or by inhaling $CHCl_3$, even at occupational exposure limits (threshold limit value and time-weighted average value of 10 ppm; ACGIH, 2003). Human CYP2E1 activity toward trichloroethylene in vitro indicated Michalelis–Menten kinetics (Lipscomb et al., 1997), and the extrapolation of these enzyme kinetic parameters into physiologically based pharmacokinetic (PBPK) models resulted in successful simulation of blood concentration–time profiles in the human (Lipscomb et al., 1998a). Since that time, new data demonstrating the variance in CYP2E1 content in adult human liver have been produced from studies with multiple liver tissue samples from human organ donors (Lipscomb et al., 2003a). The incorporation of data describing variance of enzyme content into PBPK models can aid in the determination of the degree to which variance in CYP2E1 metabolic capacity (a function of enzyme content and enzyme activity) influences the formation of reactive metabolites and thus impact risk (Kedderis & Lipscomb, 2003). However, data on enzyme kinetic parameters, also expressed at the level of CYP2E1 itself, rather than at more crude measures (e.g., product per time per milligram microsomal protein; MSP), need to be available. Because Michaelis–Menten kinetic parameters for $CHCl_3$ biotransformation were not available in any species, the present investigation was undertaken to determine these as well as specific activity of CYP2E1 for $CHCl_3$ in samples of hepatic microsomal protein derived from human organ donors and rats. Once determined, the enzyme kinetic parameters for $CHCl_3$ can be combined with data describing the tissue content of CYP2E1 with tissue concentrations of $CHCl_3$ to predict rates of $CHCl_3$ metabolism in the tissue.

METHODS AND MATERIALS

Chemicals

Chloroform (CAS number 67-66-3, 99%), D-glucose 6-phosphate (98%), β-NADP (97%, sodium salt), toluene, acetonitrile, and benzyl alcohol were obtained from Sigma (St. Louis, MO). D-Glucose-6-phosphate dehydrogenase (99%, type VII, 372 U/mg protein) was purchased from Fluka Chemie AG (Buchs, Switzerland). Bradford reagent (Coomassie blue dye) and bovine serum albumin protein standard were obtained from Bio-Rad Laboratories (Hercules, CA).

Animals

Male Fischer-344 rats (217–243 g) were obtained from Charles River Laboratories (Raleigh, NC) and were employed in $CHCl_3$ metabolism studies. Animals were maintained in an AAALAC-accredited animal facility according to National Institutes of Health Guidelines for Care and Use of Laboratory Animals (National Research Council, 1996). Animals were housed in polycarbonate cages on cedar bedding, maintained at 25 °C and 50% relative humidity with a 12-h light/dark cycle. Animals were supplied with Purina rat chow 5001 and tap water ad libitum for 5 d prior to euthanization.

Rat Microsomes

Rats were euthanized with CO_2 and bled by cardiac puncture. Livers were excised, minced, and washed 4 times with 10- to 15-ml aliquots of ice-cold 0.1 *M* sodium phosphate buffer (pH 7.4). A 13.3% (w/v) crude homogenate was prepared in a glass Potter–Elvehjem homogenizer by combining 7.5 ml freezing buffer/g rat liver. The freezing buffer consisted of 10% glycerol (v/v), 250 m*M* sucrose, 1 m*M* dithiothreitol, 0.5 m*M* ethylene diamine tetraacetic acid (EDTA), and 35 m*M* KCl (pH 7.4). After homogenization, the preparation was centrifuged at 15,000 × *g* for 15 min at 4 °C, and the supernatant (S9) was stored at –80 °C. On the day of the experiment, S9 was thawed and centrifuged at 105,000 × *g* for 60 min at 4 °C. The resulting pellet was suspended in 5 ml of 0.1 *M* sodium phosphate buffer (pH 7.4) and designated "microsomes." The protein concentration was 3.62 mg/ml according to the method of Bradford (1976). Microsomal oxidation activity, based on the rate of hydroxylation of *p*-nitrophenol (Reinke & Moyer, 1985), was 1.4 nmol/min/mg MSP. Liver microsomes used for studies with toluene were derived from adult male Sprague-Dawley rats were a generous gift from Dr. Marybeth Genter, University of Cincinnati.

Human Microsomes

Three samples of hepatic MSP from human organ donors were obtained from the International Institute for the Advancement of Medicine (IIAM, Exton, PA) and used to determine the specific activity of human CYP2E1 toward $CHCl_3$. A description of their characteristics is presented in Table 1. The protein content of each sample was 20 mg protein/ml. Demographic information was not available for the 10 samples used to determine toluene metabolism; they were graciously provided by IIAM.

Determination of Individual P-450 Forms in Rat and Human Liver Samples

Content of specific P-450 forms was estimated by a direct enzyme-linked immunosorbent assay (ELISA) (Snawder & Lipscomb, 2000). Briefly, 0.5 µg microsomal protein or homogenate protein per well was plated to microtiter

TABLE 1. Human Organ Donor Demographic Information

Age/sex[a]	Donor	HHM[b]	COD[c]	Height/weight	BMI[d]	Alcohol consumption	Cigarette smoking
45 CM	730961	226	SAH	6′1″/200 lb	26.4	12 Beer/wk	5–6 ppd
54 CM	628951	234	SAH	5′7″/135 lb	21.2	1 q/day × 30–40 yr	2 ppd × 30–40 yr
42 CF	625961	244	Anoxia	6′6″/209 lb	24.2	NR	1.5 ppd × 25 yr

Note. Adapted from Lipscomb et al. (2003a).

[a] Samples identified by age in years, ethnic background, and sex: C, caucasian; M, male; F, female. Cigarette smoking is reported in packs per day (ppd); ethanol consumption was subjectively reported. NR, a negative response was given for cigarettes or ethanol.

[b] Human hepatic microsome sample number, Tissue Transformation Technologies, Edison, NJ.

[c] Cause of death: SAH, subarachnoid hemorrhage.

