Université de Montréal

Modélisation toxicocinétique d'un mélange de composés organiques volatils dans l'eau potable

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Mémoire présenté à la Faculté des études supérieures en vue
de l'obtention du grade de Maîtrise ès Sciences (M.Sc.)
en santé environnementale et santé au travail

Avril 2012

Université de Montréal

Faculté des études supérieures

Ce mémoire intitulé :

Modélisation toxicocinétique d'un mélange de composés organiques volatils dans l'eau potable

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Mémoire accepté le………………..

RÉSUMÉ

L'évaluation des risques de l'exposition aux mélanges de produits chimiques par voies multiples peut être améliorée par une compréhension de la variation de la dose interne due à l'interaction entre les produits. Les modèles pharmacocinétiques à base physiologique (PBPK) sont des outils éprouvés pour prédire l'ampleur de ces variations dans différents scénarios.

Dans cette étude, quatre composés organiques volatils (COV) (toluène, n-hexane, cyclohexane et isooctane) ont été choisis pour représenter des produits pétroliers (essence) pouvant contaminer l'eau potable. Premièrement, les modèles PBPK ont simulé l'exposition à un seul COV par une voie (inhalation ou gavage). Ensuite, ces modèles ont été interconnectés pour simuler l'exposition à un mélange par voies multiples. Les modèles ont été validés avec des données in vivo chez des rats Sprague-Dawley (n=5) exposés par inhalation (50 ppm ; toluène, hexane, et 300 ppm ; cyclohexane, isooctane; 2-h) ou par gavage (8,3; 5,5; 27,9 et 41,27 mg/kg pour le toluène, l'hexane, le cyclohexane et l'isooctane, respectivement). Des doses similaires ont été utilisées pour l'exposition au mélange par voies multiples. Les AUC (mg/L x min) pour le toluène, l'hexane, le cyclohexane et l'isooctane étaient respectivement de 157,25; 18,77; 159,58 et 176,54 pour les données expérimentales, et 121,73; 21,91; 19,55 et 170,54 pour les modèles PBPK. Les résultats des modèles PBPK et les données in vivo (simple COV par voies multiples vs. mélange par voies multiples) ont montré des interactions entre les COVs dans le cas de l'exposition au mélange par voies multiples. Cette étude démontre l'efficacité des modèles PBPK pour simuler l'exposition aux mélanges de COV par voies multiples.

Mots clé :
Analyse de risque, Mélange, Voies multiples, PBPK

ABSTRACT

Risk assessment focusing on exposure to mixtures by multiple routes can be improved with an understanding of the changes in internal doses due to interaction among chemicals. Physiologically based pharmacokinetic (PBPK) models are proven tools to predict the magnitude of interaction in various scenarios. In this study, four volatile organic compounds (VOCs) (toluene, n-hexane, cyclohexane and isooctane) were chosen to represent petroleum products that could contaminate the drinking water (e.g. gasoline). PBPK models were used first to simulate exposure to a single chemical by a single route (inhalation, gavage) and simulate exposure to a mixture by multiple routes. PBPK models were validated by comparing simulations with *in vivo* data. These data were collected from groups of male Sprague-Dawley rats (n=5) exposed by inhalation (50 ppm of toluene, hexane; 300 ppm of cyclohexane and isooctane; 2-hr) or gavage (8.3, 5.5, 27.9, and 41.27 mg/kg, respectively, for toluene, hexane, cyclohexane and isooctane). For exposure to the mixture by multiple routes, same doses were used. The AUCs (mg/L x min) based on experimental data were 157.25, 18.77, 159.58 and 176.54 and the AUCs of the PBPKs model were 121.73, 21.91, 19.55 and 170.54, respectively, for toluene, hexane, cyclohexane and isooctane. Results from both PBPK models and *in vivo* data (single VOC, multiple routes *vs.* mixture, multiple routes) showed interactions between VOCs in the case of exposure to the mixture by multiple routes. This study demonstrated that the PBPK model is an effective tool to simulate exposure to mixtures of VOCs by multiple routes.

Keywords:
Risk assessment, Mixture, Multiple routes, PBPK

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ABBREVATIONS

Aab	Amount of solvent being absorbed during the oral exposure
ADI	Acceptable daily dose
AUC	Area-under the concentration *vs* time curve
Ca	Arterial blood concentration
Calv	Alveolar air concentration
CHL	Chloroform
Cinh	Inhaled air concentration
CNS	Central nervous system
Cv	Venous blood concentration
dA/dt	Rate of change in the amount of chemical
DCP	1,1-dichloropropanone
Fabs	Absorbed fraction
Faw	Transfer coefficient air: water
IR	Daily water intake rate
Kab	The constant of competitive metabolism inhibition of chemical *b* by chemical *a*
Kaw	Partition coefficient air: water
Km	Michaelis-Menten constant
Ko	Absorption rate in stomach
Kow	n-octanol: water partition coefficient
Kp	Dermal Permeability constant
Kx	Rate of substance transferred from stomach to intestine

