DISSERTATION

PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODELING OF SIMPLE AND COMPLEX MIXTURES OF GASOLINE AND THE GASOLINE COMPONENTS N-HEXANE, BENZENE, TOLUENE, ETHYLBENZENE, AND XYLENE

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JAMES E. DENNISON, JR. ENTITLED "PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODELING OF SIMPLE AND COMPLEX MIXTURES OF GASOLINE AND THE GASOLINE COMPONENTS N-HEXANE, BENZENE, TOLUENE, ETHYLBENZENE, AND XYLENE" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODELING OF SIMPLE AND COMPLEX MIXTURES OF GASOLINE AND THE GASOLINE COMPONENTS N-HEXANE, BENZENE, TOLUENE, ETHYLBENZENE, AND XYLENE

Gasoline consists of hundred of hydrocarbon components, some of the principal ones being *n*-hexane, benzene, toluene, ethylbenzene and xylenes. Various components of gasoline cause different toxicological effects, including depression of the central nervous system, irritation, and cancer. The assessment of the dose response of different components in various mixtures of the components or in complex mixtures such as gasoline is, however, confounded by the fact that the components are capable of interacting within the mixture. One of the best understood interactions occurs during metabolism, a pharmacokinetic process that results in clearance of the parent chemical from the body and production of metabolites that may be more or less toxic than the parent. The studies described herein were intended to clarify the extent of these interactions, and to develop a quantitative description of the pharmacokinetics of gasoline and mixtures of some of its components.

A simple mixture can be described as a well-defined mixture of two or more chemicals. The interactions of a simple mixture of the gasoline components benzene, toluene, ethylbenzene, and xylene (BTEX) have been previously described. However, the significance of the interactions in a real-world context had not been explored. Using established physiologically-based pharmacokinetic (PBPK) models, we studied the effect of exposing humans at the Threshold Limit Values and Permissible Exposure Limits, and included the effect of exercise. Depending on the accepted measure of toxicity, the PBPK model indicates that over-exposure to these chemicals can occur under a variety of real world scenarios.

If a simple mixture is well-defined, a complex mixture is a mixture of many compounds that are not present in specified amounts. Existing approaches for building PBPK models rely on building single chemical PBPK models for each component and then mathematically joining them in a biologically relevant way. This is only practical for mixtures that have a limited number of components. A method was therefore developed to cope with the hundreds of components in gasoline. Specific components were handled in the conventional manner. To account for mixture interactions between remaining components and the specifically identified ones, the remaining components were assumed to act like a single additional chemical with properties that were averaged over the range of components in the group.

These models were developed using *in vivo* pharmacokinetic data from rat studies. Rats were placed in sealed chambers and the rate of disappearance of chemicals as the animals absorbed them was determined. Existing chamber designs were improved by developing a method for measuring carbon dioxide in the chamber. While not used in the studies herein, an additional method that allows simultaneous sampling of blood was also developed. The pharmacokinetic data supported the development of a PBPK model for gasoline, with BTEX, *n*-hexane and an additional lumped component. A winter blend and summer blend of gasoline were analyzed and a good pharmacokinetic description of the mixture and the interactions between its components is presented. To assess the ability of the approach to deal with variable composition mixtures, volatile fractions of gasoline were also studied. The model successfully described the pharmacokinetics of various fractions of gasoline that were weighted by volatility.

These pharmacokinetic descriptions of gasoline were limited to the pharmacokinetics of the parent chemicals and did not address metabolites. To take a closer look at some metabolite issues, the chemical *n*-hexane was explored in more depth. *n*-Hexane is metabolized to several intermediates. The downstream intermediate hexanedione is responsible for *n*-hexane's neurotoxicological effects. Counter-intuitively, more hexanedione is formed in rats acutely exposed to 1000 ppm than exposed to 3000 ppm, *i.e.*, the chemical has a negative dose-response curve when

hexanedione is the response metric. A PBPK model was developed, based on data from previous studies, to determine the underlying cause of this effect. It was found that, if inhibition of downstream oxidative metabolic steps is assumed to be caused by *n*-hexane and other metabolites in blood, hexanedione production will be substantially depressed during exposure. After exposure ends, a large increase in hexanedione formation can occur as the inhibition is released. A reasonable correlation between the area-under-the-curve of hexanedione in the body and neurological outcomes was found.

These studies thus evaluate several types of mixtures that occur with this group of compounds. The initial study assessed interactions in a simple mixture of toluene, ethylbenzene, and xylene, three important constituents of gasoline. The next studies assessed interactions on a broader scale, in a complex mixture of gasoline components. Finally, interactions between a chemical and two of its metabolites was investigated with *n*-hexane. While all of these mixtures exhibit similar pharmacokinetic interaction properties, we found that significant impacts on the pharmacokinetics of each element of each mixture can occur.

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Chapter 1

Introduction

The importance of the present research studies is best captured by a simple statement made a few years ago.

> *"... humans are not exposed to single agents... the environment provides exposure to a complex daily mixture of agents ..."* (Calabrese 1991).

Toxicology is the study of the effects of chemical agents on organisms, in particular human beings. The discipline attempts to uncover not only the effect of chemicals, but how the chemicals cause their effects, *i.e.*, their mode of action in the body. The discipline of risk assessment is an applied science that seeks to determine, quantitatively if possible, what the impact of chemical exposure is, *i.e.*, how much death, disease, or impairment will result from various exposures to chemicals. Much work has been performed over recent years to improve the understanding of toxicology of particular agents, and to describe the resulting risks. Progress is gradually made, but may seem infinitesimally slow when considered against the backdrop of innumerable mixtures of chemicals that need consideration.

In a sense, Calabrese was paraphrasing Paracelsus, who stated in 1567 that "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy" (Paracelsus 1567). In the current risk assessment paradigm, dose is not the amount taken, but the amount of the proximal toxicant (chemical or its metabolite) in the target tissue of the body. Many examples

have been characterized in which the "dose" as defined above is affected by other elements of the mixture, and the toxicity is thereby modified. These chemical interactions are described as "antagonistic," "synergistic," and in other ways. Mixture toxicology is concerned with understanding and describing these interactions.

The mixtures to which Calabrese was referring include chemicals taken at the same time, chemicals that exist in multiple media (air, water, etc.), different chemical classes, *e.g.*, solvents, metals, and particulates, as well as chemicals that already exist in our bodies. We are chemicals. Toxicology is a long way perhaps from understanding all of the mixtures we experience, but progress is made in steps.

Many chemicals are subjected to various toxicity tests to estimate the toxicity of the compound. However, it is impossible to fully understand a chemical's toxicity prior to using the chemical for two reasons: (1) society does not demand that a chemical is fully tested prior to use, as evidenced by studies showing that few chemicals are ever subjected to even a minimal test battery (NRC 1984; Roe *et al.* 1997), arguably because conducting every test would be cost-prohibitive; and (2) even if every current test was conducted, our knowledge would be incomplete since interpretation of results is often not straightforward. Furthermore, interpreting animal tests in terms of human risk is not simple. These issues are exacerbated when mixtures are concerned. It is even less possible to consider testing all potential mixtures. Minimal toxicology testing of every possible combination of one 25-chemical mixture would cost three

trillion dollars; yet, there are approximately 100,000 chemicals being used in commerce (Yang *et al.* 1995).

To address this situation, much research has been performed in recent years to develop innovative approaches to determining toxicity. One such approach involves mathematical simulations of the body (humans or other species) and the key processes in the body that serve as determinants of toxicity. Such approaches include the development of physiologically based pharmacokinetic (PBPK) models that describe the absorption, distribution, metabolism, and elimination of a chemical from the organism. Corresponding or integrated pharmacodynamic models that describe what the chemical or its metabolites do to the organism have also been developed. These models have found great utility in toxicological research and in health risk assessment (Clewell 1995). Again, Calabrese pointed out that "... *predictive systems are desperately needed since it is impossible to study all interactions [of chemical mixtures]"* (Calabrese 1991).

This dissertation builds on earlier work in the area of PBPK modeling as a form of computational toxicology. Specifically, the dissertation research involved studies of gasoline, a complex mixture of many chemicals, and several of its principal components. While a number of toxicity tests have been performed on gasoline and some of its individual constituents, no adequate description of its pharmacokinetics has been obtained. Some of the chemicals in gasoline that have been studied in some detail include *n*-hexane, benzene, toluene, ethylbenzene, and xylene. The interactive

mixture effects of some of these chemicals have been studied to an extent. For example, the interactions between mixtures of benzene, toluene, ethylbenzene, and xylene have been described with PBPK models (Tardif *et al.* 1995; Tardif *et al.* 1997; Haddad *et al.* 1999). However, the most "complex" solvent mixture that has been described based on the underlying biological processes (*i.e.*, with a PBPK model) has included only five chemicals (Haddad *et al.* 2001). Building a PBPK mixture model one component at a time is labor intensive, requiring a series of *in vivo* pharmacokinetic (PK) studies of single chemicals and various sets of the mixture components and iterative modeling to develop the pharmacokinetic description. The approach works with simple mixtures, but clearly is not feasible for components of many chemicals.

Gasoline is a refined petroleum product containing numerous components. Depending on the analytical method used, over 400 components can be detected; the actual number of components detected is a function of the sensitivity of the method used. Some components are present at percent levels, while most are in the sub-percent level. One way to simplify the problem of characterizing the PKs of gasoline would be to look at the PKs of each component present at significant levels in the mixture. However, in order to do so, one would likely want to capture a large fraction of the total, *e.g.*, 90 – 95%. This would permit ignoring minor components that sum to less than 5-10% of the total. Still, this would require evaluation of fifty or more chemicals and would not be a practical approach (Dennison *et al.* 2003).

Moreover, toxicity of gasoline is in part dependant on the toxicity of individual components. For example, benzene is a leukemogen (USEPA 2003) and much work has been performed to determine how benzene causes this effect. But the question then becomes "How much risk does benzene pose when exposure to it comes as part of a mixture of chemicals that interact with benzene?" Again, accounting for 90-95% of the mixture's effect is a possible cutoff. Clearly, this would require an impractical amount of research to build a PBPK model using the bottom up approach.

This dissertation investigated the feasibility of a novel, alternative approach that involved chemical lumping. By chemical lumping, we mean that more than one chemical is mathematically described as a single chemical with respect to a biological process. In a PBPK model, rate equations are used to describe some pharmacokinetic processes in a way that the concentration of the chemical can be simulated in each part of the organism. Indeed, the model calculates these concentrations over the timecourse of the virtual experiment in each tissue. Ordinarily, the concentration of each chemical is thus predicted. With chemical lumping, the same approach is used, except that the values of the rate equations for a lumped chemical reflect the chemical properties of the lumped chemical as opposed to a specific chemical. In other words, the chemical properties are described in an empirical manner reflecting mean values for the lump. One such property is the blood:air partition coefficient for the chemical. The lumped PBPK model uses an average value of this parameter that estimates the mean for the lumped component.

The simplest kind of chemical lumping analysis would be to treat the whole mixture as a lump. Based on our results, a reasonable PBPK model could likely have been constructed for gasoline as one lump. However, if only one lump was used, no interactions could be evaluated. The study design that was selected involved evaluating the PKs and interactions between five components of gasoline. Thus, the PBPK model incorporated five chemicals (*n*-hexane, benzene, toluene, ethylbenzene, and *o*-xylene (BTHEX)) and a lumped component which was comprised of all remaining components in the gasoline mixture. Thus, the structure of the model reflected a six-component mixture, which in and of itself made the model one of the more complex mixtures to be described yet with PBPK. The model of course included a special component consisting of hundreds of components lumped together, and included binary interactions based on known biology.

One of the principal reasons for PBPK modeling described above is the need to be able to predict, or extrapolate models to new situations. In the context of mixtures, one of the extrapolations of concern is from one type of mixture to another. For example, if we have data on mixture A and mixture B, can we extrapolate to mixture A + B? The impossibility of testing all potential combinations has been pointed out as unrealistic. Suppose toxicity testing has been performed with one blend of gasoline. Can the results be extrapolated to another blend?

In the U.S., at least 32 blends of gasoline are produced (Economides 2002). Gasoline produced for use in warm months is engineered to be less volatile than that produced

for winter month use. There are different grades available (premium, etc.) In addition, various additives are used in different locations of the country. In the rest of the world, more diversity can be anticipated. Clearly, some differences in toxicity can be anticipated, but testing all blends is impractical. One part of this research was therefore to investigate the differences in PKs between different blends. The studies specifically involved testing of one summer blend of gasoline, one winter blend, and one other random blend. The winter blend of gasoline also contained MTBE and the summer blend contained no oxygenate. A reasonable PK description of all of these blends at multiple levels of exposure in the F344 rat was obtained.

An additional question that was posed took in consideration the manner in which humans are exposed to gasoline. The route of human exposure to gasoline is generally via inhalation, so first gasoline must evaporate and then be inhaled. As different components of gasoline evaporate at different rates, the composition of the vapor mixture will generally not be the same, or even similar to, the composition of the liquid. Part of the research therefore addressed the question of whether the lumping approach would work for quickly evaporating fractions of the whole mixture as well as the complete mixture.

The gasoline PBPK models that were developed in this thesis project demonstrate the overall feasibility of the approach. Due to the complex nature of the research, it was not feasible to include additional studies that would be necessary to transform the PBPK model, developed for the F344 rat, into a valid model for humans exposed to

gasoline. This extrapolation would be a logical next step in development of complex mixture PBPK models. When such models are built, they will be useful in supporting risk assessment for gasoline and any number of its particular components. For example, the question raised above regarding benzene can be addressed. "What is the cancer risk of benzene when it is in gasoline as compared to benzene as a single chemical?" Calculations of these risks will be available through the PBPK model that describes how the gasoline-benzene interaction affects the biological process resulting in the cancer risk.

Toxicological studies, even if scientifically satisfying, may not be put to maximum use if they are not put in terms that risk managers can use. We were interested in applying some of our understanding of the mixture interactions for these chemicals to real world scenarios, *i.e.*, performing risk assessment. One problem that comes to mind is the question of the interaction effect of some solvents that act as depressants of the central nervous system (CNS). In particular, toluene, ethylbenzene, and the xylenes act as CNS depressants and were known to exhibit interactions that increase toxicity in some dose ranges (Haddad *et al.* 2001). The three chemicals have occupational exposure levels set by the Occupational Safety and Health Administration (OSHA) referred to as the Permissible Exposure Limits and by the American Conference of Governmental Industrial Hygienists (ACGIH®) which are called Threshold Limit Values (TLVs®). For the three chemicals, the PELs are all equal to or higher than the TLVs. Using a mixture PBPK model they developed for the three chemicals, Krishnan and co-workers assessed the potential for cumulative

toxicity during exposure to the three compounds (Haddad *et al.* 1999; Haddad *et al.* 2001). Slight apparent potential toxicity was found for various mixtures of the chemicals. However, Haddad *et al.* (1999; 2001) set the exposures to approximately 1/3 of the TLV and used dose additivity as the integration method. In the U.S., exposures can range up to the PEL for each chemical and sometimes exceed the PEL. This raised the question of whether the interactions were more significant than observed in the Haddad studies. In addition, we noted that most of the studies used to set the exposure limits were conducted with animals or humans in a resting state. However, workers may perform manual tasks during the exposure period. The exercise increases the workers breathing rates, which increases uptake of the chemical. This also may increase toxicity. Finally, we noted that the TLV for xylene did not explicitly state that CNS depression was an outcome for mixture evaluation. This ambiguity raises the question of whether xylene should be included in the integrated evaluation of dose, and what its impact would be if it was not controlled to a level below the PEL and served as a potentiator of toluene and ethylbenzene toxicity.

In order to evaluate these questions, we used the same PBPK model that had been used in the Haddad *et al.* work (Haddad, 1999). PBPK model simulations were conducted for a broad array of exposure scenarios, including conditions where workers were performing manual work and had increased ventilation rates. In these analyses, much more significant potential toxicity was found to occur. This analysis

is presented in Chapter 3 of this dissertation and may be found as a published paper at some point (Dennison *et al.* 2004c).

In the next chapter of the dissertation, methods that were developed for conducting inhalation pharmacokinetic experiments are reported. In the gasoline studies (Chapters 5-6 of the dissertation), the pharmacokinetic experiments were conducted using the gas uptake chamber approach. This approach has been used since the 1970s (Andersen *et al.* 1979) to conduct inhalation studies and has several advantages over the alternative constant exposure approach. In the gas uptake system, the animal is placed in a sealed system and an initial amount of chemical vapor is added to the system. The animal absorbs the chemical over the course of the experiment, so the decline in chemical concentration in the chamber is related to the PKs of the chemical in the animal. Usually, the partition coefficients and other parameters other than metabolism are measured separately. PBPK models can be then used to estimate values for the missing metabolic parameters. Generally, the system is a good method for determining metabolism *in vivo.*

The actual method used in the gasoline studies was based on a gas uptake system that includes an autosampler that provided automatic injections of chamber air into the gas chromatograph. The system used was similar to ones previously used in these types of studies (Gargas *et al.* 1986). However, during the course of the experimental work with gasoline, three observations were made. (1) The animal's breathing rate was highly dependent on the concentration of carbon dioxide (CO_2) in the chamber, which

depended on the efficacy of the CO_2 scrubber in the chamber system. (2) The non-systemic absorption of chemicals by the animal, which is usually assumed to be a first-order process, was not first order when the concentration range was large and indeed absorption was reversible. (3) There was potential with this chamber system to perform simultaneous blood and chamber air sampling to collect more data for PBPK model development.

To address these concerns, real-time CO_2 monitoring was performed to identify the CO_2 scrubber that would maintain CO_2 concentration s at a known consistent level. The loss rates were measured, and it was found that the loss rate could be affected by chemicals absorbed to the animal's fur and thus available for desorption. A paper summarizing the problems identified and possible ways to avoid or solve the problems were described in another publication (Dennison *et al.* 2004b). This paper also provides a more detailed description of the gas uptake system than could be provided in a typical manuscript.

The results of two gasoline studies are contained in Chapters 5-6 of this dissertation. The initial study evaluated the PKs of the two gasoline blends (summer and winter blends) and provided an initial PBPK model. This was published as a manuscript in 2003 (Dennison *et al.* 2003). Subsequent investigations undertook the issue of the evaporated fractions of gasoline. In these investigations, the most volatile components of a sample of gasoline were used in separate inhalation studies along with whole gasoline. PBPK model were developed for each volatility class of

gasoline components. This work has been submitted as a manuscript as well (Dennison *et al.* 2004a).

Finally, another mixture was investigated, a mixture of *n*-hexane and its metabolites. How is it that a single chemical can exhibit mixture pharmacokinetics? The answer lies in the metabolism of *n*-hexane. In mammals, *n*-hexane is metabolized to a variety of products in a series of parallel and serial metabolic pathways that are not generally unusual. With some of the metabolites, further metabolism is mediated in the organism by the same metabolic enzymes that initially metabolize the parent chemical *n*-hexane. Thus, the potential exists for *n*-hexane and some of its metabolites to inhibit each other's metabolism. This was suggested in a preliminary PBPK model for *n*-hexane and two of its metabolites, methyl-*n*-butyl ketone (MBK) and 2,5-hexanedione (HD) in a paper given a number of years ago (Andersen and Clewell 1983). The model was able to generally reproduce the blood levels of *n*-hexane, MBK, and HD at four levels of exposure. In addition, the model recapitulated the data for HD that found that HD levels after 1000 and 3000 ppm exposure (6 hours) were not very different, and that at the higher exposure levels, a distinct pattern occurred in which HD levels did not approach steady state during the exposure and, after *n*-hexane was cleared from the body, HD levels increased rapidly, suggesting that metabolism of MBK was inhibited by *n*-hexane. These data were consistent with other studies as well, including studies of neurotoxicity at these exposure levels. The existing *n*-hexane model (Andersen and Clewell 1983) was

refined with additional parameter optimization. It appears in Chapter 7 as the manuscript submitted for publication (Andersen *et al.* 2004).

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Chapter 2

Literature Review

The studies undertaken in this thesis build on the work of many other's published in various forms over the years.

Use of and Exposure to Gasoline and BTHEX

Gasoline and the gasoline components benzene, toluene, *n*-hexane, ethylbenzene, and xylene (BTHEX) are widely produced and used. Approximately 140 billion gallons are annually produced in the U.S. (Dement *et al.* 1997). Most individual exposure occurs during refueling at gasoline stations (Wixtrom and Brown 1992) but occupational exposure to higher levels also occurs during production, distribution and use of gasoline. The limited data available suggest that exposures range widely. Occupational exposures occur up to 3,000 mg/m^3 (Wixtrom and Brown 1992; Hakkola and Saarinen 1996; Dement *et al.* 1997), and environmental exposures in the range from 4 to 9,000 mg/m^3 have been reported (Wixtrom and Brown 1992; Dement *et al.* 1997; Hakkola and Saarinen 2000).

Exposure to BTHEX occurs during use of gasoline and also as exposure to the individual chemicals or mixtures thereof. Toluene, *n*-hexane, ethylbenzene and xylene in particular are broadly used solvents in industrial processes, construction and consumer products (OEHHA 1997a, b, 1999; NIH 2004). Occupational workshift exposures to toluene have been reported up to 160 ppm (Muijser *et al.* 1996; Hammer

et al. 1998). Occupational exposures to *n*-hexane ranging up to 325 ppm have been reported with exposures over 100 ppm occurring in several industries (Mutti *et al.* 1984; Kawai *et al.* 1991; Cardona *et al.* 1993). Exposures to ethylbenzene tend to be lower than for these two chemicals, but have been reported up to 29 ppm (de Medinilla and Espigares 1988). Xylene exposures vary widely with occupation. Exposures ranged between 1 and 200 ppm in one study (Gardner 1996) and between 2 and 7000 ppm in another (Miller and Edwards 1999).

Gasoline is a complex mixture containing hundreds of hydrocarbons mostly between butane and decane in carbon number. It is comprised primarily of isoalkanes, with substantial amounts of *n*-alkanes and aromatic compounds and smaller fractions of alkenes and alkynes (Kreamer and Stetzenbach 1990). At least 32 different blends of gasoline are used in the U.S. (Economides 2002); each blend is optimized for different climatic and driving conditions. Most U.S. gasoline also contains additives, including either ethanol or methyl-*t*-butyl ether (MTBE). Based on analysis of samples used in the dissertation studies, about 20 compounds were present in the percent range, and 20% of the mixture was comprised of components in the subpercent range. To account for 95% of the total mixture, approximately 75 compounds would have to be aggregated (SWRI 2001). The gasoline used in these studies contained between 1.2- 3.6% benzene, 6-13% toluene, 1-3% *n*-hexane, 2-4% ethylbenzene, and 2.5-5% *o*-xylene (SWRI 2001).

Toxicological Effects of Gasoline and BTHEX

Epidemiological studies of gasoline have provided inconsistent results. However, gasoline was classified by IARC as a 2B carcinogen (possibly carcinogenic) (IARC 2002). Many of the cancers that may be caused during exposure to gasoline are leukemia or other cancers of the hematopoietic system, which raises the possibility that the cancer is caused principally by benzene. Gasoline is also an irritant (ACGIH 1999b) and contains many components that are depressants of the central nervous system.

Benzene is a known human carcinogen (ACGIH 1999a; USEPA 2003) and causes cancer at least in the hematopoietic system. The mode of action is not clearly established, but one or more of benzene's metabolites are involved (Smith 1996). Benzene is metabolized by cytochrome P450 (CYP), primarily in the liver and mostly by CYP2E1 (Valentine *et al.* 1996). Glucuronide and sulfate conjugates of the alcohol and diol metabolites are excreted in the urine.

Toluene is metabolized in a similar manner, primarily by CYP2E1 in the liver (Nakajima *et al.* 1992). It is first metabolized to benzyl alcohol, then oxidized to benzoic acid, conjugated with glycine, and excreted as hippuric acid (ATSDR 2000). Ethylbenzene and xylene follow similar metabolic profiles. All three chemicals are lipophilic compounds that are readily absorbed and are generally of a low order of

toxicity, although toluene and ethylbenzene and probably xylene are also CNS depressants (OEHHA 1997a, b, 1999).

n-Hexane is a neurotoxicant with a different mode of action than toluene, ethylbenzene and xylene. While *n*-hexane conceivably could cause CNS depression, the critical effect (*i.e.*, the effect that occurs at the lowest exposure level) is peripheral axonopathy (ATSDR 1999). The current hypothesis for the mode of action is as follows. *n*-Hexane is metabolized, primarily by CYP2E1 in the liver, to 1-, 2-, or 3-hexanol. 2-hexanol is biotransformed into methyl-*n*-butyl ketone (MBK). It can be further biotransformed into pentanoic acid or into an alcohol-ketone which is then transformed into 2, 5-hexanedione (HD). This compound is considered the toxic metabolite. It reacts with cellular constituents if not cleared by further metabolism. Many of the alcohol moieties are found as glucuronide conjugates in the urine. If HD is not excreted, it may react with lysine residues and after several metabolic steps, form pyrroles that can cross react with additional proteins. Proteins in the axonal transport system appear to be unusually sensitive to the interaction with HD. Mostly in the peripheral nervous system, crosslinked proteins will aggregate in the distal portions of large nerves, disrupt the transport of nutrients in the nerve fiber, and ultimately cause neurological dysfunction and cell death. Symptoms of excessive exposure include muscle weakness, twitching, paralysis and related effects (ATSDR 1999).

Pharmacokinetic studies of Gasoline and BTHEX

Numerous pharmacokinetic studies of gasoline and BTHEX have been conducted; only a sample of the available data is discussed here in order to provide representative background information.

An investigation of the pharmacokinetics of MTBE alone and in a gasoline mixture was conducted in male F344 rats. In this study, gasoline affected the pharmacokinetic behavior of MTBE in a dose dependent manner. In another study, the pharmacokinetics of benzene in gasoline were investigated (Bond *et al.* 1997). At 300 ppm gasoline exposure, benzene metabolism was modestly reduced, while at 2000 ppm gasoline, benzene metabolism was only 10-50% of uninhibited metabolism, depending on the fraction of gasoline.

Metabolism of BTHEX compounds has been studied *in vitro* and *in vivo* in animals and humans. In microsomes, CYP2E1 was found to be the major isoenzyme for benzene metabolism in lung and liver and CYP2F was found to play a role in the lung (Powley and Carlson 2000). Benzene oxide was measured in liver microsomes from mice, suggesting that it may be stable enough to be distributed to bone marrow (Lovern *et al.* 1997). In human microsomes, metabolic rates ranged between about 0.3 – 4.4 nmol/mg/min and was proportional to the CYP 2E1 substrate *p*-nitrophenol metabolic rate in each sample (Seaton *et al.* 1994). In another study, Schlosser and co-workers measured the *in vitro* metabolic rates of benzene metabolism to phenol, phenol metabolism to catechol and hydroquinone, and catechol and hydroquinone

metabolism to trihydroxybenzene (Schlosser *et al.* 1993). The kinetic data was used in a simulation model of the *in vitro* process and included competitive inhibition by phenol and benzene. However, Nakajima and coworkers indicated CYP 2C11/6 was an important isoenzyme, especially in rats, based on studies with monoclonal antibodies directed to each isozyme (Nakajima *et al.* 1993). Different isozymes were inducible to different extents (Johansson and Ingelman-Sundberg 1988; Nakajima *et al.* 1990).

In vitro studies with BTHEX

n-Hexane metabolism was studied in microsomes from rat liver, lung, muscle, and brain (Crosbie *et al.* 1997) and in cell lines expressing CYP2E1 or CYP2B6. In rat liver and muscle, 2-hexanol was the major first metabolite, whereas in lung, mostly 1-hexanol was formed. Production of all hexanols was induced by phenobarbital. In another study with induced microsomes, phenobarbital increased the rate of formation of 2- and 3-hexanol only, but 3-methylcholanthrene only stimulated the production of 3-hexanol (Morohashi *et al.* 1983).

Toluene metabolism was investigated in rat liver microsomes from developing male and female pups (Shimamoto *et al.* 1999). Ethnic variation in CYP2E1 content and activity toward toluene was described (Kim *et al.* 1997). At low concentrations, toluene metabolic activity correlated with CYP2E1 content, but at high concentrations, the activity correlated with CYP2C8. The involvement of CYP2E1

was confirmed by Nakajima *et al.* who also showed that mice had higher rates of metabolism (per milligram liver protein) than rats as with benzene (Nakajima *et al.* 1993). Induction was analyzed in liver microsomes in another study (Wang and Nakajima 1991). In control microsomes and in microsomes induced with 3-methylcholanthrene and phenobarbital, metabolites were found to covalently bind to microsomal protein (Pathiratne *et al.* 1986a) and this interaction was reduced by glutathione or cystein.

The *in vitro* metabolism of ethylbenzene was investigated in two studies (McMahon and Sullivan 1966; Maylin *et al.* 1973). Xylene was found to be a non-competitive inhibitor of antipyrene metabolism in rats (Toftgard 1983). Metabolic inhibition between *m*-xylene, benzene, and toluene was found *in vitro* (Nakajima and Sato 1979).

In vivo animal studies with *n*-hexane

A number of studies of the pharmacokinetics of *n*-hexane and its metabolites have been conducted in animals, usually rats and mice. Baker *et al.* assayed *n*-hexane, MBK, and/or HD in blood, sciatic nerve, kidney, liver, brain, testes and lung, and MBK, dimethylfuran (DMFU), 2-hexanol (2HOH) and 5-hydroxy, 2-hexanone (5OH2H) in urine and/or some tissues (Baker and Rickert 1981) in F344 rats. Bus *et al.* completed PK studies in pregnant animals (Bus *et al.* 1979), measuring *n*-hexane, MBK, and HD in blood, liver, kidney, brain, and sciatic nerve after *n*-hexane

exposure for 1 or five days (Bus *et al.* 1981), and a radiolabeled *n*-hexane study measuring exhaled *n*-hexane and carbon dioxide and label in several tissues (Bus *et al.* 1982). Dolara *et al.* measured the levels of 1HOH and MBK in urine after *i.p.* dosing of *n*-hexane (Dolara *et al.* 1978). Fedtke *et al.* assayed various metabolites in urine, including 4,5-dihydroxy-2-hexanone after *n*-hexane treatment (Fedtke and Bolt 1987). In another study, urinary excretion of 2HOH, 3HOH, MBK, DMFU, γ-valerolactone, and HD was measured in rats, rabbits and monkeys (Perbellini *et al.* 1982a). An enzyme induction study was performed after *n*-hexane exposure in hens (Lapadula *et al.* 1991).

In vivo animal studies with MBK or HD

Granvil *et al.* treated mice with MBK, 2HOH, or HD and measured the three chemicals in blood and brain. The appearance of 2HOH after MBK treatment indicated the reversible nature of the oxidation of 2HOH (Granvil *et al.* 1994). DiVincenzo *et al.* measured label in exhaled breath, urine, feces, liver, kidney, brain and the carcass after MBK treatment. The group also measured 5OH2H, MBK, and HD in blood (DiVincenzo *et al.* 1977). Eben *et al.* treated rats orally with MBK and measured MBK, HD, 2HOH, and 2,5-hexanediol in blood and/or urine (Eben *et al.* 1979). Duguay *et al.* measured MBK, 2HOH, and HD in blood, liver and lung after inhalation or oral treatment of MBK for three days (Duguay and Plaa 1995). Pyrroles in rat hair were measured after treatment with HD (Johnson *et al.* 1995).

Human studies with *n*-hexane

Humans were treated to 130 – 300 ppm *n*-hexane via inhalation for 2-3 hours and HD was assayed in urine (Filser *et al.* 1996). The rat experiments also conducted in this study suggested that *n*-hexane was metabolized by a high affinity, low capacity as well as (at higher exposures) a low affinity, high capacity pathway, with HD in urine correlating with the former. In another volunteer study, humans exposed to *n*-hexane by inhalation were monitored for *n*-hexane in blood and exhaled breath after exposure (Veulemans *et al.* 1982). Humans were also exposed to MBK at 10 -100 ppm for 4-8 hours and MBK and HD was measured in blood and urine (DiVincenzo *et al.* 1978).

Several human occupational studies have been performed. Workers exposed to 10 - 50 ppm *n*-hexane had linearly-related levels of HD in urine (Kawai *et al.* 1991). Workers in shoe factories were exposed to *n*-hexane, and DMFU, 2HOH, HD, and/or γ-valerolactone was measured in urine (Iwata *et al.* 1983b; Mutti *et al.* 1984; Ahonen and Schimberg 1988; Cardona *et al.* 1993).

PK studies of toluene, ethylbenzene and xylene

Engstrom *et al.* studied the pharmacokinetics of ethylbenzene in rats (Engstrom 1984). *In vivo*, ethylbenzene treatment induced rat metabolism of ethylbenzene and expression levels of CYPs 1A1, 2B, 2C11, 2E1, and 3A, with different temporal patterns (Yuan *et al.* 1997a; Yuan *et al.* 1997b). However, with CYP 3A, the

induction was found to be by non-transcriptional mechanisms (translation and/or stabilization) (Yuan *et al.* 1994). Gender differences in induction were also described (Sequeira *et al.* 1992). However, in another study, 600 ppm ethylbenzene exposure (6 hours/day for up to 16 weeks) did not increase CYP concentrations (Elovaara *et al.* 1985).

The three xylene isomers and ethylbenzene and a mixture of all four chemicals induced CYPs and metabolism of *n*-hexane and 7-ethoxyresorufin in a tissue specific manner (Toftgard and Nilsen 1982). Limited evidence of tumorigenic activity of ethylbenzene in rats and mice was reported (Stott *et al.* 2003). Reduction in growth hormone in rats abolished some of the CYP2E1 induction caused by ethylbenzene (Zhang *et al.* 2002).

Studies of interactions between BTHEX compounds

Studies of the interactions between benzene, toluene, *n*-hexane, ethylbenzene and xylene have been performed *in vitro*, in animals, and in humans. Xylene, naturally, exists as the ortho, meta, or para isomer, but will be referred to simply as xylene except where important to distinguish the specific form.

In vitro studies of BTHEX compounds

Inhibition between mixtures of BTHEX compounds were investigated in *in vitro* models (Mortensen *et al.* 1998). Mutual inhibition was found but it was also found that net metabolism could be compensated for by *in vivo* pre-treatment induction.

In vivo studies of BTHEX compounds

Takeuchi *et al.* studied the combined effect of *n*-hexane and toluene on blood levels and urinary excretion of *n*-hexane metabolites (Iwata *et al.* 1983a, 1984; Takeuchi *et al.* 1993). Urinary metabolites from *n*-hexane exposure was monitored in rats in another study (Perbellini *et al.* 1982b). In a controlled human study, subjects were exposed to *n*-hexane alone or with toluene and the urinary excretion of HD and toluene metabolites was measured (Baelum *et al.* 1998). Rats were treated with HD with or without toluene, and HD in urine, blood and nerve tissue was measured (Zhao *et al.* 1998b, a).

Studies involving *in vivo* metabolic interactions between benzene and toluene include those by Inoue *et al.* and Nakajima *et al.* (Nakajima and Sato 1978; Inoue *et al.* 1988). Studies of the interactions between ethylbenzene and xylene have been performed (Elovaara *et al.* 1984; Engstrom *et al.* 1984; Jang *et al.* 2001). Studies are available to describe the PK interactions between toluene and xylene (Ogata and Fujii 1979; Tardif *et al.* 1991; Tardif *et al.* 1992; Vodickova *et al.* 1995). Pathiratne *et al.* assessed interactions between benzene, toluene, and xylene (Pathiratne *et al.* 1986b).

These studies generally found metabolic interactions to occur, although they varied in degree.

PBPK Models for Gasoline or BTHEX Components

Several existing models of BTHEX components have been developed. A brief description of the models is provided below. Several of the models were models of "simple mixtures," *i.e.*, mixtures of two to four chemicals. The mixture models generally include a model for each single chemical as well as the mixture, but will be described under the simple mixture model section below.

n-Hexane and its metabolites

The first PBPK model for *n*-hexane was developed by Andersen and colleagues. In this model (Andersen and Clewell 1983), a three compartment structure was used and *n*-hexane metabolism in the liver followed a two-enzyme system. One of the metabolites (MBK) was further metabolized to HD or, via a separate pathway, to pentanoic acid. The model incorporated competitive inhibition of metabolism by *n*-hexane on MBK→ HD metabolism and possibly by MBK on *n*-hexane→ MBK metabolism. The model was used to determine the blood concentrations of *n*-hexane, MBK, and HD using published data for acute *n*-hexane exposure to 500 – 10,000 ppm. However, the short report in which the model was described did not provide most model details.

Filser *et al.* developed a one compartment model of the rat using gas uptake data after *n*-hexane treatment using naïve animals and animals pretreated with a CYP2E1 inhibitor (Filser *et al.* 1987). The model indicated that metabolism was mediated by high-affinity low capacity as well as low affinity high capacity enzymes.

The first published (in the open literature) PBPK model for *n*-hexane was developed by Perbellini *et al.* Nominally, this model (Perbellini *et al.* 1986) was an "eight compartment" model, but in reality, it was a standard four compartment model with the addition of a lung compartment. *n*-Hexane was metabolized by a first order process into "total metabolites," a portion of which was HD, created by a first-order process as well. HD was transferred into the body water and was then excreted into a "urine compartment," both as first order processes. The model was developed and/or validated with a very limited amount of data. In the rat version of the model, the liver:blood partition coefficient (PC) "increased throughout the exposure period," an unusual modeling strategy. In the human version of the model, the blood:air PC (PB) used was 0.8 (measured by Perbellini *et al.*).

In an early model, Angelo *et al.* developed a PBPK model for HD. Experiments were performed using labeled HD, and measured levels of exhaled carbon dioxide and label in various tissues and urine after single and multiple *i.p.* treatments were used to develop the model (Angelo 1981; Angelo and Bischoff 1983). The model also included label recovered as bound to blood components.

develop the model (Angelo 1981; Angelo and Bischoff 1983). The model also included label recovered as bound to blood components.

Benzene PBPK Models

Numerous models have been developed for benzene, more than for the other chemicals described in this dissertation. An early steady-state PBPK model for benzene was described (Hilderbrand *et al.* 1981). A model by Travis *et al.* used a standard structure except added bone marrow (Travis *et al.* 1990). The model incorporated saturable metabolism of benzene in the bone marrow and liver, and assumed that 80% of total benzene metabolites would be excreted as phenol in the urine. The model was validated with several existing data sets. A PBPK model was used to evaluate the adequacy of model fit when metabolic parameters values measured *in vitro* were used (deJongh and Blaauboer 1996).

Research at the Chemical Industry Institute of Toxicology (now CIIT Centers for Health Research) has developed a large body of benzene PBPK models. Many of the models incorporated relatively detailed descriptions of benzene metabolism. In the first such model, benzene was metabolized to phenol, phenol to phenol conjugates, mercapturic acid species, muconic species, and hydroquinone conjugates (Medinsky *et al.* 1989b). Subsequently, similar models were used to scale to humans and to calculate some dose metrics (Medinsky *et al.* 1989a; Medinsky *et al.* 1989c).

A PBPK model for benzene that included metabolite –hemoglobin binding by two processes was developed (Sun *et al.* 1990). A model for young and older mice with renal clearance of metabolites was developed (McMahon *et al.* 1994). Gender differences were later examined in a benzene PBPK model (Kenyon *et al.* 1996). The most metabolically detailed benzene model published to date (Cole *et al.* 2001) included most of the significant metabolites and intermetabolite-inhibition of metabolism, a concept employed in the *n*-hexane model described in Chapter 7 of this dissertation.

Most benzene PBPK models assume that benzene is metabolized directly to phenol, when what actually happens is that benzene is metabolized to benzene oxide which rearranges to phenol. A PBPK model that treated benzene oxide as a measured metabolite was developed (Lindstrom *et al.* 1997). Another model designed to examine gender differences used very simple metabolic descriptions (Brown *et al.* 1998). Several models have been used to examine issues related to parameter sensitivity and the variability of model output (Droz *et al.* 1989a; Droz *et al.* 1989b; Bois *et al.* 1991a; Bois *et al.* 1991b; Spear *et al.* 1991; Bois and Paxman 1992; Cox and Ricci 1992; Woodruff *et al.* 1992; Woodruff and Bois 1993; Spear and Bois 1994; Krewski *et al.* 1995; Bois *et al.* 1996; Thomas *et al.* 1996; Weisel *et al.* 1996).

Toluene PBPK Models

An early PBPK model for toluene was developed by Tardif *et al.* using gas uptake data. This model contained four-compartments with simple metabolism of the parent compound in the liver.

Two PBPK models were developed for toluene to determine dose metrics (toluene in brain) for comparison to neurotoxicity data in rats. The first model used a simple structure to estimate the arterial blood concentration that would occur after exposures that were used in various different studies reported in the literature (Benignus *et al.* 1998). While the studies results were fairly inconsistent when compared on the basis of external exposure level, when compared based on arterial blood concentration, the different studies were relatively consistent. The second study used a toluene PBPK model for rats to assess the impact of fluctuating exposure levels on neurological performance (van Asperen *et al.* 2003).

A seven compartment model for toluene was developed to perform uncertainty analysis (Jang *et al.* 1999). Model output was found to be affected by the values chosen for a number of input parameters including metabolism and partitioning parameters.

Ethylbenzene PBPK Models

No single chemical models for ethylbenzene have been identified in the open literature.

Xylene PBPK Models

Kaneko *et al.* developed a PBPK model for *m*-xylene (Kaneko *et al.* 2000). This model used a six compartment description of the rat (standard four compartments plus lung and tail) with single enzyme saturable metabolism in the liver. The model was compared with data for the *m*-xylene concentration in tail blood and total methylhippuric acids in urine.

A PBPK model for *m*-xylene was developed and used to assess PK differences between Asian and Caucasian males (Jang and Droz 1997). The model was a seven compartment structure (four standard plus lung, kidney, and brain) with saturable metabolism in the liver. The model was validated with *m*-xylene concentrations in breath and blood and methylhippuric acids in urine data. Significant differences were found in the PKs of *m*-xylene between the two ethnic groups.

PBPK Models of Simple Mixtures of BTHEX Compounds

Tardif and colleagues at the University of Montreal developed a series of PBPK models for BTHEX compounds. These models employed a four compartment model structure, saturable metabolism of in the liver, and competitive metabolic inhibition of substrate metabolism by other chemicals in each of various mixtures investigated. The models were generally developed using PK data for the parent chemical in venous blood of rats after four hour constant-level exposures to each chemical in the 50 – 500 ppm range. The models were then scaled to humans allometrically and validated against low level human data. Metabolic inhibition was usually described by allowing each chemical to have a different inhibition constant *vis á vis* each substrate it inhibited. The mixture models with more components were developed using the models for fewer components by adding one chemical at a time, using experimental data for each combination of chemicals engendered by adding a new component.