[d] BMI, body mass index, calculated as $kg/(m^3)$.

plates (carbonate–bicarbonate buffer, pH 9) along with microsomes containing a known quantity of the P-450 form (Gentest, 1000 fmol–1 fmol cytochrome P-450/50 µl) of interest for a standard curve. Plates were incubated overnight at 4 °C and the plating solution was removed the following morning. One hundred microliters of 50% fetal bovine serum (FBS) in phosphate-buffered saline (PBS) was added as a blocking agent and plates were incubated for 1 h at 37 °C. The blocking agent was removed and plates were washed 3 times (TBS-Tween, 10% FBS) and incubated at 37 °C for 1 h with primary antibody (anti-CYP2E1, Gentest, Woburn, MA). Primary antibody was removed, plates were washed and then incubated 1 h with 200 µl/well of anti-goat alkaline phosphate conjugate. The secondary antibody was removed, plates were washed, and 150 µl K-Gold premixed ELISA phosphatase substrate (ELISA Technologies, Lexington, KY) was added to each well. After 30 min each plate was read at 405 nm. Absorbance of sample-containing wells was compared to a standard curve. Values were expressed as picomoles P-450 formed per milligram protein.

Chloroform Exposure System

The exposure system as described in Tornero-Velez et al. (2003) consisted of a 118-ml three-necked glass vessel immersed in a water bath maintained at 37 °C, 1/4 in (ID) stainless-steel tubing, and a recirculating pump (Figure 1). All internal surfaces consisted of glass, stainless steel, or Teflon, and the total system volume was 135 ml. The headspace atmosphere was zero grade air (air at 20.5–21.5% oxygen, containing no more than 1 ppm CO, 5 ppm CO_2, 5 ppm water, and 0.1 ppm total hydrocarbons), and was recirculated through an autosampling device equipped with a 200-µl sample loop attached to a Hewlett-Packard (Palo Alto, CA) model 5890 series II gas chromatograph (GC). The GC was equipped with 1/8-in ID × 6-ft length stainless-steel column packed with 0.1% SP-1000 on 80/100 mesh Carbopack C (Supelco; Bellefonte, PA). A flame ionization detector (FID) was used to quantify $CHCl_3$ in the headspace. Operating conditions were as follows: column/oven temperature 110 °C, and FID temperature 250 °C. The flow of the carrier gas, helium, was 10.5 ml/min;

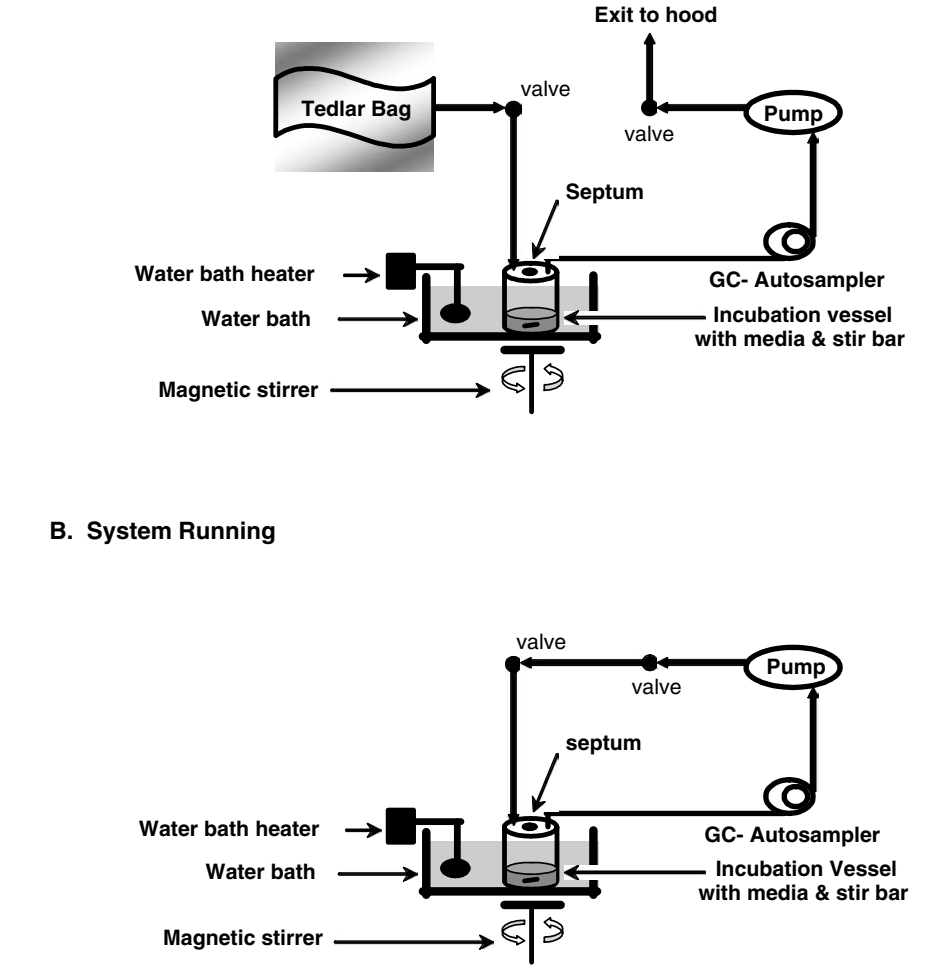


FIGURE 1. Diagram for the automated recirculating gas uptake system.

air, 400 ml/min; and hydrogen, 40 ml/min. The retention time of $CHCl_3$ was 1.28 min, allowing ample time for serial headspace sampling at 3-min intervals.

Incubation of Fischer F-344 Rat Microsomes with Chloroform

Initial experiments to establish the in vitro gas uptake system were performed with rat hepatic microsomal protein (MSP). In these exposures the incubation medium consisted of 14 ml 0.1 *M* Tris buffer, containing 5 m*M* $MgCl_2$ (pH 7.4) and 1 ml rat liver microsomal suspension (3.62 mg MSP).