Leq	Litre-equivalent
MC	Methyl chloroform
MW	Molecular weight
n	number of samples/ subjects
NOAEL	No observed adverse effect level
PBPK	Physiological based pharmacokinetic model
Pf	Fat: blood partition coefficient
Pk	Skin: blood partition coefficient
Pl	Liver: blood partition coefficient
PNS	Peripheral nervous system
ppm	Part per million
Psw	Skin: water partition coefficient
Qalv	Alveolar ventilation rate
Qc	Cardiac output
Qf	Blood flow to fatty tissues
Qi	Blood flow to tissue
Qinh	Absorbed dose by inhalation
Ql	Blood flow to liver
Qp	Alveolar ventilation rate
Qr	Blood flow to richly-perfused tissues
Qs	Blood flow to slowly-perfused tissues
RAM	Rate of the amount metabolized
RON	research octane number

RfD   Reference dose

Rr   Richly-perfused tissue: blood partition coefficient

Rs   Slowly-perfused tissue: blood partition coefficient

SAF   Source allocation facteur

T   Temperature

t   Time interval

TCE   Trichloroethylene

TCP   1,1,1-Trichloropropanone

TDI   Tolerable Daily Intake

TLV$^{®}$ Threshold limit value$^{®}$

TMP   2,2,4-Trimethypentane

U.S. EPA   United States environmental protection agency

Vmax   Maximum velocity of metabolism

VOC   Volatile organic compound

Vf   Volume of fatty tissues

Vl   Volume of liver

Vr   Volume of richly-perfused tissues

Vs   Volume of slowly-perfused tissues

Acknowledgments

Chapter 1: General Introduction

'All people, whatever their stage of development and their social and economic conditions, have the right to have access to an adequate supply of safe drinking water'
WHO

Canada is the home of one of the biggest natural resources on earth, namely water. About seven per cent of the world's fresh water is in Canada. The St Lawrence River and the Great Lakes in the east, the Mackenzie River in the north-west are important to the Canadian economy and supply drinking water for millions of people. However, these resources are susceptible to contamination by different sources such as human activities, animal waste, industrial activities (i.e., pesticides and fuel leakage). Therefore, the cleaning up of contaminated rivers (e.g., the St. Lawrence) and lakes (e.g., the Great Lakes) and protecting people by developing guidelines for drinking water quality (i.e., pollution prevention, managing toxic chemicals) should continue to be priorities in Canada (Environment Canada).

1.1. Development of guideline values for drinking water

To date, guideline values for drinking water are developed considering only oral route exposure by Health Canada and the United States Environmental Protection Agency (U.S. EPA) (Krishnan and Carrier, 2008). In this regard, the guideline value for a substance in water is calculated as the ratio between the maximal allowable amount consumed (i.e., Tolerable Daily Intake by Health Canada (TDI; mg/kg/day) or Reference Dose by U.S. EPA (RfD; mg/kg/day) x Body Weight (BW; kg) x Source Allocation Factor (SAF; 0.2 for each source of contamination: water, air, soil, food and consumer product)) and the daily water intake rate (1.5 L/day used by Health Canada; 2 L/day used by U.S. EPA):

$$\text{Guideline value} = \frac{\text{(TDI or RfD) x BW x SAF}}{\text{water intake rate}} \qquad [1]$$

However, studies as early as 1984 have shown the importance of dermal and inhalation routes, especially in the case of water contamination by volatile organic compounds (VOCs) (Brown et al., 1984; Andelman, 1985; McKone, 1987; Weisel et al., 1996; Kim et al., 2001). Among different chemical