The initial model by Tardif *et al.* included toluene and *m*-xylene in rats and humans (Lapare *et al.* 1993; Tardif *et al.* 1993). To this binary mixture, ethylbenzene was then added, creating a ternary mixture model (Tardif *et al.* 1997). Then, benzene was added to the ternary mixture (Haddad *et al.* 1999).

A later model by Tardif *et al.* was developed for toluene and *n*-hexane (Ali and Tardif 1999). In this model, a standard four compartment structure was used with

metabolism in the liver by a single saturable enzyme. *n*-Hexane was assumed to be metabolized to HD in a single step; no intermediates were considered. Different inhibitory constants were estimated for each mode of inhibition by toluene on *n*-hexane metabolism (competitive, non-competitive, uncompetitive). A similar model for toluene and *n*-hexane by Yu *et al.* involved somewhat more complex metabolism (Yu *et al.* 1998). In the Yu *et al.* model, *n*-hexane was metabolized to hexanols by a saturable pathway. Hexanols were metabolized either to HD or to "others" via two linear pathways. HD was excreted to body water and then to urine by two liner pathways in series. Inhibition between *n*-hexane and toluene was assumed to be non-competitive, affecting only the first metabolic step for *n*-hexane. Some data was used to validate the model for the single chemicals, but no validation of the mixture model was included. While Yu *et al.* used the PB (blood:air partition coefficient) for *n*-hexane of 0.8 (from Perbellini 1986), Ali *et al.* used a PB of 2.3 from another source for rats and 0.8 for humans.

Jang *et al.* developed a PBPK model for ethylbenzene and *m*-xylene following the same seven-compartment structure the group used for single chemical models (Jang *et al.* 2001). They assumed single enzyme saturable metabolism of each component under metabolic inhibition in the liver.

A PBPK model for toluene and benzene was developed using gas uptake PK data (Purcell *et al.* 1990). This model assessed the metabolic interactions between toluene

and benzene using a four compartment PBPK model with single saturable metabolism in the liver. The PK data were best described with non-competitive inhibition.

Gasoline PBPK Models

One PBPK model that involved gasoline has been previously developed (Travis *et al.* 1992). This model used gas uptakes studies using gasoline that was enriched to 30% benzene in F344 rats. This was not actually a mixture model, however. Travis *et al.* determined the best fit metabolic constants (Vmax and Km) for benzene under the single chemical and chemical mixture condition, so the model only incorporated benzene as a single chemical.

Prior Uses of Chemical Lumping

These studies, in part, apply the concept of chemical lumping to pharmacokinetic modeling. The concept of chemical lumping is not new, although the application of lumping in PBPK modeling has not previously been investigated. In a sense, chemical lumping has been performed in PBPK models in terms of metabolites. For example, Medinsky *et al.* combined various sulfate and glucuronide conjugates for benzene model development (Medinsky *et al.* 1989b). However, this approach was used for terminal metabolites (*i.e.*, metabolites that did not get further metabolized according to the model); parent compounds have never been lumped in a PBPK model. Other forms of lumping in PBPK models include tissue and blood flow

lumping (always performed) and enzymatic pathway lumping (also always assumed to some extent). Lumping of parent chemicals in complex mixtures was suggested in previous literature (Bond *et al.* 1997; Medinsky 1997; Verhaar *et al.* 1997).

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Chapter 3

Evaluation of Potential Toxicity from Co-Exposure to Three CNS Depressants (Toluene, Ethylbenzene, and Xylenes)

Abstract

Under OSHA and ACGIH guidelines, the Mixture Formula (Unity Calculation) provides a method for evaluating exposures to mixtures of chemicals that cause similar toxicities. According to the formula, if exposures are reduced in proportion to the number of chemicals and their respective exposure limits, the overall exposure is acceptable. This approach assumes that responses are additive, which is not the case when pharmacokinetic interactions occur. To determine the validity of the additivity assumption, we performed unity calculations for a variety of exposures to toluene, ethylbenzene, and/or xylene using the concentration of each chemical in blood in the calculation instead of the inhaled concentration. The blood concentrations were determined using a validated physiologically-based pharmacokinetic (PBPK) model to allow exploration of a variety of scenarios where the classical unity calculation was 1.0. For various mixtures of the three chemicals, the analysis indicates that a modest overexposure occurs due to pharmacokinetic interactions. However, if a pharmacokinetic inhibitor such as xylene is omitted from a classical unity calculation, pharmacokinetic interactions are more significant, and the tissue doses of toluene and

ethylbenzene increase about 50%. If xylene also causes some CNS depression directly, a greater cumulative toxicity would occur. The exposure limits for these chemicals were primarily based on studies where individuals were resting, unlike what may occur in the workplace. Analysis of allowable exposures indicates that effective exposure was approximately twice as high during light work as at rest. The present study illustrates the utility of PBPK modeling in occupational health risk assessment.

Introduction

In the workplace, employees are often, or perhaps normally, exposed to more than one chemical at a time. Co-exposure to chemical mixtures can result from products that contain multiple chemicals or exposure to more than one product, even at different times if there is an overlap between the clearance of one chemical from the body and exposure to a second chemical. If the chemicals cause toxicity via similar mechanisms, the cumulative toxicity from the mixture will be greater than from either of the individual chemicals. Also, if the chemicals interact within the body, their cumulative toxicity will differ from the sum of toxicity of the individual components of the mixture.

Examples of chemicals with similar mechanisms of toxicity exist for essentially every chemical class. Specifically, many organic solvents cause CNS depression after entering the brain and diffusing into membranes of neurons or supporting brain cells. Examples of CNS depressants are thought to include most hydrocarbons, halogenated hydrocarbons, alcohols, ethers, and similar compounds (Caprino and Togna 1998;

Bruckner and Warren 2001; Dobrev *et al.* 2002). Many organic solvents are also capable of interacting with other organic solvents. Two types of interactions can be considered, pharmacokinetic (PK) or pharmacodynamic (PD). "Pharmacokinetic" refers to the adsorption, distribution, metabolism, and excretion (ADME) of chemicals, and "pharmacodynamic" refers to the chemical's mechanism of action. In another parlance, pharmacokinetics refers to "what the body does to the chemical," and pharmacodynamic refers to "what the chemical does to the body." By "PK interaction", we mean that the tissue dose (*i.e.*, the concentration of a relevant chemical or metabolite in a target tissue) of one chemical is altered by the presence of another chemical.

Organic solvents commonly interact with other solvents because they are principally metabolized by a specific enzyme (primarily in the liver), cytochrome P450/2E1 (Guengerich and Shimada 1991; Lof and Johanson 1998). If the concentration of the solvent is high enough, it can compete with other solvents for metabolism. This competition can, depending on exposure levels, serve to delay the rate of elimination of the solvents and alter the potential toxicity of exposure. The literature is replete with examples of inhibition of metabolism with common organic solvents such as with mixtures of alkyl benzenes (Purcell *et al.* 1990; Tardif *et al.* 1995; Imbriani and Ghittori 1997; Tardif *et al.* 1997; Haddad *et al.* 1999a; Thrall and Poet 2000), chlorinated ethanes and ethylenes (Andersen *et al.* 1987b; Barton *et al.* 1995; El Masri *et al.* 1996; Dobrev *et al.* 2002), and other chemicals (Pelekis and Krishnan 1997). However, below some exposure level, the effect becomes insignificant. The

challenge in mixture toxicology is to delineate the threshold of interactions and to offer a means of determining the net effect of mixture exposure when exposures exceed this level.

Current approaches to assessing the risk of mixed exposures generally suggest making the assumption that cumulative effects are additive for the same mechanism of action, unless there are data available indicating that a significant interaction occurs and also supporting a means of quantifying such interactions. Physiologically-based pharmacokinetic (toxicokinetic) descriptions of solvent behavior in humans can be used as an alternate approach to assess the quantitative differences in chemical exposure to mixtures (Filser *et al.* 1993; Krishnan *et al.* 1994; El-Masri *et al.* 1997). The objective of this paper is to present an analysis of the effect of mixture exposure to a set of organic solvents (toluene, ethylbenzene, and xylene) under a variety of exposure circumstances. This analysis is intended to clarify the extent of interactions under various exposure scenarios and to suggest ways to avoid unintentional overexposures.

Many organic solvents have an unclear dose response relationship with respect to CNS depression. There are several reasons for this, including the limited number of available studies of the relationship, conflicting results of available studies, and differences in study designs (including measurements of different endpoints and design features that result in PK differences between studies). Therefore, defining the acceptable level of exposure to many solvents is a difficult process. For the solvents

included in this analysis, the American Conference of Governmental Industrial Hygienists (ACGIH) has established Threshold Limit Values (TLVs) and the U.S. Occupational Safety and Health Administration (OSHA) has established Permissible Exposure Limits (PELs). Most PELs coincidentally are based on the 1969 TLVs and as such as viewed by many as outdated, but still have the force of law. The current TLVs and PELs for the solvents in this analysis are listed in Table 3.1. The choice of occupational exposure limit (OEL) to use in a mixture assessment will have a significant impact on the result of the analysis.

While OSHA suggests that all chemicals listed in SubPart Z of 1910.1000 (General Industry standard) should be included in the assessment of mixture exposures (OSHA 2003), typical

Table 3.1
TLVs and PELs for Toluene, Ethylbenzene, and Xylene

	Toluene	Ethylbenzene	Xylenes
PEL	200	100	100
TLV	50	100	100
Critical Effect[1]	CNS	Irritation, CNS	Irritation, CNS?[2]

[1] Per TLV handbook. [2] Per other sources.

practice is to include chemicals that have the same mechanism of action. At times, this is crudely determined on the basis of target organ or tissue, *i.e.*, if two chemicals are liver toxins, they would be included in a mixture assessment, although a more detailed analysis of the mechanism of action might also be used. One source of information on target organ effects is the ACGIH's handbook listing of the TLVs (ACGIH 2002a) which mentions the "critical effect" that served as the basis for

setting the TLV. Despite the convenience of the handbook, ACGIH recommends reviewing the Documentation of the TLVs (ACGIH 2002b) to more thoroughly assess the potential for common mechanisms of action or interactions. Other sources of information are also broadly available.

The need for an evaluation of these more detailed sources is underscored by examining the critical effects of toluene, ethylbenzene, and xylene. Toluene is generally accepted as a CNS depressant at relevant exposure levels. Numerous studies support the presence of effects at levels near the current TLV (Bruckner and Warren 2001). Indeed, CNS is the listed critical effect in the TLV handbook. Studies of CNS depression are limited with xylene and particularly limited with ethylbenzene. Nevertheless, many sources considered these two chemicals capable of causing CNS depression (OEHHA 1997a, b; Bruckner and Warren 2001; ACGIH 2002b). Based on available information, the TLV handbook lists irritation and CNS effects as the basis for the TLV for ethylbenzene and irritation alone as the basis for the xylene TLV. Thus, for xylene, irritation appears to be the principal effect and CNS depression appears to be a secondary effect, occurring at the same exposure level or perhaps at a level slightly higher. Thus, three scenarios can be considered for xylene: the chemical causes CNS depression at a level consistent with the TLV; it causes CNS depression at a level higher than the TLV; or it cannot cause CNS depression at any relevant exposure level. The analysis in this paper will evaluate the effect of xylene under each scenario.

TLVs and PELs are established for specific substances, typically those in common-enough use to stimulate toxicological study. Other chemicals may exist that can cause CNS depression for which no TLVs or PELs are established. In addition, chemicals may exist that do not cause CNS depression but are capable of competitively inhibiting the metabolism of other solvents. For any of these reasons, chemicals that are capable of enhancing the toxicity of other chemicals may be omitted from consideration under mixture assessment.

Another issue that arises when interpreting toxicological studies for these chemicals relates to the ventilation rate of the subjects. At high ventilation rates, much more chemical is inspired and available for absorption through the lung. Blood flow to the liver may decrease as well, limiting metabolic clearance of the chemical. Thus, alveolar air and blood levels of inhaled solvents tend to increase during exercise (Pezzagno *et al.* 1988; Baelum 1990; Lapare *et al.* 1993). However, many of the studies of CNS depression in human volunteers were conducted at resting conditions, when CNS levels of the chemical would be lower (Ogata *et al.* 1970; Andersen *et al.* 1983; Olson *et al.* 1985; Hastings *et al.* 1986). Therefore, TLVs based on resting conditions may not be adequately protective of workers who are performing manual tasks that constitute exercise.

We have reviewed several conditions in this Introduction that can be examined for their impact on potential toxicity. These include: PK interactions that increase target tissue concentrations of chemicals, assumed potency under different OELs (TLVs or

PELs), omission of chemicals that have the same mechanism of action or cause PK interactions, and increased body burden due to exercise. In this paper, an analysis of the potential impact of these issues with respect to CNS depression caused by toluene, ethylbenzene, or xylene will be provided.

Application of the Mixture Formula

Many health and safety professionals use the "Mixture Formula" to evaluate the potential for cumulative overexposure when mixtures are present (Klonne and Miller 1997). Implementing the Mixture Formula, which is also referred to as the "Unity Calculation" and denoted as EM, is a two-step process: 1) identification of chemicals that cause similar kinds of toxic responses, and 2) for those chemicals, calculating the sum of the ratios of the employee exposure level to each chemical to the OEL for each chemical. A sum of these ratios exceeding unity (1.0) suggests that exposures should be reduced (Eq. 1).

$$EM = \sum \frac{Exposure_i}{OEL_i} \qquad \text{Eq. 1}$$

For instance, if exposure to Chemical A is 35 ppm and to Chemical B is 50 ppm, and both chemicals have an OEL of 100 ppm, the EM would be 35/100 + 50/100 = .85 and the cumulative exposure would not appear to be more significant than an exposure to either chemical alone at its OEL. This method is recommended by ACGIH (ACGIH 2002a) and is technically required for compliance with OSHA's General Industry standard (OSHA 2003). It is also equivalent to the Hazard Index

method suggested by USEPA for some risk assessment applications (Mumtaz and Hertzberg 1993; USEPA 2000).

The Mixture Formula assumes additivity and linear PKs by pro-rating exposures to the OEL, and it also ignores PK interactions between components. As indicated above, non-linear PKs and interactions commonly occur in solvent exposures. Physiologically-based pharmacokinetic (toxicokinetic) models (PBPK or PBTK models) can be used to evaluate these issues. Such PBPK descriptions have been used to describe the ADME of chemicals and chemical mixtures in laboratory animals and humans for many years (Andersen *et al.* 1987a; Andersen *et al.* 1987b; Bois *et al.* 1991; Filser *et al.* 1993). Basically, such models are able to provide descriptions of the fate of specific chemicals in various parts of the body, such as in blood, brain, or liver, by simplifying the structure of the organism based on anatomical and physiological principles, applying measured partition coefficients to determine absorption and distribution, using measured rates of metabolism and excretion, and validating the models with timecourse data for chemicals in available parts of the organisms. Validated PBPK and similar models are now the preferred basis for chemical risk assessment performed or used by the USEPA and other organizations (USEPA 1996). One of the benefits of such models is that they permit calculation of the concentration of chemicals or their metabolites in the body tissue of choice (limited of course by the structure of the model) and allow calculation of these *internal* measures of dose under flexible exposure scenarios within the model's validation range. They can therefore be used to evaluate the impact on the

biologically effective dose of a chemical when other chemicals are present by incorporating relevant interaction mechanisms.

The present analysis uses a unity calculation that is based on internal tissue doses instead of the traditional unity calculation based on external exposure level, thus taking into account non-linear PKs and PK interactions' influence on cumulative dose. The calculation is based on the pharmacokinetically-derived EM, or for short, the EM_{PK} (Eq. 2):

$$EM_{PK} = \sum \frac{(C_i @ EL)}{(C_i @ OEL)} \qquad \text{Eq. 2}$$

where C_i is the concentration of a chemical (or a relevant metabolite) in a target tissue at the Exposure Level (EL) or, for the denominator in the equation, at the OEL for the single chemical. The EM_{PK} can be used to assess the mixture exposure based on actual tissue exposures instead of assuming that the PKs are linear and that no interactions occur.

This formulation has previously been used to assess the impact of PK interactions (Ogata *et al.* 1993; Haddad *et al.* 1999b). In previous work, Haddad and co-workers evaluated the EM_{PK} for toluene, ethylbenzene, and xylene mixtures where the concentrations of each chemical were constrained so that the Mixture Formula (EM) was less than unity based on TLVs (Haddad *et al.* 1999b; Haddad *et al.* 2001). In their analysis (Haddad *et al.* 1999b), the EM_{PK} ranged from 4% to 11% over unity when the EM was at unity, suggesting a slight overexposure could occur. The increased EM_{PK} was caused by a modest inhibition of metabolism of each chemical

by the others present in the mixture. The present paper seeks to extend this analysis to address circumstances that involve alternate OELs, the omission of components based on limitations in the toxicological database, and increases in body burden with exercise.

For this analysis, the same set of solvents was selected based on their common mechanism of action, the availability of validated PBPK descriptions of their behavior, and evidence that significant PK interactions occur at exposure levels higher than those studied by Haddad *et al.* The chemicals also serve as a good case study to examine the effect of differing interpretations of the existing toxicological database.

For CNS depression by solvents, the mechanism of action, though not completely understood, involves diffusion of chemicals from the blood into brain tissue and disruption of membrane fluidity and/or membrane ion channels (Bruckner and Warren 2001). For rapidly diffusible compounds such as the alkyl benzenes, the chemical concentration in the brain is expected to be closely equilibrated with that in the venous blood, governed by the brain:blood partition coefficient. Since the concentrations in blood and brain are proportional, essentially identical results would be obtained in the EM_{PK} equation whether using chemical concentration in the venous blood or in the brain. Therefore, venous blood concentrations were used in this analysis because the available PBPK model did not separate brain from other tissues.

Methods

The PBPK model previously published by Tardif *et al.* was used in this analysis (Tardif *et al.* 1997). This model was a standard PBPK model similar to many model constructs used for other chemicals in the past. The model was initially developed for rats and then modified for male adult humans. It contained four compartments, fat tissue, slowly perfused tissues, rapidly perfused tissues (which incorporates the brain), and the liver, where all metabolism is based on a single saturable enzyme representing CYP/2E1. The tissues are perfused by the arterial blood which equilibrates with the alveolar concentration of inspired chemical in accordance with the blood:air partition coefficient. Venous blood returning from each tissue compartment is similarly equilibrated with the tissue according to their tissue:blood partition coefficients. Inhibition of metabolism is addressed as competitive inhibition in the saturable metabolism equation.

The Tardif *et al.* model for toluene, ethylbenzene, and *m*-xylene was initially developed using data for single chemicals and two-chemical mixtures (three binary mixtures.) The model used literature values for physiological parameters and partition coefficients that were measured *in vitro*. Metabolic parameters were determined using the experimental data that consisted of venous blood data collected from the rats at five 30-minute intervals after cessation of a four-hour exposure to 100 – 200 ppm of toluene, ethylbenzene, and/or *m*-xylene. The model was validated first by testing it with venous blood data for toluene, ethylbenzene, and xylene after similar exposures to all three chemicals at the same time. Then, the model was re-

scaled to humans by altering the body weight and other physiological and metabolic parameters in accordance with literature values. The model was then exercised and compared with experimental data obtained during controlled human exposures to mixtures of the three chemicals at levels below the TLV. Reasonable agreement was obtained between the human model and the data.

This model was used in the present analysis with no modification other then the exposure concentrations and in some analyses, the alveolar ventilation and blood flows. For the "working" subject, the alveolar ventilation and blood flow values were re-scaled to values reported for "light work" at 50 watts (Kumagai and Matsunaga 1995). The parameter values used for the PBPK model are listed in Table 3.2. For the present simulation, the biomarker of exposure used in the EM_{PK} calculations was the maximum venous blood concentration observed during an eight-hour exposure to the chemical(s). Generally, acute CNS depression is regarded as related to the peak concentration of chemical in the brain. Based on the proportionality between brain concentration and venous blood concentration, CNS depression is equally linked to peak blood levels. As the exposures were simulated for constant levels, the blood concentration rises throughout the exposure, reaching a quasi-steady state by the end of the period. Between Hour 7 and Hour 8, the concentration of chemicals only increased 1-2%, but the increases during earlier periods was larger. Therefore, the concentration at the end of the exposure period best represents the maximum concentration.

Table 3.2
Model parameters

	Resting	Light Work
Alveolar ventilation	18 L/hr/kg^0.75	40 L/hr/kg^0.75
Cardiac Output	18 L/hr/kg^0.75	26 L/hr/kg^0.75

	Fraction body	Fraction blood flow at rest	Fraction blood flow, light work
Fat tissue	19%	5%	7%
Liver tissue	2.6%	26%	13%
Richly perfused tissue	5%	44%	30%
Slowly perfused tissue	62%	25%	50%

Chemical Parameters	Blood:air	Liver	Fat	SP	RP	Vmax	Km	Ki1	Ki2
Toluene	15.6	5.36	65.4	1.78	5.36	4.8	0.55	.79	.17
Ethylbenz.	28.0	2.99	55.6	.93	2.15	7.3	1.39	.33	.23
Xylene	26.4	3.44	70.4	1.59	3.44	5.5	.22	.77	1.5

KI1 is the KI towards the substrate that appears highest in the table.

Many previous studies have indicated metabolic inhibition occurs between toluene, ethylbenzene and xylene (Engstrom *et al.* 1984; Tardif *et al.* 1993; Mortensen *et al.* 1998). Most literature describes this interaction as being caused by competitive inhibition of metabolism by CYP/2E1 in the liver (Tardif *et al.* 1995; Tardif *et al.* 1997; Haddad *et al.* 1999a). *m*-Xylene is one of three xylene isomers. Toxicological effects of the three isomers are generally considered to be similar (OEHHA 1997b) and the PKs of *o*-xylene have been found to be similar to the PKs of *m*-xylene (Dennison *et al.* 2003). Therefore, the results of the present evaluation, based on *m*-xylene, should be similar to results obtained for other isomers of the compound.

The following equations describe the method of EM_{PK} calculations when xylene is considered to be non-neurotoxic, or equipotent at 200 ppm (2x TLV) or 100 ppm (1x TLV) to toluene and ethylbenzene at their OELs.

$$EM_{PK} = \frac{C_{t,EL}}{C_{t,OEL}} + \frac{C_{e,EL}}{C_{e,OEL}}$$

Xylene is not neurotoxic.

Eq. 3

$$EM_{PK} = \frac{C_{t,EL}}{C_{t,OEL}} + \frac{C_{e,EL}}{C_{e,OEL}} + \frac{C_{x,EL}}{C_{x,200ppm}}$$

Xylene is equipotent at 200 ppm.
Eq. 4

$$EM_{PK} = \frac{C_{t,EL}}{C_{t,OEL}} + \frac{C_{e,EL}}{C_{e,OEL}} + \frac{C_{x,EL}}{C_{x,100ppm}}$$

Xylene is equipotent at 100 ppm.
Eq. 5

Exposure Scenarios and Risk Evaluation

A series of assessments are presented in this paper. In each of the following cases, we will examine three possible scenarios concerning the contribution of xylene to CNS depression. Xylene may only act as a CNS depressant at such high levels that it practically contributes nothing to the cumulative CNS depression directly. However, in this scenario, xylene still impacts the PKs of toluene and ethylbenzene, so it indirectly increases CNS depression through this interaction. Thus, to examine this scenario, we include xylene in the PBPK model to account for PK interactions, but it does not appear as a term in the EM_{PK} equation because it is assumed to not act as a CNS depressant (Eq. 3 above).

Since the OELs of toluene and ethylbenzene are based, in part, on CNS depression, we can say that at the OEL, CNS depression will occur to some extent, albeit possibly

a minimal one. In other words, toluene and ethylbenzene have some degree of potency at their OEL. The second scenario for xylene's impact assumes that xylene has an equivalent neurotoxic potency at its OEL as toluene and ethylbenzene have at theirs. In other words, 100 ppm xylene is "equipotent" to 100 ppm ethylbenzene or 50 ppm toluene (Eq. 5 above). The third scenario for xylene's impact lies in between the first two, assuming that xylene has an equivalent potency at 200 ppm (2X OEL), *i.e.,* 200 ppm xylene is "equipotent" to ethylbenzene and xylene at their OELs. For each scenario, the exposure concentrations of toluene and ethylbenzene are set at 50% of their OEL so that an EM calculation would be 1.0, ignoring xylene. Xylene is then introduced at concentrations between 0 and 125 ppm. Thus, the three scenarios in this evaluation of the effect of xylene are (1) xylene is not neurotoxic (but may increase the neurotoxicity of toluene and ethylbenzene by increasing their concentrations in blood, (2) xylene is less neurotoxic at its OEL than toluene and ethylbenzene are at theirs, and (3) xylene is equally neurotoxic at its OEL as toluene and ethylbenzene are at theirs. These three scenarios are combined with analyses of toluene and ethylbenzene under differing assumptions regarding the adequacy of TLVs, PELs, and the effect of exercise. Evaluation of the mixture of toluene, ethylbenzene, and xylene at ratios where the EM is less than 1.0 is not performed here, as this scenario was evaluated in the previous study described above (Haddad *et al.* 2001).

Using these scenarios, we examined the impact of PK interactions caused by metabolic inhibition, at both TLVs and PELs. First, exposures relative to TLVs are

assessed, with toluene and ethylbenzene at 50% of their TLV and xylene ranging between 0 and 125 ppm. Then, exposures relative to PELs are assessed, again with xylene ranging between 0 and 125 ppm. The EM_{PK} calculations for this analysis presume that the PEL is set at an appropriate level with respect to CNS depression.

In the next set of analyses, we evaluate the impact of using "inappropriate" OELs. In other words, if the TLV is set at the appropriate level to protect a worker from CNS depression, what would the impact be if the PEL was used as the exposure standard? Thus, in this analysis, exposures are set at 100 ppm for toluene and 50 ppm for ethylbenzene (50% of PEL) and 0 to 125 ppm for xylene. However, the EM_{PK} calculations use the venous blood concentration of each chemical at the TLV as the benchmark.

The impact of light work was assessed by altering the physiological parameter values according to previous reports (Kumagai and Matsunaga 1995). For light work, the alveolar ventilation and cardiac output are increased in the PBPK model and cardiac output is redistributed (Table 3.2).

Finally, a worst-case analysis is presented. In this analysis, exposure concentrations of toluene and ethylbenzene are permitted at the full value of their PELs (EM = 2.0) but it is assumed that the TLV is the more appropriate OEL, and light work is performed. Again, xylene is varied between 0 and 125 ppm and assumed to be non-neurotoxic, equipotent at 100 ppm or equipotent at 200 ppm.

Results

In the first scenario, toluene and ethylbenzene exposures are permitted to occur at 50% of their TLV. This scenario could occur if a health professional were to deem toluene and ethylbenzene but not xylene to be CNS depressants. If xylene is actually not neurotoxic, the EM_{PK} ranges from 1.16 to 1.6 for xylene between 0 and 125 ppm (Figure 3.1, Curve A). The low value is obtained when no xylene exposure occurs at all, *i.e.*, the worker is exposed to a binary mixture of toluene and ethylbenzene within the EM limit. Thus, the result is equivalent to previous studies finding a modest overexposure to these mixtures when the EM is obeyed for TLVs (Haddad *et al.* 1999b). Higher apparent overexposures occur when xylene is present, up to 1.6 (*i.e.*, a 60% increase in dose), because even if xylene is not a CNS depressant, it increases CNS depression by raising blood concentrations due to inhibition of toluene and ethylbenzene metabolism. Thus, at 100 ppm (the xylene TLV), the EM_{PK} for toluene and ethylbenzene is 1.5, indicating a 50% overexposure.

If 200 ppm xylene is equipotent to toluene and ethylbenzene at their TLVs, the EM_{PK} ranges from 1.16 to 2.2 (Figure 3.1, Curve B). At 0 ppm xylene, the EM_{PK} is identical to that in the first scenario. However, at 100 ppm, the EM_{PK} of 2.0 is higher than the EM_{PK} when xylene is assumed to confer no direct CNS depression. This calculation indicates that in addition to indirectly causing more CNS depression (EM_{PK} of 1.5), the additional CNS depression caused by xylene directly would

increase the EM_{PK} by another 0.5. When 100 ppm xylene is assumed to be equipotent to toluene and ethylbenzene, the EM_{PK} ranges from 1.16 to 3.7 (Fig. 3.1, Curve C). At 100 ppm xylene, the EM_{PK} is 3.1, indicating a very significant overexposure caused by direct and indirect effects of xylene.

Altering the ratios of toluene and ethylbenzene within the EM formula does not significantly change the EM_{PK} calculations. However, if no unity calculation is performed at all (toluene and ethylbenzene are allowed to range up to their full TLVs), the EM_{PK} would start at about 2 and go up from there.

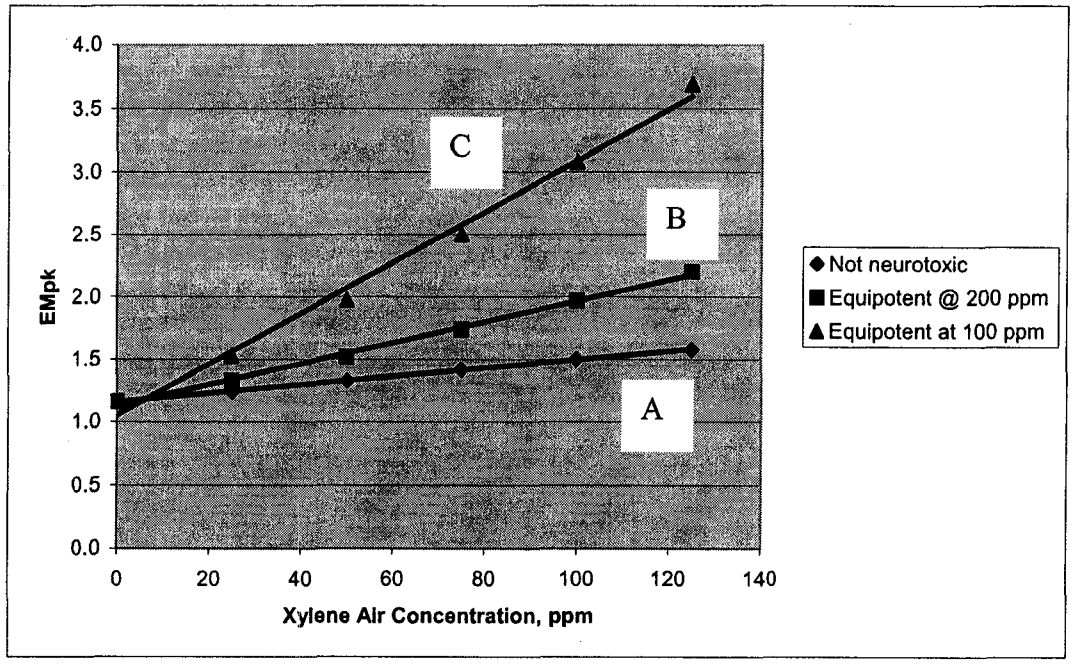

Figure 3.1 EM_{PK} calculations based on TLVs as an appropriate OEL. The EM_{PK} is based on exposures to 25 ppm toluene and 50 ppm ethylbenzene (EM = 1.0 if xylene is not included.) "EM_{PK}" represents the unity calculation using the concentration of the chemical in the venous blood, an internal measure of dose. For xylene between 0 and 125 ppm, the curves shown assume A) xylene does not cause CNS depression, B) xylene would be equipotent at 200 ppm to toluene and ethylbenzene at their TLVs, and C) xylene is equipotent at 100 ppm to toluene and ethylbenzene at their TLVs.

Similar calculations were also conducted for PELs (Fig. 3.2) rather than TLVs. In these assessments, the toluene concentration is increased to 100 ppm based on its 200 ppm PEL, but based on PELs, the EM calculation is still 1.0. In other words, this would be an allowable exposure under OSHA regulations if the EM calculation is applied to toluene and ethylbenzene. If xylene is assumed to not be a CNS depressant, the EM_{PK} ranges from 1.16 to 1.4 (Curve A). When xylene is 100 ppm, the EM_{PK} is 1.3, slightly lower than when the toluene exposure was based on its TLV. This behavior occurs because the PEL exposure to toluene is more permissive. In

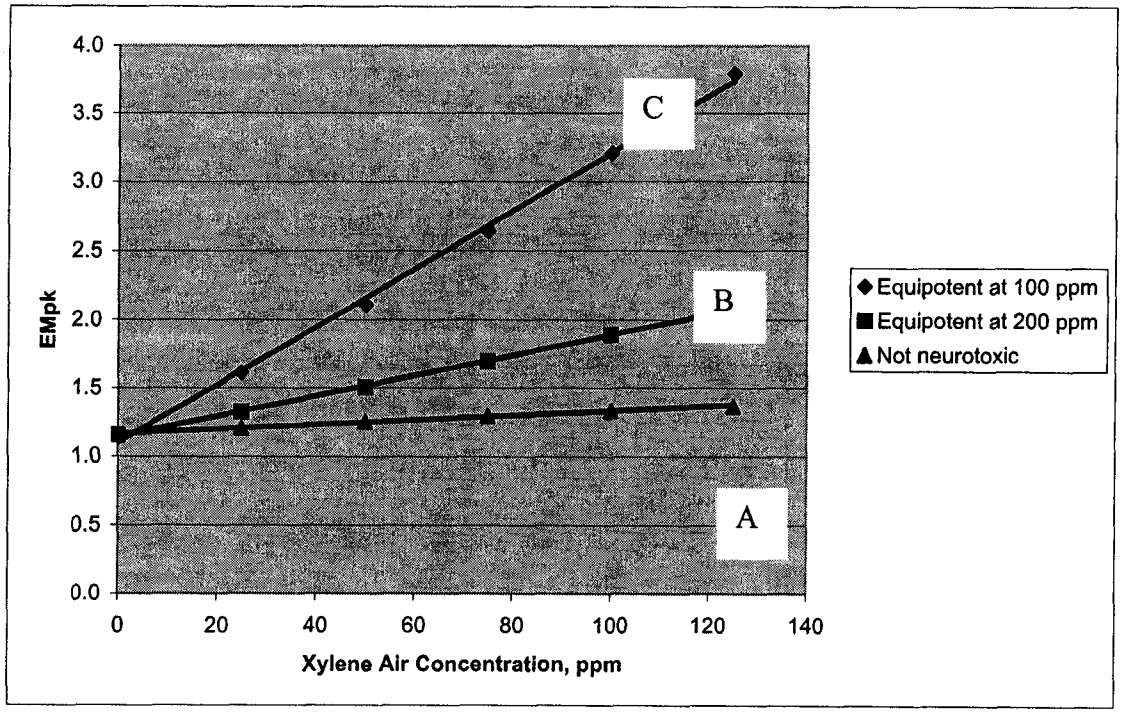

Figure 3.2. EM_{PK} based on PELs as an appropriate OEL. The EM_{PK} calculations are based on exposures to 100 ppm toluene and 50 ppm ethylbenzene (EM = 1.0 based on PELs if xylene is not included.) For xylene between 0 and 125 ppm, the curves shown assume A) xylene does not cause CNS depression, B) xylene would be equipotent at 200 ppm to toluene and ethylbenzene at their TLVs, and C) xylene is equipotent at 100 ppm to toluene and ethylbenzene at their TLVs.

other words, the venous concentration at the OEL is higher for toluene at its PEL (denominator term in the EM_{PK} calculation), and the resulting EM_{PK} is lower. When xylene is assumed to be equipotent at 200 ppm, the EM_{PK} ranges from 1.16 to 2.1 and is 1.9 when xylene is 100 ppm (Curve B). When xylene is assumed to be equipotent at 100 ppm, the EM_{PK} ranges from 1.16 to 3.8 (Curve C). These EM_{PK} calculations are similar to the EM_{PK} calculations when TLV exposures were used (Figure 3.1).

A third set of calculations was performed with exposure to toluene and ethylbenzene at 100 and 50 ppm respectively (50% of PEL) so that an EM calculation based on PELs would be 1.0 (Fig. 3.3). In this scenario, we assume that the PEL is an inappropriate OEL to protect against CNS depression and that the TLV would be an appropriate limit. Thus, the exposure is based on the TLV. Even without any xylene exposure, this premise produces an estimate of significant overexposure (EM_{PK} >4). This is primarily caused by the fact that the toluene PEL is four times the TLV. Further modification of the EM_{PK} is also caused by PK interactions between toluene and ethylbenzene. When xylene is also present, a more significant overexposure occurs. If xylene is not a CNS depressant (Curve A), the EM_{PK} ranges up to 4.7 due to additional metabolic inhibition of toluene and ethylbenzene. If xylene is equipotent at 200 ppm (Curve B), the EM_{PK} ranges up to 5.4 and if xylene is equipotent at 100 ppm (Curve C), the EM_{PK} ranges up to 7.1. These calculations suggests that if the PELs are inappropriate standards and xylene is not addressed in limiting the mixture exposure with the EM, a very substantial overexposure occurs.

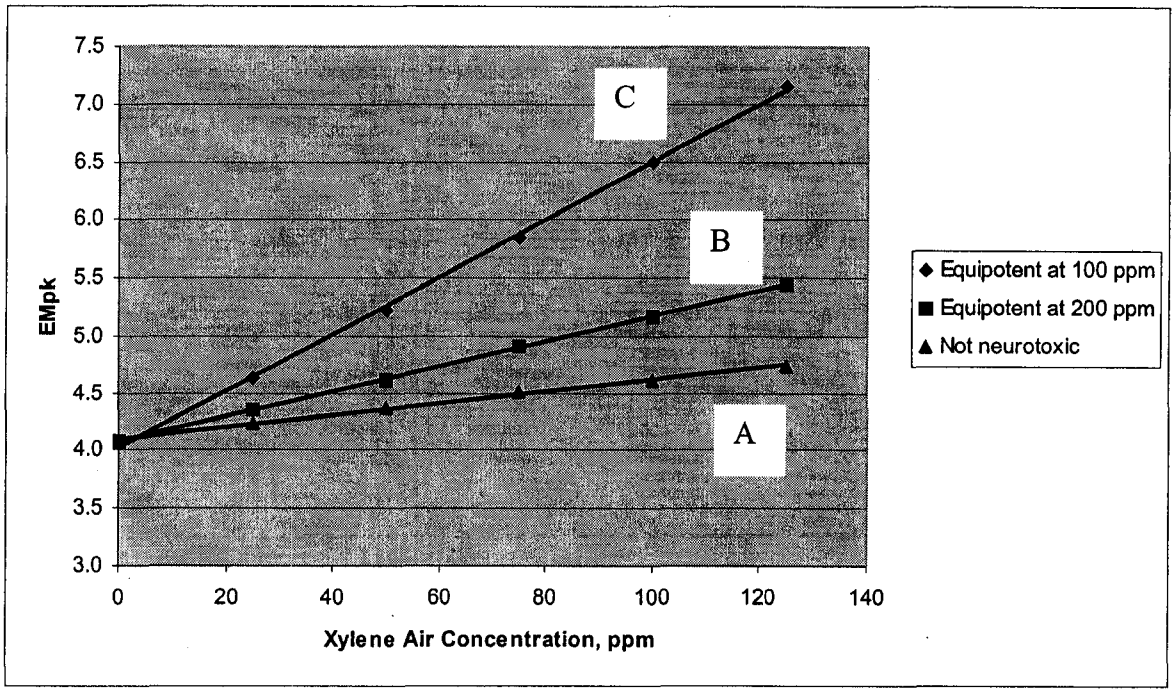

Figure 3.3. EM_{PK} based on TLVs as the appropriate OEL, but exposure being controlled by a unity calculation based on the PELs for toluene and ethylbenzene. EM_{PK} calculations for an exposure to 100 ppm toluene and 50 ppm ethylbenzene (EM = 1.0 based on PELs if xylene is not included.) However, the assumption is made that the TLVs are appropriate standards to protect against CNS depression (EM based on TLVs is 2.0). For xylene between 0 and 125 ppm, the curves shown assume A) xylene does not cause CNS depression, B) xylene would be equipotent at 200 ppm to toluene and ethylbenzene at their TLVs, and C) xylene is equipotent at 100 ppm to toluene and ethylbenzene at their TLVs.

The impact of light work activity was also evaluated (Fig. 3.4). Panels A-C show the venous blood concentration of the single chemicals during resting states and under light work. In each case, the blood level of the chemical is significantly increased by light work. Under light work loads, the exposure concentration that would yield the same venous blood level as would occur under resting condition exposure to the TLV is 28, 59, and 58 ppm for toluene, ethylbenzene and xylene respectively. In Panel D, the EM_{PK} is plotted against the EM with all three chemicals included and assuming

that each is a CNS depressant at the TLV. At rest, the EM_{PK} is slightly higher than the EM, as previously mentioned. These would represent allowable exposures under ACGIH and OSHA guidance. However, if light work is performed, a more significant blood concentration is reached, represented by the upper EM_{PK} curve. For an EM of 1.0 (each component is at 1/3 the TLV), the light work EM_{PK} is 2.0, representing a doubling of actual exposure. These results are similar to previous findings (Pezzagno *et al.* 1988; Nihlen and Johanson 1999).

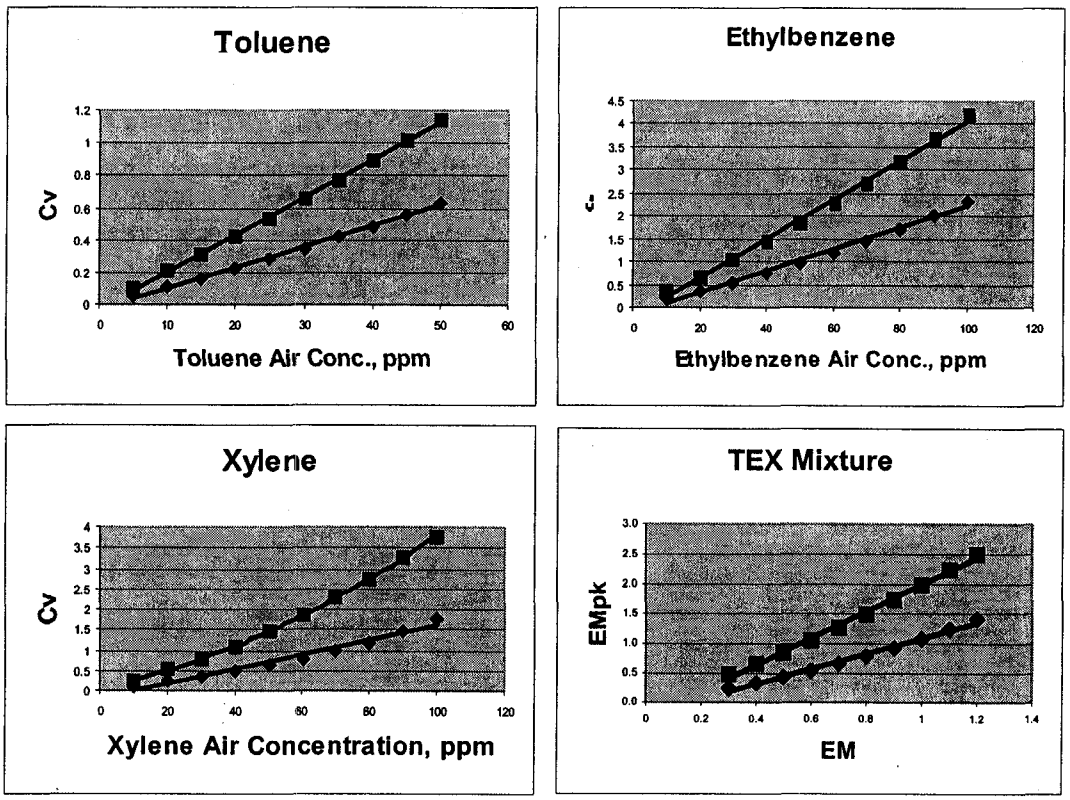

Figure 3.4. Impact of light work on the blood level of chemicals and EM_{PK}. A-C. Comparison of venous blood concentrations (Cv) for toluene, ethylbenzene, or xylene under resting and light work activity levels. D. Comparison of EM_{PK} versus EM for mixtures of toluene, ethylbenzene, and xylene (TEX) under resting or light work. In all panels, light work is shown in the upper curve and resting condition is shown in the lower curve.