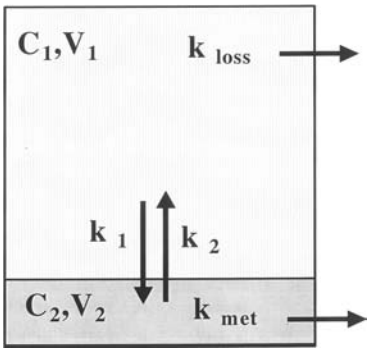
Chloroform was added as vapor into the system to bring headspce concentrations to 2.9, 2.3, 2.1, 7.5, 7.6, 7.7, 25.6, 26.1, 25.4, 95.3, 95.0, 94.5, 302, 303, or 304 ppm. After $CHCl_3$ achieved equilibrium between gas and liquid, metabolism was initiated (at 66–90 min) by injection of 1000 µl of a standard NADPH-generating system in 0.1 *M* potassium phosphate (Lipscomb et al., 1997). The final concentration of $NADP^+$ was 0.66 m*M*; glucose 6-phosphate, 13.7 m*M*; and glucose-6-phosphate dehydrogenase 2.8 units/ml. The final rat liver MSP concentration in the medium was 0.23 mg/ml. Concentrations of $CHCl_3$ in headspace were assessed (1) without buffer, MSP, or NADPH regenerating system, (2) containing only buffer and MSP, and (3) containing buffer, MSP, and NADPH regenerating system.

Incubation of Human Microsomes With Chloroform

The incubation medium consisted of 160 µl microsomal suspension and 2.84 ml buffer. Because stock suspensions of human MSP were at 20 mg MSP/ml, this resulted in 3.2 mg human MSP in the incubation, similar to that employed in the rat studies. The reduction in volume between the rat and human exposure systems was done to reduce the ratio of buffer:MSP in human studies. Chloroform was added as vapor into the system resulting in equilibrium headspace concentrations of 1.24, 1.24, 9.6, 9.6, 90.6, or 92.6 ppm for human donor 226; 1.16, 1.42, 9.67, 9.35, 90.6, or 91.4 ppm for human donor 234; and, 1.17, 1.09, 9.7, 9.42, 90.1, or 91.9 ppm for human donor 244. Metabolism was initiated at 30 min by injection of 300 µl of NADPH-generating system, resulting in a final reaction volume of 3.3 ml and a final microsomal protein concentration of 0.97 mg/ml.

Calculation of Chloroform Metabolic Rate Constants

Concentration–time course were analyzed according to the two-compartment model of Kedderis and Held (1996), modified to account for system losses and the loading of vapor-phase CHC3 into the system (Figure 2). The uptake of substrate ($k1$) into the medium and the liquid–gas partition coefficient (P) of $CHCl_3$ were obtained independently from the metabolic parameters. For rat microsomal preparations it was estimated that $k_1 = 1.8\,h^{-1}$ and $P = 2.6$. For human microsomal preparations it was estimated that $k_1 = 1.25 h^{-1}$ and $P = 1.75$. Microsomal metabolism of $CHCl_3$ was assumed to follow Michaelis–Menten kinetics. The apparent maximum rate of metabolism, V_{max}, and Michaelis–Menten constant, K_m, were obtained by fitting the two-compartment model to sets of measured concentration-time courses. For rat microsomal preparations, an overall system loss rate ($k_{loss} = 0.05\,h^{-1}$) was used to account for nonspecific system losses. For human microsomal preparations a loss rate of $0.0585\,h^{-1}$ ($SE = 0.0063\,h^{-1}$) was observed. Loss rates were determined by regressing log (concentration) on time, for the portion of the concentration–time courses between equilibration and cofactor addition (the time period between 15 to 30 min after the addition of $CHCl_3$). Coupled differential equations were written in Matlab (The MathWorks, Natick, MA) and solved



Differential equations related to kinetic model:

Gas phase (1): $V_1 dC_1/dt = S_1 Q C_0 G + k_1 V_1 C_2/P - k_1 V_1 C_1 - k_{loss} V_1 C_1$

Liquid phase (2): $V_2 dC_2/dt = -k_1 V_1 C_2/P + k_1 V_1 C_1 - S_2 C_2 V_{max}/(K_m + C_2)$

V_1 volume of gas phase (L)
V_2 volume of incubation medium (L)
C_1 substrate concentration in the gas phase (μmol/L)
C_2 substrate concentration in the medium (μmol/L)
k_1 rate constant of substrate uptake into medium (h)
k_2 rate constant of substrate distribution into the incubation medium (h^{-1})
k rate constant of substrate loss in headspace compartment (h^{-1})
P equilibrium partition coefficient; [$P = C_2/C_1 = (k_1 V_1/k_2 V_2)$]

System Charging (2 min)

S_1 switch indicating system open [1 = open (Figure 1A)]
C_0 substrate concentration in Tedlar bag (Figure 1)
Q flow rate of pump (L)
G $G = \exp(-Q/V_1)t$

System Running

S_1 system closed; [0 = closed (Fig. 1B)]
S_2 switch indicating presence of cofactor; [0 = no, 1 = yes]
V_{max} maximum rate of metabolism (μmol/h)
K_m apparent Michaelis-Menten constant (μmol/L)

FIGURE 2. Conceptual and mathematical depiction of the two-compartment model used to describe the kinetics of the chloroform clearance the in gas uptake system.

with a variable-step, variable-order integration routine. The Nelder–Mead simplex optimization algorithm was employed to determine the parameter values that minimized a weighted least-squares criterion. Chloroform metabolism was calculated from data describing the removal of $CHCl_3$ from headspace. This is justified by the correction of rates of removal in NADPH-stimulated microsomal protein by rates of loss observed in the same system containing buffer only and heat-inactivated microsomal protein. Data on the CYP2E1 content of MSP samples employed were used to convert maximally observed rate values to units of specific activity (pmoles $CHCl_3$/min/pmol CYP2E1).