contaminants, VOCs have interested regulators because of their potential for multiple routes of exposure due to their ubiquitous distributions in the various environmental compartments (Pohl and Scinicariello, 2011). Large numbers of VOCs are used as additives in fuel in order to increase its burning capacity (Lee et al., 2002; Lin et al., 2005). Their particular physiochemical characters are low water solubility, high vapor pressure and low molecular weight (Liu and Zhou, 2011). These specific characteristics of VOCs give them a marked capacity to transfer from fuel to air and to contaminate surface and underground water sources (U.S. EPA, 1990). As a result, the general population can be exposed to these chemicals in water supplies not only via the oral route but also through the dermal route by bathing, showering and by inhalation of indoor air (Mckone, 1999). Because of its large absorptive surface area (50- 100 m^2) (Deshpande, 2005), the lungs are important routes for absorption of VOCs in ambient air. Brown et al. (1984) indicated that VOC absorption via skin can occur as a result of direct contact with water, such as showering, bathing or swimming. In addition, several studies demonstrated the contribution of inhalation and dermal routes to total internal dose of VOCs in water. For example, in 1996, Weisel and Jo designed a well-controlled exposure scenario (i.e., via oral, inhalation and dermal routes) to chloroform (CHL) and trichloroethene (TCE), in humans to evaluate the contribution of each exposure route to the internal dose of VOCs. Eleven subjects participated in a total of 25 experiments which included a 10-minute shower (dermal contact: CHL, 10-41 µg/l; TCE, 16-150 µg/), a 60-minute bath (dermal contact; T= 40°C) and a 10-minute inhalation exposure (CHL, 10-50 µg/l; TCE, 28-41 µg/l) during shower. The subjects were also exposed by the oral route by drinking 0.5 liter of tap water (CHL, 20 µg/l; TCE, 20-40 µg/l). The CHE and TCE were present in the exhaled breath following inhalation exposure (0.25-0.05 µg/l CHL and 1.0-0.1 µg/ TCE, 30-180 minute following exposure) and dermal exposure (0.33-0.00 µg/l CHL and 0.9-0.01 µg/l TCE, 30-180 minutes following exposure). However, results of breath samples taken minutes after ingestion of tap water, showed no trace of CHL. This indicates that CHL is totally metabolized through the first pass in the liver. On the other hand, TCE was found

seconds to minutes after ingestion of water, in the exhaled breath. The authors indicate that this is an outcome of the low water/air partition coefficient, which results into rapid transfer of TCE from water to air in the oral cavity. Based on their observations, after oral ingestion, the absence of continued elevation of the VOC in the exhaled air shows that blood:air exchange in the alveolar sac has no importance. They concluded that the rate of metabolism of VOCs and therefore their potential toxicity is dependent on the exposure route.

Another study by Xu and Weisel (2005) on human subjects, also demonstrated that the dermal route has an important effect on the absorption of VOCs such as CHL, 1,1-dichloropropanone (DCP) and 1,1,1-trichloropropanone (TCP) used to disinfect tap water. In a well-controlled dermal exposure environment, subjects (n=6) took baths in water (T: 38±1 ºC) with 40 µg/l of CHL (well below the concentration found in tap water) and 25 µg/l of DCP and TCP (higher than concentrations that can be found in a poorly controlled water system) for 30 minutes. Results show that maximum breath concentration of CHL during dermal exposure varied between 20 to 90 percent (16-34 µg/l) of the initial concentration among subjects. At the same time, maximum breath concentration of detected DCP was as little as two percent (0.1-0.9 µg/l) whereas for TCP, it was less than one percent of the initial dose (0.07-0.25 µg/l).

These studies suggested that traditional approaches to evaluate guidelines for drinking water are inadequate to account for multiple routes of exposure. Consequently, guideline value derivation for drinking water contaminants (DWC) has been improved as follows (Krishnan and Carrier, 2008):

$$\text{Guideline value} = \frac{(\text{TDI or RfD}) \times \text{BW} \times \text{SAF}}{\text{intake rate} + \text{L-eq}} \qquad [2]$$

In the above equation, L-eq (liter equivalent) corresponds to the number of liters of the dermal and inhalation exposure equivalent to oral exposure. In other

words, L-eq is the fraction of the daily dose received by inhalation or dermal route relative to the dose absorbed by the oral route. The L-eq values are obtained on the basis of dose received via either inhalation or dermal route as follows:

$$\text{L-eq inhalation (L)} = \frac{\text{dose absorbed via inhalation route (mg) x IR (L)}}{\text{dose absorbed via oral route (mg)}} \quad [3]$$

$$\text{L-eq dermal (L)} = \frac{\text{dose absorbed via dermal route (mg) x IR (L)}}{\text{dose absorbed via oral route (mg)}} \quad [4]$$

IR: daily intake rate
Therefore the total L-eq is:

$$\text{L-eq total (L) = L-eq inhalation (L) + L-eq dermal (L) + IR (L)} \quad [5]$$

The L-eq can be calculated if the absorbed dose for the various routes can be estimated using human biomarkers or pharmacokinetic data (i.e., blood concentration, expired alveolar breath) after dermal or inhalation exposure in a controlled environment. However, conducting the necessary human experiments in order to compute the L-eq for all VOCs is not feasible (Krishnan and Carrier, 2008). In this regard, the use of mathematical models would be practical. The capacity of physiologically based pharmacokinetic (PBPK) models to predict absorption, distribution, metabolism and excretion of VOCs has already been proven (Ali and Tardif, 1999). Also, these models are increasingly used in regulatory risk assessment processes for VOCs in drinking water. For example, as early as 1988, Bogen and Hall developed the PBPK model to predict the safe concentration of methyl chloroform (MC) in drinking water based on a no observed adverse effect level (NOAEL) from experimental data in rodents. In the same study, they also applied a PBPK model for multiple routes of exposure (i.e., inhalation, ingestion and dermal) to MC in humans. They concluded that the safe