In a worst-case scenario, all sources of potential overexposure described above are combined (Fig. 3.5). In this case, the exposure to toluene and ethylbenzene is set at the full PEL (not 50% of the PEL), in the event that no unity calculation is implemented to reduce exposure to the mixture. In addition, the analysis assumes that the TLV is the appropriate OEL for CNS depression for toluene and ethylbenzene. Finally, the analysis assumes that the worker is performing light work activity and calculates the EM_{PK} based on the three scenarios for xylene

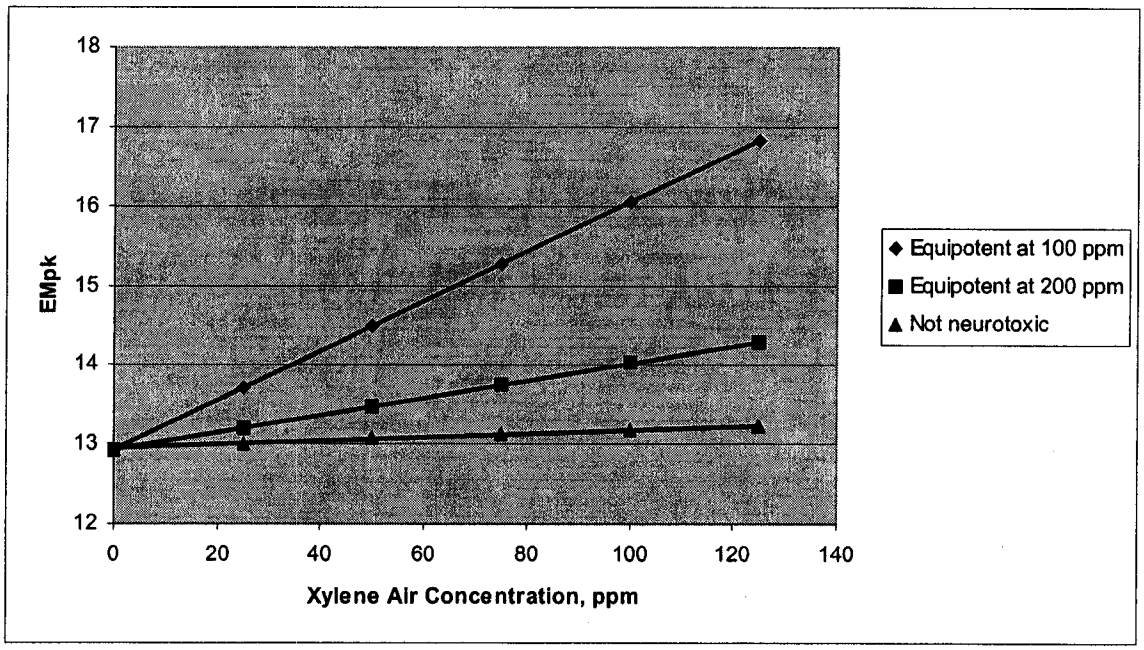

Figure 3.5. A worst case calculation of EM_{PK}. In this scenario, exposures to toluene and ethylbenzene at permitted at 100% of their PEL, but CNS depression is calculated based on the assumption that the TLV is the appropriate OEL. Xylene is not included in the EM calculation and adds to cumulative toxicity directly as a CNS depressant and indirectly by inhibiting metabolism of toluene and ethylbenzene. Finally, the TLVs are based on resting activity levels, but that the worker is performing 50 watts of activity (light work).

neurotoxicity. With no exposure to xylene, a EM_{PK} of 13 is obtained. When xylene is present, the EM_{PK} ranges from 13 to 17. In all cases, a very significant overexposure occurs.

Conclusions and Recommendations

In this paper, we used a PBPK model to calculate the concentration of chemicals in venous blood and then performed a unity calculation based on chemical concentrations in blood, thus factoring in PK interactions and other variables. We explored a variety of real-world scenarios with respect to inclusion of chemicals in a mixture evaluation, use of different exposure limits, and the effect of light work. While most health professionals do not have ready access to PBPK models, the magnitude of the overexposures indicated can be illustrative of the consequences of mixture exposure when 1) PK interactions occur, 2) chemicals with a common mechanism of action are omitted from the EM calculation, 3) inappropriate OELs are used, or 4) when work activity levels are higher than in the studies that served as the basis for setting the OEL. Each of these factors leads to increases in blood concentrations. While the only available validated PBPK model for these chemicals was based on male physiology, similar results would be expected for females.

Health and safety professionals may omit chemicals from a unity calculation for several reasons. First, they may not perform the unity calculation at all. Second, they may refer to a source of information that omits the appropriate mechanism of action. Third, the mechanism of action may not be listed because appropriate toxicity tests

have not been performed. Fourth, the effect may not be "critical;" it may occur at a level slightly higher than the effect that drives the OEL, although still contributing to the cumulative effect, to some degree. Fifth, the chemical may not have an OEL at all, although it still causes relevant toxicity. As the analysis here indicates, omission of a chemical that contributes directly to the form of toxicity can underrepresent to actual dose of chemical to the target organ or tissue, and can suggest that the actual exposure is consistent with the OEL when it is actually higher.

In this analysis, we have used xylene as an example of the impact of omitting a chemical from the unity calculation. Since there is uncertainty regarding its actual ability to contribute to CNS depression, we have used it to explore three scenarios. In the first scenario, the least-conservative assumption is made, that it contributes no significant CNS depression. We find in this case that, because application of the unity calculation for the mixture of toluene and ethylbenzene would permit exposures to toluene and ethylbenzene (combined ratios less than 1) and xylene (separately up to its OEL), a significant apparent overexposure would occur when xylene was at a high but permissible exposure level due to PK interactions alone. Xylene in this manner can be thought of as a surrogate for any chemical that is not a CNS depressant but can inhibit metabolism of other chemicals. As there are many chemicals that inhibit metabolism of solvents, this issue is not restricted to mixtures that include xylene. Such chemicals should not be ignored when part of a mixed exposure. Even higher cumulative exposures occur if xylene directly causes CNS depression, even at

levels above its OEL, as indicated when xylene was assumed to be equipotent at 200 ppm.

What is the appropriate procedure to follow if a chemical may cause the effect of concern at an exposure level higher than its OEL? The dilemma is that, if they are omitted from the unity calculation, an overexposure could occur. On the other hand, if they are included in the unity calculation using the OEL for the non-relevant critical effect, it may indicate overexposure when overexposure is not actually occurring. The most appropriate way to analyze such exposures is to use an effect-specific OEL. In other words, one may estimate what the OEL would be based solely on the effect of concern and use this in a unity calculation. This approach would be preferable to omitting the chemical entirely from the analysis of the cumulative exposure.

Omission of CNS depressants from a unity calculation and basing such unity calculations on inappropriate OELs can lead to significant overexposures to workers. For toluene, ethylbenzene and xylene, the effective dose of the mixtures of chemicals could be as high at seven times the equivalent dose at the TLVs even if OSHA regulations are followed (Fig. 3.3). Workers performing light work activities would be exposed to approximately twice as much chemical as resting workers, even when only single chemical exposures occur. These analyses underscore the need to consider the actual conditions of work when applying TLVs (or other OELs) within the workplace.

The following recommendations arise out of the present analysis. Some of these apply principally to mixtures of toluene, ethylbenzene, and xylenes although many of them are more broadly applicable.

1. It is important to use a unity calculation when mixture exposures occur with chemicals that have a similar mechanism of action. Internal dose-based approaches are preferable when available, but standard Mixture Formula calculations would minimize significant overexposures.

2. For toluene, ethylbenzene and xylene mixtures, only slight overexposures appear to occur if the EM is less then 1.0 based on TLV exposures. An additional safety factor of 10-15% can be applied to compensate for PK interactions that occur when exposures are less than the TLVs when all interacting chemicals are included in the unity calculation. For CYP/2E1-metabolized chemicals, this range probably applies to most solvents with OELs in the range of 50-300 ppm.

3. The assessment shown in this paper demonstrates that it is critical to include in any unity calculations all components of the mixture that have a similar mechanism of action or can cause PK interactions. Omissions of such components can lead to significant overexposures.

4. Up-to-date OELs should be used to determine the potential for overexposure. However, the analysis of exposure should not be limited to chemicals for which there are OELs. Chemicals for which OELs do not exist should be considered in the same manner.

5. As a start, the documentation of the applicable OELs should be consulted when evaluating whether chemicals should be included in a unity calculation. However, because newer studies may be available, detailed reviews of the toxicological information should be conducted depending on the severity of exposure.

6. Many OELs may be based on resting activity levels. For some chemicals, as with the ones in the present evaluation, a worker performing "light work" may experience significantly higher absorbed doses. Consideration of the activity level should be a part of the assessment of exposure and the allowable exposure should be adjusted accordingly.

7. Much work remains to be done to clarify the dose-response relationships of toluene, ethylbenzene, and xylene with respect to CNS depression. This work should include development of PBPK models that include biomarkers, improvement in the assessment of mixture PKs (including for other compounds) and extension to include a better quantitative understanding of pharmacodynamic effects.

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Chapter 4

Pitfalls and Related Improvements of *In Vivo* Gas Uptake Pharmacokinetic Experimental Systems

Abstract

Gas uptake chamber studies have been widely used to study inhalation pharmacokinetics (PKs) in rodents, often for the ultimate purpose of developing physiologically-based pharmacokinetic (PBPK) models that can be used to describe human PKs and to support risk assessment for the chemical. In the course of our studies of gasoline PKs, we revisited several important issues heretofore not thoroughly addressed. Here, we report several refinements which will significantly improve future studies with this type of system, relating to the understanding of loss rates, the importance of carbon dioxide removal, and sampling of blood and chamber air at the same time. Losses of chemicals in gas uptake systems consist of leakage, adsorption to system components, and adsorption to the hair and skin (fur) of experimental animals. The loss rates were experimentally determined for a series of chemicals and mixtures including *n*-hexane, benzene, toluene, ethylbenzene, *o*-xylene, gasoline, and other gasoline components. The rate of loss to the animals' fur was similar to loss rates to system components and involved absorption to both hair and skin. Most of the absorption to fur was reversible when the chamber concentration was low enough. The amount of chemical that desorbed from the animal after an experiment was significant when compared to the amount of chemical

in the chamber at the end of a gas uptake experiment, indicating that the rate of decline in concentrations can be influenced by a decrease in the fur absorption rate or desorption of chemicals. A modified gas uptake system design is described in which a steel ring improved the connections to an autosampler and allowed insertion of probes to monitor gases, such as carbon dioxide (CO_2), in the chamber. When CO_2 absorbent efficiency was inadequate, CO_2 concentrations rose to levels that significantly affected the animals' ventilation rate. Using a real-time CO_2 probe, an absorbent system was developed that adequately controlled CO_2 levels in the chamber. Attention to details of absorptive loss and CO2 scrubbing can improve the reliability of kinetic constants inferred from closed chamber studies. We then describe a method for extending gas uptake experiments by simultaneously collecting blood to be analyzed for chemicals and/or metabolites.

Introduction

Gas uptake pharmacokinetic (PK) systems have been used for several decades to study the pharmacokinetics of hydrocarbon solvents (Hefner *et al.* 1975; Andersen *et al.* 1978; Lutz and Schlatter 1978; Filser and Bolt 1979; Gargas *et al.* 1986). They are frequently used to study the kinetics of absorption and, indirectly, distribution and metabolism of volatile chemicals in acute rodent PK studies. While this experimental system is a sensitive and non-invasive method for studying inhalation PKs, a number of potential confounding issues have never been thoroughly examined. Three major points are addressed in this paper. These are: (1) the absorption of test chemicals onto and potential desorption off fur during and after exposures, (2) the impact of

accumulation of carbon dioxide (CO_2) in the chamber from inefficient CO_2 removal on ventilation rates, and (3) the benefits of using a new method for simultaneously collecting chamber air and animal blood samples.

The basic approach of gas uptake experiments is to seal an animal in the chamber, add chemical(s), and monitor the decline of chemical concentration in the chamber to determine the PKs of chemical absorption into the animal. Because the rate of absorption of chemicals is related to tissue equilibration and metabolic processes, chemical partitioning and metabolic rate constants can be estimated from these results using physiologically-based PK (PBPK) models (Gargas *et al.* 1986). A typical gas uptake PK system consists of a container, such as a glass desiccator, ports allowing for the introduction of neat or gaseous chemicals and for extraction of small samples of air from the container, and a means of eliminating CO_2 and replenishing oxygen (O_2) (Figure 4.1). A detailed description of the components a gas uptake system is found in Methods.

Two basic gas uptake system configurations have been used in the past. One type is the manually sampled system (Filser 1992; Filser and Bolt 1979). This system was built out of a glass desiccator with injection and sampling ports that were custom-blown into the glass. The animal rests on a stainless steel meshed plate about 1/3 of the way up from the chamber base. The top of the desiccator was sealed to the base with stopcock grease, although a stretchable film such as Parafilm® may also be

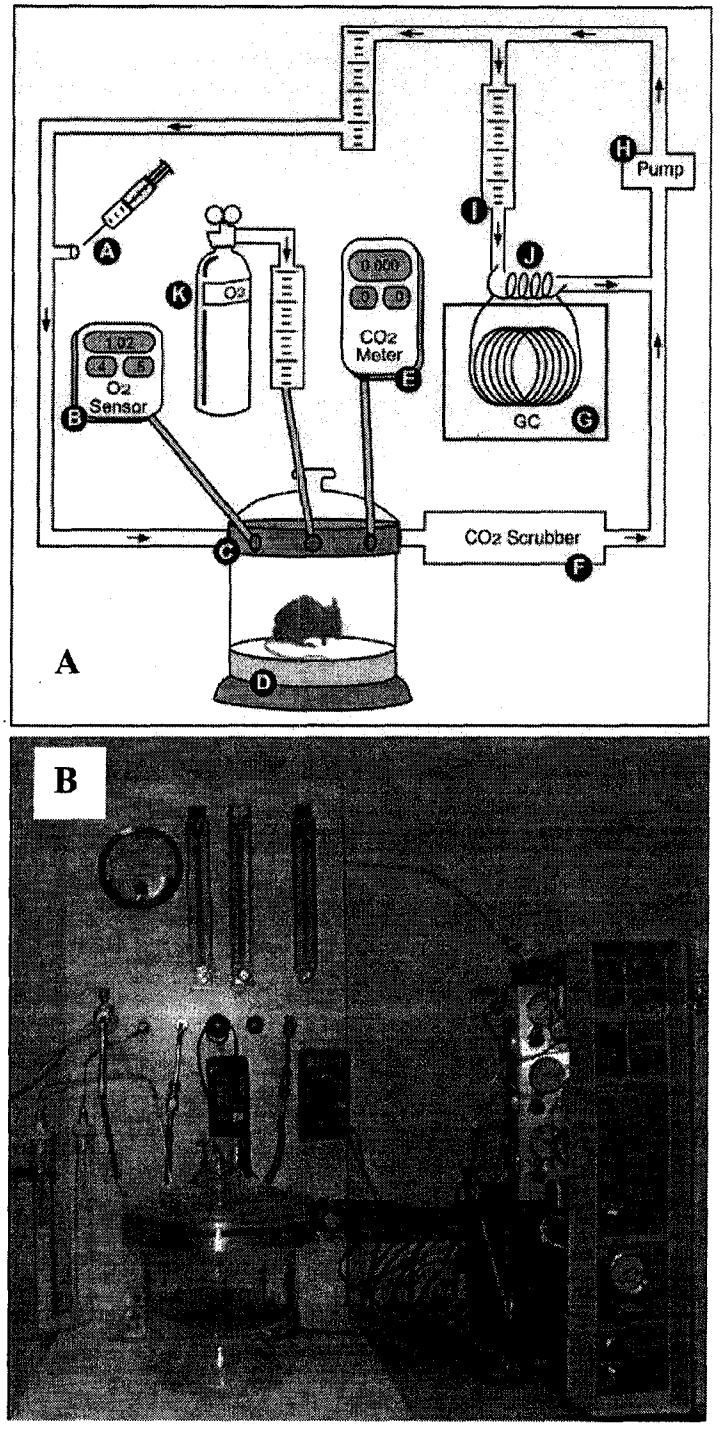

Figure 4.1. The gas uptake system used in the present experiments.

used. CO_2 is removed using an alkaline absorbent (scrubber). Soda lime pellets have frequently been used, although alternatives include Baralyme pellets and various aqueous hydroxide solutions, placed in the bottom of the chamber. The O_2 re-supply

system consisted of a bubbler through which O_2 is slowly flowing (Filser and Bolt 1979). As the animal consumes oxygen and the CO_2 is scrubbed, the pressure in the chamber declines. With a slight negative pressure, O_2 bubbles into the chamber to equalize pressure. Based on the 1:1 stoichiometry (1 mole O_2 produces 1 mole CO_2), an appropriate ambient level of O_2 inside the chamber will be maintained provided leakage into the chamber is insignificant.

This chamber design requires that a technician periodically sample the chamber and manually inject samples into the gas chromatograph. The injection is usually performed using a gas tight syringe with an injection volume of approximately 100 uL. An alternative approach was developed to allow automatic sampling of the atmosphere (Andersen *et al.* 1978; Gargas *et al.* 1986). In this system, a pump circulates the chamber atmosphere from the chamber to the gas chromatograph (GC) through stainless steel tubing and other inert materials. At determined intervals, valves on the sample loop at the GC are actuated, injecting a sample on-column. While it is more difficult to control loss rates with this system, it has some advantages over the manually-sampled system. First, the autosampling system frees the technician and can generate more reproducible results. Second, CO_2 removal should be more efficient because the chamber atmosphere can be passed directly through a scrubber rather than relying on diffusion; consequently, less scrubber material may be required and less absorption of the experimental chemicals to the scrubber may occur. O_2 replacement is usually achieved by adding O_2 via a valved flowmeter; thus,

maintaining normal O_2 concentrations does not depend on efficient removal of CO_2. However, these automated systems are prone to pressure fluctuations.

Other advantages of the autosampling system also exist. In the manually sampled system, mixing between the top and bottom of the chamber can potentially be limited by the plate upon which the animal rests. Chamber mixing can be enhanced in the autosampling system by locating the inlet or outlet tube of the chamber below the plate. The plate can also affect CO_2 diffusion to the scrubber placed in the bottom of the chamber in the manual system unless good mixing is achieved. In the autosampling system, chemicals with lower volatility can be introduced via a heated septum port located outside the chamber in the tubing before the chamber, to ensure rapid volatilization. In the manual system, chemicals are usually injected into the chamber through the top or side, and there is a risk that the chemical will contact the animal instead of evaporating. Nonetheless, the manually-sampled system is less expensive, easier to set up, and has far fewer locations where leakage can occur. Gas uptake systems have also been extended to systems for performing headspace analysis of *in vitro* metabolism of volatile chemicals (Hilderbrand *et al.* 1981; Hwang *et al.* 1996; Tornero-Velez *et al.* 2003).

Chamber losses occur in gas uptake systems for several reasons. Non-specific losses occur as the chemical(s) adsorb to surfaces within the system or on the CO_2 scrubber. Also, chemicals can be adsorbed to the skin and hair (fur) of the animals. Some leakage from the chamber can also occur, especially if the chamber operates at a

positive pressure compared to atmospheric. Generally, the total losses are reported to be in the range of 2 - 4% per hour (Gargas *et al.* 1990; Borghoff *et al.* 1996). Higher loss rates may occur with polar or less volatile chemicals. The question of whether these losses are permanent or reversible (*i.e.*, a return of adsorbed material to the chamber as the concentration of test chemical falls over time) has not been adequately addressed.

METHODS

The system described here was a modified version of one previously developed (Gargas *et al.* 1986). It has been used in PK studies of mixtures including benzene, toluene, *n*-hexane, ethylbenzene, and *o*-xylene, as well as the complex mixture gasoline. Results of these PK studies and the PBPK models developed have been published elsewhere (Dennison *et al.* 2003; Dennison *et al.* 2004). In principal, the methods described below would work equally well for other volatile hydrocarbons, but some considerations are noted that apply particularly to the chemicals in the study.

Description of the system

The picture and schematic of the system (Figure 4.1) includes a desiccator, CO_2 probe, CO_2 scrubber column, flowmeters, heated inlet port, and tubing. For the present study, a ~3 liter desiccator was used with one rat. In other studies, investigators have used larger desiccators and more animals. Generally, a desiccator

volume of about 3 liters per 200-250 g rat avoids excessive crowding. Larger volumes can also be used with a single rat; however, it is beneficial to minimize the chamber-to-animal volume ratio to maximize the rate of decline in chemical concentration in the chamber. Use of single rats reduces animal usage and expense, and also allows the experiment to provide unpooled data. However, two or more animals may reduce variability between experiments.

The chamber was connected to the GC using stainless steel tubing. Wherever possible, ¼" tubing was used to minimize pressure drop, but some shorter sections were made with 1/8" tubing. Wherever possible, non-absorptive and inert materials were used for chamber components. Earlier versions of this system used chambers constructed from desiccator jars of various sizes, but in each case, custom glassblowing was required to install ports. For the present studies, this design was modified by installing a 5 cm high stainless steel ring between the top and bottom of the desiccator. The ring was machined flat on top and bottom. Various threaded ports were machined into the side of the ring. This arrangement allows attachment of a variable number of connections that can be capped off if not needed. The glass desiccator system without the ring was not flexible; if another port was needed, the desiccator had to be sent to the glassblower. Furthermore, it was difficult to attach stainless steel tubing to the glass. Breakage during attachment of tubing or otherwise was expensive. The cost of the earlier desiccator system was approximately $700. The stainless steel ring was approximately $400 but lasts indefinitely, and can be used with desiccators costing $100. The tubing was connected to the pump, flowmeters,

and valves as indicated in the schematic (Fig. 4.1a). The chamber inlet tubing was extended past the stainless steel ring and into the bottom of the chamber to enhance the chamber's mixing characteristics (not visible in photo).

The top and the bottom of the desiccator can be sealed to the ring using stopcock grease or stretchable wrap, *e.g.* Parafilm®. Different stopcock greases were tested, but appeared to absorb a significant amount of the chemicals in our study. Therefore, stretchable wrap was tested and was found to seal the system without causing significant absorption.

When the system was first set up, the atmosphere of the chamber was pumped through the gas sampling loop on the GC. However, when the flow was set at a slow rate, the transit time between the chamber and the loop was excessive. When the flow was increased, pressure imbalances in the system occurred due to the constriction of the loop, causing a significant pressure drop at high flow rates. Therefore, a bypass loop was added, as in the original design (Gargas *et al.* 1986). Flow through this loop is driven by the pressure differential in the sample lines before and after the pump. The configuration used allowed a reasonable flow rate through the bypass when the system flow rate was ~2 liter per minute (lpm). Two lpm was selected for the system flow to additionally enhance mixing in the chamber.

CO_2 Absorbent

Initially, the CO_2 scrubber used was an aqueous sodium hydroxide solution. Twenty mL of 4.5 N NaOH was placed in the base of the chamber. Studies indicated that this arrangement did not effectively reduce CO_2 levels. At the time our initial mixture studies were performed, CO_2 probes were not routinely used and animal breathing rates increased during the exposure, possibly due to the CO_2 levels. Subsequently, other scrubbers were investigated. Solid pellet soda lime or Baralyme in the base of the chamber did not remove sufficient CO_2. Scrubber inefficiency could be due to restrictions in diffusion of CO_2 into the bottom of the chamber or because the pellet form of the scrubber was not able to absorb enough chemical. Subsequently, the scrubber was placed in a tube inline with the pump, and the entire system flow passed through. The tube was configured to be relatively long (>20 cm) in order to maximize contact time. With a tubular scrubber, soda lime caused a significant depletion of chemical from the chamber. However, Baralyme did not cause excessive absorption and CO_2 absorption was maximized using 35 g Baralyme in the tube.

After the discovery that CO_2 accumulated in the chamber, we installed a real-time CO_2 probe in the system. Most CO_2 probes were either too bulky or required continuously drawing samples of air out of the chamber into the probe's sensor. However, an infrared sensor probe was found that could be fully inserted into the system, maintaining the seal of the system (Testo 525, SKC, Fullerton, CA). The more ineffective scrubbers allowed the CO_2 level to rise above the sensors 10,000

ppm limit, but Baralyme controlled the CO_2 level to 5,000 – 6,000 ppm (*i.e.*, 5-6 %), provided that enough Baralyme was present.

As in previous versions of the system, the pump was a stainless steel bellows pump (Parker Hannifen, Sharon, MA). Flows were regulated by needle valves on flowmeters. One flowmeter regulated the O_2 delivery to the system. Another regulated the main system flow, and a third measured but did not regulate the bypass flow. The O_2 sensor was an electrochemical analog sensor (Handy O_2 Probe, Point Four Systems, Richmond, BC, Canada), connected to a voltmeter to monitor chamber O_2 concentrations. The bypass loop was connected to a 0.5 mL gas sampling loop that was controlled and pneumatically actuated by the gas chromatograph (Agilent, Palo Alto, CA). Most of the pressure drop in the bypass loop was caused by the constriction of the $1/16^{th}$ inch gas sampling loop. We also found that the temperature program for GC analysis required during our gasoline PK experiments (Dennison *et al.* 2003; Dennison *et al.* 2004), 35°C to 210°C, caused temperature fluctuations in the gas sampling loop mounted on the top of the GC. The loop temperature had to be at least 70°C to avoid fluctuations, resulting in sample volume expansion in the loop. Even at 70°C, standards analysis indicated that the sample had expanded by 5%. This volume change did not follow the ideal gas law precisely due to pressure in the system lines.

Gas uptake PK experiments were next performed with various mixtures of benzene, toluene, ethylbenzene, *o*-xylene, *n*-hexane, and/or gasoline, as previously described in

detail (Dennison *et al.* 2003; Dennison *et al.* 2004). These experiments were conducted for up to six hours or until chemicals were depleted in the chamber. The samples were analyzed by GC with flame ionization detector. Depending on run conditions, samples could be taken at 10 or 20 minute intervals.

Characterization of loss rates and absorption of the chemical by rats

Empty chamber studies were conducted to assess the characteristics of the chamber losses. These were performed in an empty chamber with or without CO_2 scrubbers. In the empty chamber studies, the chamber was set up exactly as it was used in *in vivo* experiments, except no O_2 supply was attached and no animal was in the chamber. Since the O_2 supply during *in vivo* experiments was a constant positive feed, no chemical loss would be expected through the oxygen system. After injection of test chemicals, the chamber losses in the absence of animals were quantified for six hours.

To investigate the possibility of reversible adsorption to the rats, studies were performed with dead animals. Rats were sacrificed by CO_2 and placed into the chamber. The chamber was set up exactly as it was in *in vivo* experiments, except no O_2 supply was used. Chemicals were added and the loss rates of each component chemical were determined. Loss rates were analyzed by exponential regression in Microsoft Excel.

Fur desorption studies were conducted by first conducting a dead rat study for two hours or as long as necessary for the chemical to reach a near steady-state concentration in the chamber. The chamber was then disconnected from the gas uptake system and sealed. The gas uptake system continued to run until it was purged of chemicals (~30 minutes). The chamber was then quickly opened and the animal was transferred into a clean chamber, which was re-connected to the system to monitor the increase of chemical in the chamber.

Using the ordinary differential equation solver Berkeley Madonna v. 8.0.2a8 (Macey and Oster 2002), the absorption curves were fit to the equation

$$\frac{dA_f}{dt} = V_f * K_f * (C_c - C_f / P_f) \qquad \text{Eq. 1}$$

where dA_f/dt is the rate of change in the amount of chemical in the fur, V_f is the volume of fur, K_f is the flux rate (/hr), C_c is the concentration of chemical in the chamber, C_f is the concentration of chemical in the fur, and P_f is the partition coefficient between fur and air. The equation represents simple partitioning behavior with kinetic control by the parameter K_f. The best fit values for K_f and P_f for each chemical were determined with the root mean square optimization algorithm in Berkeley Madonna. Using the absorption PBPK model, the amount of chemical in the fur compartment was determined for the time at which the animal was transferred into the clean chamber. This amount was then used as the initial condition for running the model with the desorption data.

Sampling blood during gas uptake experiments

We also developed a methodology for serially sampling blood from rats inside the chamber. Rats were obtained that were pre-cannulated in the jugular vein with polyethylene tubing exiting the top of the neck (Charles River, Wilmington, MA). The cannula were connected to narrow gauge tubing (PE50 tubing, Becton Dickinson, Franklin Lakes, NJ) which exited the chamber through one of the ports in the stainless steel ring. Syringes used to draw blood were pre-rinsed with saline plus heparin (70 units/mL) and purged of all liquid. Blood was collected from the cannulated rats every 40 minutes by withdrawing 150 uL of blood. The rat's blood was replaced by infusing saline containing 20 units of heparin/mL to prevent coagulation of blood in the tubing. When blood was drawn, the first part of the blood mixed with saline from the tubing and was discarded prior to drawing samples.

The blood samples were placed in 2 mL headspace vials and quickly sealed. After equilibration, 100 uL of headspace from the vial was injected into the GC for *n*-hexane analysis. The original blood concentration was calculated from the headspace concentrations and the blood:air partition coefficient (Gargas *et al.* 1989).

RESULTS

Chamber loss studies

Loss rates were determined for gasoline and some of its principle components in empty chambers or in studies with dead rats in the chamber (Table 4.1). In the empty chamber, loss rates were .02 - .05/hr. for individual chemicals (*n*-hexane, benzene, toluene, ethylbenzene, *o*-xylene, and various fractions of gasoline). Generally, the less volatile chemicals exhibited higher loss rates. Even chemicals with very high vapor pressures such as *n*-hexane experienced a loss rate of approximately .02/hr, suggesting that some of the loss was due to leakage. However, the increase in the loss rates as volatility declined also indicates that some adsorption to system components occurred and increased as volatility declines.

When a dead rat was placed in the chamber prior to the loss study, loss rates were higher for each chemical, generally almost twice that of the empty chamber, indicating absorption to the animal's skin and hair (fur) was occurring (Table 4.1). These losses also increased as volatility decreased. In a previous study, two blends of gasoline were tested, Gas A and Gas B (Dennison *et al.* 2003) and in another study, a third blend (Gas C) was tested (Dennison *et al.* 2004). These three blends had similar loss rates in dead rat chamber studies ranging from 0.041 to 0.044/hr. Gas C (Dennison *et al.* 2004) was divided into subsamples according to volatility. This separation was done by evaporating one-third of the gasoline and collecting the evaporated components over dry ice (a "1/3 cut" sample of gasoline.) A two-thirds cut was likewise prepared. When compared with the gasoline from which they

derived, the loss rates in the dead animal chamber study tracked with the volatility of the subsample.

Table 4.1
Loss Rates (/hr) of Chemicals in Gas Uptake System

	Empty chamber	Dead Rat	Large, Dead Rat	Shaved Large, Dead Rat
n-hexane	0.020 +/- 0.002	0.024+/- 0.002	0.037	0.026
benzene	0.028 +/- .0005	0.040 +/- 0.01	0.064	0.054
heptane	0.027 +/- 0.005	N/A	N/A	N/A
toluene	0.034+/- 0.003	0.068 +/- 0.006	0.074	0.062
octane	0.030+/- 0.002	N/A	N/A	N/A
ethylbenzene	0.043+/- 0.003	0.072 +/- 0.01	0.076	0.062
o-xylene	0.047+/- 0.005	0.081 +/- 0.02	0.084	0.057
Gas A	N/A	0.041 +/- 0.001	N/A	N/A
Gas B	N/A	0.044 +/- 0.01	N/A	N/A
Gas C	N/A	0.042	N/A	N/A
Gas C 1/3 cut	N/A	0.022	N/A	N/A
Gas C 2/3 cut	N/A	0.034	N/A	N/A

Table 4.1. Chemical loss rates (/hr) from the gas uptake chamber with an empty chamber or a chamber containing a dead rat, based on exponential regression of experimental data. Where shown, data are mean +/- SD. Other data are the result of one experiment. "N/A": data not available.

To assess the impact of rat size on the dead rat loss rates, a dead rat study was performed with a large (390 g) rat. With a large dead rat in the chamber, the loss rates of more volatile chemicals increased but not of the less volatile chemicals. In a separate experiment, the contribution of the hair to the absorption was evaluated by carefully shaving the carcass of the animal after sacrifice. When compared to the absorption to the large rat, loss rates were lower for the shaved rat. These data

suggest that part of the absorption was to the skin of the animal and part of it was to the hair.

Fur desorption studies

To explore whether absorption to the fur of the animals was reversible, we conducted dead rat studies that included an absorption phase and a desorption phase. The absorption phase indicated the expected decline in chemicals from the system as they were absorbed by the animal. The decrease over a short period and small change in chamber concentration can be regressed linearly or exponentially but has usually been described exponentially in the past (Gargas *et al.* 1990; Borghoff *et al.* 1996; el-Masri *et al.* 1996). The timecourse loss data were modeled using Equation 1 and fitting values for K_f and P_f. Parameter values for each chemical's K_f and P_f that provided best fits to the absorption phase data are provided in Table 4.2. In the desorption phase, an exponential rise in chemicals was observed in the chamber, consistent with chemical desorption from the animal (data not shown). However, during desorption, a fast early phase was also present in some cases.

The significance of the amounts being desorbed was assessed by comparing the concentration of chemical in the chamber at the end of a six-hour gas uptake experiment with the amount that was desorbed from the animal in the dead rat study (Table 4.2). The amount desorbed was determined from the peak concentration in the

Table 4.2

Absorption-Desorption Model $\frac{dA_f}{dt} = V_f * K_f * (C_c - C_f / P_f)$ Eq. 1

	Kf (/hr)	Pf [-]	Concentration at Start (ppm)	Concentration at End (ppm)	Concentration Desorbed (ppm)
n-Hexane	0.14	4.7	169	22	5.0
Benzene	0.18	15	145	16	7.0
Toluene	0.25	26	400	8.3	30
Ethylbenzene	0.25	15	71	2.1	10
o-Xylene	0.4	25	74	3.0	10
Lumped	0.25	6.5	1105	356	82

Table 4.2. Model-optimized values for K_f (flux rate) and P_f (skin:air partition coefficient) for several chemicals and a comparison of the concentration at the end of a typical gas uptake experiment and the amount that was desorbed from a dead rat. The ending concentration (ppm) was obtained in an experiment that was initiated at the starting concentrations indicated. The desorbed concentration was obtained in a dead rat study after steady state was reached. The "lumped" chemical represents all gasoline components other than *n*-hexane, benzene, toluene, ethylbenzene, and *o*-xylene, grouped as a single chemical. The volume of fur for a 250-g rat was estimated to be 47.5 g for these calculations.

chamber during the desorption experiment. For the more volatile components (*n*-hexane, benzene, and the lumped component, which consists of all gasoline components other than those listed), the amount desorbed was a significant fraction of what would be found in the chamber at the end of a live rat experiment. For toluene, ethylbenzene, and *o*-xylene, the amount desorbed was 3-5 times the amount in the chamber at the end of the live rat experiment.

Carbon Dioxide Scrubbing

CO_2 levels in the chamber were a complex function of system flow rate, scrubber configuration, scrubber type, surface area, and scrubber mass. Higher system flow rates resulted in lower CO_2 levels because more chamber air was scrubbed of CO_2 per unit time. Generally, scrubbers mounted in columns through which the system pumped air were more effective than scrubbers placed in the bottom of the chamber, requiring CO_2 to move from the animal's breathing zone to the chamber base by passive processes.

We investigated the efficacy of different scrubbers, including aqueous sodium hydroxide solution, soda lime, and Baralyme. When sodium hydroxide solution was placed in the bottom of the chamber (in excess of stoichiometric requirements), the CO_2 levels were indirectly observed by recording the animal's breathing rates. Over time during the experiment, the animal's breathing rate increased to as high as 140 breaths per minute. The rapid breathing rate was attributed to excessive CO_2 levels in the chamber. Modeling PK data from experiments conducted in this manner required extremely high alveolar ventilation rates in the vicinity of 22 L/hr/kg$^{0.74}$. Conducting experiments in this manner also placed unnecessary strain on the animal.

Attempts to increase scrubbing by increasing the surface area of the sodium hydroxide solution were only effective when the surface area was increased to several times the area available within the chamber. Placing the sodium hydroxide in a tube

with a fritted bubbler was more effective, maintaining CO_2 levels between 5,000 and 7,000 ppm, but was not used due to the concern over the possible generation of alkaline aerosols.

The effectiveness of solid scrubbers was dependent on the configuration and amount of the scrubber. If scrubber was placed in the bottom of the chamber, CO_2 levels quickly rose above to 10,000 ppm and above the limit of the CO_2 sensor. When soda lime was placed in a column in the system flow path, CO_2 levels were generally maintained in the 5,000 – 7,000 ppm range. However, chemical loss studies indicated loss rates of ~8%/hr. for all chemicals.

As an alternative, Baralyme scrubber was investigated. With approximately 35 grams of Baralyme in a flow-through tube, high levels of CO_2 were recorded when the system flow was ~1 liter/min. Subsequently, the system flow rate was increased to about 2 liters/min. At the higher flow rate, CO_2 levels were maintained in the 5,000 – 7,000 ppm range. Even lower CO_2 levels could be achieved, but only at flow rates that caused pressure imbalances in the system. The calculated steady state concentration of CO_2 in the system, assuming perfect mixing and 100% CO_2 removal by the scrubber at a 2 liters/min flow rate is 1650 ppm. The higher measured concentration at 2 liters/min is most likely a result of both imperfect mixing, i.e., the CO_2 probe is located adjacent to the CO_2 source (rats breathing zone), and incomplete CO_2 removal by the scrubber.

In previous gas uptake system descriptions, CO_2 measurements have not been reported. A 200 g rat exhales approximately 3.3 mL/min CO_2 (Fukuda 1991). With a 200 g rat in a 3 L chamber, the CO_2 levels would increase by more than 1000 ppm/min if scrubbing did not occur. This rate of CO_2 increase is observed during early periods in gas uptake experiments when CO_2 monitoring was conducted. Measured values for CO_2 ranged up to 30,000 ppm during our initial gas uptake experiments in which the scrubber was not working properly. Therefore, there is significant potential for excess CO_2 levels in a chamber experiment. Breathing rates depend primarily on CO_2 levels in blood (Ganong 1987). Based on an exponential regression of published data (Ganong 1987), the minute volume is related to inhaled concentrations of CO_2 by the equation:

$$\text{Minute Volume (L/min)} = 5.9 * \exp^{(0.26 * \%\,CO2)} \qquad \text{(See Figure 4.2)}$$

This relationship indicates that the minute volume increases by 14% at 5000 ppm CO_2 and by 30% at 10,000 ppm. As many PBPK models are relatively sensitive to the ventilation rate parameter, and most use default values of about 15 L/hr/$kg^{0.74}$, it is important to control CO_2 levels. Monitoring the CO_2 levels in the chamber is therefore a useful way to ensure CO_2 control is achieved.

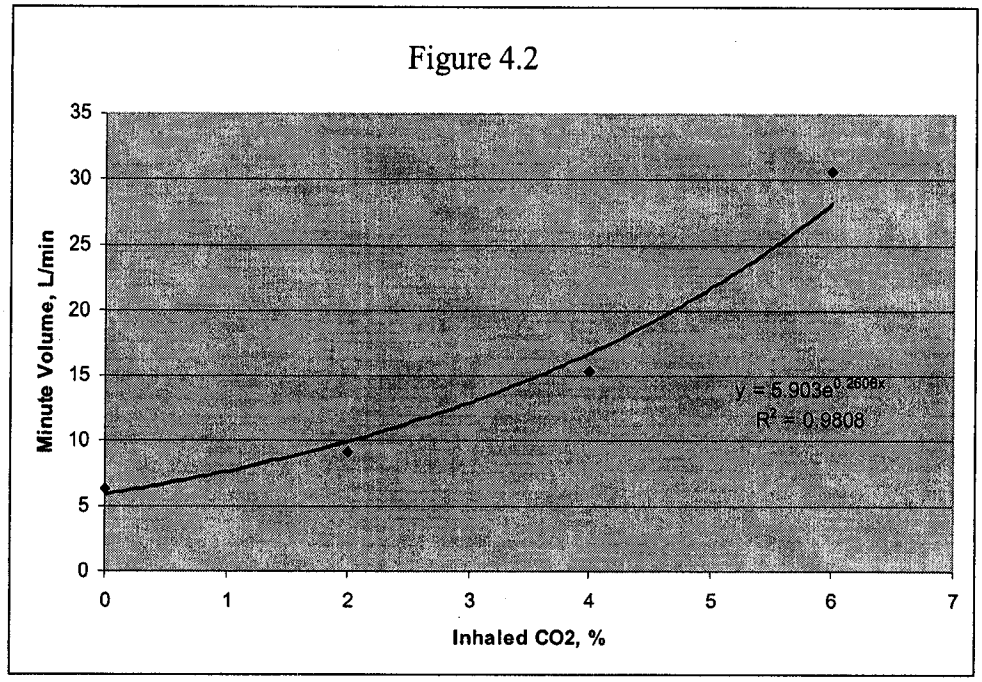

Figure 4.2. Minute volume as a function of % CO_2 inhaled. Based on data of Ganong, W.F., 1987.

PBPK Modeling of Gas Uptake and Blood Data

For the single chemical *n*-hexane, two gas uptake experiments were performed during which blood was collected and analyzed by headspace for *n*-hexane. Further refinements of collection and analysis methods are expected to result in a sampling interval of less than 40 minutes. Results of the gas uptake data and the venous blood data for *n*-hexane are shown in Figure 4.3 along with PBPK simulation results. The PBPK model shown here is similar to that reported previously (Dennison *et al.* 2003). This model adequately represents the chemical concentrations in both blood and chamber air. The ratio of chamber air concentrations and blood concentrations for *n*-hexane suggest an apparent blood:air partition coefficient of about 0.54 – 0.62.