Toluene Metabolism

Rat and human liver microsomes were incubated with toluene and benzyl alcohol (BA) formation monitored by high-performance liquid chromatography (HPLC) based on modifications of procedures of Nakajima et al. (1997) as described in Davis et al. (2002). Briefly, 200 µg MSP was incubated for 5 min in 0.1 m*M* Tris buffer containing 5 m*M* $MgCl_2$ and 1 to 10 m*M* toluene. All reactions were initiated by the introduction of an NADPH-regenerating system and were quenched 30 min later by the addition of 0.2 ml cold acetonitrile. Reverse-phase HPLC performed using an Agilent 1100 system (Palo Alto, CA) equipped with a 4.6 mm ID × 250 mm Zorbax C-18 column. BA was quantified against an external standard curve of known concentrations of authentic BA. The mobile phase was isocratic, 70/30 water/acetonitrile at pH 3, and a flow rate of 0.2 ml/min. Under these conditions, BA demonstrated a retention time of 21.7 min. Area under the curve for the peak was integrated to quantify BA present, values were expressed as nanomoles BA formed per minute per milligram MSP. Data on the CYP2E1 content of human and pooled rat liver MSP were used to convert values to nanomoles BA formed per minute per picomole CYP2E1. Linear regression on rate of BA formation was performed using Sigmaplot 5.0 (Jandel Scientific, San Rafael, CA).

RESULTS

Results from the ELISA determination of CYP2E1 demonstrated a linear detection of authentic CYP2E1 standard over the range of 0–1000 fmol/well (data not shown). The ELISA method has been previously employed to quantify CYP proteins, without regard to catalytic activity, in samples of isolated microsomal protein (Davis et al., 2002; Lipscomb et al., 2003a; Snawder & Lipscomb, 2000) and in liver homogenate (Deluca et al., 2000; Lipscomb et al., 2003a). Samples of human microsomal protein were procured from the supplier based on information of a relatively high activity toward the probe substrate, chlorzoxazone, indicative of the activity of CYP2E1, and because they had been previously demonstrated to possess a CYP2E1 content (pmol CYP2E1/mg MSP) that was appreciably higher than the mean value reported for human MSP of approximately 50 pmol CYP2E1/mg MSP (Lipscomb et al.,

2003a; Shimada et al., 1999). In this study of both rat and human MSP, the same samples of MSP were employed for CYP2E1 quantification and toluene and $CHCl_3$ metabolism. Rat MSP contained 87 pmol CYP2E1/mg MSP, and human samples contained 83 to 100 pmol CYP2E1/mg MSP (Table 2). Previous examination of the CYP2E1 content of liver homogenate from these 3 samples indicated a CYP2E1 content in intact liver ranging from 2147 (sample 628951) to 2591 (sample 625961) pmol CYP2E1/g tissue; by dividing the CYP2E1 content of intact liver by the CYP2E1 content of matching MSP, an MSP content of 27 (sample 628951) to 34.4 mg MSP/g tissue (sample 625961) was determined. Based on an assumed MSP content of 50 mg MSP/g rat liver, rat liver was estimated to contain 4350 pmol CYP2E1/g. Initial experiments with rat hepatic microsomal protein indicated that $CHCl_3$ uptake from headspace was dependent on time and on the concentration of MSP present in the system (data not shown). No evaluations of the activity of CYP2E1 toward typical probe substrates, such as *p*-nitrophenol, chlorzoxazone, or dimethylnitrosamine, were performed to verify the integrity of enzyme activity following the 3-h incubation period. However, the rate of uptake of $CHCl_3$ from headspace during the latter period of incubation appeared equivalent to that measured during the first segment of the postdistribution phase, leading to the conclusion that CYP2E1-dependent metabolic activity remained intact during the incubation period. Concentrations of $CHCl_3$ in headspace declined more rapidly in the presence of an NADPH-regenerating system system than in its absence, or in reference vials. Figure 3 demonstrates the removal of $CHCl_3$ from chamber atmosphere by rat liver microsomes stimulated by the addition of NADPH. To aid clarity of presentation, curves showing loss in the absence of NADPH were not presented. Loss rates from either those curves or from the postequilibrium, pre-NADPH stimulation period were used in calculations. Loss rates from incubations containing medium alone and medium containing heat-inactivated microsomal protein were approximately 0.05 h^{-1}, consistent with the loss rate observed before the addition of cofactor to incubations containing viable microsomal protein.

By correcting for background decreases in concentration (e.g., those resulting from nonspecific binding to protein and binding to vials), these decreases in concentration over time indicated an apparent V_{max} of 0.1 μmol/h. This value was divided by the content of rat liver MSP employed (3.62 mg) and corrected for units of time to yield a value of 0.46 nmol/min/mg rat MSP. In rats, MSP contained 87 pmol CYP2E1/mg MSP. Thus, the apparent V_{max} value for rats converts to 5.29 pmol/min/pmol CYP2E1. The apparent K_m value of 1 μ*M* in suspension was divided by the partition coefficient value of 2.6 to yield a K_m value of 9.6 ppm in headspace. Figure 4 demonstrates NADPH-stimulated removal of $CHCl_3$ from chamber atmosphere by human liver microsomes. Concentrations ranging downward to 1 ppm demonstrated substrate disappearance rates representing those under saturating substrate concentrations to those approaching first-order rates. Studies with 3 samples of human liver MSP indicate a mean specific activity of 5.24 (±0.1 S.D.) pmol/min/pmol CYP2E1.