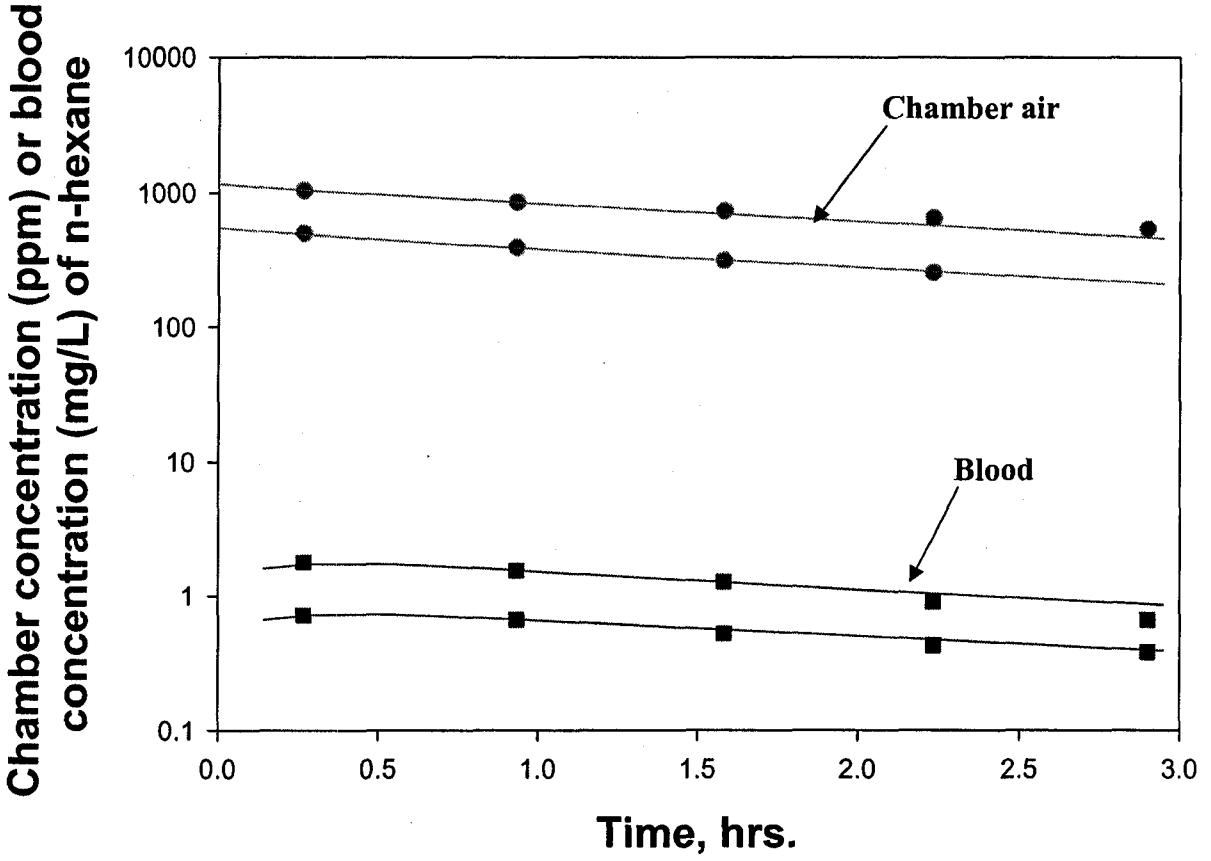

Figure 4.3. PBPK model simulations for *n*-hexane describing gas uptake chamber data and blood *n*-hexane data collected simultaneously. Symbols represent experimental data and curves represent the PBPK model simulation for each of two experiments.

DISCUSSION

In the development and testing of this closed chamber exposure system, we have found several pitfalls and noted ways to avoid many of them. It is important, for instance, to (1) maintain appropriate pressure balances in the system, (2) minimize losses, (3) configure the system to enhance mixing, and (4) have reasonable flow rates for delivery of sample to the GC. The system used in these studies was more

flexible because additional probes or attachments could be easily made through the stainless steel ring to assess chamber performance.

Chemical absorption to chamber and animal

In a gas uptake experiment, losses of chemical from the system represent both the amount of chemical that is adsorbed by the animal as well as non-specific losses. These non-specific losses include losses within the system (absorption to system components such as glass or CO_2 scrubber), leakage from the system, and loss to the fur. The absorptive losses tend to be more significant as the vapor pressure of the chemical declines. However, if these loss rates are carefully measured and incorporated in the PBPK model for the chamber compartment, they do not interfere with use of the chamber for assessing kinetic constants of metabolism.

Initial attempts to model the absorption and desorption of chemicals provided approximate fits to experimental data, although the process is probably more complex than provided for by the simple diffusion-equilibrium expression (Eq. 1). The absorption-desorption studies indicate that the amount of chemical absorbed to the fur can be a large fraction or even greater than the amount of chemical in the chamber air at the end of a six hour gas uptake study. Furthermore, the studies indicate that the absorption of the chemicals is reversible. Thus, in a gas uptake experiment where the concentration of chemical declines by 1-2 orders of magnitude, the concentration of chemical in the chamber can be expected to decrease to the point that there is no net

flux of chemical from the chamber onto the fur and possibly a net flux of chemical from the fur back into the chamber. When this occurs, the loss rate would be zero or negative. Models based on the assumption of a fixed loss rate independent of concentration level or amount accumulated in the fur will overstate the losses later in the experiment. This systematic error may lead to an overestimate of PBPK model parameters such as metabolic rates.

CO_2 exposure and ventilation rate

One of the major issues addressed in this study was consideration of the CO_2 levels in the chamber. We found that breathing rates were substantially increased unless the CO_2 was carefully controlled. Based on the relationship between the CO_2 level and minute volume (Ganong 1987), the breathing rate may be different from default rates even when CO_2 is removed to the maximum possible extent. For PBPK model development, when the model is sensitive to the breathing rate, different parameter values for the alveolar ventilation rate may be required. Thus, CO_2 levels should be routinely measured. Another approach would be use of plethysmography to determine the breathing rates quantitatively, and to determine the impact of neurological depression and/or sensory irritation on those parameters. This can be performed during the gas uptake experiment using the present chamber design.

Benefits of simultaneous collection of chamber air and blood

In inhalation pharmacokinetics, the major alternative to the gas uptake experiment is the constant exposure system, in which animal(s) are exposed to a constant level of chemical over some period of time, often also including a post-treatment period without exposure. A constant exposure system is usually used to expose animals which are then sacrificed for tissue collection including blood. The tissues are then analyzed for parent chemicals and/or metabolites or other markers of exposure and effect. In the gas uptake experimental system, the animals are exposed to a declining concentration of chemical throughout the experiment. Both systems have been used to acquire data that can be used to support the development and validation of physiologically based (PBPK) models that provide a quantitative description of the chemical PK within the animal based on underlying biological processes.

Risk assessment is often based on the amount of chemicals or their metabolites present in the animal as a function of exposure concentration. Direct measurements of tissue concentrations are generally preferred to indirect measures that are based on loss rates and fitted parameters for metabolism; thus, the advantage with experimental designs in which tissues can be collected. On the other hand, a major advantage of the gas uptake PK system is in the range of actual exposure concentrations that occur during the course of one experiment. For lipophilic chemicals that are metabolized at a reasonable rate, the chemical concentrations in the chamber may decline by two orders of magnitude during a six hour gas uptake experiment. Experiments typically

include starting exposure concentrations that are in the range where metabolic saturation occurs. As the concentration declines, metabolism becomes increasingly less saturated and may enter the linear range for metabolism (Gargas et al., 1990). Thus, a single gas uptake experiment can be regarded as equivalent to a suite of constant exposure experiments. The singular advantage of the gas uptake system has been the ease of estimation of whole body clearance kinetics for metabolism. These parameters have been valuable in providing initial bounds for parameters for individual organs and evaluating regions of non-linearities in chemical disposition.

In this paper, we have presented a method to combine these experimental designs by sampling blood during a gas uptake experiment. While there are some inherent limitations to this approach in terms of the amount of blood that can be extracted from one animal and the inability to sample other tissues, coupling gas uptake experiments with internal measurements of a chemical and metabolites in blood will allow construction of more robust PBPK models in the future than models based on either approach alone. Possibly, the most comprehensive design that could be achieved with a limited number of experimental animals would include 1) gas uptake experiments at several concentrations, *e.g.*, 3-6 different starting concentrations, 2) serial collection of blood from the animal during the experiment at intervals of 15-30 minutes, 3) analysis of the blood for parent chemical and relevant metabolites, and 4) post exposure collection of tissues and excreta and analysis for parent chemical and relevant metabolites. These analyses would provide a suite of data for evaluating the pharmacokinetics, particularly including metabolism, of chemicals. PBPK models

may also make use of additional modeling inputs, such as *in vitro* based studies of metabolism (Huang *et al.* 1996), reaction network modeling approaches to elucidate metabolic pathways and rates (Liao et al. 2002) and various pharmacodynamic submodels.

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Chapter 5

Characterization of the Pharmacokinetics of Gasoline using PBPK Modeling with a Complex Mixtures Chemical Lumping Approach

Abstract

Gasoline consists of a few toxicologically significant components and a large number of other hydrocarbons in a complex mixture. By using an integrated physiologically based pharmacokinetic (PBPK) modeling and lumping approach, we have developed a method for characterizing the pharmacokinetics (PKs) of gasoline in rats. The PBPK model tracks selected target components (benzene, toluene, ethylbenzene, *o*-xylene (BTEX) and *n*-hexane) and a lumped chemical group representing all non-target components, with competitive metabolic inhibition between all target compounds and the lumped chemical. PK data was acquired by performing gas uptake PK studies with male F344 rats in a closed chamber. Chamber air samples were analyzed every 10 –20 minutes by gas chromatography/flame ionization detection and all non-target chemicals were co-integrated. A four-compartment PBPK model with metabolic interactions was constructed using the BTEX, *n*-hexane, and lumped chemical data. Target chemical kinetic parameters were refined by studies with either the single chemical alone or with all five chemicals together. *o*-Xylene, at high concentrations, decreased alveolar ventilation, consistent with respiratory irritation. A six-chemical interaction model with the lumped chemical group was used to estimate lumped chemical partitioning and metabolic parameters for a winter blend of gasoline with methyl *t*-butyl ether and a summer blend without any oxygenate. Computer simulation results from this model matched well with

experimental data from single chemical, five chemical mixture, and the two blends of gasoline. The PBPK model analysis indicated that metabolism of individual components was inhibited up to 27% during the six-hour gas uptake experiments of gasoline exposures.

Introduction

Gasoline is an abundant chemical product used in the U.S. and elsewhere. Approximately 140 billion gallons are produced and used in the U.S. annually (Dement *et al.* 1997) and at least 660 million tons are annually produced and used worldwide (INEGI 2002). In the U.S., approximately 100,000 workers are exposed to fossil fuels during production, distribution and use (NIOSH 1999). Approximately 111 million U.S. persons are exposed during refueling at gasoline stations (Wixtrom and Brown 1992). Limited data are available regarding various widely ranging exposures. Occupational exposures can range from 1 to 3,000 mg/m^3 (Dement *et al.* 1997; Hakkola and Saarinen 1996; Wixtrom and Brown 1992), and shorter non-occupational exposures have also been measured in the range from 4 to 9,000 mg/m^3 over brief periods (Dement *et al.* 1997; Hakkola and Saarinen 2000; Wixtrom and Brown 1992). The dermal route of exposure can also be significant in some occupations (Laitinen *et al.* 1994; Jia *et al.* 2002).

As with other petroleum mixtures, the gasoline fraction contains several hundred components. The gasoline fraction ranges from C4 to C10, with smaller amounts of larger components (Kreamer and Stetzenbach 1990; King 1992), and contains

isoalkanes, *n*-alkanes, aromatic derivatives, and smaller amounts of alkenes and alkynes. Also, most gasoline, at least that which is produced in the U.S., also contains fuel additives, principally the oxygenating compounds ethanol or methyl *t*-butyl ether (MTBE). MTBE is targeted for phase out from gasoline in the U.S. due to concern with potential carcinogenic effects (USEPA 2002). Other than oxygenates, few chemicals in gasoline are present in large proportions. Depending on the blend, toluene and xylenes are frequently present in the largest proportion, but still generally constitute less than 10% of the blend. Only about 20 components are typically present in the percent range, and about 20% of the blend is comprised of components in the subpercent range. To account for 95% (v/v) of the components, about 75 components would have to be considered.

Gasoline is not a homogeneous mixture of known or constant proportions. At least 32 distinct different blends of gasoline are produced in the U.S. (Economides 2002). Moreover, human exposure can be to the whole gasoline or to vapors that are weighted (usually enriched) by its more volatile components. These exposure and toxicological issues complicate efforts to assess human health risks encountered by different populations.

Although epidemiological studies have been somewhat inconsistent, gasoline has been classified by IARC as 2B, "possibly carcinogenic" (IARC 2002). Some of the components in gasoline have been studied for toxicological effects, including benzene, toluene, ethylbenzene, xylenes (BTEX), *n*-hexane, several other *n*-alkanes,

alkenes, aromatic species, and oxygenates. Benzene's principal toxicological effect at relevant exposure levels is cancer, and its pharmacokinetics have been studied in some detail (Cole *et al.* 2001). *n*-Hexane is a neurotoxicant that causes central-peripheral axonopathy and other effects (Graham *et al.* 1995; Agrawal *et al.* 1985). A number of gasoline constituents are CNS depressants, including toluene, ethylbenzene, and the xylenes (OEHHA 1999, 1997; ACGIH 1999). Various other constituents have been evaluated for a wide variety of toxicological effects.

All gasoline components are primarily metabolized in the liver. The first step in metabolic transformation for most of gasoline's components that have been studied in this regard is oxidation by cytochrome P450/2E1. Due to the prevalence of this enzyme in initial oxidation of the compounds; it is likely that essentially all of the components of gasoline serve as competitive inhibitors of oxidation of all other hydrocarbons in the mixture (Guengerich and Shimada 1991). The significance of each compound as an inhibitor depends on both the concentration of inhibiting components at the enzyme active site and their potencies. Thus, there is no simple method for determining the extent of inhibition of metabolism of a particular component, since the inhibition depends on concentrations and inhibition potencies of numerous components.

Other groups have developed PBPK descriptions of some constituents of gasoline including benzene (Cole *et al.* 2001; Kenyon *et al.* 1996), toluene (Thrall and Poet 2000; Pierce *et al.* 1996), *n*-hexane (Andersen and Clewell 1983; Perbellini *et al.*

1986), and MTBE (Borghoff *et al.* 1996; Rao and Ginsberg 1997), among others. Efforts to develop pharmacokinetic descriptions of some mixtures that include some constituents such as BTEX compounds, *n*-hexane, and other chemicals have also been reported (Krishnan *et al.* 1994; Haddad *et al.* 1999; Yu *et al.* 1998; Dobrev *et al.* 2001). These mixture models contained between two and five chemicals. These models can be thought of as a "bottom-up" approach because they start with single chemicals and add additional components one by one until a description of the full mixture is achieved, usually including *in vivo* experimental work on nearly all permutations of the mixture. To address at least 95% of the potential interactions between components with gasoline, the bottom up approach would have to describe the interactions between 75 components. Such an approach would likely be impractical on an *in vivo* basis.

Limited pharmacokinetic studies involving complex mixtures have been published. Classical, non-physiological, PK studies have been performed to determine the PKs of some gasoline constituents such as MTBE or benzene after gasoline exposure (Kumarathasan *et al.* 1996; Popp *et al.* 1994). However, these studies do not provide a PK description that can be extrapolated to other exposure conditions or species other than the animal model tested. A PBPK model for benzene in rats with or without the influence of gasoline has been developed (Travis *et al.* 1992). This model was based on benzene uptake when the rats were exposed to the single chemical as compared to when the rats were exposed to the same concentration of benzene in gasoline. The model was fitted to experimental data by altering both the V_{max} and K_m

of benzene to reflect reduced metabolism in the mixture as compared to single chemical metabolism; no inhibitory parameter was included in the mathematical model. Other data describe the inhibition of benzene metabolism by other components of gasoline (Bond *et al.* 1997). In these studies, benzene metabolism was reduced by up to 46% at 2000 ppm of gasoline but much less at 300 ppm. In all these studies, emphasis was given to the effect of gasoline on the kinetics of a key component, not on the PKs of gasoline itself.

The objective of this study was to develop an approach for using chemical lumping in pharmacokinetic analysis of complex mixtures and to use the approach to build a PBPK model for gasoline, as previously suggested (Verhaar *et al.* 1997; Bond *et al.* 1997). Lumping can be used when it is unimportant to distinguish one component in the lump from another and the relevant properties of each lump can be described by a central estimate. Chemicals about which specific information is desired, such as the specific PKs of a component that is responsible for an effect under consideration, can be left un-lumped, i.e., they can be "split" out. For example, components such as benzene, MTBE, and *n*-hexane could be split out and the remaining components could be included in one or more lumps. In the present model, we split benzene, *n*-hexane, toluene, ethylbenzene, and *o*-xylene as five single chemicals, and combined all remaining chemicals into one lumped group. Closed chamber gas uptake experiments were used to obtain PK data for model development and validation.

Methods

Rats

Male Fisher 344 rats (Harlan Sprague Dawley) were housed in temperature controlled quarters with a 12h light-dark cycle. The animals (190-230 g and 9-10 weeks old at time of use after a minimum two-week acclimatization period) were provided NIH 07 diet and water *ad libitum* until the start of six-hour experiments, during which no food or water was provided.

Chemicals

Benzene, *n*-hexane, toluene, *o*-xylene, and ethylbenzene were selected as target chemicals for the studies because 1) they have different modes of toxicity, 2) they are all prevalent in gasoline, and 3) they represent different chemical structures (alkanes, aromatic, alkyl aromatic). These reagents (>98%, confirmed by GC) were obtained as liquids (Fisher, Chicago, IL). Gasoline samples were provided by the Southwest Research Institute (San Antonio, TX) with original analyses. One gasoline sample was described as a "winter blend" that was relatively more volatile and also contained MTBE (Table 5.1). The second sample was a "summer blend" that was relatively less volatile and contained no oxygenate. Both samples were stored in Teflon sealed glass vials at 0° C until use.

Table 5.1
Composition of Gasoline Blends

	Summer Blend	Winter Blend
aromatics	53	32
isoparaffins	24	22
naphthenes	1.8	5.1
olefins	6.2	19
paraffin	11	7.0
oxygenates	0.0	10
butanes	1.9	3.7
pentanes	7.4	23
hexanes	19	15
heptanes	23	17
octanes	24	16
nonanes	17	11
decanes	3.1	4.8
n-hexane	2.8	1.2
benzene	3.6	1.2
toluene	13	6.3
ethylbenzene	4.3	1.8
o-xylene	5.0	2.5

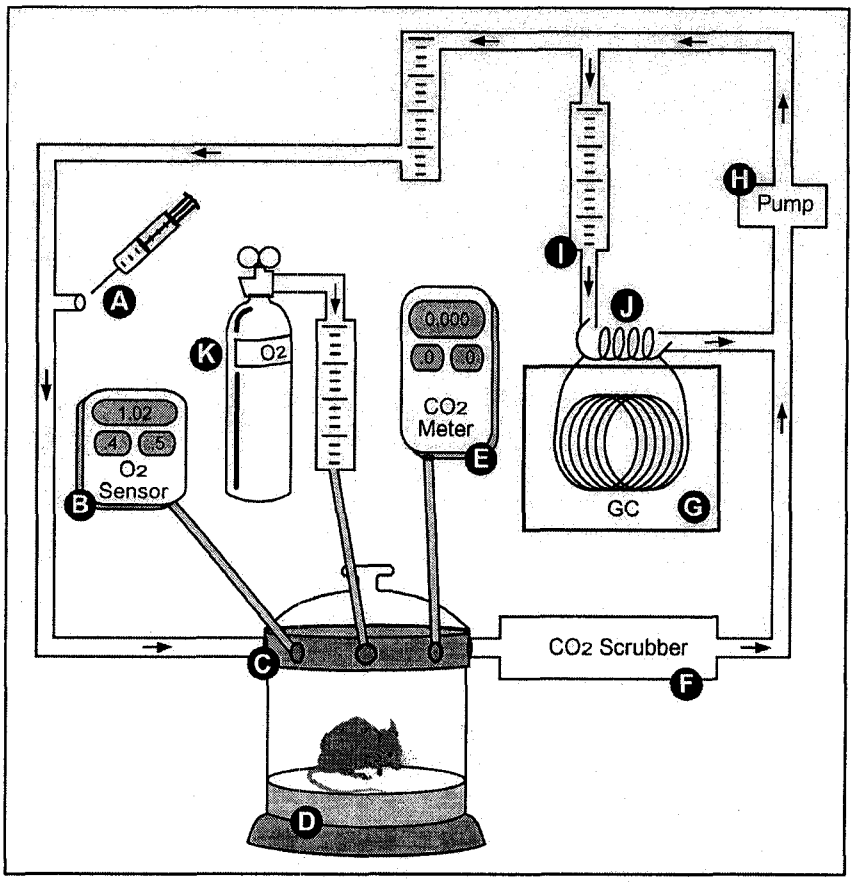
Table 5.1. Approximate composition of the summer and winter blends of gasoline used in this study (SWRI 2001). Data in current study was based on analysis of samples in the experimental system.

Exposure System

Gas uptake studies are conducted by placing animals (e.g., rats) in sealed chambers, adding chemicals to the chamber, and monitoring the decline in chemical concentration in the chamber atmosphere. For the present study, a previous chamber design (Gargas *et al.*, 1986) was modified by inserting a stainless steel ring between the top and bottom of the desiccator, sealed with Parafilm (Figure 5.1). Tubing was connected and probes were inserted through existing ports in the stainless steel ring.

The chamber was a glass desiccator with an approximate gross volume (including tubing) of 3.08 L. The chamber volume used in data analysis was adjusted by the volume of the animal, assuming a density of 1.0 g/mL. The desiccator was connected to a HP 5890 gas chromatograph through a 0.5 mL gas sampling loop. The atmosphere of the chamber was recirculated through the tubing to the loop on the GC using a stainless steel bellows pump regulated by flowmeters. Thus, the entire system consisted of stainless steel and glass with small amounts of Teflon or other materials in contact with the chemicals, in order to minimize chemical reaction and absorption. The flow through the chamber was approximately 1 L/min but the flow through the gas sampling loop was reduced to about 20 mL/min by using a bypass loop and separate flowmeter. The transit time from the chamber to the loop was approximately 2 minutes.

At the start of each experiment, chemical(s) or gasoline was introduced into the chamber in a heated port just upstream of the chamber. Immediately downstream of the chamber, carbon dioxide (CO_2) was removed from the atmosphere with an in-line CO_2 absorbent column containing 40.0 g of Baralyme (Morgan Scientific, Haverhill, MA). This amount of absorbent held the CO_2 level in the chamber to about 5000 +/- 1000 ppm. The CO_2 levels were periodically monitored using an infrared CO_2 probe (Testo 525, SKC, Fullerton, CA) that was inserted into the chamber through the stainless steel ring. Similarly, oxygen was continuously monitored using another probe (Handy O_2 Probe, Point Four Systems, Richmond, BC, Canada) also inserted into the chamber atmosphere through the stainless steel ring. As the animal breathed,



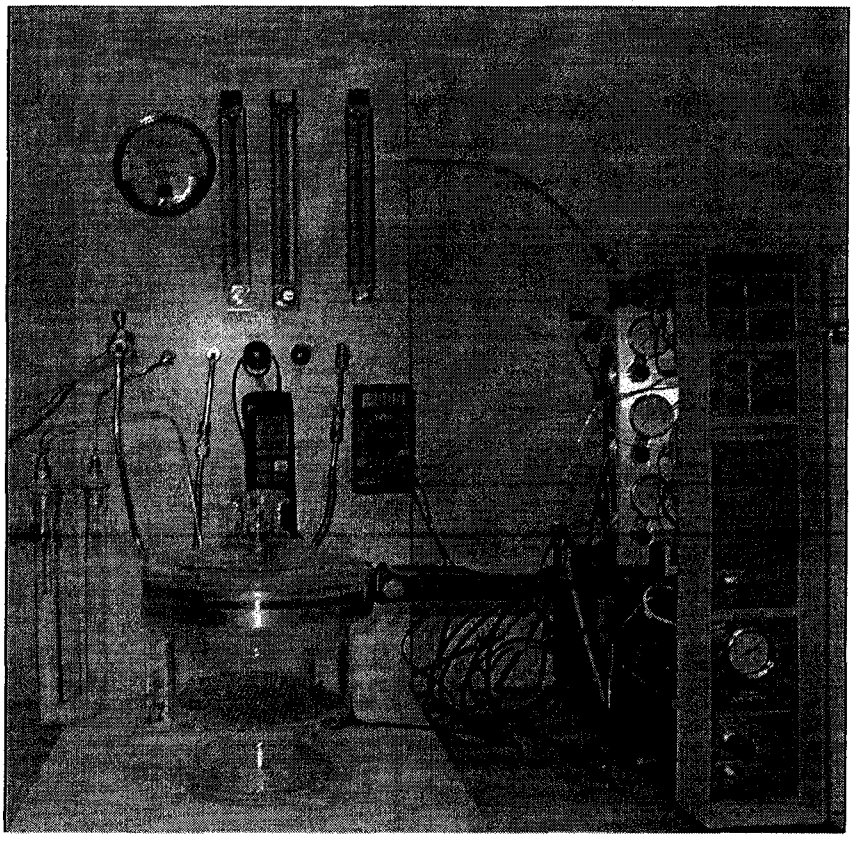


Figure 5.1 Photograph and schematic representation of system of gas uptake exposure system. A. Injection port for chemical. B. Oxygen sensor. C. Stainless steel ring with tubing or probes inserted. D. Gas uptake chamber. E. CO2 sensor. F. CO2 scrubber. G. Gas chromatograph. H. Bellows pump. I. Flowmeter. J. Gas sampling loop. K. Oxygen supply.

oxygen was re-supplied through a metering value, maintaining an ambient concentration of 20.9 +/-1%. To avoid unnecessary chemical condensation, a moisture trap was not used, but water in the system did not appear to be excessive.

The system was tested for chemical loss with no animal or oxygen supply present. Non-specific losses determined this way were small (<0.02/hr.) However, when dead animals were placed in the chamber, losses were more significant and chemical specific. A corresponding loss rate (KL) was determined by non-linear regression and ranged from 0.02 – 0.08/hr, with less volatile chemicals showing greater loss rates (Table 5.2). This loss rate was included in the model using the equation

Rate of loss (mg/hr) = Mass of chemical in chamber (mg) * KL (1/hr)

Table 5.2
Chemical Parameters for Simulations

Chemical	PB	PL	PF	PS	Vmax[1]	Km[2]	KL[3]
n-hexane	2.29	2.27	69.4	1.27	7.0	0.01	.025
benzene	17.8	0.96	22.0	0.58	5.3	0.10	.052
toluene	18.0	4.64	56.7	1.54	5.3	0.02	.070
ethylbenzene	42.7	1.96	36.4	0.61	7.6	0.10	.073
o-xylene	44.3	2.44	42.4	1.16	6.5	0.20	.086
lumped summer blend	2.9	3.16	80	0.88	2.0	0.10	.042
lumped winter blend	4.75	3.16	80	0.88	2.0	0.10	.042

[1] $mg/hr/kg^{0.74}$
[2] mg/L
[3] /hr

Analytical Conditions

Loss rate studies and *in vivo* experiment samples were analyzed on the attached GC with a flame ionization detector using a pre-programmed sampling interval of 10-20

minutes. The GC used 6 mL/min of nitrogen as carrier, a DB-1 capillary column (30 m, 0.45 mm diameter, 2.55 μm film; J&W, Folsom, CA), auxiliary flow of 25 mL/min, detector hydrogen flow of 30 mL/min, air flow of 400 mL/min, detector temperature of 250 °C, and inlet temperature of 200 °C. For single and five-chemical mixture experiments, the oven temperature was 60 °C to start, 8 °C/min ramp to 90 °C, held for 4.25 min, and chamber air samples were analyzed every 10 minutes. For gasoline experiments, the oven temperature was 35 °C to start, 10 °C/min ramp to 130 °C, 50 °C ramp to 210 °C and held one minute, and chamber air samples were analyzed every 20 minutes. The gas sampling loop temperature was held at 70 °C. Loop temperatures could not be held stably at lower levels due to oven cycling. At 70 °C, standards analysis indicated that the effective loop volume was 0.47 mL due to gas expansion in the loop.

Chromatograms were analyzed using HP Chemstation software. Initially, the intent was to include *m*-xylene as a representative of xylenes. However, during method development, it was determined that *m*-xylene could not be resolved from *p*-xylene under analytical conditions that would permit frequent chamber sampling. Therefore, *o*-xylene was selected instead. BTEX and *n*-hexane (BTHEX) peaks were determined by matched retention times and confirmed by fingerprinting analysis of the same samples run on GC/MS. After GC/MS analysis using similar run conditions, the mass spectra of BTHEX peaks were inspected for co-eluters. Toluene, ethylbenzene, and *o*-xylene were determined to run essentially uncontaminated, while there were small peaks that co-eluted with benzene and *n*-

hexane that were of minimal significance (Dick 2001). A typical gas chromatogram for gasoline analysis is shown in Figure 5.2.

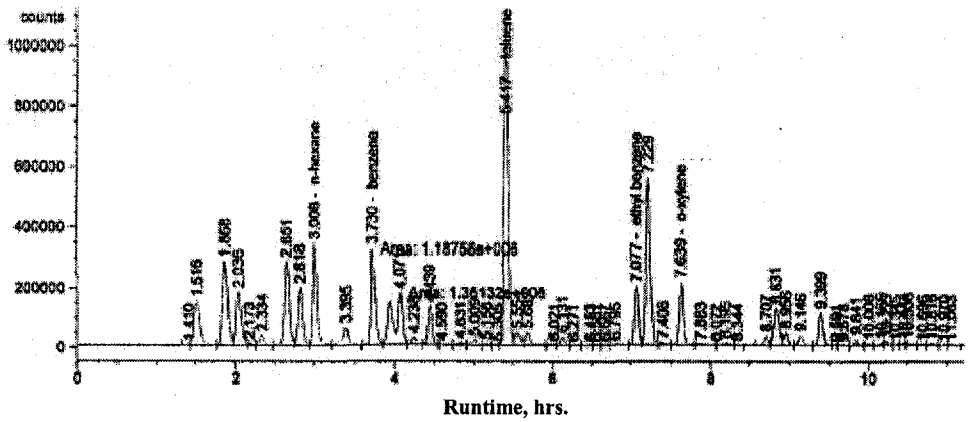


Figure 5.2. Typical gas chromatogram of gasoline at the start of a gas uptake experiment. Sample was injected onto a 30m DB-1 megabore capillary column, separated during a 12 minute runtime before analysis by flame ionization.

After data analysis, the concentrations for BTHEX peaks were determined using calibration curves for each compound. All remaining detected peaks were summed and compared with a calibration curve established for whole gasoline.

In vivo experiments

Rats were exposed for 6 hours or until chemical(s) in the chamber were essentially depleted. Initially, a series of experiments with single chemicals was performed with initial chemical concentrations between 500 and 2000 ppm. Subsequently, a series of experiments were performed with the five chemical BTHEX mixtures where one chemical was introduced at a high level and the others were introduced at lower levels

("high/low" experiments). In these experiments, the low levels were approximately 50 ppm and the high levels were approximately 1000 ppm.

These data were used in the PBPK model described below to estimate kinetic constants for BTHEX. A suite of validation studies, which were not used to estimate parameter values, was thereafter conducted with BTHEX. In three experiments, all five chemicals were introduced at approximately the same concentration, at 100, 300, and 500 ppm each. Finally, experiments with gasoline were conducted with the winter and summer blends of gasoline. For these, the initial concentration of the gasoline mixture was approximately 500, 1000, and 1500 ppm.

PBPK Modeling

All PBPK modeling was performed in Berkeley Madonna, v. 8.0.2a8, a recent beta version that includes scripting capabilities (Macey and Oster 2002). The basic structure of the four compartment PBPK model used has been previously described (Gargas *et al.* 1986; Krishnan *et al.* 1994; Purcell *et al.* 1990). In this model, the rat is comprised of fat tissue, slowly perfused tissue, rapidly perfused tissue, and liver compartments, and also includes the gas uptake chamber as an external compartment. A lung compartment is not included, but the atmospheric concentration is assumed to be in equilibrium with an additional lung blood compartment. The arterial blood perfuses the tissue compartments, which are then regarded as being in equilibrium with the venous return blood from each compartment. Cardiac output (QC) was set at 15 L/hr/kg and the default alveolar (pulmonary) ventilation (QP) was set at 12

L/hr/kg. These parameters as well as V_{max} were scaled by (body weight)$^{0.74}$. Except as noted below, all physiological parameter values were taken from the literature (Brown *et al.* 1997). It was found, however, that high levels of *o*-xylene required a reduction in alveolar ventilation as described below. The physiological values for the model are indicated in Table 5.3.

Table 5.3
Physiological Parameters for Simulations

Tissue group	Volume (%BW)	Flow (%QC)
Liver	3.7	18.3
Fat	.035*BW+.205	7.0
Richly Perfused	5.4	51.0
Slowly Perfused	91 – remaining	100 – remaining
Lung Blood	0.2	N/A
System parameters		
Cardiac output	15 L/hr/kg^0.74	
Alveolar ventilation	6.5 – 8 L/hr/kg^0.74	(*o*-Xylene at high level)
	12 L/hr/kg^0.74	(all other simulations)

Chemical specific parameters include the partition coefficients and the kinetic parameters. Literature sources for partition coefficients (PCs) were used for benzene, toluene, *n*-hexane, and *o*-xylene (Gargas *et al.* 1989) and ethylbenzene (Tardif *et al.* 1997), setting the richly perfused PC equal to the liver PC. Partition coefficients for the lumped chemical were estimated in the model, as described below. The loss rates determined for each chemical were included in the model as a first order loss. Chemical parameters values for the model are listed in Table 5.4.

Table 5.4
Chemical Parameters for Simulations

Chemical	PB	PL	PF	PS	Vmax[1]	Km[2]	KL[3]
n-hexane	2.29	2.27	69.4	1.27	7.0	0.01	.025
benzene	17.8	0.96	22.0	0.58	5.3	0.10	.052
toluene	18.0	4.64	56.7	1.54	5.3	0.02	.070
ethylbenzene	42.7	1.96	36.4	.609	7.6	0.10	.073
o-xylene	44.3	2.44	42.4	1.16	6.5	0.20	.086
lumped summer blend	2.9	3.16	80	0.88	2.0	0.10	.042
lumped winter blend	4.75	3.16	80	0.88	2.0	0.10	.042

[1] $mg/hr/kg^{0.74}$
[2] mg/L
[3] /hr

Kinetic constants were included for saturable metabolism of each chemical. All metabolism was assumed to occur in the liver, including competitive inhibition by each inhibitor present, according to the equation:

$$\text{Rate of metabolism} = \frac{V_{max} * [S]}{[S] + K_m * (1 + \sum_i \frac{[C_i]}{K_i})} \qquad \text{Eq. 1}$$

where V_{max} is the maximum rate of metabolism, [S] is the concentration of the substrate, K_m is the affinity constant, $[C_i]$ is the concentration of inhibitors, and K_i is the inhibitory constant. The values for the constants V_{max}, K_m, and K_i for each chemical were determined by visual optimization with subsequent verification with the Berkeley-Madonna software curve-fit utility, which uses an algorithm to minimize the root mean square deviation. When verifying the optimization using Berkeley-Madonna, the first and second initial values (starting points for the optimization) were set by incrementing +/- 10% from the visually-determined

parameter value. The best fits determined in this manner were reasonably consistent between different model simulations and experimental datasets. Sensitivity analysis of model parameters was performed as well. This analysis determines the sensitivity of the model, in terms of chamber concentration, as a result of a small change (1%) in an input parameter. Thus, the ratio of the fractional change in chamber concentration to 0.01 was calculated representing the sensitivity coefficient for that parameter. Parameters for which the model had a sensitivity coefficient of 0.2 to 0.5 were regarded as moderately sensitive, and above 0.5 were regarded as sensitive. Other equations used for the present model are given in the earlier versions of the model (Gargas *et al.* 1986; Ramsey and Andersen 1984).

The present PBPK model thus contained an identical structure for each of the six chemicals. With six sets of chemical specific parameters, each chemical's model influenced each other's through competitive inhibition as shown in the metabolic equation (Eq. 1). The sixth chemical was the lumped chemical representing all components of gasoline other than BTHEX, from the common integration of all non-BTHEX peaks in gas chromatograms.

The experimental design was intended to reduce the number of parameters that had to be estimated by the PBPK model at one time, in order to keep the computations tractable and to improve the confidence in the simulation. The sequence of experiments permitted the model to be used to estimate one or at most two parameters at a time for each dataset by using exposure conditions that would only be relatively

sensitive to one or two parameters. Starting concentrations were close to expected values and the model used a starting concentration that was thereafter estimated by extrapolation of the chamber decay curves back to time-zero. Single chemical runs were used to determine a starting value for V_{max}, using initial V_{max} and K_m values from previous reports (Andersen and Clewell 1983; Haddad *et al.* 1999). Once V_{max} was set for a given chemical, K_i could be determined from the "high/low" experiments by examining the effect of the chemical at high levels on the low level chemicals. Initially, each chemical's K_i value was set to equal its K_m, based on the biochemical notion of competitive inhibition. Since the inhibitory effect of the high level chemical is expected to be essentially similar on each of the low level chemicals with competitive inhibition, the K_i for the high level chemical was altered until the low level chemical simulations were consistent with their corresponding chamber loss data. Due to the fact that the K_m for subsequent chemicals were not all established until the last chemical was parameterized, this process had to be performed iteratively until the best fit was obtained.

After finding values for each of the five chemicals' V_{max}, K_m and K_i (which still equaled K_m), the simulations were run. These simulations supported our approach to equate K_i and K_m for each chemical. The parameter estimates developed in this manner were then tested with the validation datasets for BTHEX and used in the gasoline simulations without further altering any parameters.

Simulations for the complete gasoline mixture required estimation of partition coefficients, V_{max}, K_m, and K_i for the lumped hydrocarbon group. The PCs of the lumped chemical were initially set to equal measured PCs for *n*-heptane obtained from the literature (Gargas *et al.* 1989). These values permitted an adequate simulation of the winter blend, but simulation of the summer blend required a decrease in the blood:air PC. For the lumped chemical, the K_i was again set to equal the K_m. K_i (and K_m) for the lumped chemical were obtained by fitting the model to the BTHEX chemical data in the gasoline experiments. V_{max} for the lumped chemical was then obtained by fitting the model to the lumped chemical datasets in these same experiments. As with the BTHEX optimization, the process was conducted iteratively due to the interdependence of some of the parameter estimates.

Results

Single chemical experiments were initially performed to estimate V_{max} for each of the target chemicals. In all figures, the symbols are experimental data and the smooth curves are the results of the PBPK model simulations. Also, all chamber concentrations are expressed in parts per million (ppm) based on standard temperature and pressure. In Figure 5.3, data and simulations are shown for single chemical runs for *n*-hexane, benzene, toluene, and ethylbenzene. A single exposure study was deemed adequate for these compounds since PBPK models were available for each of them. Individual optimization for each compound yielded better fits to the single chemical data than shown in the Figure; however, the simulations shown represent the best fit to the single chemical and to the BTHEX high/low mixture data sets together.

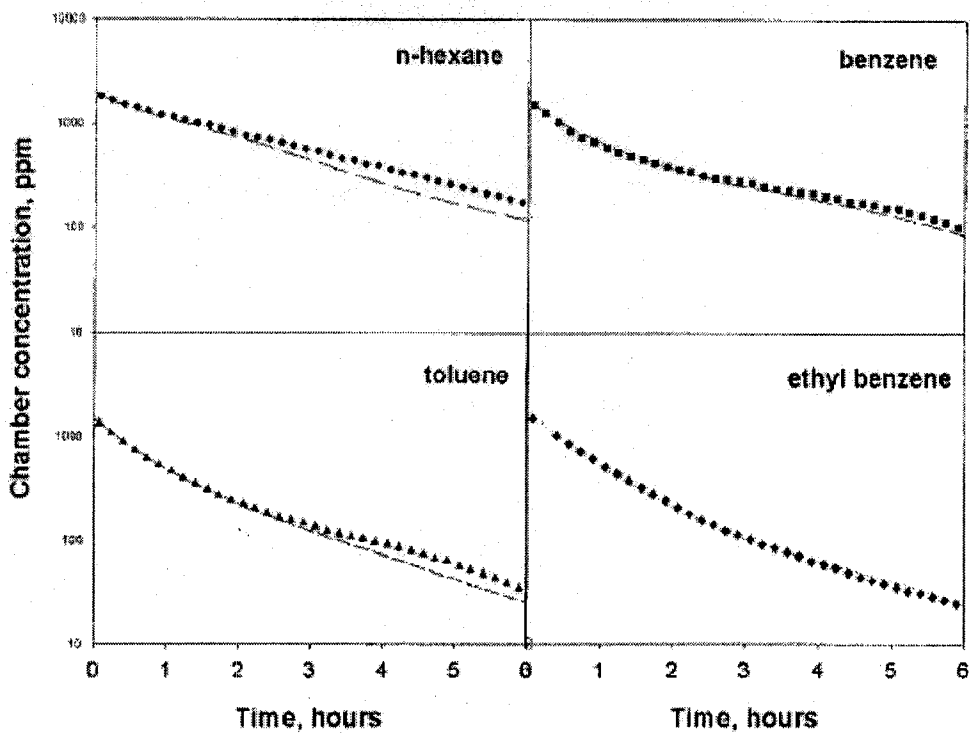


Figure 5.3. Gas uptake experiments for single chemicals in four separate experiments. In Figures 5.3-5.8, smooth curves represent model simulations obtained from the PBPK model described in this paper. Symbols represent experimental data from gas uptake experiments described in Methods. Common axes are labeled at left and bottom.

For benzene, *n*-hexane, toluene, and ethylbenzene, these data were used to develop model estimates that could be compared with existing published models. However, no PBPK model has been reported for *o*-xylene. Therefore, three experiments were performed to develop the description of *o*-xylene PKs (Figure 5.4a). Adequate simulation could not be obtained (Figure 5.4b) if the default alveolar ventilation (QP) value was used (12 L/hr/kg$^{0.74}$). Because *o*-xylene is a known respiratory irritant, the alveolar ventilation parameter was reduced until an adequate fit of the gas uptake data was obtained. In the single chemical *o*-xylene experiments, QP was set at 6.5

L/hr/kg$^{0.74}$. In the high/low experiments when *o*-xylene was the high chemical, QP was 8 L/hr/kg$^{0.74}$. In all other simulations the default value for alveolar ventilation was used.

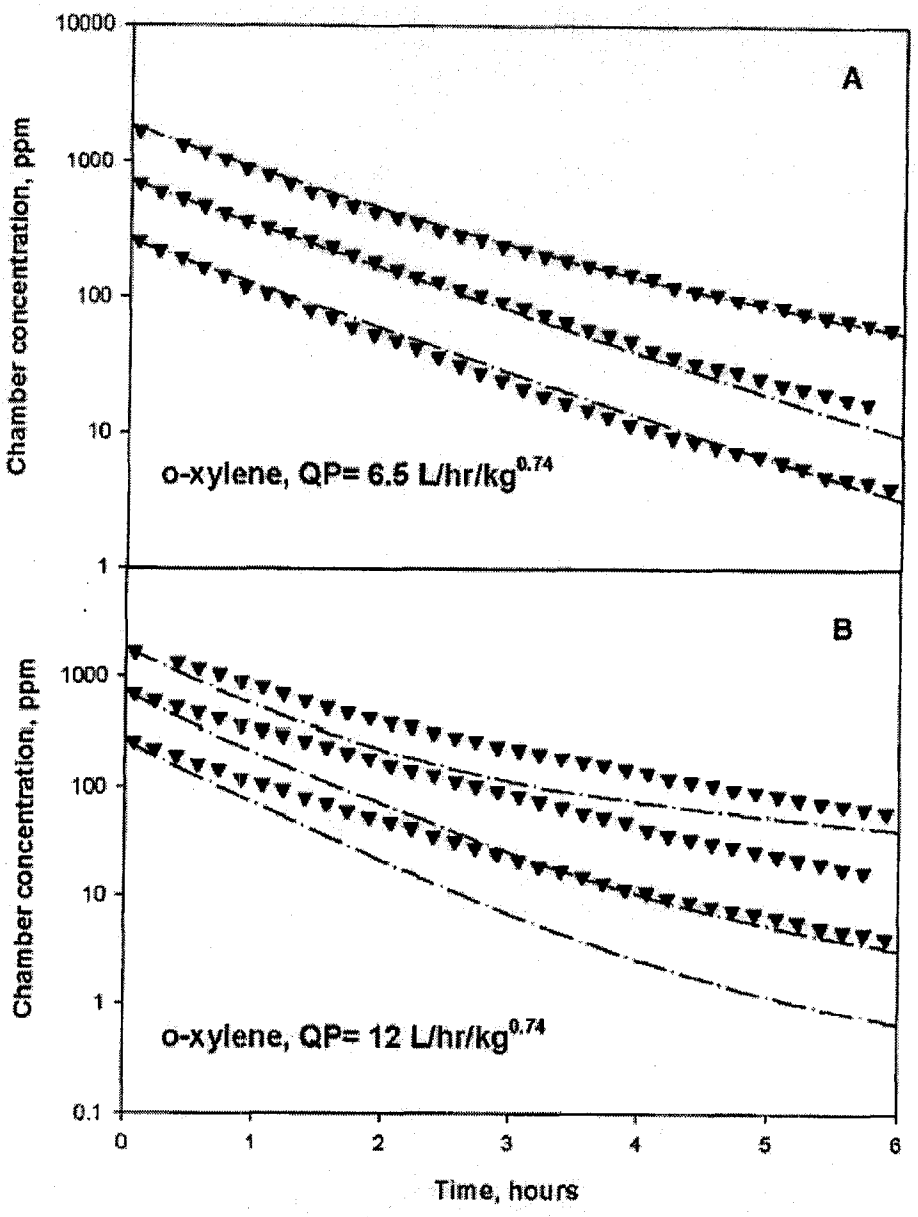


Figure 5.4. A. Gas uptake experiments for *o*-xylene at three initial starting concentrations of 500 – 1,500 ppm. The single chemical experiments were primarily used to obtain estimates of V_{max}. Simulation with alveolar ventilation set at 6.5 L/hr/kg$^{0.74}$. B. Simulation of *o*-xylene PKs with alveolar ventilation set at the default value of 12 L/hr/kg$^{0.74}$ plotted against the same data sets as in A. Adjustment of other parameters did not substantially improve the fit.

Figure 5.5 shows the model simulations of the BTHEX mixture data for the high/low experiments. To avoid excessive data and simulation overlap on each plot, three of the five chemicals were plotted in the left and two in the right hand panels. These models were obtained by using the initial V_{max} estimates from the single chemical experiments and then sequentially estimating a value for K_i for each high chemical with K_m equal to K_i. In total, these experiments provided 25 data sets for examination. With a few exceptions, the simulations closely matched the corresponding data within the range of experimental error. Lack of fit occurred for a few of the simulations, but did not appear to be a consistent problem for any chemical. In addition, inflections in the simulations also tracked with inflections in datasets. In the high/low experiments, the model was moderately sensitive to K_i, the parameter for which the data was used to estimate.

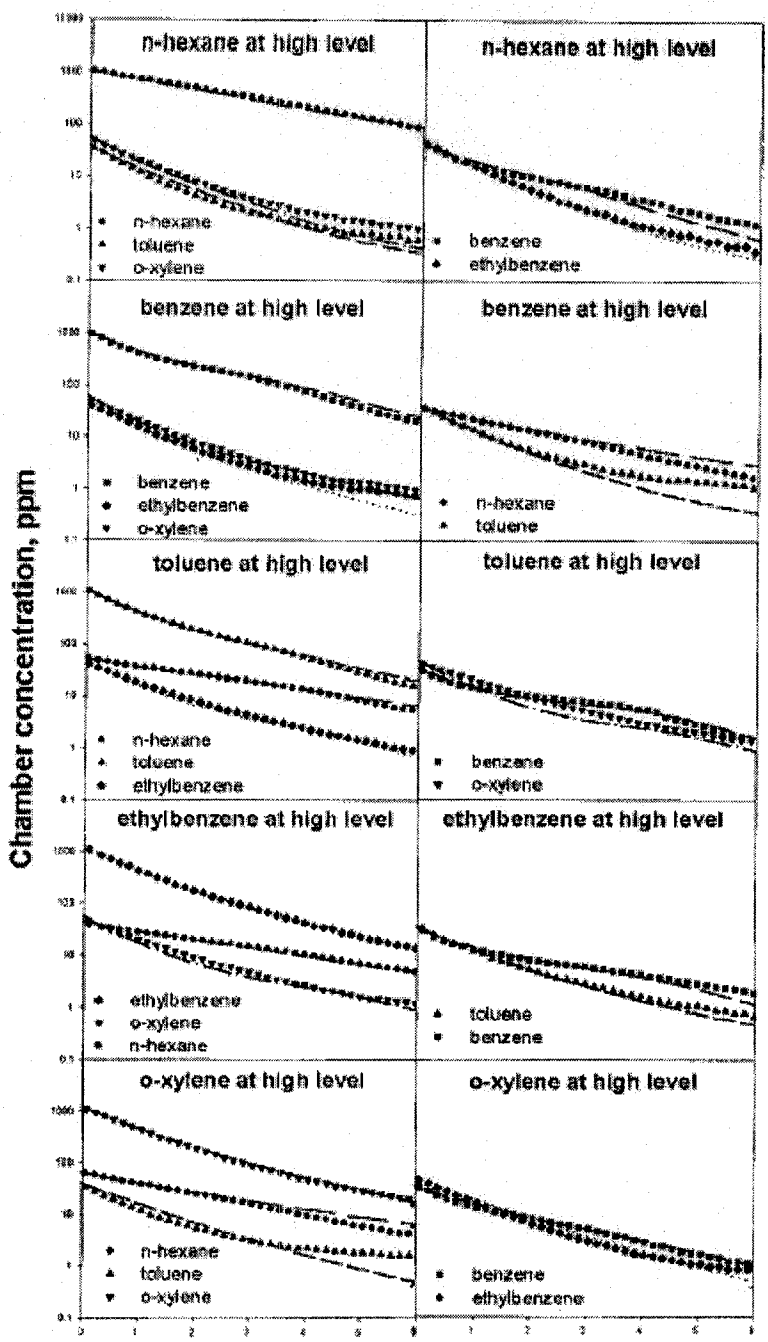


Figure 5.5. Gas uptake experiments for 5° mixtures of BTHEX components. In each experiment, one chemical was at a high level and the other four were at low levels. Each experiment was divided into two panels (left and right) to show traces of all four low-level chemicals with the order based on visual separation of data and simulations. In Figures 5.3-5.8, smooth curves are model simulations; symbols represent data.

n-hexane – – – ● benzene - - - - - ■ toluene - - - - - ▲ ethylbenzene ····· ◆

o-xylene – · – · ▼ lumped chemical —— +

A separate suite of experiments was performed to provide validation data for the BTHEX model. In these experiments (Figure 5.6), similar levels of each chemical were added to the chamber at the start of the experiments. As in Figure 5.5, three chemicals appear in the left hand panel and two chemicals appear in the right panel for each experiment to avoid cluttering. The simulations of these data using the parameter values estimated from the single and high/low experiments fit the corresponding datasets as well as the high/low simulations.

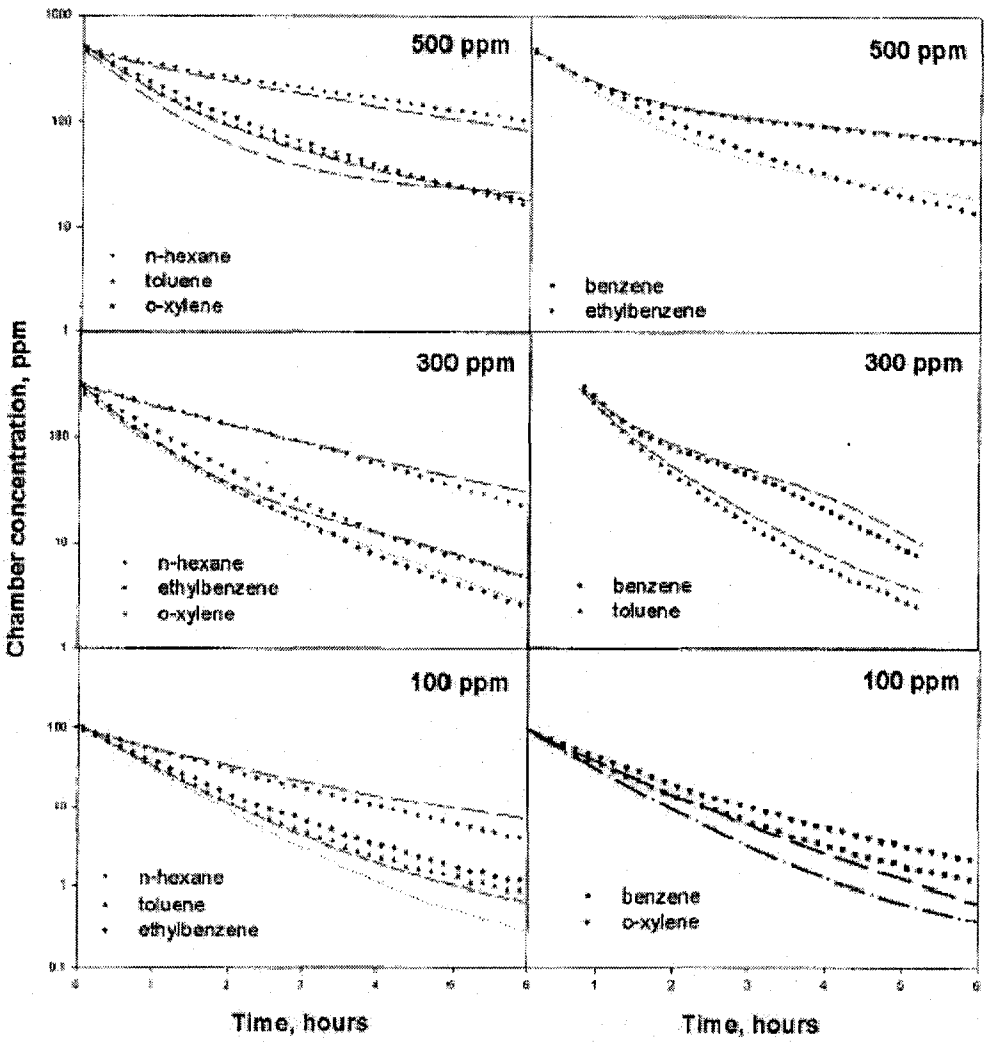


Figure 5.6. Validation experiments for BTHEX. In these three gas uptake experiments, all five chemicals were introduced into the chamber at about the same starting concentration (100, 300 or 500 ppm). Each experiments is divided into two panels (left and right) to allow discernment of the traces. These data were not used for parameter estimation and the model simulations use the same values for all parameters as used in other simulations. See Figure 5.5 for legend symbols.

For the gasoline experiments, it was necessary to estimate the kinetic constants V_{max}, K_m, and K_i, and the partition coefficients for the lumped component. The optimal fits were obtained using the parameter values indicated in Table 5.4. Also, the fit of the data for both blends was improved by adding a second linear pathway for lumped chemical metabolism in the liver, with a rate constant of 11/hr/kg, scaled to (body weight)$^{-0.3}$. This approach accounts for the presence of CYPs other than CYP2E1. The data and simulations with the summer blend of gasoline are shown in Figure 5.7 and data and simulations for the winter blend are shown in Figure 5.8. The lumped chemical was well fit by using the same parameters for both gasoline blends except for a decrease in the blood:air PC. In addition, at all three exposure levels, the same K_m and K_i for both blends were able to provide the approximate degree of inhibition of the metabolism of the target chemicals BTHEX.

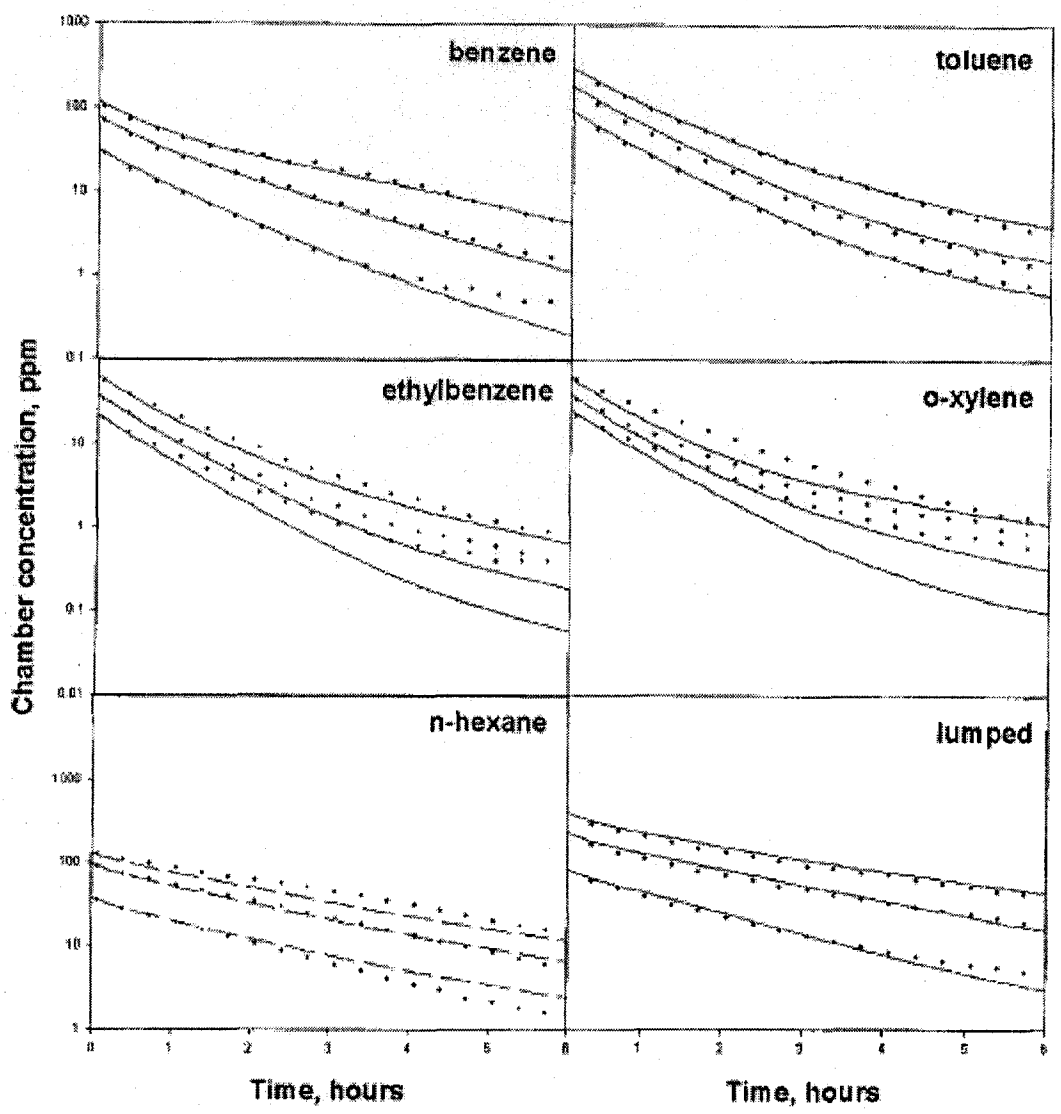


Figure 5.7. Summer blend of gasoline in gas uptake experiments at starting concentrations between 500 and 1,500 ppm. Parameter values of BTHEX chemicals are unchanged; kinetic parameters for the lumped chemical are estimated here. See Figure 5.5 for legend details.

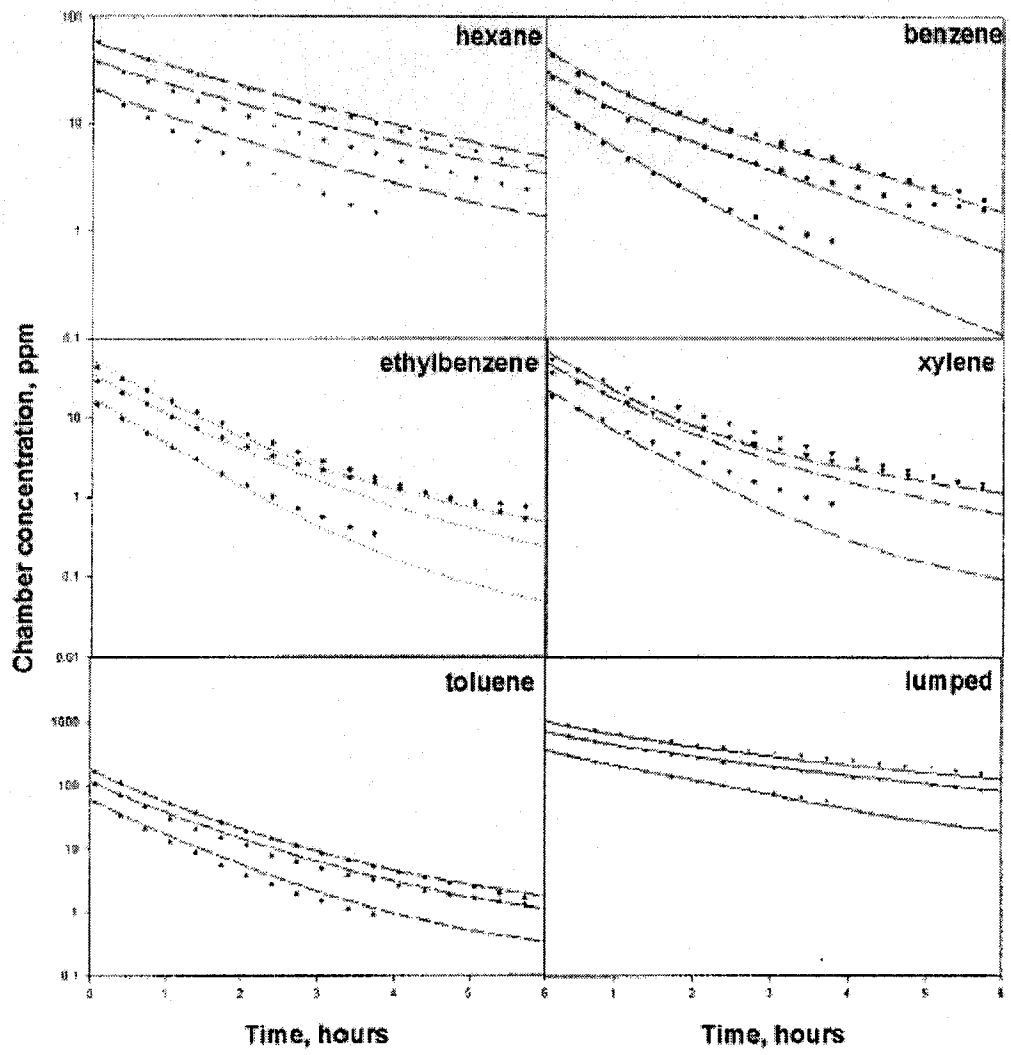


Figure 5.8. Winter blend of gasoline in three gas uptake experiments at starting concentrations between 500 and 1,500 ppm. The same parameter estimates are used as in the summer blend gasoline except for the blood:air partition coefficient (4.75 for winter blend vs. 2.9 for summer blend). See Figure 5.5 for legend details.

The model was used to determine the total metabolism of target chemicals under gas uptake conditions (Figure 5.9). As a reflection of metabolic inhibition, this figure shows the percent reduction in metabolism for each component (BTHEX and the lumped component) during a simulated gas uptake experiment with a representative gasoline containing 1.5% *n*-hexane, 2% benzene, 8% toluene, 2% ethylbenzene, 2.5%

o-xylene, and 84% lumped components at the starting exposure concentrations indicated.

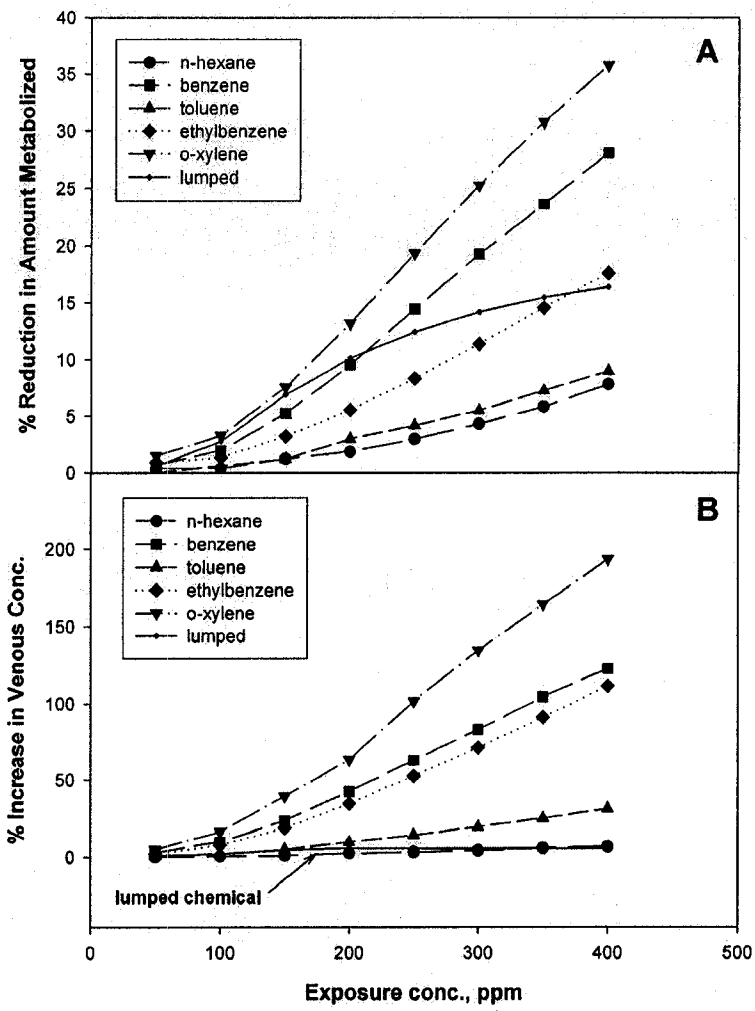


Figure 5.9. Net inhibition of metabolism for each target chemical and the lumped chemical, expressed as a ratio of the metabolism of the single chemical to metabolism of the same starting concentration of chemical in a representative blend of gasoline. See Figure 5.5 for legend details.

Metabolism of *n*-hexane was inhibited the least and *o*-xylene's metabolism was inhibited the most. The metabolism of all chemicals was somewhat inhibited, even at the lowest concentration simulated (100 ppm). These measures of inhibition would

not reflect inhibition during constant exposure. During a constant exposure, steady state concentrations are much higher than during gas uptake experiments where the chamber concentration declines by at least one order of magnitude during the experiment. Consequently, the degree of inhibition would be greater during constant exposure.

Discussion

In this paper, we present a PBPK model that describes the PKs of gasoline by focusing on five individual target components in gasoline as well as the PKs of the residual chemicals as a lump. This approach represents a first step in the development of a PBPK model for this complex mixture. From a suite of gas uptake experiments, we estimated V_{max} for each single chemical primarily from the single chemical experiment data, K_i and K_m for each chemical primarily from the high/low experiments, and the lumped chemical kinetic parameters from the gasoline experiments. The average deviation between the model and the data is in the range of experimental error, and no systematic errors appear to occur. The model adequately describes the PKs of the lumped chemical as well as the PKs of each of the five target chemicals. Nevertheless, there are some instances where some disagreement between the model and the data occur.

In contrast to PBPK models for single chemicals or for simple mixtures, lumping the majority of the components of gasoline with hybrid chemical parameter estimates may have some limitations. Our approach assumes that the properties of the lumped

chemical are constant, even though the proportion of chemicals in the lumped component are likely to vary over time in the closed chamber atmosphere and in the animals. In particular, chemicals that partition into the animal quickly and/or are rapidly metabolized would be expected to decrease in proportion in the overall lumped compound group. Rapidly metabolized chemicals, to the extent that their K_m's are lower than average for the lump, would be depleted faster, possibly resulting in an increase in the composite K_i of the lump at later times. In the future more complex approaches could be used to vary these properties over the course of the experiment.

Most of the kinetic parameter estimates compared well with those in previously published PBPK models. For *n*-hexane, the V_{max} reported here was similar to the V_{max} in a previously published model (7 vs. 8.3 mg/hr/kg when scaled to $BW^{0.74}$ (Hilderbrand and Andersen 1981)). For benzene, previous models have determined V_{max} in a range of 1 to 7 mg/hr/$kg^{0.74}$ (Purcell *et al.* 1990; Travis *et al.* 1990; Kenyon *et al.* 1996; Medinsky *et al.* 1989) that includes the current estimate. The V_{max} for toluene in the present model (5.3 mg/hr/ $kg^{0.74}$) was also in the range of previous model estimates of 3.4 – 7.5 mg/hr/$kg^{0.74}$ (Haddad *et al.* 1999; Purcell *et al.* 1990; Thrall *et al.* 2001). Few PBPK models exist for ethylbenzene, and for xylenes, the only models are for the *meta* isomer. However, our estimates compared well with that published by Krishnan and coworkers (Tardif *et al.* 1997; Haddad *et al.* 1999); our V_{max} estimate and the previous estimate were 7.6 vs. 6.4 and 6.5 vs. 6.6 mg/hr/ $kg^{0.74}$ for ethylbenzene and xylene respectively. On the other hand, our estimates for

K_m and K_i were generally lower than previous estimates (Haddad *et al.* 1999; Purcell *et al.* 1990). The model was generally fairly sensitive to V_{max} during single chemical runs (sensitivity coefficient 0.5 – 0.8). In the high/low experiments, chemicals at the low level were less sensitive to changes in the high level chemical's K_i (sensitivity coefficient 0.1 to 0.3), increasing the uncertainty of this parameter estimate.

Significant inhibitory effects of the lumped chemical affected chamber clearance of all the selected targets. Simulated chamber experiments suggest that total metabolism of BTHEX and the lumped chemical would be inhibited by 1-28% over the course of a 6-hour gas uptake experiment at starting concentrations between 500 and 1,500 ppm (Figure 5.9). *o*-Xylene was the most inhibited and *n*-hexane was the least. Notably, the degree of inhibition was a linear function of starting concentration and generally followed the rank order of blood:air partition coefficients. Other factors also enter into determining how much inhibition will occur, including the K_m for the component being inhibited. Low K_m components tend to be inhibited less; thus toluene is less affected than benzene and ethylbenzene is less affected than *o*-xylene even while their blood:air partition coefficients are similar. More soluble compounds reach higher concentrations and are more likely to interfere with the metabolism of other compounds. Components that have toxicity related to the presence of the unaltered parent compound will tend to show increased toxicity due to inhibition as indicated here, whereas components that are biotransformed into more toxic metabolites will tend to have reduced toxicity.

As a six-chemical model with a lumped component, this model used a top-down approach that could be implemented because it did not depend on performing numerous 1^{o}, 2^{o}, 3^{o}, 4^{o}, and 5^{o} mixture combination experiments. We performed a simple suite of single chemical and five chemical mixture experiments and then went straight to the full mixture. The justification for this is that the strength of a component as an inhibitor is an intrinsic property of the chemical and doesn't depend on the concentration of the chemical itself or of the other chemicals that it is inhibiting. Therefore, inhibitory parameter estimates determined in binary experiments should be valid for any higher order mixture containing the chemical, as found in previous studies (Tardif *et al.* 1993; Tardif *et al.* 1997; Haddad *et al.* 1999).

In the present work, we retained the constraint that K_i equals K_m. Most previous reports suggest that the mode of interaction between many of the components of gasoline is competitive inhibition (Backes *et al.* 1993; Hargreaves *et al.* 1994; Haddad *et al.* 1999). Although the K_m and K_i determined *in vivo* are *apparent* values that are influenced by multiple factors, including second isozyme metabolic pathways, under theoretical competitive inhibition they should be related parameters. Regardless of this argument, forcing K_m to equal the optimized values of K_i did not result in a significant decline in the fit of the model. This approach was consistent with earlier studies (MacDonald *et al.* 2002) and significantly reduced the numbers of parameters that were evaluated by optimization.

During method development, we evaluated the adequacy of the CO_2 absorbent to adequately control CO_2 levels by conducting real time CO_2 measurements in the chamber. Below a minimum amount of absorbent, CO_2 levels in the gas uptake chamber would rise rapidly to a level above the sensor range of 10,000 ppm (data not shown). Analysis of the chemical data from these experiments indicated that CO_2 stimulation of the animal's breathing rate has a significant effect on absorption of the chemical(s) from the chamber, as noted in the literature (Johanson and Filser 1992). Conversely, we noticed that the animal's breathing rate was reduced during exposure to *o*-xylene. This observation was consistent with the known irritation effects of *o*-xylene (OEHHA 1997). *m*-Xylene was found to reduce pulmonary ventilation by 50% (RD_{50}) at a concentration of 1360 ppm (Korsak *et al.* 1993). For *p*-xylene and *o*-xylene respectively, the RD_{50} was 1300 ppm and 1500 ppm in another study (Muller and Greff 1984), consistent with the general statement that acute toxicity assays for various xylene isomers have generally found similar results (OEHHA 1997). However, the effect of pulmonary irritants on respiratory parameters over longer exposures or in declining exposure levels is not easily quantifiable.

Gasoline and some of its constituents are also pulmonary irritants, but to a lesser extent than xylene (ACGIH 1999). This is consistent with the reduction in our model of alveolar ventilation to 12 $L/hr/kg^{0.74}$, as generally proposed originally by Filser and coworkers (Johanson and Filser 1992). As with *o*-xylene, the effect of longer duration exposure to other pulmonary irritants is not well established. The alveolar ventilation rate used for exposure to *o*-xylene alone (6.5 $L/hr/kg^{0.74}$) and in mixtures

(8 L/hr/kg$^{0.74}$) as well as the standard rate used for other chemicals and mixtures (12 L/hr/kg$^{0.74}$) were within the range reported in previous studies when chemical exposure was present (Johanson and Filser 1992). Additionally, the range of alveolar ventilation in unexposed rats in the Brown *et al.* review (Brown *et al.* 1997) is quite wide (13-58 L/hr/kg$^{0.74}$ for a 250 g rat), a 4.4-fold range. The fact that the model is sensitive to alveolar ventilation (sensitivity coefficients average approximately 0.5) in this range suggests that better estimates or actual measurements of the alveolar ventilation would be useful in future studies.

Partition coefficients for the lumped chemical were estimated during model optimization. PCs for hydrocarbons have been related to hydrocarbon chain length both in predictions and experiments (Poulin and Krishnan 1996; Fiserova-Bergerova and Diaz 1986; Gargas *et al.* 1989). Most of the lumped component consisted of alkanes and the average molecular weight of the two samples as provided by the originating laboratory (SWRI 2001) was 96 g/mole, just slightly less than the molecular weight of heptane. For this reason, this simulation was initiated by using published PCs for *n*-heptane (Gargas *et al.* 1989). These worked reasonably well for the winter blend, but the blood:air PC for the summer blend had to be decreased to 2.9 to get an acceptable fit.

Better fits of the model to the chamber clearance curves were obtained by including a second pathway for metabolic clearance of the lumped group of chemicals. Many hydrocarbons exhibit more than one pathway of metabolism for initial oxidation

(Guengerich and Shimada 1991; Nakajima *et al.* 1989; Kim *et al.* 1997). For example, liver metabolism of *n*-hexane appears to be caused by two isoforms of CYP P450 (Toftgard *et al.* 1986). A second pathway may be more likely for paraffins than for alkyl aromatic species that have fewer locations for insertion of molecular oxygen given the preference for alkyl groups over ring carbons. As the lumped component was relatively poor in aromatics relative to paraffin compounds, the second pathway would be more likely for the lumped group of hydrocarbons. In addition, this lumped group of compounds has many different hydrocarbons that may have different affinities and sites for oxidation leading to different clearance behaviors from the chamber atmosphere. A number of existing PBPK models have included second linear pathways (Clewell *et al.* 2000; Andersen and Clewell 1983; Andersen *et al.* 1987).

The current model structure for gasoline had five split components and one lump, which behaves like a six-chemical mixture in terms of the mathematic model. Gasoline was treated as "perfectly lumpable" in that the same lumping paradigm (i.e., a single lump) was used with respect to all of the PK processes (absorption, distribution, metabolism, and elimination). By default, gasoline was also treated as properly and continuously lumpable (Verhaar *et al.* 1997). Lumping approaches have been used for many years in chemical engineering, often for kinetic process analysis (Ancheyta-Juarez and Sotelo-Boyas 2000; Ancheyta-Juarez *et al.* 1999) in petroleum refining. The approach has been extended to atmospheric contaminant modeling (Harley *et al.* 1993) as well. Lumping in a sense has also been used in structure-

activity relationship concepts leading to risk assessments for classes of compounds such as dioxins and furans. PBPK models have successfully employed tissue lumping approaches in the past. Tissues with similar time constants (a property related to the partition coefficient and to the ratio of the tissue volume to blood perfusion) can generally be lumped without a significant sacrifice in model accuracy (Andersen 1991). Chemical lumping employs a similar approach in terms of using average parameter values for lumped components, but prior to developing the model, it was unclear whether such a simplification would be acceptable. Previous efforts to develop PBPK descriptions of benzene in gasoline did not use a lumping analysis (Travis *et al.* 1992). In these analyses, the inhibitor (gasoline) was not included in the model or in the model's calculation of benzene metabolism. Instead, new empirical kinetic constants were assigned to benzene itself. Therefore, extrapolation of these constants to different blends of gasoline may be difficult. Using lumping in a complex mixture PBPK model was suggested earlier (Medinsky 1997; Bond *et al.* 1997; Verhaar *et al.* 1997).

In closed chamber studies, clearance is determined by the initial steps in parent compound metabolism. Toxicity frequently is associated with more downstream metabolites in complex sequences of xenobiotic processing. More complete descriptions of benzene metabolism are available (Cole *et al.* 2001; Medinsky *et al.* 1989), but for most chemicals, more work is needed to improve the single chemical metabolic descriptions. With at least *n*-hexane and benzene, at high exposure concentrations, the presence of the parent chemical can inhibit further metabolism of

the first metabolite to second metabolites (Cole *et al.* 2001). In the case of *n*-hexane, metabolites can actually inhibit metabolism of the parent chemical to some extent (Clewell and Andersen, 1984). Inhibitory interactions within metabolic pathways may become much more significant in the case of a complex mixture, when the concentration of parent chemicals is lower compared to the aggregate concentration of the numerous metabolites that are jointly present.

As a first effort, the lumping approach used in this model was successful in predicting the PKs of target components in gasoline. Other target components such as MTBE or ethanol could be split out from the lumped group and treated in a similar manner, usually on the basis that the component exerts a specific toxicity as a single chemical. Generally, the simplest lumping approach would be to split those chemicals where individual pharmacokinetic information is needed, for risk assessment or some other purpose, and the include all remaining components in the lump. Other complex hydrocarbon mixtures such as diesel fuel, jet fuels, and heating fuels are similar in that they consist of a broad array of hydrocarbons with properties that differ qualitatively. If there are target components within some of these mixtures, the present approach may be useful in developing a PK description for these other complex mixtures. However, gas uptake methods are unlikely to work with higher boiling distillates. For these mixtures, approaches should be pursued that are based on constant concentration inhalation exposures and evaluation of blood concentrations of individual components during and after the period of exposure.

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Chapter 6

Development of a PBPK Model for Volatile Fractions of Gasoline Using Chemical Lumping Analysis

Abstract

Physiologically-based pharmacokinetic (PBPK) models have often been used to describe the absorption, distribution, metabolism and excretion of chemicals in animals, but have been limited to single chemicals and simple mixtures due to the difficulty in obtaining the necessary pharmacokinetic data for each component. To solve this difficulty, we used a chemical lumping approach with a PBPK model for the complex mixture gasoline. Our previous gasoline model consisted of five individual components (benzene, toluene, ethylbenzene, xylene, and hexane) and a lumped chemical that served as a surrogate for all remaining components of whole gasoline. Despite being comprised of hundreds of components, the lumped component was well described using single chemical parameters that depended on the blend of gasoline. In the present study, we extend this approach to volatility-weighted fractions of whole gasoline, such as may evaporate during brief exposures. The PBPK model that was developed for whole gasoline performed well in describing the pharmacokinetics of different gasoline fractions. Parameter adjustments were made for the lumped component's partitioning and rates of metabolism, while model

equations and all other parameters were invariant. The model serves as the first example of how the engineering concept of chemical lumping can be used in pharmacokinetics.

Introduction

Gasoline is a large-production chemical product with significant human exposure potential (Wixtrom and Brown 1992; Laitinen *et al.* 1994; Hakkola and Saarinen 1996; Dement *et al.* 1997; NIOSH 1999; Hakkola and Saarinen 2000; INEGI 2002). It contains several individual components that cause toxicity in humans at sufficiently high exposures (Graham *et al.* 1995; OEHHA 1997a, b, 1999; IARC 2002). Yet, studying the toxic effects of gasoline is complicated for many reasons, including (1) toxicity studies cannot be performed on every component and every blend of gasoline; (2) interactions between components of complex mixtures such as gasoline can alter toxicity; (3) these alterations will depend on exposure concentrations and the blends of gasoline examined; and (4) exposures occur to whole gasoline and fractions of these fuels, i.e., low boiling volatiles or high-boiling residues after evaporation. Predictive approaches to toxicity of gasoline blends, such as the approach presented in this paper, will be important to understanding the fate of the chemical in organisms and the risks associated with exposures.

Pharmacokinetic aspects (absorption, distribution, metabolism, and excretion) of chemical toxicity have been elucidated in recent years using physiologically-based pharmacokinetic (PBPK) modeling for various animals, including humans (Andersen 1991; Fisher *et al.* 1991; Filser *et al.* 1993). These models are fundamentally similar

to models used for many years to describe multimedia environmental processes, *i.e.*, groundwater models or fate and transport models (Paterson and Mackay 1986). In simple PBPK models, the organism is conceptualized as consisting of homogeneous (well stirred) compartments between which chemicals are transported advectively (sometimes diffusively as well) by the blood. At most interfaces, equilibrium is usually assumed. In PBPK models, metabolism of chemicals is analogous to chemical reaction in multimedia models. The models are codified using equations often similar to those in multimedia compartmental models and can be solved using similar differential equation solvers. Thus, there are distinct parallels between the two model types (Paterson and Mackay 1986). PBPK models have also been directly linked to environmental fate models (Georgopoulos *et al.* 1997; Cahill *et al.* 2003).

Over the past 20 years, PBPK models have been used to evaluate a large number of individual chemicals (Leung 1991) and simple chemical mixtures (Andersen *et al.* 1987b; Filser *et al.* 1993; Krishnan *et al.* 1994). Mixture models yield different results from single chemical models when chemicals interact. The most common pharmacokinetic (PK) interaction between organic chemicals is metabolic inhibition, e.g., when chemicals are metabolized by the same enzymes, they can competitively inhibit (reduce the rate of) metabolism of the other compound. Many components of gasoline have this effect on other gasoline components (Guengerich and Shimada 1991).

A "biologically effective dose" can be thought of as the amount of chemical (parent chemical or metabolites) that reaches the target organ in the body where it exerts its principal effect. Recently, human health risk assessments have been frequently based on the biologically effective dose rather than the ambient exposure level since the former basis yields a measure of the true internal dose of the chemical (Andersen *et al.* 1987a; USEPA 1996). Computational methods for determining the internal dose are important, particularly with compounds that may interact in the body, and can be provided by PBPK modeling (Clewell and Andersen 1985). PBPK models therefore allow PK interactions to be taken into account when performing mixture risk assessments and have received increasing emphasis by the USEPA (Yang and Dennison 2003).

The simple mixture models that have been developed typically describe the PKs of mixtures of two to five components (Andersen *et al.* 1987b; Krishnan *et al.* 1994; Tardif *et al.* 1997). These mixture models are developed by conducting suites of experiments with all single chemicals and various combinations of the mixture, including binary, ternary, etc. combinations. A practical limit is soon reached for the size of the mixture that can be accommodated this way, a limit that is well below the number of components in gasoline that need to be included for a reasonable analysis. An alternative approach that incorporates chemical lumping into PBPK modeling has also been used to address this issue. In our previous work, we developed a PBPK model for two blends of whole gasoline that described the PKs of specific components of gasoline (*n*-hexane, benzene, toluene, ethylbenzene, and *o*-xylene

(BTHEX)) and a lumped component that represented the bulk of the mixture (Dennison *et al.* 2003). Interactions between all of the components, including the lumped components, were incorporated into the model.

Not only is PBPK modeling analogous to various types of environmental modeling, the present model's addition of a chemical lumping approach is analogous to the chemical lumping approach used in the chemical engineering field, and specifically in petroleum refining processes. In PBPK modeling, each chemical requires data for thermodynamic parameters (partition coefficients) and kinetic parameters (metabolism, inhibition, excretion, etc.) In particular, kinetic parameters require extensive experimentation to determine. Since it is not feasible to conduct these experiments on the numerous components in gasoline, we determined a set of parameter values for a group of chemicals that are lumped together in the PK analysis. A similar approach has been used in petroleum refining and in kinetic models of environmental processes, where blends of feedstock, refined product, or pollutants are segregated into a small number of lumps according to the thermodynamic or kinetic properties of the lump (Harley *et al.* 1993; Ancheyta-Juarez *et al.* 1999; Ancheyta-Juarez and Sotelo-Boyas 2000). Hybridized parameter values are then determined for the lump, generally in an empirical manner.