TABLE 2. Cytochrome P-450 2E1 Content and Activity Toward Chloroform

Sample	K_m (μ*M*)	V_{max} (μmol/h/mg MSP)	pmol CYP2E1/mg MSP	V_{max} (nmol/min/mg MSP)	mg MSP/g liver[b,c]	Specific activity (pmol/min/pmol CYP2E1)	pmol CYP2E1/g liver[b,d]	Metabolic capacity (nmol/min/g liver)
Human 730961	0.256	0.0257	83[b]	0.428[a]	34.3	5.16	2439	12.59
Human 628951	0.113	0.0269	86[b]	0.448	27.0	5.21	2147	11.19
Human 625961	0.090	0.0322	100[b]	0.537	34.4	5.36	2591	13.89
Rat	1.0	0.0276	87	0.460	50	5.29	4350	23.01

[a] Because the three human MSP samples used in this study contained much higher CYP2E1 concentrations than previously reported mean values, these apparent V_{max} values should not be used to represent population mean values for human MSP activity toward $CHCl_3$; see Discussion.
[b] From Lipscomb et al. (2003a).
[c] Determined by dividing the CYP2E1 content of intact liver by the CYP2E1 content of MSP.
[d] Determined in homogenate protein and volumetrically corrected to intact tissue mass.

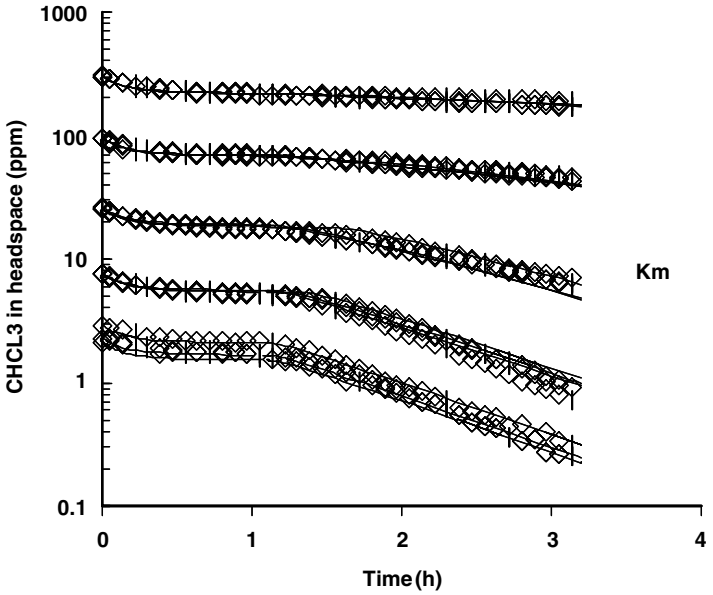


FIGURE 3. Concentration–time courses of $CHCl_3$ in the gas phase of the recirculating headspace system with incubation medium containing rat liver microsomes (0.23 mg protein/ml, pH 7.4, 37 °C). Experimental data are indicated with diamonds and the simulation with solid lines. Initial concentrations in headspace (average of triplicate runs) were 2.4, 7.6, 25.7, 94.9, and 303 ppm. An apparent K_m of 1.0 μ*M* (liquid phase concentration) was derived and converted into concentration in the gas phase from [(1.0 μ*M* in suspension × 24.45 M^{-1})/P = 2.6] = 9.4 ppm in headspace.

To examine the impact of variability in CYP2E1 content in MSP on metabolism of CYP2E1 substrates, BA formation from toluene was examined in 10 samples of human MSP obtained from individual, otherwise healthy, human organ donors. CYP2E1 content in these samples ranged from 34 to 151 pmol CYP2E1/mg MSP. Toluene metabolism demonstrated an increase proportional to time and MSP concentration (data not shown). Results in Figure 5 demonstrate the relationship between BA formation and CYP2E1 content. BA formation was 1.7 ± 1.06 (mean ± SD, range 0.81 to 3.82) nmol/min/mg MSP (CV = 62%), and 23.06 ± 3.85 (mean ± SD, range 19.31 to 30.8) pmol/min/pmol CYP2E1 (CV = 16%). Results from human samples (Figure 5, squares) demonstrate a marked correlation between CYP2E1 content and BA formation (R^2 = .909), and subjectively that the activity of rat CYP2E1 is similar to that of human CYP2E1.

DISCUSSION

Results describing $CHCl_3$ metabolism in preparations from rats and from individual human organ donors are presented in Table 2. Apparent V_{max} values among these human samples (rate/mg MSP) are similar in this investigation because human organ donors were selected with a high expression of CYP2E1