Our previous PBPK model (Dennison *et al.* 2003) adequately described the PKs of whole gasoline and several individual components. However, actual inhalation exposures to "gasoline" will frequently be weighted towards the more volatile

components, raising the question of whether this lumping approach would work for a low boiling fraction of gasoline. A variety of exposure scenarios occur where exposure is to the more volatile components: emissions from gas tank refilling, fugitive emissions from contained products, and evaporation from spills. This wide variety of scenarios suggests that a method for predicting the PKs of gasoline components that can be adapted to different subsets of gasoline components based on volatility would be useful. The present work extended the existing model to examine a series of samples of the most volatile components of gasoline that represent the fractions of gasoline components that would be inhaled after release of gasoline to the environment.

Methods

The methods for this study were similar to those previously reported (Gargas *et al.* 1986; Dennison *et al.* 2003) except as described below, and will be summarized briefly. Experimental work was based on gas uptake pharmacokinetic studies. In these studies, a rat (male Fisher 344 weighing 190-220 grams) is placed in a closed chamber experimental system. A sample of the test chemical (gasoline or fraction thereof) is injected into the system at the start of the experiment and rapidly evaporates by gentle heating to mix in the chamber atmosphere. The concentrations of chemicals in the chamber decline as the rat absorbs them from the atmosphere, so the declining concentration reflects the PKs of the chemical in the organism. When metabolism is inhibited by the presence of other chemicals, the chemical is cleared from blood more slowly, which reduces the rate of absorption of chemical from the chamber. The atmosphere in the chamber is serially sampled and analyzed by gas

chromatography. Peaks corresponding to BTHEX, confirmed by GC/MS, are identified and all remaining peaks are integrated as a whole.

Intrinsic losses of chemical in the chamber are pre-measured (including losses to hair and skin) and incorporated into the model as first order rates. Carbon dioxide (CO_2) expired by the animal is removed by a CO_2 scrubber (Baralyme) and oxygen is replaced as consumed. Both gases are monitored with direct reading probes.

A sample of gasoline was obtained from a local gasoline station (BP "Regular Unleaded"; 2/24/03) and maintained at 0°C until prepared for use. Two volatile fraction samples were prepared from the gasoline stock blend ("whole" gas) by evaporating an aliquot of whole gasoline until a pre-determined amount had evaporated. The headspace vapors were removed and collected over dry ice. One sample was prepared by allowing the first third of whole gas to evaporate ("1/3 cut"). Using a fresh subsample of whole gasoline, another sample was prepared by allowing the first two-thirds to evaporate ("2/3 cut"). These two samples and the whole gasoline were then used in PK experiments. The concentration of BTHEX in each sample differed greatly (Table 6.1) with the volatile fraction samples containing more *n*-hexane and less toluene, ethylbenzene, and *o*-xylene than the whole gas. With the 1/3 cut and 2/3 cut samples, ethylbenzene and *o*-xylene were below the limit of detection initially or within the first hour of the PK experiment, so were not included as model data.

For each fraction, three gas uptake experiments were performed at approximate starting concentrations of 500 ppm, 1000 ppm, and 1500 ppm. Due to rapid absorption by the animal, chemical concentrations decline during the six-hour experiment by roughly one to two orders of magnitude. Thus, the average exposure levels for the six-hour period are much lower than the initial concentration. Hence the advantage of the gas uptake experimental design over constant exposure designs: in addition to maintaining a mass

Table 6.1
Component Analysis of 1/3, 2/3 cut and Whole Gas
(percent by weight)

Component	1/3 cut	2/3 cut	whole
n-Hexane	4.8%	5.5%	4.0%
Benzene	0.7%	2.0%	1.5%
Toluene	0.2%	2.1%	4.7%
Ethylbenzene	0.2%	0.1%	1.1%
o-Xylene	0.2%	0.2%	1.7%
Lumped component	94%	90%	87%

balance, the gas uptake design requires fitting model behaviors over a wide range of concentrations that are differentially affected by different parameter estimates.

All PBPK modeling was performed in Berkeley Madonna, v. 8.0.2a8 (Macey and Oster 2002) using a basic four-compartment structure that has been previously used in many PBPK models (Ramsey and Andersen 1984; Dennison *et al.* 2003). The model (Figure 6.1) includes fat tissue, liver tissue, slowly perfused tissue (muscle, skin etc.)

and rapidly perfused tissue (other internal organs etc.) compartments. Metabolism is represented in the liver by a single enzyme metabolic pathway representing the predominant enzyme for initial hydrocarbon oxidation (Cytochrome P450 2E1). Michaelis Menten (saturable) metabolic rate equations were used, i.e., rate of metabolism = Vmax * [substrate]/([substrate] + Km), where Vmax is the maximum

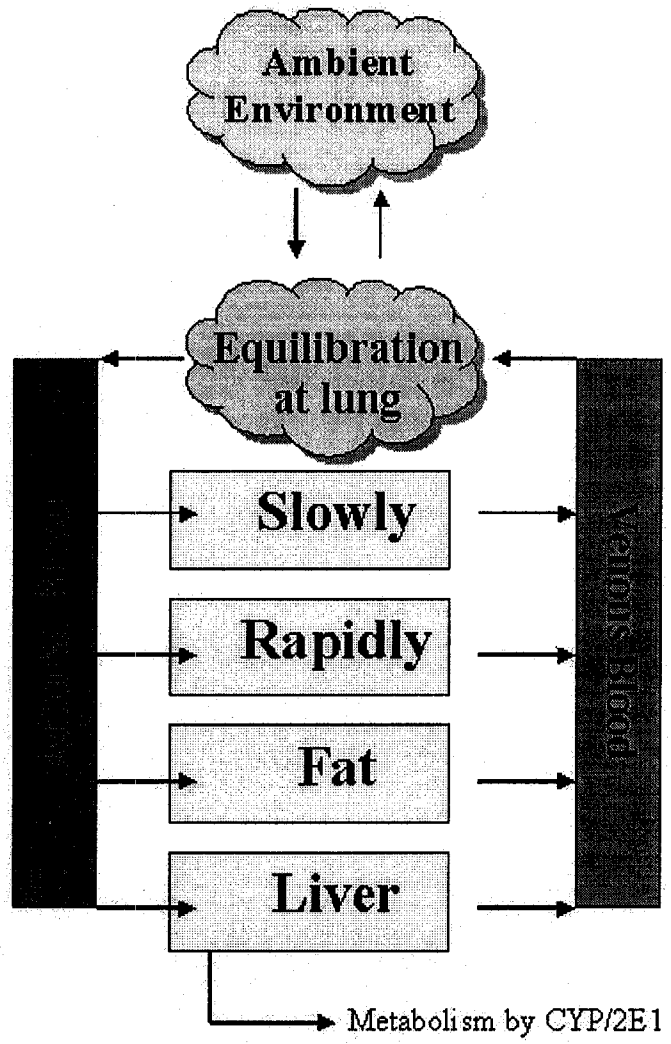

Figure 6.1. Structure of the PBPK model used in this study, showing four compartments, fat, liver, slowly perfused tissues ("slowly") and rapidly perfused tissues ("rapidly").

rate of metabolism and Km is the affinity constant. Inhibitors were assumed to act by competitive inhibition, modifying a substrate's Km: Km*(1+ [inhibitor]/Ki]) where Ki is the inhibitory constant (Segel 1993). The compartments are linked to an arterial blood compartment, which is in equilibrium with the chamber atmospheric concentrations for each chemical, representing the blood leaving the lung. The venous blood leaving each tissue compartment is represented as in equilibrium with the compartment. An external chamber compartment is also included for mass balance.

Anatomical and physiological parameter values, including tissue volumes and blood flows for each compartment, were taken from the literature ((Brown *et al.* 1997); Table 6.2). Based on observations in this and other studies (Johanson and Filser 1992), the alveolar ventilation rate was treated as an adjustable parameter, within the range previously reported.

Parameter values for BTHEX-specific parameters were maintained the same as in the previous gasoline PBPK model (Dennison *et al.* 2003). For all single chemicals, partition coefficients (Table 6.3) were taken from the literature (Gargas *et al.* 1989) and metabolic parameters (Vmax, Km, and Ki) were determined through optimization of single chemical and simple mixture PK data in the previous study (Dennison *et al.* 2003). As the gasoline blends used in this study varied, the lumped chemical parameters for each blend were determined through simulation. The alveolar

ventilation rate (QP), cardiac output, and Vmax were allometrically scaled to body weight$^{0.74}$. Model equations can be found in previous PBPK modeling papers (Andersen *et al.* 1987b; Krishnan *et al.* 1994).

Table 6.2

PBPK Model Parameters

Tissue group (%QC)	Volume (%BW)	Flow
Liver	3.7	18.3
Fat	.035*BW+.205	7.0
Richly Perfused	5.4	51.0
Slowly Perfused	91 – remaining	100 – remaining
Lung Blood	0.2	N/A
System parameters		
Cardiac output	15 L/hr/kg^0.74	
Alveolar ventilation	14.9 L/hr/kg^0.74	low exposures
	13.5 L/hr/kg^0.74	medium exposures
	12.5 L/hr/kg^0.74	high exposures

Table 6.3

Chemical Parameters

Chemical	PB	PL	PF	PS	Vmax[1]	Km[2]	Ki[2]
n-hexane	2.29	2.27	69.4	1.27	7.0	0.01	.01
benzene	17.8	0.96	22.0	0.58	5.3	0.10	.10
toluene	18.0	4.64	56.7	1.54	5.3	0.02	.02
ethylbenzene	42.7	1.96	36.4	.609	7.6	0.10	.10
o-xylene	44.3	2.44	42.4	1.16	6.5	0.20	.20
1/3 Cut	1.6	3.16	80	0.88	2.5	0.15	.06
2/3 Cut	2.3	3.16	80	0.88	3.5	0.10	.20
Whole Gas	3.0	3.16	80	0.88	2.7	0.30	.10

[1] $mg/hr/kg^{0.74}$ [2] mg/L

The optimization of parameter values was done by visual best-fit methods with Berkeley Madonna, an ordinary differential equation solver. The optimization started with data from PK experiments with the 1/3 cut sample. First, the data for the lumped chemical in three experiments was fit by adjusting the available parameters (QP, Vmax, and Km). During this optimization with the 1/3 cut sample, it was observed that the best fit was obtained by slightly decreasing QP for experiments starting at higher concentrations. This adjustment is biologically consistent with known effects of gasoline in terms of central nervous system depression and respiratory irritation, as previously noted (Johanson and Filser 1992). The best fits occurred with QP = 14.9 $L/hr/kg^{0.74}$ for the lowest concentrations, consistent with the default values used in other PBPK models (Clewell 1995; Haddad *et al.* 1999). QP was decreased to 13.1 $L/hr/kg^{0.74}$ for the medium concentration and to 12.5 $L/hr/kg^{0.74}$ for the highest

concentration, consistent with previous reports (Johanson and Filser 1992). Using these rates for QP, the parameter values for Vmax and Km for the lumped component were determined across all three data sets (Table 6.3). After setting the parameters for QP, Vmax, and Km, the inhibitory parameter Ki was adjusted until a best fit for the other components of the mixture (BTHEX) was obtained for the three experiments.

The data for the 2/3 cut and whole gasoline was simulated using a similar approach, except that QP was not re-optimized; the same values for QP at each respective concentration were used for the other blends. For optimization of the 2/3 cut and whole gasoline, the lumped chemical's Vmax and Km were first determined. Then, the lumped chemical's Ki was determined by fitting the BTHEX data.

The PBPK model was then exercised to determine the alteration in venous blood concentrations and the amount of each chemical metabolized due to inhibition under constant exposure conditions. The exposure scenario for this analysis was a six-hour exposure to constant levels of 100 – 500 ppm of the 1/3 cut, 2/3 cut and the whole gasoline.

Results and Discussion

Model simulations are plotted against the PK data for each of the three experiments with each of the blends of gasoline components (Figures 6.2-6.4). The 1/3 cut was well represented by the PBPK model (Fig. 6.2) at all exposure levels with slight deviations for *n*-hexane. A very good fit was obtained for toluene and the lumped

component at all exposure levels. A good fit was achieved for benzene at the high and medium levels, while the model overpredicted benzene chamber concentrations somewhat at the low level. *n*-Hexane fits the data well at the high level, but is overpredicted at the medium and low levels.

A good representation of the PKs of the 2/3 cut was also achieved (Fig. 6.3). As with the 1/3 cut, the toluene and the lumped component data were well described at all exposure levels. Benzene data was well described although was somewhat underpredicted at the high exposure level. *n*-Hexane was well described except at the low exposure level where the model overpredicted. As indicated in Methods, ethylbenzene and *o*-xylene were not detected in the PK experiments for the 1/3 and 2/3 cuts.

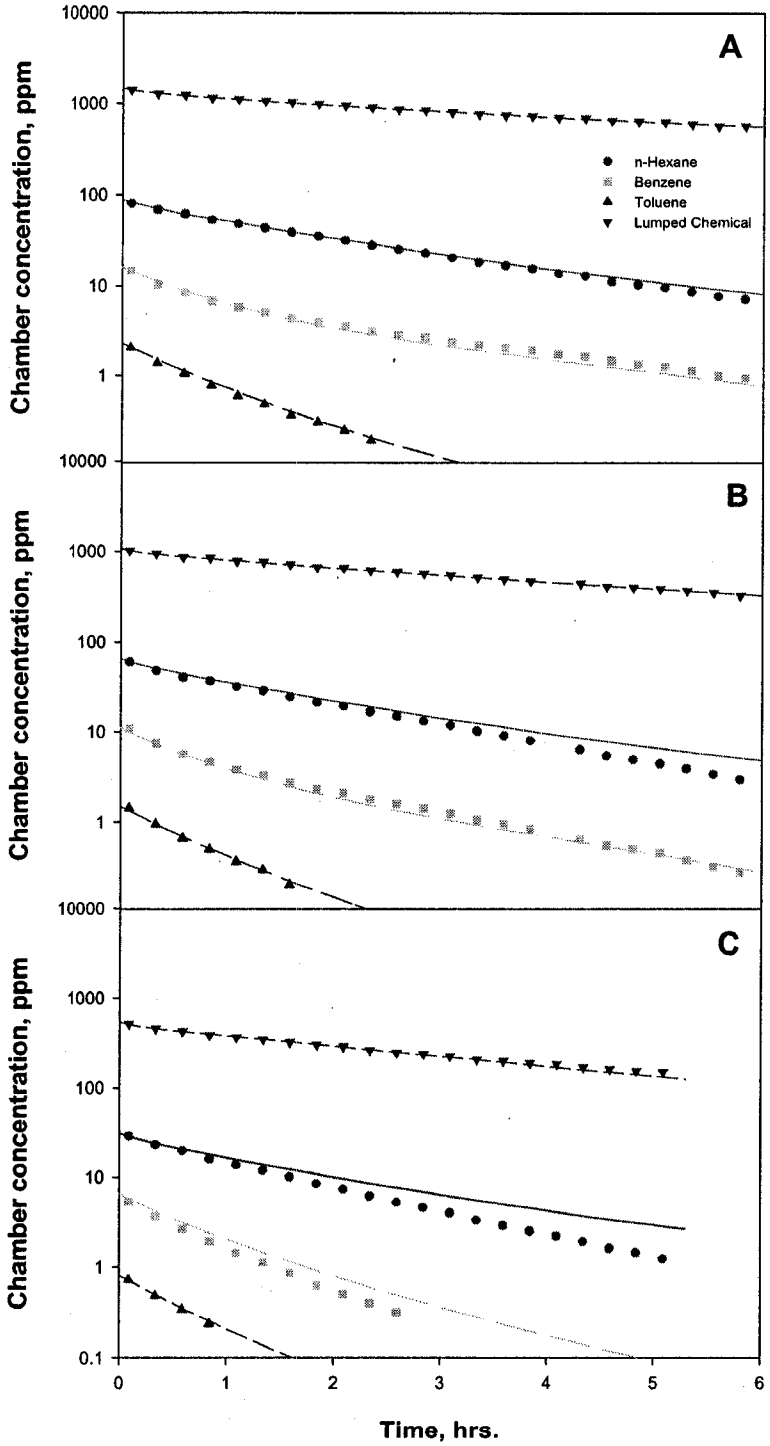

Figure 6.2. Gas uptake studies with the 1/3 Cut sample. In Figures 6.2-6.4, curves are simulations of the model described in this paper and symbols are experimental data. Panels A (high exposure), B (moderate exposure) and C (low exposure).

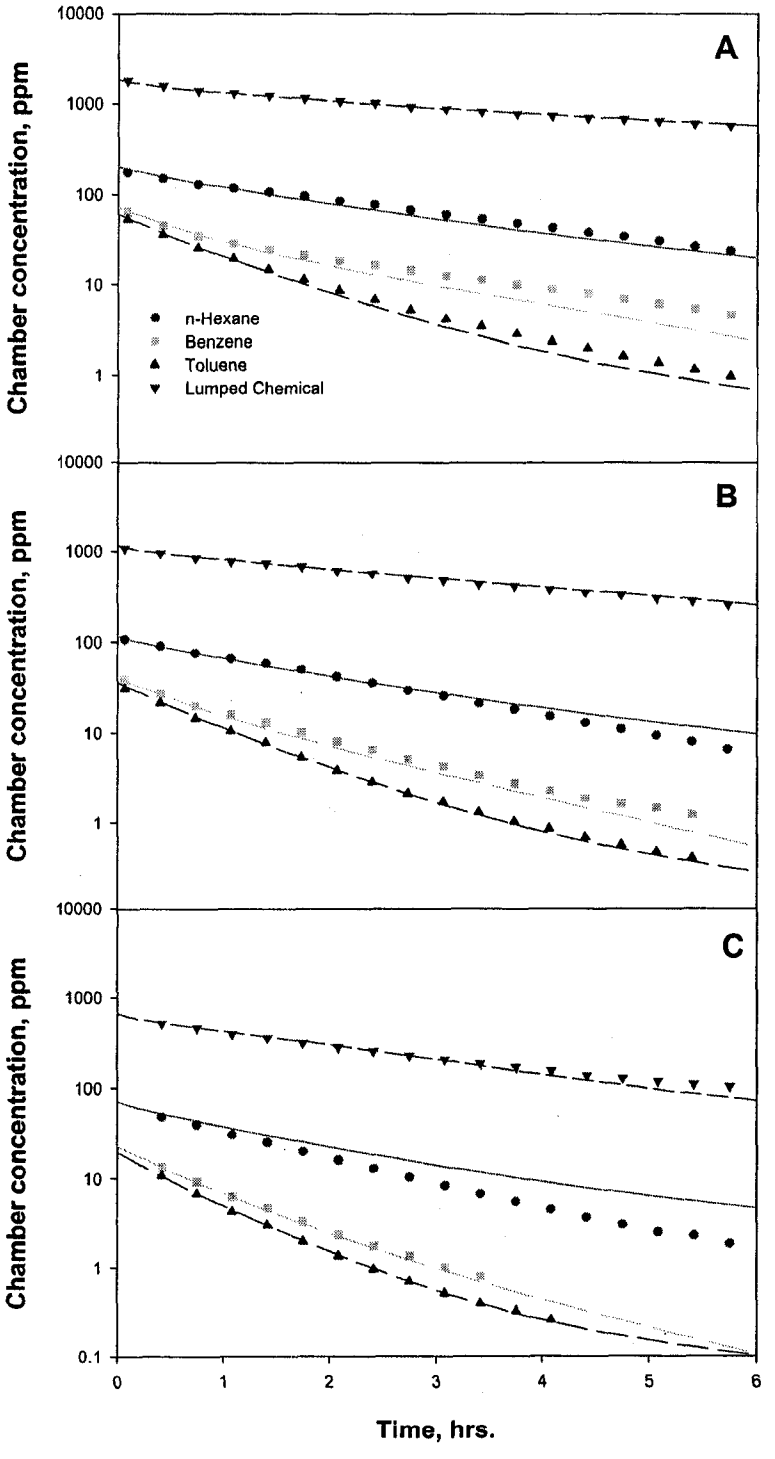

Figure 6.3. Gas uptake studies with the 2/3 Cut sample. The upper curve is the lumped component that includes all components except BTHEX.

The whole gasoline contained quantifiable amounts of each of the BTHEX components and all could be detected in all three experiments. The model provides a close fit to the data for toluene, ethyl benzene and the lumped component at all three exposure levels (Fig. 6.4). *n*-Hexane is overpredicted at the low level and small deviations are observed for benzene. Generally, the *o*-xylene data are more linear than the model on a semilog scale.

In most of the simulations, *n*-hexane was overpredicted by the model, although not always. This discrepancy could be due to imprecision in one of the *n*-hexane-specific parameter values. The parameters used for *n*-hexane were taken from the literature (partition coefficients (Gargas *et al.* 1989)) or the previous study (Dennison *et al.* 2003), where some deviations were also noted. In the previous study, a limited dataset was used for model parameterization. Additional studies are being used in our lab to improve the description of the single chemical pharmacokinetics for this chemical. Another possibility lies, in part, with the partition coefficient for *n*-hexane. The experimentally determined value (Gargas *et al.* 1989) was measured *in vitro*. However, it has been previously noted that the PB measured *in vitro* may differ from the apparent *in vivo* PB (Andersen and Clewell 1983). Furthermore, the *n*-hexane model overpredicts by a greater margin at low exposure levels, which could be attributable to the impact of saturable blood binding's effect on the measured PB, as suggested by Krishnan and co-workers (Poulin and Krishnan 1996).

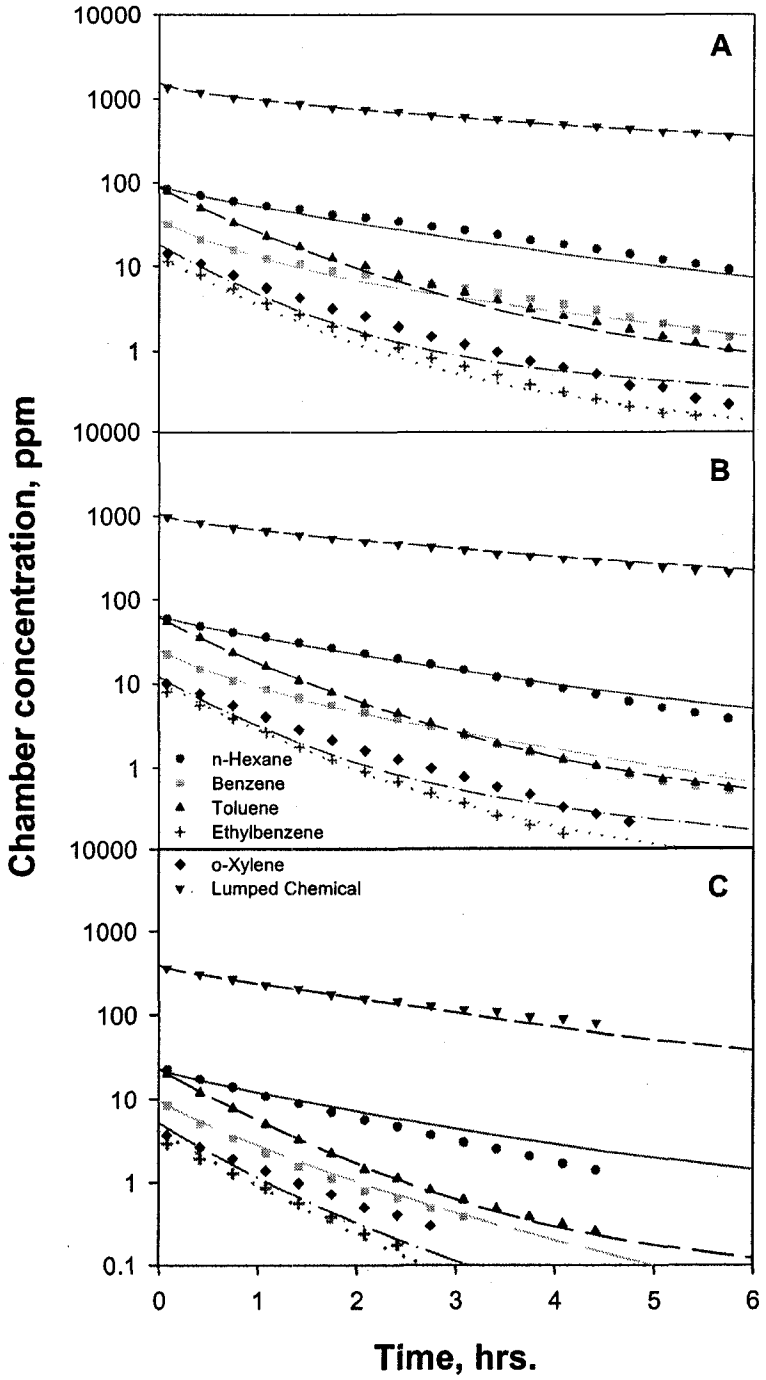

Figure 6.4. Gas uptake studies with whole gasoline.

PB is an important parameter governing the uptake of chemical from the chamber. As the PB for some hydrocarbons is related to vapor pressure (Poulin and Krishnan 1996), which in turn is related to molecular weight (Schwarzenbach *et al.* 1993), we expect that the PB for the lumped component will be near the PB for that gasoline component with the median molecular weight. Indeed, a reported molecular weight of gasoline (~95 g/mol) lies between the molecular weights of *n*-hexane and *n*-heptane (86 and 100 g/mol respectively), whose measured PBs are 2.3 and 4.75 respectively (Gargas *et al.* 1989). Thus, our values for PB (1.6 (1/3 cut), 2.3 (2/3 cut), and 3.0 (whole gas)) are in the appropriate range. More significantly, the PB increased as the volatility of the blend decreased, as expected.

Kinetic parameter estimates for the lumped component are also similar to those reported for gasoline in the previous study (Dennison *et al.* 2003). Here, Vmax was 2.5 – 3.5 L/hr/kg$^{0.74}$ as opposed to 2.0 L/hr/kg$^{0.74}$ in the previous study. Km in this study (0.1 – 0.3 mg/L) compares well to the previous value (0.1 mg/L). Thus, the lumped component is relatively well-metabolized. Ki's in this study (0.06 to 0.2 mg/L) also agreed with the previous value (0.1 mg/L). As each cut of the whole gasoline contained different proportions of components, metabolic parameters for the lumped component are expected to vary slightly.

The power and perhaps the principal purpose of PBPK models are to allow extrapolation of the model to exposure scenarios of interest. Ultimately, this gasoline

PBPK model should be extrapolated to humans, but sufficient data to support such extrapolation is not available at this time. However, the model was used to determine the degree of alteration in metabolism of BTHEX in rats. Of most interest are the change in blood concentrations and the degree of reduction of BTHEX metabolism during exposure to relevant levels of gasoline in the environment. The gasoline concentration in the gas uptake chamber at the end of the PK experiment that started at the lowest concentration was approximately 150 ppm; extrapolation of this PBPK model well below this level to environmental exposure levels would be associated with greater uncertainty. However, the exposure levels for several components allowed by the U.S. Occupational Safety and Health Administration were within the exposure levels used in these studies. Also, the Threshold Limit Value for gasoline is 300 ppm over an eight-hour workshift (ACGIH 2003). Therefore, the model can be used to determine internal measurements of dose, i.e., biologically effective doses, in this range (Figure 6.5).

Using a significance level defined as a 10% increase in a biologically effective dose (Dobrev *et al.* 2002), inhibition in gasoline was frequently found at concentrations of 200 ppm and above and occasionally at 100 ppm. The blood concentrations of ethylbenzene, *o*-xylene and benzene tended to be affected more than *n*-hexane, the lumped component and toluene. The 2/3 cut affected blood concentrations the least as the lumped component in this fraction was a weaker inhibitor than in the other fractions. For chemicals that have toxicity mediated through metabolites, the amount of chemical metabolized is a better marker of biologically effective dose than levels

of the parent compound. For the 1/3 and 2/3 cuts, the amount metabolized is decreased for most BTHEX components at 300 ppm and above. For the whole

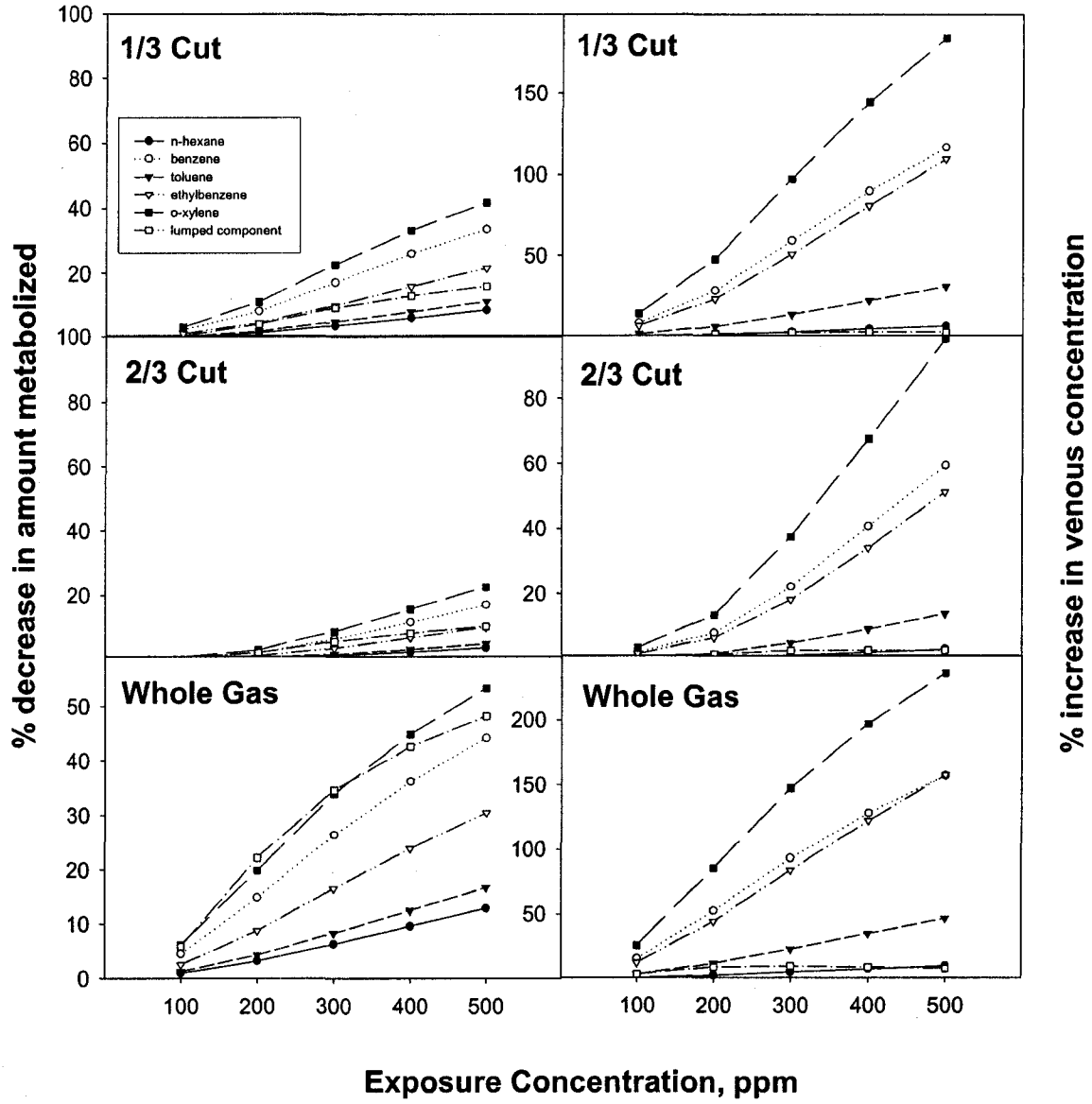

Figure 6.5. Percent decrease in amount metabolized (left panels) and percent increase in venous blood concentration (right panels) of BTHEX and the lumped component during a six hour exposure at constant level, as determined with the PBPK model.

gasoline, a decrease in the amount metabolized is seen for most components at 200 ppm and above.

The present model suggests that lumping approaches will work for whole gasoline and for various volatile fractions of gasoline, such as may be inhaled by workers exposed to gasoline vapors in various workplaces. Lumping approaches could also be useful with respect to the PKs of complex mixtures via other routes of entry into the body, e.g., dermal exposures. PBPK lumping can also be applied to classical (non-PBPK) PK studies and to other complex mixtures, e.g., diesel fuel, jet fuel, combustion products (polycyclic aromatic hydrocarbon mixtures), or asphalt fumes and may serve as the basis for cumulative risk assessments of components exerting similar kinds of toxicological action within the mixture.

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Chapter 7

A PBPK Model for n-Hexane and its Neurotoxic Metabolite 2, 5-Hexanedione with Parent Compound-Metabolite Inhibition

Abstract

N-hexane is a neurotoxicant that causes central-peripheral axonopathy due to reaction of the metabolite 2, 5-hexanedione (HD) with crosslinking proteins in nerve fibers. Previous studies have indicated that *n*-hexane may undergo complex metabolism, due to the findings that blood and urinary levels of the neurotoxic metabolite HD either decline or cease to increase at exposure levels somewhat above 1000 ppm, depending on the length of the exposure. Mechanistically, this was primarily due to competitive inhibition by *n*-hexane of metabolism of the intermediate methyl -*n*-butyl ketone (MBK) to HD. To develop a quantitative description of *n*-hexane's metabolism that includes metabolic interactions with metabolites, a PBPK model for *n*-hexane, MBK, and HD was developed for rats. Two versions of this model are described, and both can recapitulate most of the *n*-hexane, MBK, and HD data from a previous study (Baker and Rickert, 1981). However, HD data from high and low exposure levels could not be adequately simulated. Additional clearance terms and inhibitory terms were subsequently added to the model, and a reasonable representation of all metabolite timecourses was obtained. At higher exposure concentrations, MBK is pooled in the blood due to suppression (inhibition) of metabolism to HD. The

inhibition of metabolism of MBK to HD is primarily caused by high levels of *n*-hexane in the blood, but other metabolites also contribute to inhibition in this model. Once *n*-hexane exposure is ended, the competitive inhibition is released, and the rate of metabolism of MBK to HD increases dramatically. At 10,000 ppm exposure levels, the rate of metabolism increases by approximately 900% over the suppressed level. At 3000 and 10,000 ppm exposures, both the rate of metabolism of MBK to HD and the concentration of HD substantially increases after the exposure ends, even though most of the *n*-hexane is already eliminated. The 24-hour area under the curve of HD after 1000 and 3000 ppm exposures is roughly equivalent, offering an explanation for why some neurotoxic effects do not substantially increase at the higher exposure level.

Introduction

n-Hexane (HX) is a neurotoxic hydrocarbon that is widely used as an industrial intermediate and multipurpose solvent, with applications in agriculture, laboratories, industry, and construction, and is also found in numerous household products, hobby goods and gasoline (ATSDR 1999; NIH 2004). HX exposure also occurs intentionally during "solvent sniffing" (Smith and Albers 1997). Occupational exposures ranging up to 325 ppm have been reported with exposures over 100 ppm occurring in several industries (Mutti *et al.* 1984; Kawai *et al.* 1991; Cardona *et al.* 1993).

HX causes several toxic responses, but the driver for risk assessment is toxicity to the peripheral nervous system (ATSDR 1999; USEPA 2003). The histopathology includes accumulation of neurofilaments in the distal regions of sensory and motor nerve axons resulting in clinical signs of impairment such as muscle weakness. If the exposure is sufficient, damaged axons can't be repaired and may degenerate (ATSDR 1999). HX undergoes several biotransforming steps into the proximate toxic metabolite 2, 5-hexanedione (HD). HD reacts with transport protein lysine residues, undergoes additional biotransformation, and then forms protein-pyrrole adducts. The pyrrole can then crosslink additional proteins, disrupt axonal transport, and cause the accumulations of proteins in the nerve fiber (Graham *et al.* 1985).

HX is subject to complex biotransformation in the liver and other tissues (Fig. 7.1). The parent compound is oxidized by cytochrome P450s, CYP 2E1 and other CYPs, to 1 – 2- or 3-hexanol (Crosbie *et al.* 1997; Iba *et al.* 2000). The principal hexanol (2-hexanol) is oxidized to methyl-*n*-butyl ketone (MBK) which can be oxidized again, presumably by CYP2E1 and/or other CYPs, to 5-hydroxy, 2-hexanone or may be cleared through an alternate pathway to pentanoic acid. 2-hexanol can also be transformed into 5-hydroxy, 2-hexanone through an alternate intermediate. 5-hydroxy, 2-hexanone can be oxidized to HD, oxidized again to 4, 5 dihydroxy, 2-hexanone, or cleared through other pathways. Each of the alcohols may be excreted to urine, either as the alcohol or glucuronide conjugate. The metabolites also distribute in the blood and tissues. Overlapping substrate specificity of the CYPs and

Figure 7.1

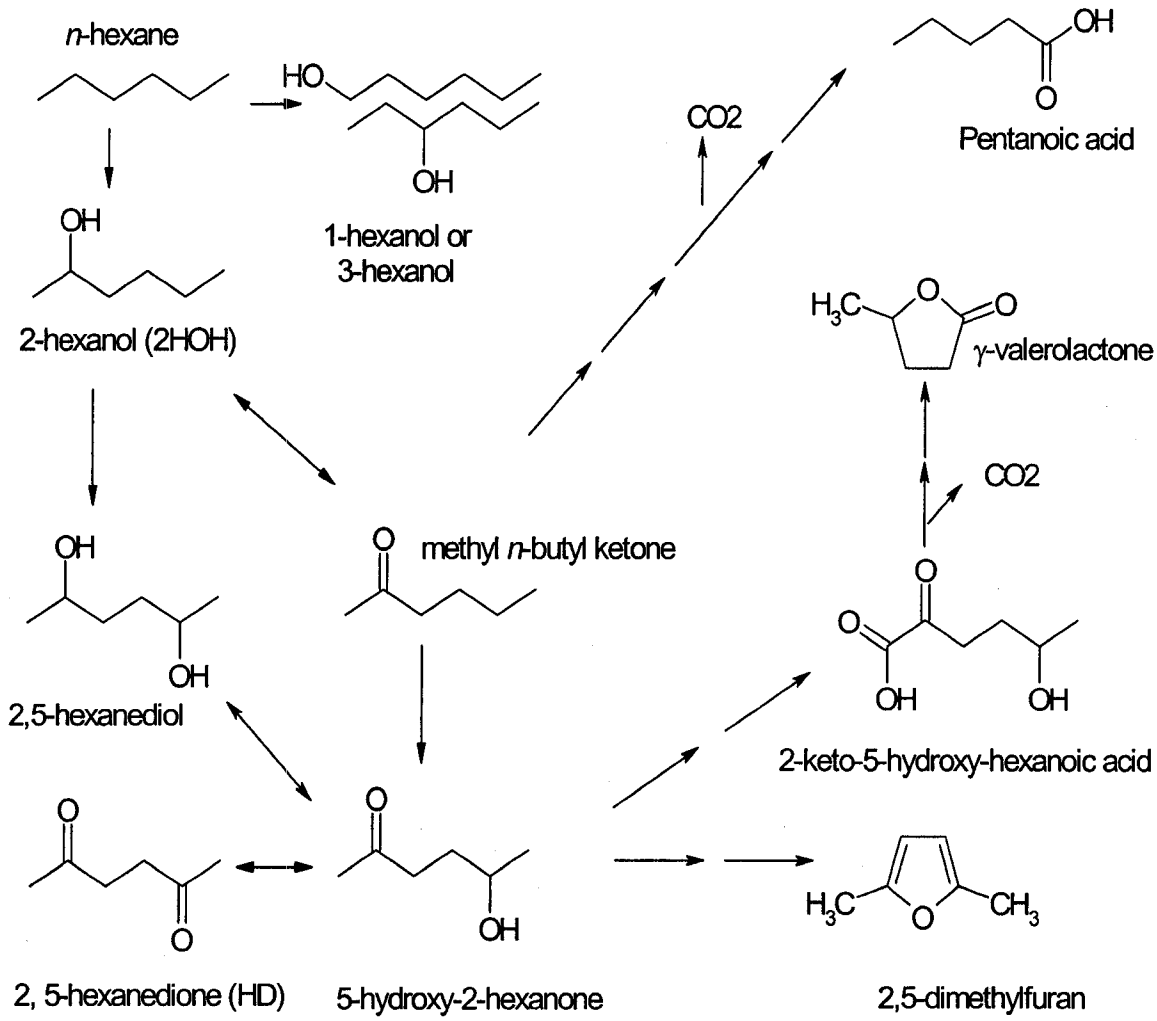

Fig. 7.1. Metabolism of n-hexane. Adapted from Couri and Milks (1982) and Fedtke and Bolt (1987).

possibly other enzymes involved in metabolism of HX and its metabolites suggests the possibility that HX and its metabolites could serve as inhibitors of each other's metabolism.

Numerous pharmacokinetic studies have previously been performed with HX, HX metabolites, and HX with other interacting chemicals (including DiVincenzo *et al.* 1977; Baker and Rickert 1981; Bus *et al.* 1981; Fedtke and Bolt 1987; Shibata *et al.* 1990; Granvil *et al.* 1994). One study found that the total amount of the urinary metabolites 2,5-dimethylfuran, MBK, 2-hexanol, HD, and γ-valerolactone only increased by 9% between exposures at 1000 and 3000 ppm (Iwata *et al.* 1983). In another study, the blood levels of HD at the conclusion of a 3000 ppm exposure were actually less than after exposure at 1000 ppm (Baker and Rickert 1981). In addition, both of these studies suggested that after 3000 ppm exposures, HD concentrations in blood and urine actually increased after exposure ceased, during a period when HX blood levels were declining rapidly and it was effectively not producing more metabolite. In sum, the studies suggest that HX undergoes complex pharmacokinetics possibly with some unusual features.

Several toxicological studies have also been conducted with HX or its metabolites to examine dose response for a number of endpoints (Takeuchi *et al.* 1981; Nachtman and Couri 1984; Misumi and Nagano 1985; Planas and Cunningham 1987; Pryor and Rebert 1992; Madeja *et al.* 1997). At times, the results of these studies have been difficult to interpret, partly due to the lack of quantitative information on the tissue dose of HD. In one study, neurotoxicity at 3000 ppm was not much greater than at 1000 ppm, i.e., the dose response curve flattens out, but at levels without significant mortality (Takeuchi *et al.* 1981). It has been suggested that metabolic saturation could be responsible for this flat dose response curve (Takeuchi *et al.* 1981).

The interest in understanding the pathophysiology of n-hexane stimulates a need to be able to determine tissue doses of target metabolites under varying exposure scenarios. Several existing physiologically-based pharmacokinetic (PBPK) models exist for *n*-hexane, but to date, metabolism has been simplified in the models to one or two metabolic steps (Perbellini *et al.* 1986; Perbellini *et al.* 1990; Yu *et al.* 1998; Ali and Tardif 1999). An early PBPK model was also developed for HD itself (Angelo and Bischoff 1983), but no models are available that describe the complex metabolic pathways seen in Figure 7.1. The present model is an extension of an earlier version (Andersen and Clewell 1983) that included several interactions between *n*-hexane and metabolites. The objective of this study was two-fold: to develop a PBPK model for HX and HD that incorporate more metabolic detail and to use this model to evaluate neurotoxicity data in relation to HD concentrations. As data are not presently available for the complete suite of metabolites suitable for PBPK modeling, the present model focuses on two principal metabolites, HD and MBK.

Methods

Blood data

Blood data were taken from an existing study (Baker and Rickert 1981; Baker 1983). In this study, male Fisher 344 rats (150-200 g) were exposed to 500, 1000, 3000, or 10,000 ppm HX for six hours via inhalation. The animals were sacrificed via decapitation and blood was collected in heparinized tubes. HX and MBK were

analyzed in blood samples collected at 0.5, 1, 2, 3, 4, and 6 hours during exposure and at 0.5, 1, 2, 4, 6 and 8 hours post-exposure. HD was analyzed in blood samples collected 0.5, 1, 2, 4, 6 and 8 hours post-exposure. Data were taken from a previous publication (Baker and Rickert 1981) or kindly provided from the authors (Rickert 1983).

PBPK model for HX, MBK, and HD

A PBPK model was constructed that is conceptually similar to previous models for volatile organic solvents (Ramsey and Andersen 1984; Clewell *et al.* 2001). The model used a four compartment structure (Fig. 7.2), consisting of the liver, fat tissues, richly perfused tissues, and slowly perfused tissues. These tissue groups add up to 91% of total body weight and the balance of the body was considered non-perfused. During exposure periods, HX is assumed to be in equilibrium with the blood leaving the lung, which then perfuses the four compartments. Perfusion rates, ventilation rates, and compartment volumes were taken from a standard PBPK modeling reference (Brown *et al.* 1997) and are shown in Table 7.1. Two different versions of the PBPK model are presented in this paper, a preliminary model that includes several interactions and a final model that included additional interaction features as described below.

Figure 7.2

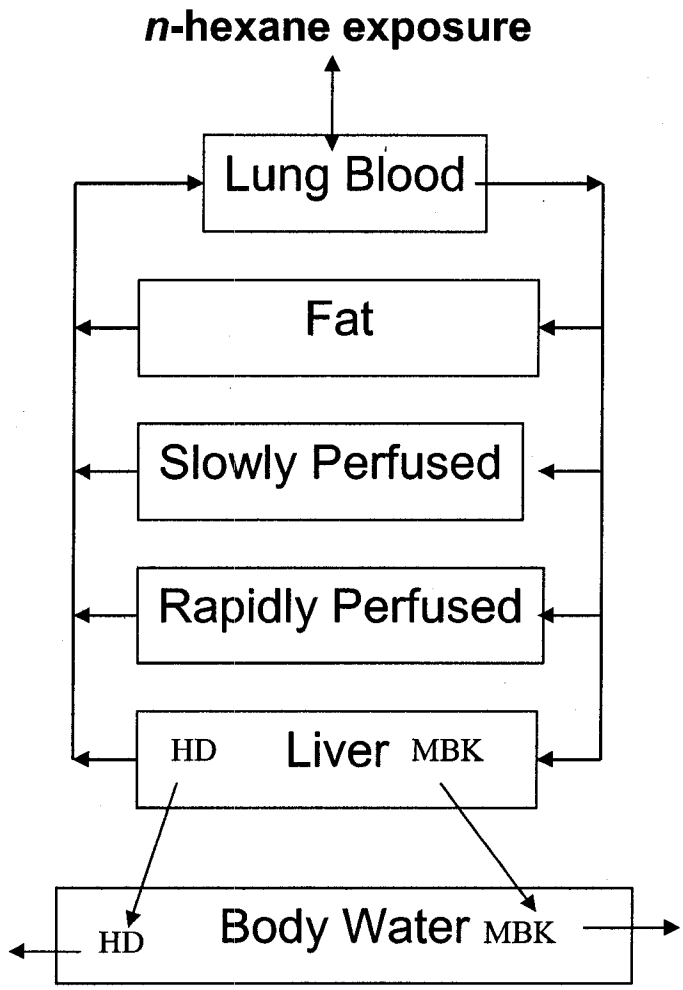

Fig. 7.2. Structure of the PBPK model used in this study for describing the pharmacokinetics of HX, MBK, and HD. The same model structure is used for both preliminary and final models. Body water is represented as a volume of distribution.

Table 7.1.

Parameter	Units	Scaling	Value
Body weight	Kg	--	0.175
Alveolar ventilation, scaled	L/hr/kg	BW^ 0.74	14
Cardiac output, scaled	L/hr/kg	BW^ 0.7	15
Liver volume, scaled	Kg	BW	0.04
Fat volume, scaled	Kg	BW	0.05
Richly perfused tissue volume, scaled	Kg	BW	0.05
Slowly perfused tissue volume, scaled	Kg	BW	0.77
Liver blood flow, scaled	L/hr	QC	0.3
Fat blood flow, scaled	L/hr	QC	0.05
Richly perfused blood flow, scaled	L/hr	QC	0.73
Slowly perfused blood flow, scaled	L/hr	QC	0.19
Blood:air PC, n-hexane	--	--	0.8
Blood:air PC, MBK	--	--	125
Liver:blood PC, n-hexane	--	--	2.3
Fat:blood PC, n-hexane	--	--	67
Richly perfused:blood PC, n-hexane	--	--	2.3
Slowly perfused:blood PC, n-hexane	--	--	1.3
Liver:blood PC, MBK	--	--	1.0
Liver:blood PC, HD	--	--	1.0
Volume body water	L	BW	1.25

Table 7.1. Values of anatomical, physiological, and chemical parameters for Models 1-3 in this paper. Anatomical and physiological parameters were obtained from Brown *et al.* (1997). The blood:air partition coefficient and liver:blood PCs for MBK and HD was determined in this study. Other partition coefficients were obtained from Gargas *et al.* (1987).

Basic PBPK model

Initial and further metabolism of HX was assumed to occur in the liver alone. The metabolic scheme used in this model is shown in Figure 7.3. HX is assumed to be

Figure 7.3

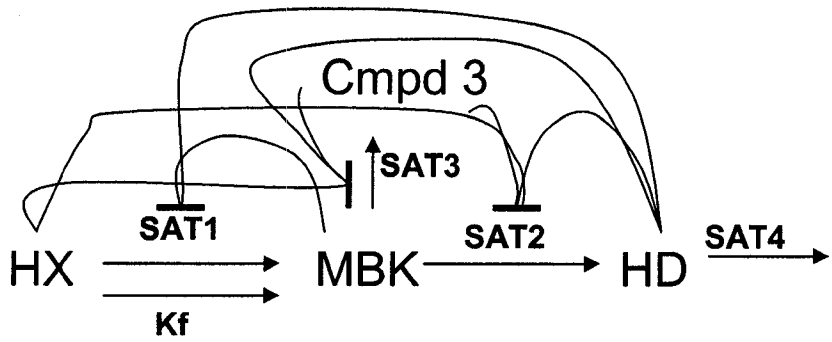

Fig. 7.3. Description of HX metabolism used in the PBPK models in this study.

metabolized to MBK in a single step. In the Baker and Rickert study, the intermediate 2-HOH was not detected in blood at any exposure concentration (Baker and Rickert 1981), suggesting that the oxidation of 2-HOH to MBK is sufficiently fast to ignore. Metabolism of HX to MBK is assumed to be a two-pathway process, mediated by a high-affinity, low capacity enzyme (*i.e.*, CYP2E1) and a low-affinity, high capacity enzyme, producing the same product. For high exposure concentrations, a two-enzyme system for *n*-hexane metabolism has been suggested based on experimental data (Filser *et al.* 1996) and the assumption has been previously used in PBPK models (Clewell *et al.* 2001). The high affinity process is considered to be saturable, and is referred to in the model as "SAT1." For the low affinity pathway, substrate concentrations are usually lower than the affinity constant, so this pathway is modeled as a linear (non-saturable) process (Clewell *et al.* 2001).

According to the data from Baker and Rickert, a significant fraction of MBK is cleared via a process that does not produce HD (Baker and Rickert 1981). This is likely to be the metabolic pathway to pentanoic acid, as shown in Figure 7.1. This biotransformation was included in the model using saturable metabolism (SAT3). Metabolism of MBK to HD was also assumed to be saturable (SAT2). Like the transformation of HX to MBK, this was modeled as a single step, assuming that the second step is fast. Similarly, data were not available to incorporate the pathway involving 2, 5-hexanediol, which, from inspection of the Baker and Rickert data, appears to be of lesser importance. MBK can also be cleared via exhalation or excretion which was included in the PBPK model as a linear rate equation. SAT4 is an additional metabolic clearance process that is included in the final model.

HD can be cleared from the body via excretion (especially after conjugation) and several further metabolic steps. As data are not available to parameterize each individual step, these clearance processes were lumped into a single linear process (KE3). After being produced in the liver, MBK and HD were considered to partition to the body water (a volume of distribution) as blood:tissue partition coefficients for the metabolites were not available.

Metabolism of HX to MBK and MBK to both pentanoic acid and HD were included as saturable metabolic processes described by Vmax (maximum rate of metabolism) and Km (affinity constant). In addition, the saturable metabolic steps were assumed to be subject to competitive inhibition according to the equation: Rate of metabolism

= Vmax* [CVL]/([CVL] + Km*(1 + [CVL*i*]/Ki)) where the [CVL] terms are the concentrations of the chemicals in the venous blood (HX) or body water (MBK and HD) as they leave the liver, the subscript *i* refers to the inhibitor, and Ki is the inhibition constant. HX was assumed to inhibit the MBK → HD reaction, MBK and HD inhibited the HX → MBK reaction, and HD also inhibited the MBK → HD and MBK → pentanoic acid reactions. Inhibitory constants were assumed at first to equal the affinity constant but were then optimized for the model.

Chemical parameters are listed in Table 7.2. Model 1 was the basic model with parameter estimates from visual optimization. As discussed below, an alternate parameter set estimate was obtained by weighting the first post-exposure data point and using numerical optimization. This (Model 2) used the same model equations as Model 1, but resulted in improved fits to the data based on revised parameter estimates.

Revised Model

Model 3 was produced by making revisions to the basic model. In the basic model, metabolism of MBK to pentanoic acid results in clearance of MBK, but the concentration of pentanoic acid is not needed and therefore is not calculated. In order to allow inclusion of additional inhibition of MBK metabolism by pentanoic acid, the concentration of pentanoic acid was explicitly modeled, although experimental data were not available for calibration. Note that the term "pentanoic acid" means

pentanoic acid and intermediates. The rate of accumulation of pentanoic acid (referred to as Compound 3) is the net of formation from MBK and linear clearance. Compound 3 was then assumed to competitively inhibit SAT2 and SAT3. The revised model explored whether allowing the Ki for Compound 3 to vary for SAT2 and SAT3 would significantly improve the model.

Table 7.2

Variable	Definition	Units	Model 1	Model 2	Model 3
Vmax1	Max. velocity SAT1	mg/hr/kg	6	6	6
Vmax2	Max. velocity SAT2	mg/hr/kg	7	3.63	2
Vmax3	Max. velocity SAT3	mg/hr/kg	10	13.8	10
Vmax4	Max. velocity SAT4	mg/hr/kg	--	0.71	1.5
KM1	Affinity constant SAT1	mg/L	0.3	0.3	0.3
KM2	Affinity constant SAT2	mg/L	0.6	0.6	0.05
KM3	Affinity constant SAT3	mg/L	1	1.1	1
KM4	Affinity constant SAT4	mg/L	--	1.38	10
KM1-2	Inhibition constant for H on SAT2	mg/L	0.3	0.42	0.1
KM1-3	Inhibition constant for H on SAT3	mg/L	1000	1000	10
KM2-1	Inhibition constant for MBK on SAT1	mg/L	0.6	0.62	0.6
KM3-1	Inhibition constant for HD on SAT1	mg/L	1	0.57	0.6
KM3-2	Inhibition constant for HD on SAT2	mg/L	1	1.1	1
KM3-3	Inhibition constant for HD on SAT3	mg/L	0.6	0.43	0.43
KF	Rate constant for H metabolism	/hr.	2	3.22	2
KE2	Rate constant for MBK elimination	/hr.	0.084	0.084	0.084
KE3	Rate constant for HD elimination	/hr.	0.12	0.072	0.08
KMCPD3	Inhibition const. for Cmpd 3 on SAT2, SAT 3	mg/L	N/A	N/A	10000
KECpd3	Rate constant for Cmpd 3 elimination	/hr.	N/A	N/A	1
n	Exponent on [HX] as inhibitor	--	N/A	N/A	1.5
n1	Exponent on [CPD3] as inhibitor	--	N/A	N/A	2

Table 7.2. Metabolic and clearance rate and constants determined in this study. Vmax values are scaled to BW^0.7. Linear rate constants are scaled to BW^-0.3.

The other modification made in the revised model was to use an exponential on the concentration of the inhibitor in the rate equation for metabolism of MBK → HD.

This modification was made to allow an increase in the suppression of this metabolic step, based on the observation of the Baker and Rickert data. In other words, the rate of metabolism (R) of MBK to HD in the revised Model 3 was

$$R = \frac{[MBK] * V\max(SAT2)}{[MBK] + Km * (1 + \frac{[CVL]^n}{Ki1-2} + \frac{[Cmpd3]^{n1}}{KiCMP3} + \frac{[HD]}{Ki3-2}}, \quad \text{where}$$

[MBK] is the concentration of MBK in the body water

Vmax(SAT2) is the maximum rate of metabolism of MBK in the SAT2 pathway

Km is the affinity constant for MBK in the SAT2 pathway

[CVL] is the concentration of HX in the venous blood leaving the liver

n is the exponent of [CVL] representing an increase in inhibition with concentration

Ki1-2 is the inhibitory constant for HX (Chemical 1) on SAT2

[Compound 3] is the concentration of Compound 3 in the body water

n1 is the exponent of [Compound 3] representing an increase in inhibition with concentration

KiCMP3 is the inhibitory constant for Compound 3 on SAT2

[HD] is the concentration of HD in body water

Ki3-2 is the inhibitory constant for HD on SAT2

In the basic model, one HD clearance pathway was included in the model representing the sum of metabolic and excretory processes to which HD is subjected. 4,5-dihydroxy 2-hexanone has been shown to be an important HD metabolite (Fedtke and Bolt 1987). The revised model included two clearance mechanisms, metabolism

to 4,5-dihydroxy 2-hexanone, and other clearance, which includes excretion (all forms of HD) and other metabolic clearance processes. Metabolism of HD→ 4,5-dihydroxy 2-hexanone was incorporated as saturable metabolism not subject to inhibition (Vmax4, Km4).

The model was coded and run in Advanced Continuous Simulation Language (ASCL) version 11.8.4 (Aegis Simulation, Inc., Huntsville, AL) an ordinary differential equation solver. Parameter estimation was performed by visual optimization except where noted. Anatomical and physiological parameters and tissue:blood partition coefficients were not adjusted from the starting values reported in Table 7.1. The parameters that were subjected to optimization include the metabolic, inhibitory, and clearance parameters listed in Table 7.2 and the blood:air partition coefficient for HX.

Partition coefficients of HX were taken from the literature (Gargas *et al.* 1989). The initial value of the blood:air partition coefficient (PB) for HX (2.29) was too high and produced blood concentrations that were well above those in the data from Baker and Rickert. Increasing metabolism to the maximum possible extent (*i.e.*, all chemical reaching the liver was eliminated) still resulted in blood levels that were much too high. Therefore, the PB was reduced until good agreement was found between the exposure level and blood levels. The value of PB used was similar to that used in previous HX PBPK models (Perbellini *et al.* 1986; Yu *et al.* 1998; Ali and Tardif 1999).

Dose response data

Dose response data were taken from a previous report (Takeuchi *et al.* 1981). Takeuchi *et al.* exposed Wistar rats (310 g.) to 0, 1000, or 3000 ppm for 12 hours per day for 16 weeks. The nerve conduction velocity was measured in the exposed rats' tail nerves every four weeks.

Results

Simple inspection of the data from Baker and Rickert (Baker and Rickert 1981) reveals some interesting features (Fig. 7.4). HX concentrations in blood increase very quickly after the onset of exposure and rapidly reach a steady state level. These levels also decline very quickly after exposure is ended, in large part because the chemical is cleared by both rapid metabolism and exhalation. MBK levels increase more slowly, generally not reaching steady state, but also decline rapidly in the post-exposure period. HD also increases more slowly and does not reach steady state, but also declines more slowly than either HX or MBK. The post-exposure blood levels of HD are higher after 1000 ppm exposure than after 500 ppm exposure. However, the immediate post-exposure blood levels of HD are lower after 3000 ppm exposure than after 1000 ppm and then actually increase for about three hours into the post-exposure period, rising to a level that almost as high as the peak post-exposure blood concentration after 1000 ppm. This effect is even more pronounced after 10,000 ppm

exposure, where the initial blood concentration after exposure ends is lower than for any of the other dose groups, but the concentration then rises by over 200% over the next six hours. Thus, blood concentrations of HD after 3000 and 10,000 ppm exposure appear to be depressed as compared to exposures at 500 and 1000 ppm, and there appears to be a substantial delay before HD is being produced at the maximum rate in the post-exposure period. The HD concentration at the end of exposure for both the 500 and 10,000 ppm exposure groups is about 4 mg/L even while the blood concentration of HX increases from about 1 to 26 mg/L for the 500 and 10,000 ppm groups. Clearly, simple metabolism does not provide a rationale explanation for these findings.

A simple PBPK model was developed to examine whether inhibition of metabolic processes by HX and some of its metabolites can provide a reasonable description of the pharmacokinetics of HX and two principal metabolites. Model 1 (Fig. 7.4) included a basic metabolic description and inhibition of several steps. Despite some scatter in the data, Model 1 is able to describe much of the HX pharmacokinetics. In this and other versions of the model, the model generally predicts faster clearance of HX from blood in the post exposure period than shown by the data. Model 1 does a reasonable job of simulating the MBK concentrations during and after exposure. A good correspondence between the model and the HD data is obtained for 1000 and 3000 ppm exposures, but the HD timecourse for 500 ppm is overpredicted and for 10,000 is overpredicted by yet more. No set of parameters could be found to resolve

this problem in the context of the basic model; indeed, the figures presented show the best overall fit that could be obtained.

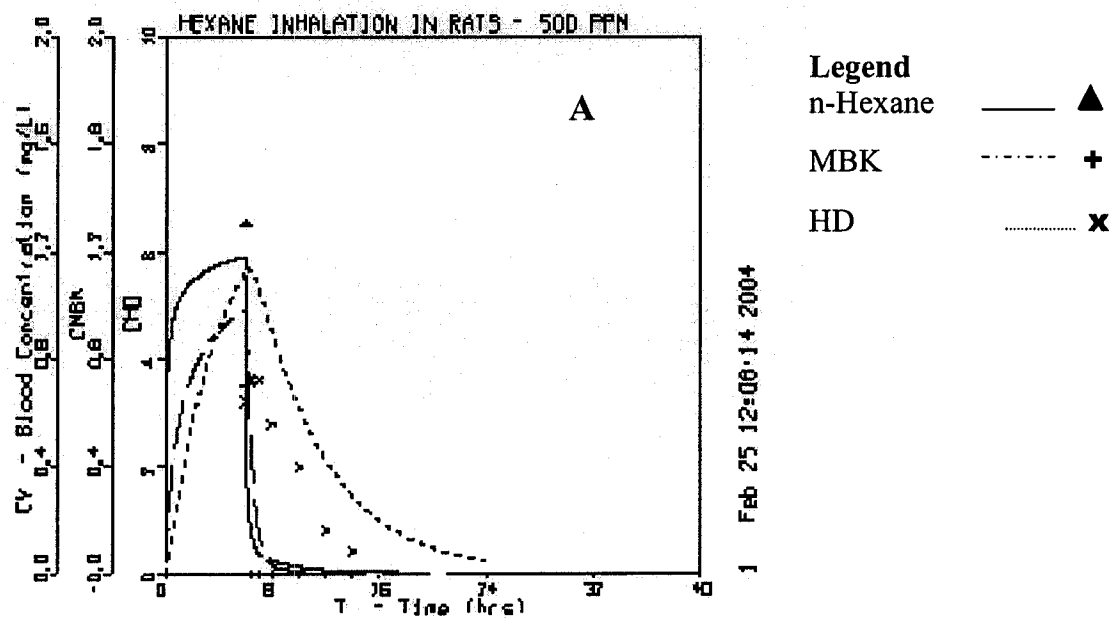

Fig. 7.4a. PBPK model results for the Model 1. In Panels A-D, data shown in symbols are from Baker and Rickert (1981); Rickert (1983) for exposures of rats to 500, 1000, 3000, or 10,000 ppm *n*-hexane for 6 hours. Concentrations of *n*-hexane (CV), MBK (CMBK), and HD (CHD) are shown along with corresponding simulations (curves). A reasonable correspondence between data and simulations was achieved at all but the 10,000 group, where the concentration of HD was much too high. Small inflections can be observed in the HD simulation at 3000 and 10,000 ppm at the point when HX concentrations are rapidly declining. These represent release of inhibition by HX.

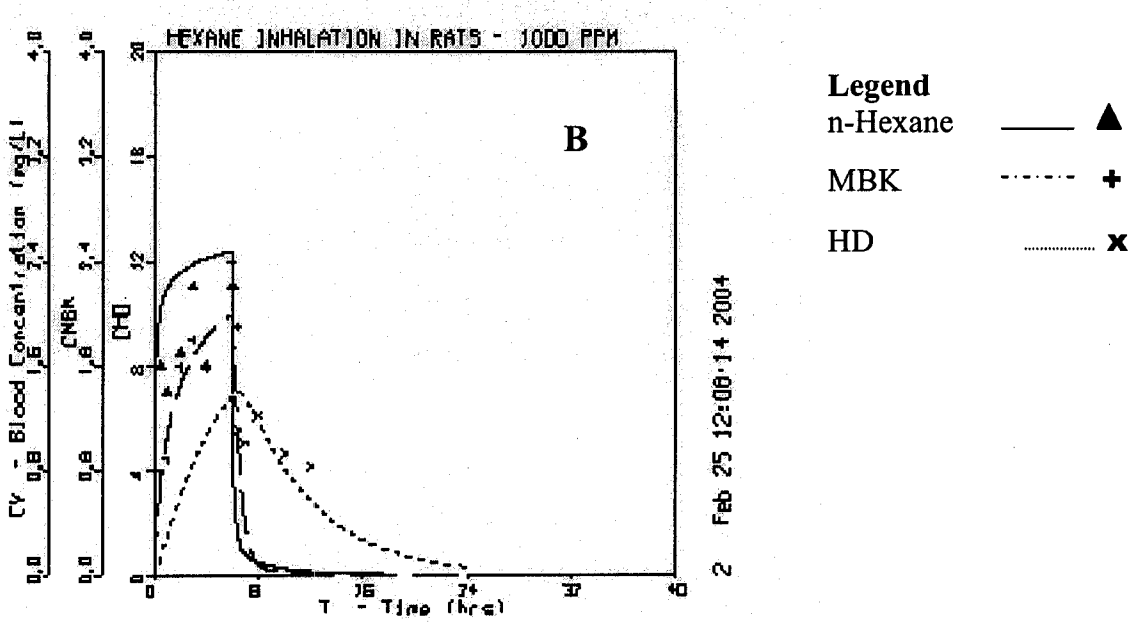

Fig. 7.4b. Model 1 simulation for 1000 ppm exposure. See explanation in legend for Fig. 7.4a.

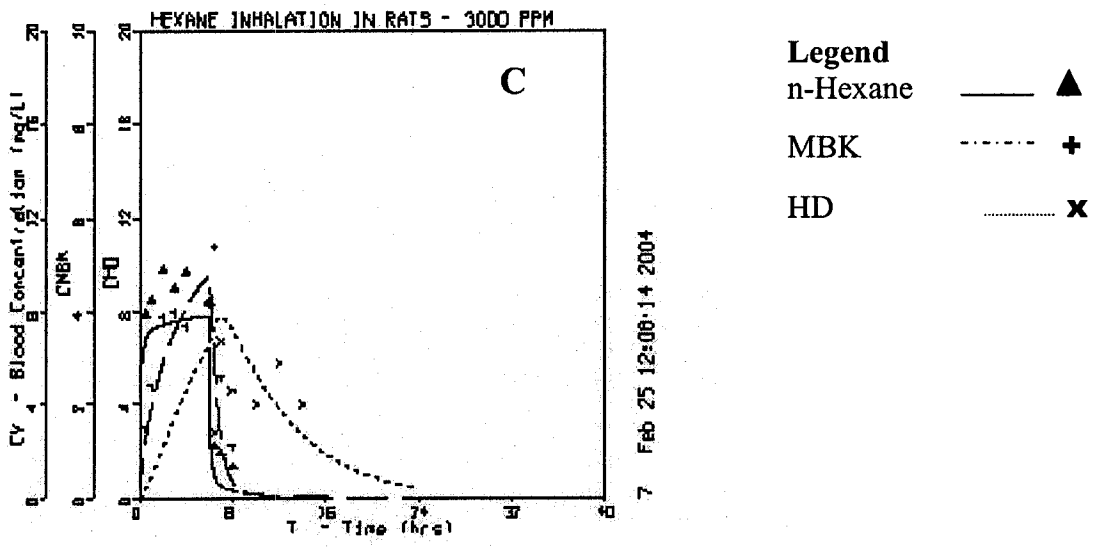

Fig. 7.4c. Model 1 simulation for 3000 ppm exposure. See explanation in legend for Fig. 7.4a.

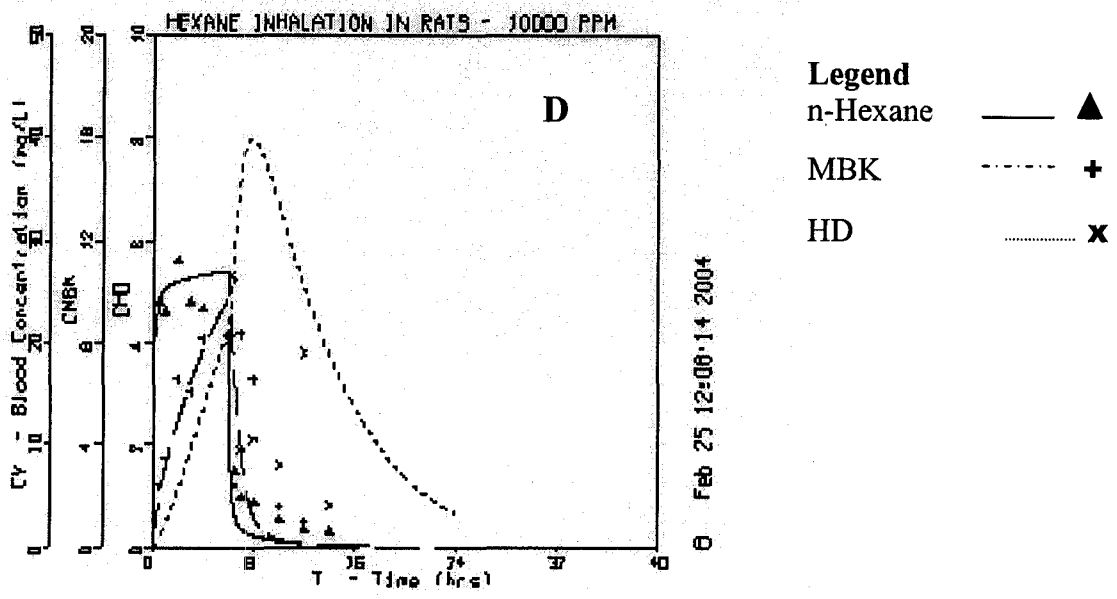

Fig. 7.4d. Model 1 simulation for 10,000 ppm exposure. See explanation in legend for Fig. 7.4a.

A second strategy was employed for fitting the parameters to the Baker and Rickert data sets. Generally, Model 1 (Fig. 7.4) tended to overshoot the first post-exposure HD timepoint. In order to better recapitulate the behavior of HD during the post-exposure period, numerical optimization emphasizing this timepoint was conducted by weighting the first post-exposure time point by 10 (Fig. 7.5). When this was done, the HD data for 10,000 ppm could be represented fairly well without disturbing the adequacy of the HX and MBK simulations by too much, but HD was then underpredicted at all other exposure levels.

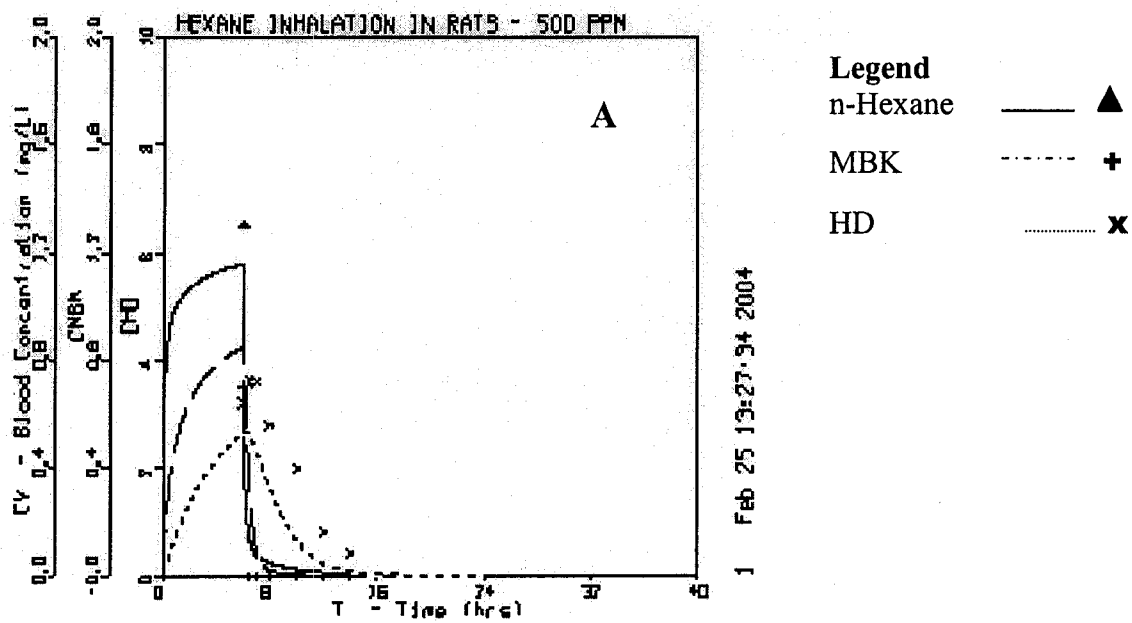

Fig. 7.5a. Model 2. PBPK model for HX, MBK, and HD using a numerically-optimized weighted approach to parameter estimation. In Panels A-D, data shown in symbols are from Baker and Rickert (1981); Rickert (1983) for exposures of rats to 500, 1000, 3000, or 10,000 ppm *n*-hexane for 6 hours. Concentrations of *n*-hexane (CV), MBK (CMBK), and HD (CHD) are shown along with corresponding simulations (curves). Panel A: Exposure at 500 ppm. A reasonable fit of the simulation was achieved at 10,000 ppm, but HD was too low at lower exposure levels. Inflections at the end of exposure at 3000 and 10,000 ppm groups reflect the release of inhibition as in Figure 7.4.

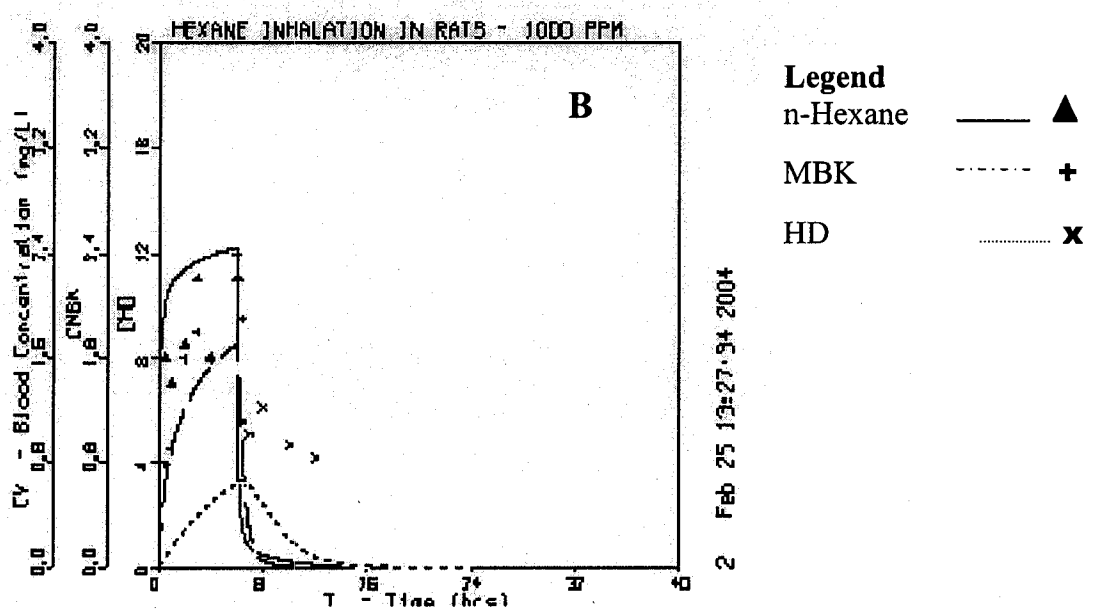

Fig. 7.5b. Model 2 simulation for 1000 ppm exposure. See explanation in legend for Fig. 7.5a.

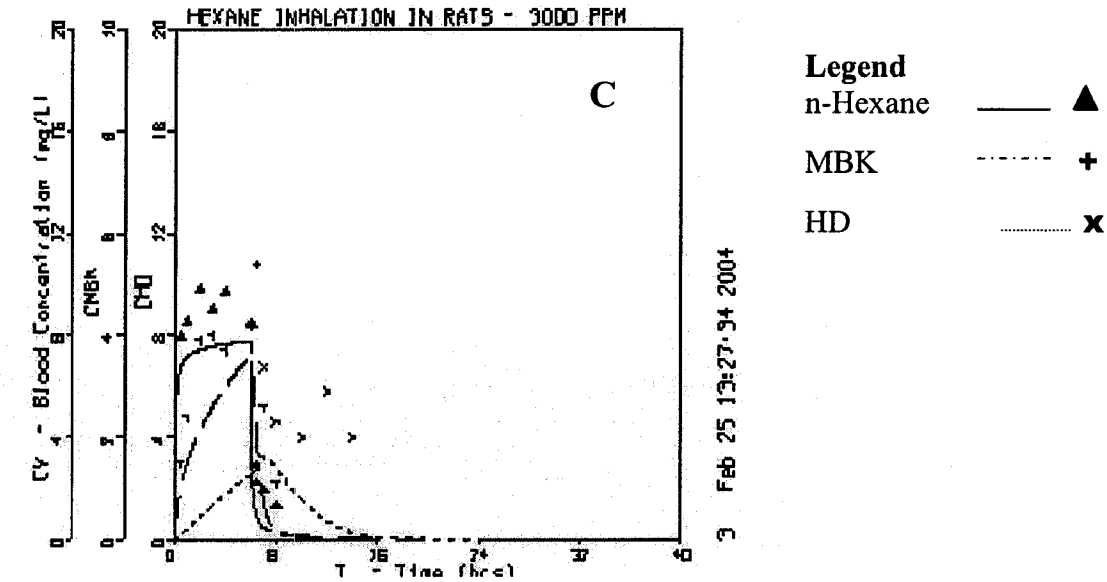

Fig. 7.5c. Model 2 simulation for 3000 ppm exposure. See explanation in legend for Fig. 7.5a.

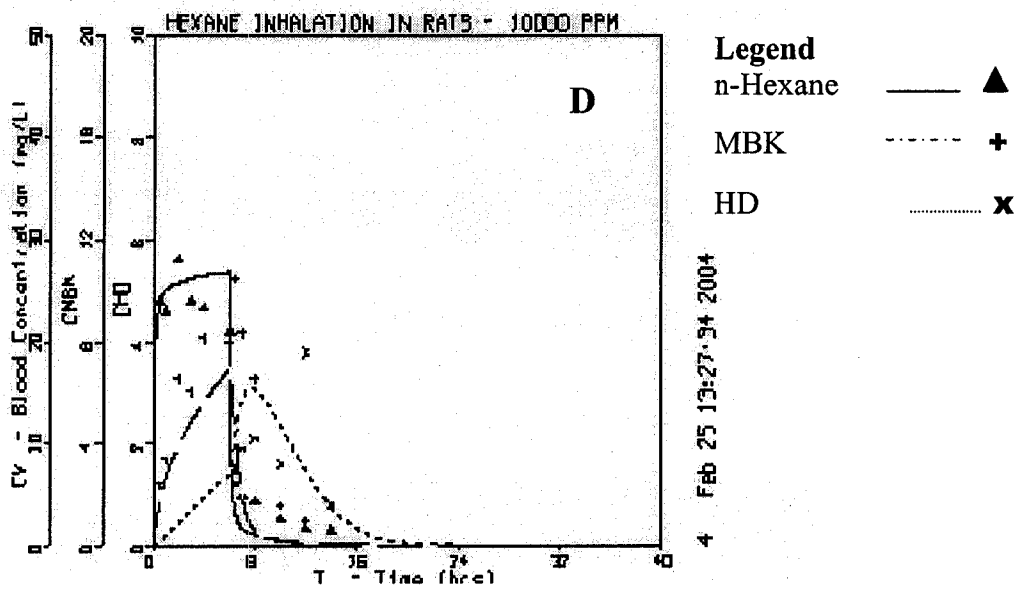

Fig. 7.5d. Model 2 simulation for 10,000 ppm exposure. See explanation in legend for Fig. 7.5a.

Models 1 and 2 are mathematically identical except for alteration of certain parameters values denoted in Table 7.2. In both versions of the model, a post-exposure increase in the HD blood concentration is observed at 3000 and 10,000 ppm, as suggested in the Baker data. Thus, the model is able to capture this important and unusual feature of the pharmacokinetics of HX, MBK, and HD. Model 2 shows this as slightly more pronounced than Model 1. Particularly after 10,000 ppm exposure, the blood concentration of HD roughly doubles over a few minutes as HX (and MBK) are cleared from the animal.

Model 3 incorporated additional inhibition and clearance parameters, as described in the Methods. A substantially better description of the Baker and Rickert data was obtained with Model 3 than with either Model 1 or 2 (Fig. 7.6). HD was better predicted across all exposure groups with HX and MBK are still reasonably represented. The HD simulations shows the rapid increase in the post-exposure period for the 3,000 and 10,000 exposure groups.

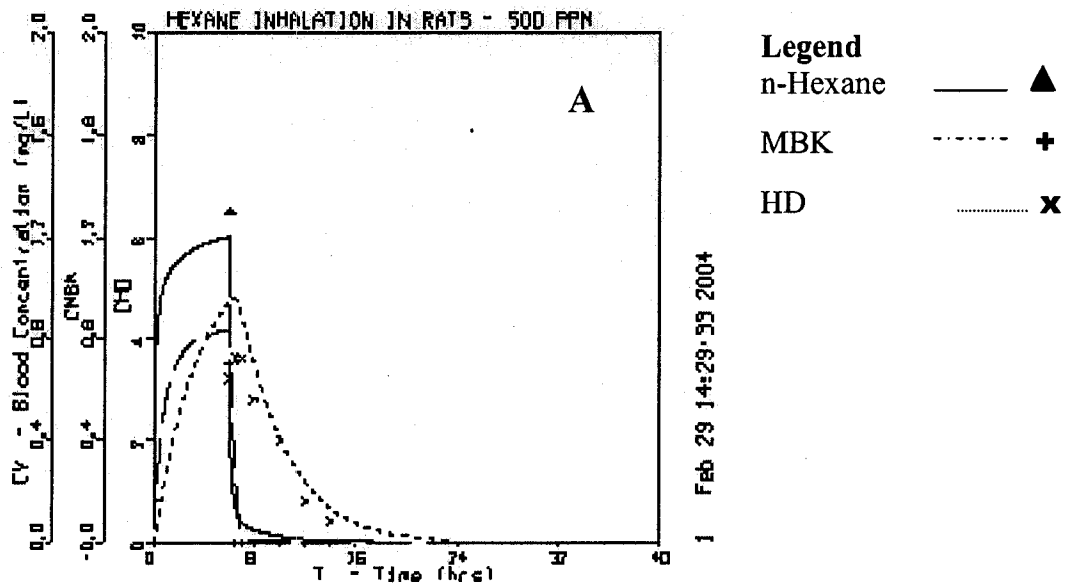

Fig. 7.6a. Model 3. PBPK model for HX, MBK, and HD at 500, 1000, 3000, and 10,000 ppm using the revised PBPK model described above. In Panels A-D, data shown in symbols are from Baker and Rickert (1981); Rickert (1983) for exposures of rats to 500, 1000, 3000, or 10,000 ppm *n*-hexane for 6 hours. Concentrations of *n*-hexane (CV), MBK (CMBK), and HD (CHD) are shown along with corresponding simulations (curves). Panel A: Exposure at 500 ppm. This model included additional inhibitory steps, and the potency of some inhibitors was an increasing function of concentration. An overall better fit was obtained with this model. Inflections in the post-exposure concentration of HD are apparent at 3000 and 10,000 ppm. Following the Baker *et al.* data, the concentration of HD after 10,000 ppm is increasing even as the concentration of its precursor (MBK) has dropped off, indicating that the relative proportions of MBK going through different clearance pathways is rapidly changing.