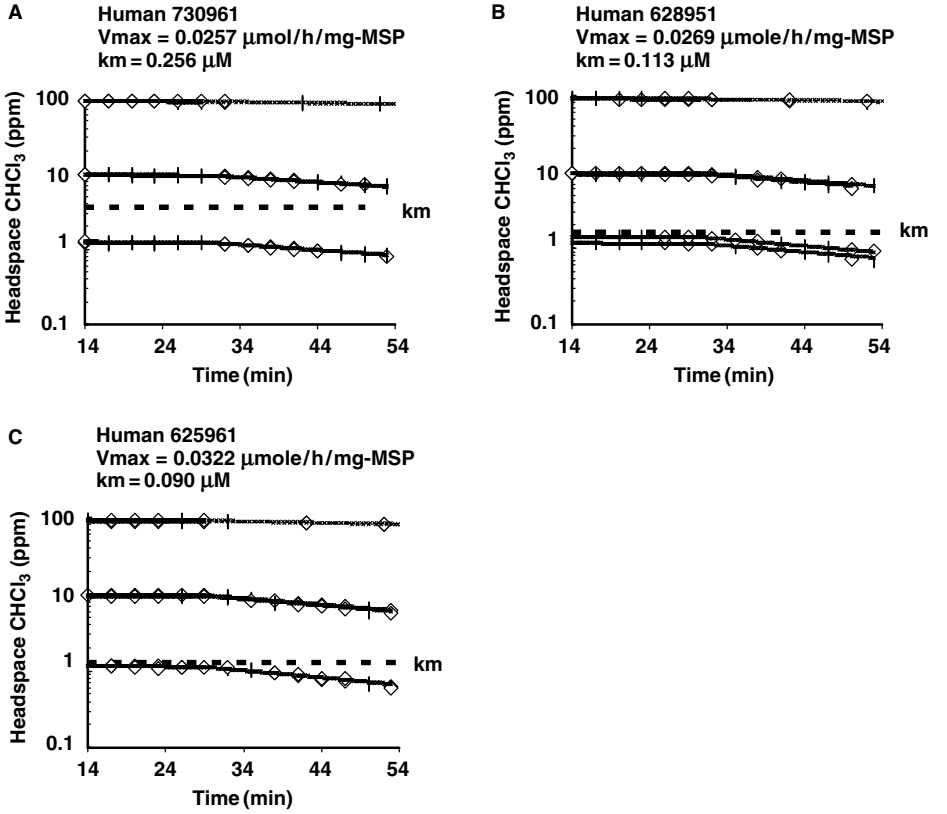


FIGURE 4. Concentration–time courses of $CHCl_3$ in the gas phase of the gas-uptake system with incubation medium containing liver microsomes of human donor samples 730961, 628951, and 625961. Experimental data, indicated with diamonds, and simulations, in solid lines, are shown postequilibration (14 min). Apparent K_m values in the gas phase are 3.58, 1.58, and 1.26 ppm, respectively.

per milligram MSP to better facilitate derivation of metabolic rate constants in incubations of rat and human MSP preparations containing roughly the same concentrations of lipid associated with MSP. Consistent with a conservation of the gene and protein across mammalian species, the specific activity of rat CYP2E1 for $CHCl_3$ is similar to the specific activity determined for human CYP2E1. The application of the specific activity of human CYP2E1 and the liver tissue content of CYP2E1 in adult human organ donors results in an estimation of tissue V_{max} (nmol/min/g tissue) roughly similar to that presented by Corley et al. (1990). Corley et al. (1990) calculated a V_{max} value for humans, expressed as 307 nmol/h/liver. Previously, the geometric mean value for CYP2E1 content of adult human liver was reported to be 2482 pmol CYP2E1/g (Lipscomb et al., 2003a). This value, combined with the specific activity value of 5.24 pmol/min/pmol CYP2E1, results in a V_{max} value of 780 nmol/h/g liver, roughly 2.5-fold higher than the value reported by Corley et al. (1990).

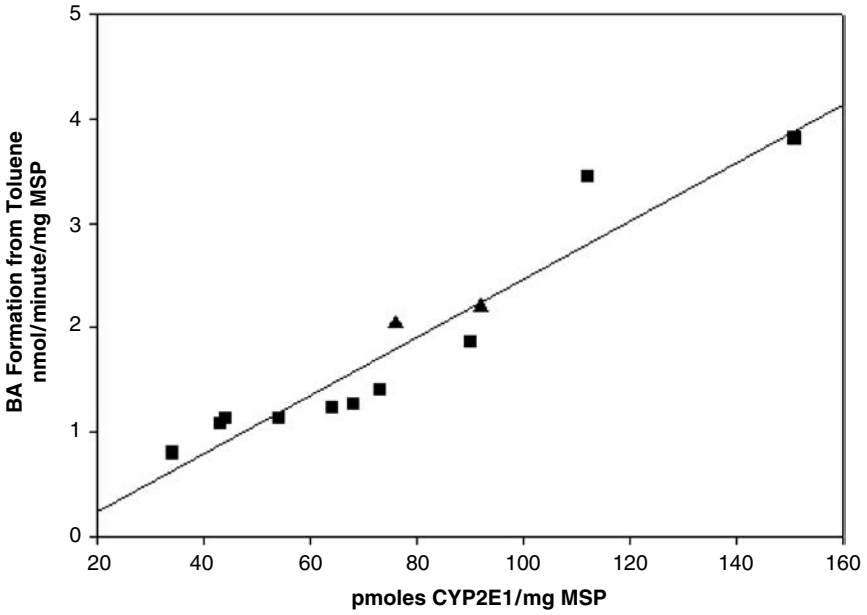


FIGURE 5. Benzyl alcohol (BA) formation from toluene in microsomal protein from rats and humans. Squares represent BA formation in samples of human MSP; triangles represent BA formation in two samples of pooled rat liver MSP. The solid line represents the results of a regression analysis of only human data. Results indicate that variability in CYP2E1 content of MSP significantly correlates with differences in toluene metabolism and subjectively, that the activity of rat CYP2E1 is very similar to that of human CYP2E1.

The human samples employed in our study contained CYP2E1 at concentrations above 80 pmol/mg MSP, a value appreciably higher than the geometric mean of 48.9 pmol/mg MSP demonstrated from a sample of MSP preparations from 60 adult human organ donors, including these 3 (Lipscomb et al., 2003b; Shimada et al., 1999). Therefore, a best estimate for the average V_{max} value in human MSP might be developed by combining the specific activity of 5.24 pmol/min/pmol CYP2E1 with a more central measure of CYP2E1 content in human MSP. The product of these values indicates a mean V_{max} value (48.9 pmol CYP2E1/mg MSP$\times$5.24 pmol $CHCl_3$/min/pmol human CYP2E1) of 256 pmol/min/mg human MSP, roughly 55% that of the V_{max} observed in rat MSP in this investigation. The magnitude of this difference roughly approximates the difference in the CYP2E1 content of liver MSP from these species. Further, the observed species differences in $CHCl_3$ metabolism in this study are consistent with species-dependent differences observed for other CYP2E1 substrates. Previously, rat, mouse, and human MSP were seperately employed to determine the kinetic parameters governing rates of trichloroethylene oxidation, a reaction catalyzed by CYP2E1. The values for the apparent V_{max} in pooled MSP from adult male Fischer 344 rats and adult humans were reported to be 4826 and 1440 pmol TCE/min/mg MSP, respectively (Lipscomb

et al., 1998b). The CYP2E1 content of these MSP preparations was not available. Further investigation of the human interindividual variability of MSP-dependent TCE oxidation in 15 individual human MSP samples indicated that the specific activity of human CYP2E1 for TCE oxidation ranged five-fold from 20 to 100 pmol/min/pmol CYP2E1, with a mean value approximating 32 pmol TCE/min/pmol CYP2E1 (Lipscomb et al., 2003b). Assuming a similar CYP2E1 content in MSP derived from Fischer 344 rats from these two sources, the apparent V_{max} for TCE oxidation can be converted by the value for CYP2E1 content in rat liver MSP derived in this study (87 pmol CYP2E1/mg MSP) to estimate a specific activity for TCE oxidation of 56 pmol TCE oxidized/min/pmol CYP2E1. This value is approximately two fold higher than the mean value in humans, but is near the middle of the observed range of values in humans.

Toluene is another occupationally and environmentally important solvent and substrate for CYP2E1-mediated oxidation. The study of toluene metabolism was a part of a larger, separate, and temporally remote project, which accounts for methodological differences in capturing chemical metabolism. Toluene metabolism by virus-expressed human recombinant CYP2E1 was also assessed by Nakajima et al. (1997). Among 11 CYP forms examined, CYP2E1 was the major contributor to toluene metabolism. In these studies, BA formation at 5 m*M* toluene was 140 pmol/min/mg microsomal protein, and the content of expressed CYP2E1 was 68.3 pmol/mg MSP. Together, these data indicate a specific activity, under these conditions, of 2.05 pmoles BA formed/min/pmol expressed CYP2E1. These values are substantially lower than those indicated in our present study (19–30 pmol BA/min/pmol CYP2E1). Nakajima et al. (1997) also demonstrated rates of BA formation in authentic human MSP (1.5 nmol/min/mg MSP) that were similar to our results. Differences in rates of BA formation between CYP2E1 expressed in MSP isolated from virus expression systems and CYP2E1 contained in samples of human MSP may be explained, in part, by differences in test systems employed, the effect of a lower substrate concentration employed in the Nakajima et al. (1997) studies (5 v. 10 m*M*), and the consistent relationship between reductases and CYP enzymes in our authentic MSP samples. Using rat liver MSP, Wang and Nakajima (1991) demonstrated kinetic constants for BA formation from toluene in control and ethanol-treated (CYP2E1-induced) male Wistar rats. Their results indicated the contribution of two separate enzymes, and that the low-affinity form was the same form whose activity was induced by ethanol treatment. K_m values of 0.13 m*M* and 0.17 m*M* and V_{max} values of 1.58 and 8.42 nmol/min/mg MSP were found in control and ethanol-treated rat MSP, respectively. For comparison, data showed rates of BA formation in Sprague-Dawley rats of approximately 2.1 nmol/min/mg MSP, corresponding to a specific activity of approximately 25 pmol BA formed/min/pmol CYP2E1 (see Figure 5). Together, these results support a similar V_{max}, and suggest a similar *K*m value for the CYP2E1-catalyzed oxidation of toluene, regardless of strain or species. This finding of similarity in metabolic activity of CYP2E1 across species mirrors that demonstrated for $CHCl_3$.

Together with the values for $CHCl_3$ metabolism determined in the present study, three important conclusions can be drawn. First, CYP2E1 varies in catalytic activity with respect to the substrate under investigation; second, CYP2E1 appears to have similar specific activity toward a given substrate, regardless of whether expressed in rats or humans; and third, species differences in maximal metabolic rates for CYP2E1 substrates are dependent on the CYP2E1 content of the preparation or system under investigation, and not based on differences in the intrinsic activity of the enzyme between these mammalian species. In addition, this demonstration of remarkably similar specific activity of rat and human CYP2E1, respectively, toward chloroform and toluene also indicates a lack of detrimental effect of human liver procurement conditions on the activity of CYP2E1, and should bolster confidence placed in results from using these metabolic preparations.

Finally, these results demonstrate, for the first time, Michaelis–Menten kinetic paremeters for $CHCl_3$ metabolsim in humans and rats and characterize the specific activity of CYP2E1 in humans and rats toward $CHCl_3$. These results demonstrate that differences in CYP2E1 content of MSP among species are largely reponsible for differences in $CHCl_3$ metabolism in vitro (especially at higher but physiologically relevant concentrations), and demonstrate a marked similarity in the specific activity of CYP2E1 from these two species toward $CHCl_3$.

REFERENCES

Agency for Toxic Substances and Disease Registry. 2001. *Managing hazardous materials incidents. Vol.III. Medical management guidelines for acute chemical exposures: Toluene*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

American Conference of Governmental Industrial Hygienists. 2003. *Threshold limit values for chemical substances and physical agents and biological exposure indices*. Cincinnati, OH: American Conference of Governmental Industrial Hygienists.

Bradford, M. M. 1976. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72:248–254.

Brady, J. F., Li, D., Ishizaki, H., Lee, M., Ning, S. M., Xiao, F., and Yang, C. S. 1989. Induction of cytochromes P450 IIE1 P450 IIB1 by secondary alcohols and the role of P450 IIE1 in chloroform metabolism. *Toxicol. Appl. Pharmacol*. 100:342–349.

Brown, B. R., Jr., Sipes, I. G., and Sagalyn, M. A. 1974. Mechanisms of acute hepatotoxicity: Chloroform, halothane, and glutathione. *Anesthesiology* 41:554–561.

Corley, R. A., Mendrala, A. L., Smith, F. A., Staats, D. A., Gargas, M. L., Conolly, R. B., Andersen, M. E., and Reitz, R. H. 1990. Development of a physiologically based pharmacokinetic model for chloroform. *Toxicol. Appl. Pharmacol*. 103:512–527.

Davis, R. R., Murphy, W. J., Snawder, J. E., Striley, C. A. F., Henderson, D, Khan, A., and Kreig, E. F. 2002. Susceptibility to the ototoxic properties of toluene is species specific. *Hearing Res*.166:24–32.

DeLuca, J. G., Doebber, T. W., Kelly, L. J., Kemp, R. K., Molon-Noblot, S., Sahoo, S. P., Ventre, J., Wu, M. S., Peters, J. M., Gonzalez, F. J., and Moller, D. E. 2000. Evidence for peroxisome porliferator-activated receptor (PPAR) alpha-independent peroxisome proliferation: Effects of PPAR gamma/delta-specific agonists in PPAR alpha-null mice. *Mol. Pharmacol*.58:470–476.