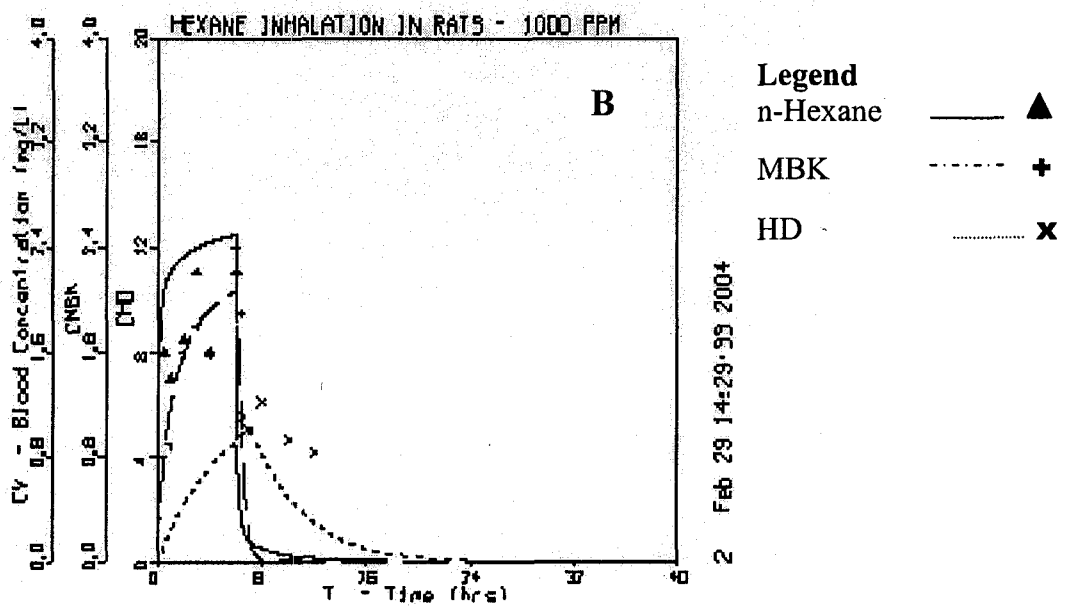

Fig. 7.6b. Model 3 simulation for 1000 ppm exposure. See explanation in legend for Fig. 7.6a.

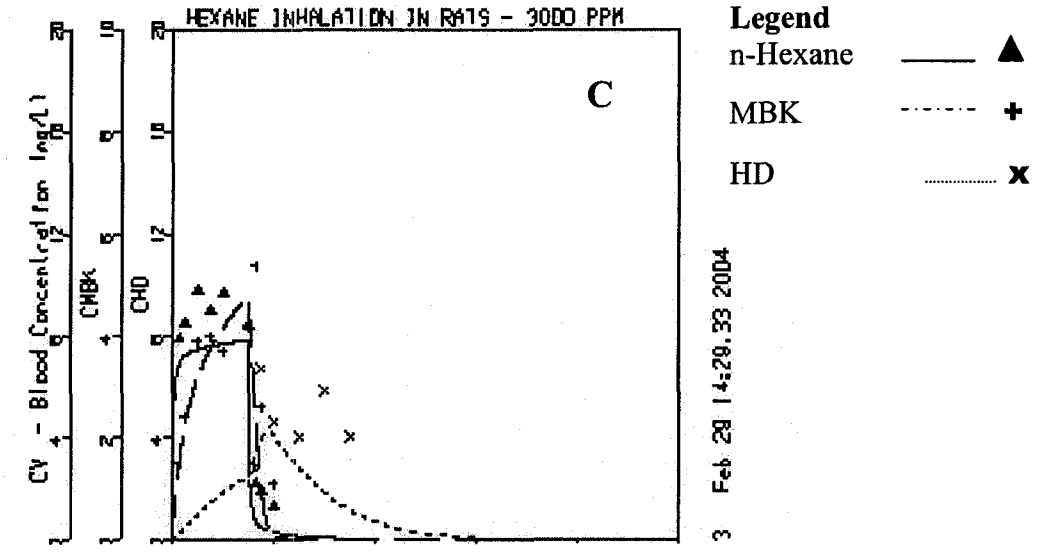

Fig. 7.6c. Model 3 simulation for 3000 ppm exposure. See explanation in legend for Fig. 7.6a.

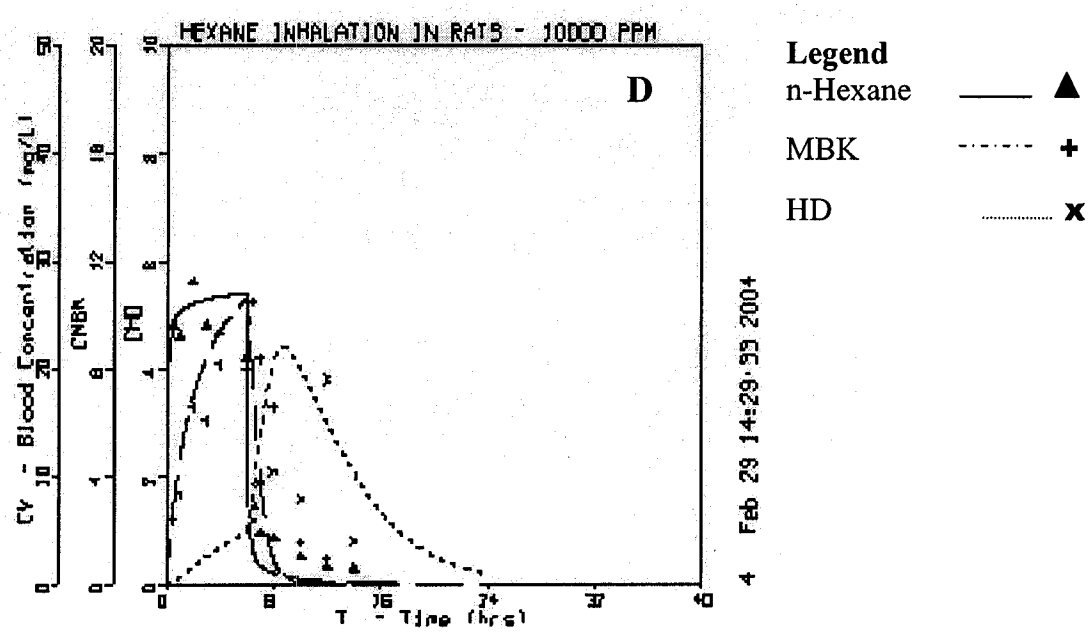

Fig. 7.6d. Model 3 simulation for 10,000 ppm exposure. See explanation in legend for Fig. 7.6a.

A comparison was made between the response measured by Takeuchi *et al.* (1981), motor nerve conduction velocity (MCV) and two dose metrics. One dose metric consists of the cumulative exposure (applied or administered dose). This was calculated from the experimental exposure, described as exposure to 1000 or 3000 ppm HX for 12 hours/day, 7 days/week for 4-16 weeks. The loss of MCV (percent relative to control) shown in Figure 7.7 was linearly related to either cumulative exposure (ppm-hrs) or AUC of HD in body water (mg-hr/L) at both 1000 and 3000 ppm. However, the slope of the lines for AUC at 1000 and 3000 ppm are roughly parallel, suggesting a relationship between AUC and loss of MCV. The slope of the

lines for cumulative exposure and MCV are divergent. Therefore, AUC of HD in blood serves as a better dose metric for the neurotoxicologic effect.

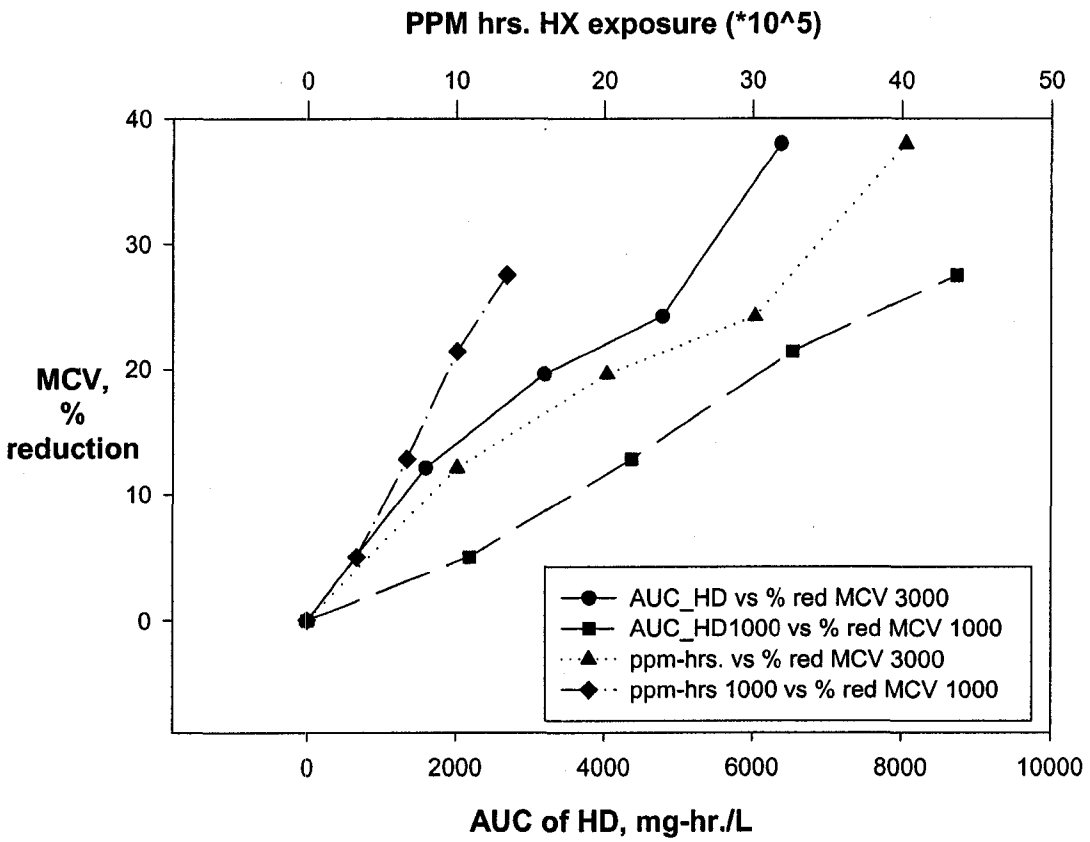

Fig. 7.7. The percent reduction in motor nerve conduction velocity (MCV) as a function of the HD in body water AUC (mg-hr/L) or cumulative exposure in ppm-hrs. AUC data were estimated with Model 3 as described in the paper, adjusting body weight and exposures to the Takeuchi *et al.* experiments (top axis). MCV and cumulative exposure (bottom axis) are from Takeuchi *et al.* (1981). Generally linear curves suggest that the extent of response is related to cumulative exposure and that dose rate is not important. Parallel curves for AUC suggest a correspondence in the response in relation to AUC of HD. Different slopes for cumulative exposure (ppm-hrs.) suggests that response is not directly related to this dose metric.

Discussion

Three versions of a PBPK model for HX and its metabolites MBK and HD are presented in this paper. The challenge of the models was to recapitulate very low levels of HD in blood during very high exposures to HX. The first two models, which were mathematically identical but varied in some parameter estimates, reproduced the Baker and Rickert data to an extent, but the timecourse of HD could not be achieved without some errors; either the 10,000 ppm HD timecourse was too high or timecourses at lower exposures were too low. Consideration of additional interactions and an inhibitor potency that increased at higher doses was required to bring the HD timecourse into range at all exposure levels.

In general, the PBPK parameters selected for this model were in the ranges of parameters used previously. The anatomical, physiological, and chemical parameter values were from literature sources except for the blood:air partition coefficient (PB) for HX. Reported values for PB are 2.29 for rats (Gargas *et al.* 1989) and 0.8 for humans (Perbellini *et al.* 1986). Previous PBPK models have used these values for rats and humans respectively (Perbellini *et al.* 1986; Yu *et al.* 1998; Ali and Tardif 1999), but a difference this large in PB between species is greater than previously measured for several chemicals (Gargas *et al.* 1989). The present model did not use either value *a priori*, but found that, due to the sensitivity of the HX concentration in blood to this parameter, values above 1.0 were excluded. Thus, it was by coincidence

that the best estimated value ended up as the same value used previously in some human models.

Comparable values for Vmax and Km are not available, because the previous models lumped all metabolism into one or two steps. In a rat/human HX model, Ali and Tardif reported a Vmax value of 1.35 mg/hr/kg$^{0.75}$ (Ali and Tardif 1999) corresponding to our Vmax1 of 6.0 mg/hr/kg$^{0.7}$, although the Km values were similar. However, the Ali and Tardif model did not incorporate inhibition of metabolism by the metabolites and was designed to evaluate the pharmacokinetics at lower exposure levels, so the basis for setting the metabolic parameters was not equivalent. Our value of KEC (0.13 – 0.20/hr.) was similar to the value calculated from the excretion data in a previous study of 0.26/hr. (Eben *et al.* 1979).

The use of the constants n and n1 is semi-empirical in nature, incorporated in the model to improve the ability of the model to recapitulate the complex behavior of HD after HX exposure. However, these constants were close to the default value of 1.0. They may act as surrogates for an undefined process or for interactions that were not included in the design of the model.

We explored the ability of different interactions to improve the model's description of the Baker and Rickert data. Interactions that did not observably improve the model were omitted. For example, inhibition of SAT2 and SAT3 by Compound 3 were incorporated in the model. Optimization of the value for the inhibitory parameter for

Compound 3 (KmCPD3) did not yield an observable improvement in the model, including when the affinity of the inhibitor for the enzyme in SAT2 and in SAT3 were allowed to vary from each other. Therefore, in the final Model 3, the inhibitory parameter for Compound3 was set at 10,000 (no inhibition).

Previous studies had shown that the AUC of HD in blood and urine was similar after 3000 ppm and 1000 ppm exposures (Baker and Rickert 1981; Iwata *et al.* 1983). However, the present model suggests that metabolic saturation is only partially responsible for the effect. Saturation will reduce the rate of metabolism, but does not directly result in a disproportionate post-exposure increase in the blood concentration of HD. The rate of metabolism of MBK to HD is shown in Figure 7.8 over the course of the exposures to 500, 1000, 3000, and 10,000 ppm HX for six hours. During exposure, the rate of metabolism of MBK to HD is actually lower as the concentration increases even though the concentration of MBK increases with exposure level. The increase in MBK concentration will tend to increase the metabolic rate. As the metabolic rate for the 500 ppm exposure group was about 60% of Vmax, there was remaining metabolic capacity that was not used by MBK. However, the rate of metabolism during exposure was approximately .32, .30, .20, and .07 mg/hr for 500, 1000, 3000, and 10,000 ppm exposure groups respectively. The reason for the decrease is clearly the inhibition of metabolism.

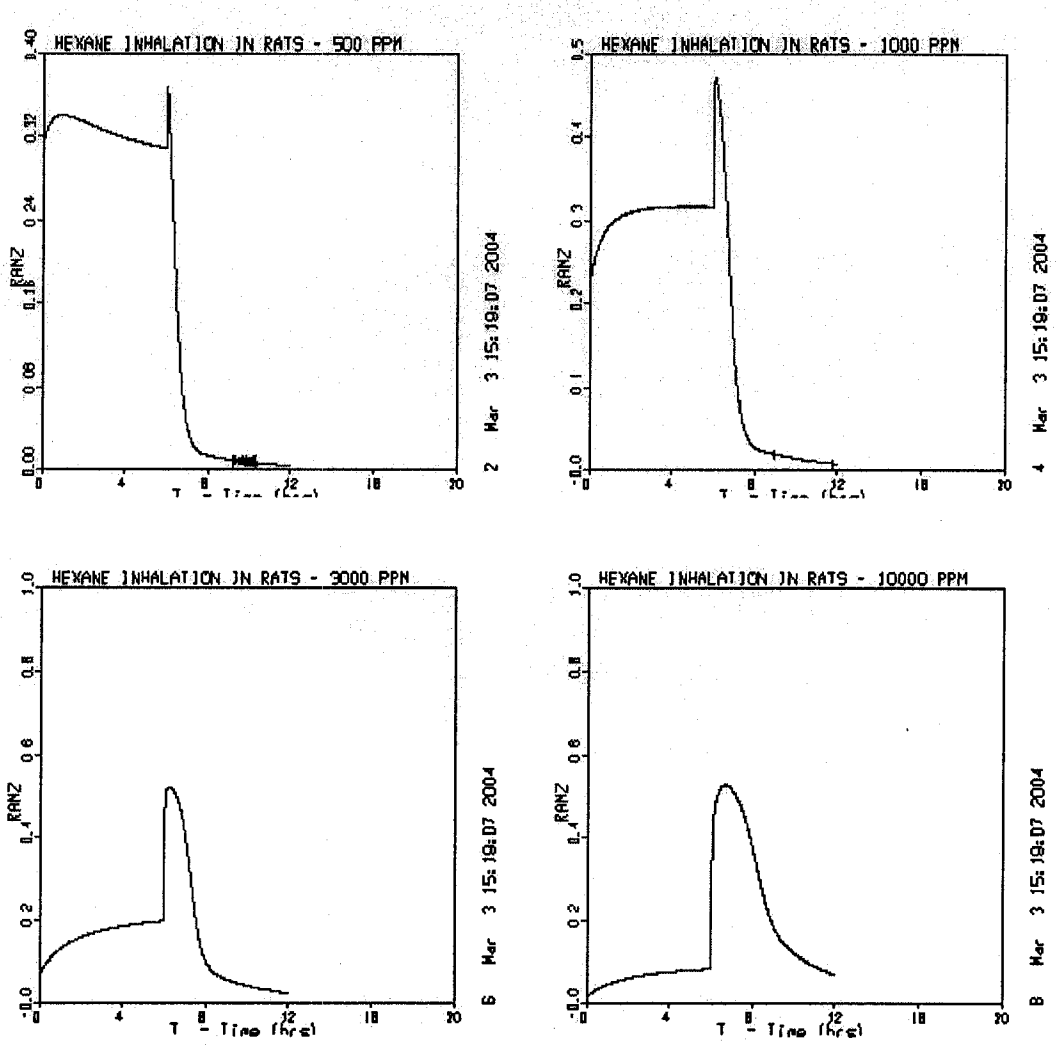

Fig. 7.8. Rate of metabolism of MBK→ HD. The rate of metabolism (mg/hr) for the second metabolic step is plotted vs. time where rats were exposed to the concentration of HX indicated for six hours. Vmax in this model (Model 3) was 0.59 mg/hr. As concentration increased, the rate of metabolism at steady state (~5 hours) was reduced by inhibition. The magnitude of the post-exposure spike was related to the amount of pooled MBK and then available for metabolism as HX was rapidly cleared after exposure ended. The breath of the spike was related as well to the amount of pooled MBK and the length of time it took to produce HD from MBK.

Another interesting feature of the pharmacokinetics of HX revealed by Figure 7.8 is the spike that occurs after exposure ceases. As concentration increases (Panels A-D), the relative height of the spike and the breath of the spike increases. When the reaction MBK→ HD is inhibited, the concentration of MBK rises above the steady state level and serves as a pool. When exposure ends, HX is cleared from the blood and liver very quickly, and the concentration of other metabolites also declines. This clearance releases the inhibition of MBK metabolism to HD and the rate of metabolism increases until the pool of MBK is depleted. A small pool of MBK and inhibition of its metabolism occurs at 500 ppm exposure leading to a short-lived increase in the metabolic rate, on the order of 10%. After exposure to 1000 ppm HX, the increase in metabolism after release of inhibition is about 50%. After 3000 ppm exposure, the increase is over 100% and lasts for approximately two hours. Finally, after 10,000 ppm exposure, the increase in metabolism is about 900% and lasts for approximately six hours. The duration of the spike is related to the length of time it takes to clear the pool of MBK. While most of the reduction in metabolism during exposure is due to inhibition instead of saturation, during the spike, saturating rates do occur. The peak velocity after 1000 ppm exposure is about 80% of Vmax and after 3000 and 10,000 ppm exposure is about 90% of Vmax. Thus, as more MBK is metabolized to pentanoic acid when metabolic saturation limits the rate of metabolism to HD, some of the depression of AUC of HD metabolites in blood and urine may be attributable to saturation.

The present model suggests that the inhibition of metabolism of MBK to HD is primarily caused by HX. While HD may serve as an inhibitor of its own production from MBK, the inhibitory constant for HD on MBK metabolism estimated in this model (1.0) was higher than the inhibitory constant for HX (0.1 - 0.4). Likewise, the concentration of HX was generally higher than the concentration of HD, so HX will serve as the more effective inhibitor.

While most of the model behavior reasonably recapitulates the Baker and Rickert data, future improvements in the model may be possible. Some of the interactions could be studied *in vitro* to determine the nature of inhibition. However, these *in vitro* studies will be complicated by the fact that once a substrate and inhibitor are introduced to the enzyme(s), they may both act as inhibitors of each other and may also produce additional metabolites that also inhibit some reactions. However, some efforts to determine the *in vitro* rates of each step, including steps that were not included in the present model, may be fruitful. In addition, obtaining a full suite of metabolite data *in vivo* would allow the inclusion of a fuller description of metabolism.

The *n*-hexane data were reasonably simulated by each version of the model. However, the model tended to overstate the rate at which HX would be cleared from blood after exposure. The rate of HX clearance in the PBPK model is controlled by the blood:air partition coefficient and the ventilation rate. The PB is largely governed by the steady state concentration of HX in blood relative to the exposure

concentration and the ventilation rate is based on the literature. Alternatively, the fact that the data indicate that clearance of HX from the blood is slower than predicted suggests that some of the HX may reside in deep tissue compartments. The present model assumed that tissues were in equilibrium with the blood as the blood leaves the tissue. A deep compartment is one where some of the chemical enters the tissue deep enough that diffusional time is long enough to prevent equilibrium. Thus, chemical enters the tissue but when exposure ends, some of the chemical remains longer in the tissue and keeps the blood concentration up as it leaves the tissue more gradually. Representing HX clearance by incorporating deep tissue compartments in the model, however, is unlikely to substantially improve the description of MBK and HD data.

The AUC for HD calculated with the PBPK model developed in this study did not perfectly correlate with the neurotoxicity data in Takeuchi *et al.* (1981). There are several possible reasons for this. Some differences may exist between the strain of rats used in the studies (F344 and Wistar). HD may not be the only metabolite that is directly involved with formation of pyrroles. Also, the rate of pyrrole formation and clearance may not be a linear function of HD concentration. However, the fact that the degree of loss of motor nerve conduction velocity was linearly related to the length of exposure suggests that damage is cumulative and irreversible.

In the Takeuchi *et al.* study (1981), rats were dosed for 4 – 16 weeks, raising the possibility that enzyme induction occurred. When the present model was exercised at induction rates of 200%, the AUC of HD was affected, but the relative AUC at

different exposure levels was quite similar (data not shown). However, the possibility remains that induction would be greater at 3000 ppm than at 1000 ppm exposures, and the resulting difference would improve the correlation between the AUC of HD and reduction of MCV.

As described in this paper, the timecourse of HD can be somewhat counter-intuitive at times. The inhibition that is responsible for altered blood levels of HD occur when the blood levels of HX are above about 1 mg/L, a range that is often used in studies of the effects of HX and/or HD. As neurotoxicity is directly related to the production of HD, these issues may make the interpretation of effect studies more difficult, as in the case of the Takeuchi *et al.* study of neurotoxicity at 1000 and 3000 ppm (Takeuchi *et al.* 1981). Therefore, models such as the present ones, that permit direct evaluation of the tissue dose of HD under varying types of exposures (acute gavage, repeated dose, etc.) should be useful. These could be applied to various experimental situations, such as experiments in different species, dosing by different routes of exposure, or *in vitro* experiments. Effects could then be interpreted in the context of the more relevant dose metric, HD AUC.

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Chapter 8

Conclusions and Future Directions

In this dissertation, PBPK models for BTHEX compounds and gasoline mixtures have been described. The work was successful at developing approaches that can be applied to practical problems. In this chapter, general conclusions will be discussed and suggestions for several additional lines of investigations will be made.

In Chapter 3, a PBPK model for toluene, ethylbenzene, and xylene was used to examine several pertinent issues. These issues included and evaluation of the extent of interactions at the Permissible Exposure Limits (PELs), comparison of the tissue dose at exposures that were either at the PEL or consistent with the unity calculation at the PEL, omission of xylene from the unity calculation when it causes CNS depression or simply inhibits metabolism of toluene and ethylbenzene, and potential for overexposure when a worker is exercising.

The analysis conducted indicated several salient points. First, modest potential overexposures occurred if xylene was not in fact neurotoxic or if it was included in the unity calculation. More extensive potential overexposures could occur if xylene was neurotoxic or if it was neurotoxic and not included in the unity calculation. Serious potential overexposures would occur if the unity calculation was based on the

PEL assuming that Threshold Limit Value (TLV) was the appropriate standard. Also, if the worker was exercising, a serious potential overexposure could occur. Any combination of these factors could also cause a serious potential overexposure. The American Conference of Governmental Industrial Hygienist emphasizes that the TLVs are not a fine line that defines a safe exposure. The general idea is that they serve as a goal for controlling exposure, and that reduction below the TLV is a good idea, and that exposures above the TLV carry a greater risk of toxicity. Therefore, the issues raised here regarding interpretation of exposures with respect to PELs or TLVs should be regarded as sources of additional risk.

Specifically, we recommended that health and safety professionals consider the following:

1. Be sure to include in the unity calculation all chemicals that cause neurotoxicity or interfere in the metabolism of others that cause neurotoxicity.
2. Apply safety factors to the unity calculation as appropriate or conduct PBPK modeling of applicable exposure scenarios to determine appropriate mixture exposure limits.
3. Use up-to-date occupational exposure limits and understand the basis used in setting the standards.
4. Consider work activity level and whether the exposure limit was designed to protect accordingly.

Additional research could be usefully conducted in this area. First, there are numerous PBPK models for toluene, ethylbenzene, and xylene, as indicated in Chapter 2, but the models vary somewhat and none may be the definitive model. Additional research to develop more robust models, particularly for humans, for these compounds would provide greater confidence in the calculation of tissue doses. The PBPK models should be extended to determine internal metabolite levels and to deal with the issues of human variability to better understand pharmacokinetic issues with biomarkers, so that the Biological Exposure Indices (BEIs) can be applied more routinely. This would address issues of the effect of personal protective equipment, variability in uptake and activity levels, dermal exposure, and other factors that serve as shortcomings in performing exposure assessment on the basis of applied dose. While a number of investigators have assessed the impact of work activity level on tissue doses, a consensus on how this should be done and how it should be applied to occupational exposure risk assessment is lacking.

In Chapter 4, some improvements in gas uptake systems were described. Specifically, a method for monitoring carbon dioxide (CO_2) in the chamber, for assessing the reversibility of chemical absorption to the fur of the animal, and for sampling blood during a gas uptake experiment were provided. Gas uptake studies are a very useful method for performing inhalation PK studies and will likely be used for many additional chemicals. The recommendations in Chapter 4 should help improve such experiments.

Based on the observation that the rat's breathing rate was sensitive to CO_2 in the chamber, and that PBPK models are often very sensitive to the alveolar ventilation rate parameter, it is important to control CO_2 levels, and moreover, to keep them consistent from one experiment to the next. The relationship between the parameter value used in PBPK models for the alveolar ventilation rate (QP; often set to 15 liters/hr/kg body weight $^{\wedge 0.75}$) and the actual minute volume should be established. This would reflect a certain CO_2 level. Then, the relationship between that parameter and the value of the parameter at different CO_2 levels should be determined. This information would enable the modeler to determine the acceptable range of CO_2 levels in a chamber. The information would also possibly help explain possible differences between inhalation studies conducted at ambient CO_2 levels (open chamber studies) and those conducted in gas uptake systems. In the future, monitoring the CO_2 in the chamber should be a routine operation.

We determined in the gasoline studies that the chemicals absorbed to the fur of the animal in gas uptake studies could desorb from the animal. Chemicals will desorb if the ambient concentration is low enough relative to the concentration in the fur and the fur:air partition coefficient. Based on the data from experiments performed during these studies, the concentration of chemicals in this study at the end of a gas uptake experiment were low enough that either desorption or at least a decrease in the rate of absorption was expected. Further research should be performed with other chemicals. If this issue is confirmed to be a problem, the significance of the problem

should be assessed. If the problem is significant, mathematical corrections should be determined for use in the PBPK models. The solution could be as simple as using a loss rate for a specified period of time, and then turning off the loss process for the remainder of the experiment.

Many PBPK models are developed from different types of data from different experiments. For example, a PBPK model can be developed with gas uptake data from one lab, gavage data from another, etc. This has the benefit of avoiding or minimizing the problem of "bad data." However, data sets can be "good data" and just be inconsistent for several reasons. For example, body weights may be different. The animals may be of different ages or genders or strains. Conditions may exist that affect the animals in terms of oxidative balance, stress, etc. When developing an initial model, it would then be preferable to have data sets that derive from a single set of animals. Other data can always be incorporated later, but when model accuracy is in question, it is convenient to know that variation in laboratories is not the issue. Thus, our method for sampling the animals' blood during a gas uptake study should be of interest.

Additional development of the blood sampling method needs to be performed. Specifically, for each chemical assayed, the minimum amount of blood to be sampled should be determined. Better sensitivity could be obtained by using a gas chromatograph with a narrow bore capillary column, solid phase microextraction, or other techniques. This would enable more frequent blood sampling.

It should also be considered whether sampling the blood for metabolites would provide useful information. With frequent blood sampling, it is unlikely that current analytical methods will be sensitive enough to detect some of the more trace metabolites. However, the more significant metabolites can probably be determined. This will provide excellent data for calibrating metabolic processes in the model.

Similarly, methods for collecting urine during a gas uptake experiment should be investigated. For chemicals primarily excreted in the urine, lack of urine data is often the biggest data gap in PBPK models. In other words, the model may have gas uptake data and blood data, but no data regarding elimination of metabolites. Thus, PBPK models often determine the creation of a metabolite from disappearance of the parent chemical and clearance of a metabolite from its timecourse in blood, but lack any verification of elimination processes if the urine data is not used. A possible reason that urine data is not used is that, the way it is normally collected, it represents the area-under-the-curve (AUC) for the excretion process, not a timepoint and as such, is more difficult to use in a model. Usually, urine is collected at specified timepoints, such as at the end of the experiments and every 12 hours thereafter. This is for convenience as well as for getting an adequate volume of liquid. Thus, each sample represents all the fluid urinated by the animal over some period of time. It doesn't even represent the entire amount of chemical that was excreted by the kidney, because some is still in the bladder. When incorporated into a PBPK model, the rate of excretion is usually controlled by a rate constant (such as a first order rate

constant) multiplied by some measure of the chemical's concentration in the blood (which perfuses the kidney). The determination of the rate constant is usually performed by optimization of urine data. However, if the urine concentration is higher than the model predicted, was the rate constant too low at the beginning of the period or at the end of the period (or was there some left in the bladder?) Moreover, uncertainty in the validity of the model in describing the rates of formation of the metabolite and in describing other clearance processes (metabolite may be further metabolized instead of being excreted), complicate the optimization of the excretion rate constants. Many of these issues would be helped if continuous urine data were available. Depending on the rate of formation of urine and the minimum sample volume required for analysis, samples of urine could be collected at more frequent time intervals. If these time intervals are frequent enough, the data could be more or less continuous. At a minimum, more frequent data would make it easier to cope with the issues described above.

In Chapter 5, we reported our preliminary model for gasoline. This model split out *n*-hexane, benzene, toluene, ethylbenzene, and *o*-xylene and lumped the remaining components of gasoline. In effect, the model was a six-component model with binary interactions accounted for between each pair of chemicals. The model was developed with data from gas uptake experiments with one blend of gasoline and validated with data from another blend. Parameter estimates for metabolism were refined in single chemical experiments and the parameters defining the metabolic inhibition between each BTHEX component were estimated in five chemical mixtures experiments. The

gasoline experimental data was used to estimate parameter values for the blood:air partition coefficient (PB) and metabolic and inhibitory parameters for gasoline. Several parameter values were constrained in the model. This included that only the PB varied between blends, and that the inhibitory parameter was equal to the metabolic affinity constant.

An adequate fit to much of the pharmacokinetic data was obtained with the simple model. Several areas were found to deviate but overall, the model indicated that the chemical lumping approach would offer a reasonable way to predict and describe the pharmacokinetics of a complex mixture. The model recapitulated the experimental finding that a substantial inhibition of metabolism of gasoline components occurred during some exposure conditions.

Since humans are rarely exposed to the stoichiometric equivalent of gasoline, we explored whether the approach would work for various fractions of gasoline, such as might be inhaled during spills or vehicle fueling. We evaporated gasoline until one-third of the whole gasoline was evaporated and collected the vapors as a sample of the light ends (a one-third cut). Likewise, we evaporated two-thirds of whole gas and collected the vapors as a two-thirds cut. These samples were much more volatile than whole gas, which was reflected by their empty chamber and dead rat loss studies. The samples were then used along with the whole gas in gas uptake experiments.

The one third and two third cut samples did not contain detectable amounts of ethylbenzene and *o*-xylene, so the PBPK model for these fractions was a four-chemical model (*n*-hexane, benzene, toluene, and the lumped chemical). We used the same parameter values that were determined in the previous model with only the expected exceptions. These exceptions were the blood:air partition coefficients and metabolism parameters for the lumped fractions. As these fractions contained very different chemical compositions than whole gas, one would not expect the partition coefficient to be the same and it seems likely that metabolic parameters could vary, as they do from chemical to chemical. These values were re-optimized for each blend. Initial modeling suggested another idea as well—that the animal's alveolar ventilation rate differed as a function of exposure concentration. When simulations were performed using differing ventilation rates, much better correspondence between the model and data were obtained. This serves as an example of the iterative benefit of modeling and experimental data analysis: conceptually, one would expect that ventilation rates would be affected, at least to some extent and possibly to a significant extent, during exposure to a CNS depressant and sensory irritant such as gasoline. Other PBPK models, to the best of our knowledge, have never contended with this issue, even though some of them have been developed for chemicals that are CNS depressants and/or irritants. Thus, a new hypothesis was developed: that increased concentrations of chemical exposure led to reduced rates of ventilation. The improved correspondence of the model supports this idea and underscores the importance of better understanding the role of ventilation rates in pharmacokinetics. Given the intended simplification of lumping many chemicals as though they were

one component, the final model provided an excellent description of the pharmacokinetics of gasoline and the components that were split from the lump.

This research effort was the first to use chemical lumping in PBPK models and essentially in pharmacokinetics overall. Yet, many additional issues could be investigated further. It has already been mentioned that the issue of alveolar ventilation deserves attention. In our studies, the concentration of chemical(s) in the chamber declines over the course of the experiment. Even while it may be expected that the alveolar ventilation rate would be affected at higher exposure concentration, the effect would not be expected to result in a constant ventilation rate over the whole experiment. Further studies, possibly including actual ventilation measurements with plethysmography or other techniques, could be used to determine the actual rates. This is not a simple issue, however, because PBPK model parameters are already adjusted to compensate for other factors, such as airway dead space. The default parameter values used are essentially empirical estimates anyway, so it would be necessary to develop a detailed understanding of a number of physiological processes to develop a better estimate. However, significant improvement in model and resulting risk assessments may be available if this is done. For example, extrapolation of exposure limits to emergency conditions (Acute Exposure Guideline Levels) can be based on resting conditions or simple assumptions about the ventilation rates of people attempting escape. The choice made for these assumptions has a large impact on the exposure guidelines that result.

The gasoline lumping approach is potentially applicable to other complex mixtures, such as for other fractions of petroleum distillates, including jet fuel, kerosene, diesel fuel, natural gas, and asphalt liquids. Some of the fractions are not significant inhalation risks, but present dermal exposure hazards. The lumping approach could be used in dermal PBPK models in an analogous manner. For chemicals that are inhaled, the lumping approach is equally amenable to aerosols and vapor inhalation.

The structure of the lumps in the models developed should be based on the chemistry of the compounds as well as the intended purpose of model. Some models are used to help predict the pharmacokinetics and resulting risks of the mixture exposure. In the event that particular chemicals are important in the risk assessment, they can be split from the lump, as was done in these studies with benzene and other chemicals. In some cases, it is possible that fractions of the mixture present specific risks. In a hypothetical example, suppose that the hydrocarbons from C4 – C7 present risk for CNS depression and hydrocarbons from C8 – C12 present immunotoxicity risk. In this case, one would want to create two-lump model. This could be easily achieved with the existing experimental approach. Further suppose that hydrocarbons from C4 – C7 present risk for CNS depression and hydrocarbons from C7 – C12 present immunotoxicity risk, so there is an overlap at C7. In this case, a three lump model could be developed for hydrocarbons between C4-C6, C7, and C8-C12. The pharmacokinetic description could then be used to support risk assessment for either endpoint (CNS depression = Lump 1 + Lump 2 and immunotoxicity risk = Lump 2 + Lump 3).

In our experiments, a reasonable analytical quantification of *n*-hexane, benzene, toluene, ethylbenzene, and *o*-xylene was obtained in the chromatography. However, the analysis did involve a tradeoff. Gas uptake experiments usually sample the chamber every 10 minutes. This was not feasible for analysis of gasoline, as the separation of chemical peaks was inadequate with that run time. Samples every 20 minutes gave better separation. If samples were only collected every 30 minutes, even better separation could be achieved, but at the expense of obtaining experimental data on a sufficiently frequent interval for model development. Thus 20 minutes was selected as the sampling interval. It was observed, however, that some of the peaks for BTHEX were not "clean" and actually represent the identified chemical and a co-eluter. At the beginning of the experiment, the co-eluter seemed of minimal significance, but some of the chromatograms indicated that they were more significant at the end of the experiment (which could occur if they were slowly cleared from the chamber due to slow uptake by the rat). This issue should be resolved if possible in future experiments.

One potential solution would be to collect samples for later analysis instead of analyzing samples in real-time. This solution would allow more frequent sampling, if desired, and would permit extended run times and essentially complete resolution of all components. It would have the further advantage of allowing flexibility in definition of the lumps. For instance, all components could be included in one lump except the component of interest, e.g., benzene. Later, another lump could be defined

that included all components of interest except toluene. Based on future needs, additional lumps or components could be defined without repeating the experiments to evaluate other hypotheses that are not included in the present investigation.

Additional areas where the gasoline model could be improved also exist. First, partition coefficients for gasoline and the various fractions should be measured in vial equilibrium experiments. Measurements of kinetics *in vitro* should be performed. The blood sampling system described above should be used to allow serial collection of blood samples. Blood should be analyzed for gasoline components and metabolites if possible. The possibility of sampling urine should also be considered.

After the model is further validated and refined, it should be scaled up to humans. It is highly preferable to do this with actual human data. Such data is not presently available in the open literature. If experiments need to be performed to collect it, an issue will occur with institutional approval of the experimental protocol. While gasoline is not regarded as a very toxic substance, it contains carcinogens. Depending on the requirements of the institutional review panel, it may or may not be possible to conduct the experiments. However, *de minimus* exposures may be permitted.

The human version of the gasoline PBPK model could be coupled with existing PBPK models for chemicals such as benzene, when available. In other words, if the single chemical model has been developed to the point that it could be used to support

risk assessment (benzene's hasn't so far), it can be coupled to the gasoline model to modify the risk assessment calculation for gasoline exposures.

In Chapter 7, a PBPK model for *n*-hexane and two of its principal metabolites was provided. This model provided a reasonable description of the pharmacokinetics of *n*-hexane, methyl-*n*-butyl ketone (MBK) and 2, 5-hexanedione (HD) during and after constant exposures to rats of 500 to 10,000 ppm. The model successfully predicts the restriction in formation of HD above 1000 ppm, due to metabolic suppression of the second oxidation of the chemical. The model also successfully predicts the delay before the peak exposure to HD occurs at the higher exposure levels. These data were consistent with the data from neurotoxicity tests that determined that neurotoxicity at 3000 ppm was not more extensive than at 1000 ppm. Thus, the PBPK model provides a rational explanation of the counter-intuitive flat dose-response curve.

In Chapter 2, a sample of available pharmacokinetic literature on *n*-hexane is provided. The striking thing about the body of literature is that there is so much of it. Each study was somewhat insular, however, investigating one piece of the toxicity of *n*-hexane. What is also striking is that there is so much literature, but no real way for it to be organized into a complete description. Moreover, no single study or set of connected studies examined *n*-hexane metabolism in a truly comprehensive manner. In other words, one study looked at one set of metabolites, and another study looked

at a different set. PBPK models could serve as the way to organize all this information, but would be hampered by the lack of any comprehensive dataset.

One approach to solving this problem would be as follows. Define a method to work out the pharmacokinetics of *n*-hexane and pertinent metabolites using experiments and PBPK modeling in an iterative manner. The approach would require some experiments, then some modeling, then more experiments, and so forth. At each stage of the process, existing experimental data could be used if available, or the experiments could be performed.

The difficulty with *n*-hexane stems in part from the multiple interactions that occur and from the fact that numerous metabolic pathways occur. While one can draw a conceptual description of the metabolism and interactions, incorporating this into a PBPK model requires estimates of the values of dozens of parameters. Thus, it will be necessary to simplify the problem as much as possible.

The approach should start with looking at the pharmacokinetics of terminal metabolites first. Most of the chemicals can be purchased commercially. PK experiments should be conducted with the terminal metabolites to determine their rates of elimination. The second-to-last metabolites can then be examined. PBPK models can be built in this manner, adding one metabolic step at a time. In some cases, performing metabolism studies *in vitro* may be useful, to get a first estimate of kinetic parameter values and to define which interactions are important. Either *in*

vitro or *in vivo*, some of the metabolism studies may be benefited from use of selective inhibitors to suppress alternate metabolic pathways. As the data are acquired, they should be incorporated into a PBPK model to estimate the rate constants and to design the next suite of experiments. Determination of partition coefficients experimentally is also necessary. Consideration of the possibility that the blood:air partition coefficient for *n*-hexane may be concentration dependent is also necessary. Finally, consideration of the question of enzyme induction during repeated dose studies should be investigated.

In this dissertation, we have described the development and use of PBPK models that address interactions between the chemicals in chemical mixtures on several levels. An existing three-chemical mixture PBPK models was used to examine, on a quantitative basis, the potential for increases in the concentration of each chemical in blood above the level that would occur during exposure to the chemicals within various Occupational Exposure Limits. To extend the ability of PBPK models to address mixtures of greater complexity, we developed a new approach for dealing with mixtures of numerous chemicals, which we refer to as "complex mixtures". The new approach was necessary because the existing bottom-up approach is far too data-intensive to permit consideration of the pharmacokinetics of complex mixtures. Our new approach uses the concept of chemical lumping to make the problem tractable. Finally, we have extended the concept of mixture interactions to the problem of interactions between a chemical and one or more of its metabolites in the body. This permitted exploration of the pharmacokinetics of *n*-hexane and its neurotoxic

metabolite, 2, 5-hexanedione (HD). We found that, in order to successfully describe the pharmacokinetics of HD, it was necessary to incorporate these interactions in the PBPK model, an approach not previously used.

Many PBPK models have been developed for various chemicals over the years. Over time, the models have become more biologically rigorous in some ways. The addition of the concept of modeling the interactions between chemicals and their metabolites is one example of how the present work provides an approach for introducing the necessary biology into PBPK models. The approach for coping with complex mixtures is another. We also developed methods as part of this research to better control carbon dioxide concentrations in gas uptake experiments and a method to simultaneously sample blood and gas uptake chamber air. However, a number of other issues were brought to light during this research which, as they are answered in time, will serve to enhance the science in the pharmacokinetic arena.