Foo, S., Jeyaratnam, J., and Koh, D. 1990. Chronic neurobehavioral effects of toluene, *Br.J.Ind.Med*. 47:480–484.

Kedderis, G. L., and Held, S. D. 1996. Predictions of furan pharmacokinetics from hepatocyte studies: comparison of bioactivation and hepatic dosimetry in rats, mice, and humans. *Toxicol. Appl. Pharmacol*. 140:124–130.

Kedderis, G. L., and Lipscomb, J. C., 2003. Application of *in vitro* biotransformation data and pharmacokinetic modeling to risk assessment *Toxicol. Ind. Health* 17:315–321.

Lipscomb, J. C., Garrett, C. M., and Snawder, J. E. 1997. Cytochrome P450-dependent metabolism of trichloroethylene: Interindividual differences in humans. *Toxicol. Appl. Pharmacol*., 142:311–318.

Lipscomb, J. C., Fisher, J. W., Confer, P. D., and Byczkowski, J. Z. 1998a. In vitro to in vivo extrapolation for trichloroethylene metabolism in humans. *Toxicol. Appl. Pharmacol*. 152:376–387.

Lipscomb, J. C., Garrett, C. M., and Snawder, J. E. 1998b. Use of kinetic and mechanistic data in species extrapolation of bioactivation: Cytochrome P-450 dependent trichloroethylene metabolism at occupationally relevant concentrations. *J. Occup. Health* 40:110–117.

Lipscomb, J. C., Teuschler, L. K., Swartout, J. C., Striley, C. A. F., and Snawder, J. E. 2003a. Variance of microsomal protein and cytochrome P450 2E1 and 3A forms in adult human liver. *Toxicol. Mech. Methods* 13:45–51.

Lipscomb, J. C., Teuschler, L. K., Swartout, J., Popken, D., Cox, T., and Kedderis, G. L. 2003b. The impact of cytochrome P450 2E1-dependent metabolic variance on a risk relevant pharmacokinetic outcome in humans. *Risk Anal*. 23:1257–1275.

Lucas, D., Ferrara, R., Gonzales, E., Albores, A, Manno, M., and Berthou, F. 2001. Cytochrome CYP2E1 phenotyping and genotyping in the evaluation of health risks from exposure to polluted environmenta. *Toxicol. Lett*. 124:71–81.

Nakajima, T., Wang, R.-S., Elovaara, E., Gonzalez, F. J., Gelboin, H. V., Raunio, H., Pelkonen, O., Vainio, H., and Aoyayma, T. 1997. Toluene metabolism by cDNA expressed human hepatic cytochrome P450. *Biochem. Pharmacol*. 53:271–277.

National Research Council. 1996. *Guide for the care and use of laboratory animals*. Washington, DC: National Academy Press.

National Toxicology Program. 1989. *Toxicology and carcinogenesis studies of toluene in F344/N rats and B6C3F1 mice*. Tech. Rep. Ser. 371. Research Triangle Park, NC: NTP.

Reinke, L. A., and Moyer, M. J. 1985. *p*-Nitrophenol hydroxylation. A microsomal oxidation which is highly inducible by ethanol. *Drug Metab. Dispos*. 13:548–552.

Richardson, S. D. 1998. Identification of drinking water disinfection by-products. In *The encyclopedia of environmental analysis & remediation,* ed. A. A. Meyers, vol. pp.1398–1421. New York: John Wiley.

Rook, J. J. 1974. Formation of haloforms during chlorination of natural waters. *Water Treat. Exam* 23:234– 243.

Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F. P. 1994. Interindividual variations in human liver cytochrome P450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: Studies with liver microsomes of 30 Japanese and 30 Caucasinas. *J. Pharmacol. Exp. Ther*. 270:414–423.

Shimada, T., Tsumura, F., and Yamazaki, H. 1999. Prediction of human liver microsomal oxidations of 7-ethoxycoumarin and chlorzoxazone with kinetic parameters of recombinant cytochrome P-450 enzymes. *Drug Metab. Dispos*. 27:1274–1280.

Sipes, I. G., Krishna, G., and Gillette, J. R. 1977. Bioactivation of carbon tetrachloride, chloroform and bromotrichloromethane: Role of cytochrome P-450. *Life Sci*.20:1541–1548.

Snawder, J. E., and Lipscomb, J. C. 2000. Interindividual variance of cytochrome P450 forms in human hepatic microsomes: Correlation of individual forms with xenobiotic metabolism and implications in risk assessment. .*Regul. Toxicol. Pharmacol*. 32:200–209.

Testai, E., de Curtis, V., Gemma, S., Fabrizi, L., Gervasi, P., and Vittozzi, L. 1996. The role of different cytochrome P-450 isoforms in in vitro chloroform metabolism. *J. Biochem. Toxicol*. 11:305–312.

Tornero-Velez, R., Ross, M. K., Granville, C., Laskey, J., Jones, J. P., DeMarini, D. M., and Evans, M. V. 2004. Metabolism and Mutagenicity of source water contaminants 1,3-dicholoropropane and 2,2-dichloropropane. In press, *Drug Metab. Dispos*. 32:123–131.

U.S. Environmental Protection Agency (US EPA). 2001. Toxicological review of chloroform. U.S. Environmental protection Agency, Washington, D.C. Available on-line at http://www.epa.gov/iris/toxreviews/0025-tr.pdf.

U.S.Environmental Protection Agency. 2003. *Integrated Risk Information System. IRIS Summary for toluene*. Available online at http://www.epa.gov/iris/toxreviews/0025-tr.pdf.

Wang, R.-S., and Nakajima, T. 1991. Kinetic studies of toluene metabolism in ethanol- and phenobarbital-induced rat liver microsomes in vitro. *Arch.Toxicol*. 65:39–44.