concentration of MC in the water based on their modeling and data evaluations would be lower than that calculated based on the traditional method. Haddad et al. (2007) established a PBPK model to analyze the contribution of each route of exposure to the total absorbed dose and total metabolized amounts of five VOCs (CHL, TCE, bromodichloromethane, dibromochloromethane, bromoform). They developed the model to predict human exposure via single route, for individual chemicals in tap water. They assumed that exposure occurs in the bathroom and elsewhere in the house during daily activities such as drinking, bathing and showering. In this study, total amounts absorbed and metabolized were calculated by adding separate amounts for inhalation, dermal and ingestion routes. They concluded that dermal and inhalation (showering) exposures contribute as much as, or even more than, the ingestion of water in terms of total absorbed dose of VOCs. They also indicated that these PBPK models can be useful to establish guidelines for different VOCs in the water, to account for multi-route exposures.

Although previous studies on single chemicals have demonstrated the usefulness of PBPK models, the evaluation of possible interactions during mixed exposure to chemicals via multiple routes has not yet been undertaken. Human exposure to chemical mixtures found in the environment has been well documented (Newill, 1989; Saéz et Rittman, 1993; Eskenazi et al. 1999; FSA, 2002; Wolff et al. 2008). The U.S. EPA, National Institute of Occupational Safety and Health and National Center for Toxicological Research have developed risk assessment methods for mixtures, in the early 1980s (RAMAS, 2003). Since 1996, U.S. EPA has been required by the Food Quality Protection Act, to include the evaluation of mixture risk assessment of chemicals (RAMAS, 2003; Rider et al., 2010). However, as discussed before, all regulatory threshold limits, such as tolerable daily intakes (TDI), are based only on individual chemical risk assessment. Furthermore, there is the question of whether it is safe to be exposed to chemicals in mixture at low levels; although Carpenter et al. (2002) pointed out that there might not necessarily be interactions among all chemicals

in a mixture. Those chemicals that share the same site of action could result in non-additive effects. Despite the importance of exposure to mixtures, until now, risk assessments have largely focused on single and multiple routes of exposure to individual chemicals (Sexton and Hattis, 2007).

It is particularly important in the case of drinking water contamination to consider both mixture effects and multi-route exposure while establishing guideline values; however, this has not yet been done. The interactions could occur to different degrees according to the route of exposure. The present study focused on the use of *in vivo* data and PBPK models to evaluate the impact of multiple routes of exposure on the kinetics of a chemical mixture. For this purpose, VOCs representative of the substances found in large volume petroleum products (e.g., gasoline, kerosene, gas oil) that could potentially contaminate water supplies were selected (toluene, n-hexane, cyclohexane and isooctane). In general, petroleum products contain mostly carbons (84-87%) and hydrogens (11-14%), other elements being present in much lower proportions. Table 1 represents the major classes of chemicals present in petroleum hydrocarbon products (Hyne, 2001).

Table 1: Major classes of chemicals present in petroleum hydrocarbon products.

Chemical class	Weight (%)
Paraffin (alkane)	30
Naphthenes (cycloalkanes)	49
Aromatic	15
Asphaltic	6

Substances chosen in this study belong to different families of hydrocarbons found in crude oil, as well as gasoline. For instance, while toluene represents the aromatic hydrocarbon family, n-hexane, cyclohexane and isooctane belong to

straight-chain paraffins, naphthenes and branched-chain paraffin, respectively (Chen et al., 1998). The following section presents a brief summary of the toxicokinetics and toxicity characteristics of these VOCs.

1.2. Toxicokinetics and toxicity of toluene, n-hexane, cyclohexane and isooctane

Table 2: Summary of key toxicokinetics and toxicity features of toluene, n-hexane, cyclohexane and isooctane.

	Toluene	n-Hexane	Cyclohexane	Isooctane
Principal absorption route	Respiratory Oral	Respiratory	Respiratory Oral	Oral
Metabolite	Hippuric Acid	2,5-hexanedione	Cyclohexanol	2,4,4-trimethylpentanoate
Site of metabolism	Liver	Liver	Liver	Liver
Target Site	CNS	PNS	CNS	Kidney In male rat
TLV$^®$(ppm)	50	50	300	300

TLV$^®$: threshold limit value$^®$; CNS: central nervous system; PNS: peripheral nervous system

1.2.1. Toluene ($C_6H_5CH_3$)

1.2.1.1. Toxicokinetics

Toluene is a colorless liquid, volatile, miscible and almost insoluble in water (U.S. EPA, 2005). Its ability to prevent engine knocking and its high research octane number (RON=111) makes it a good fuel additive in order to achieve a better performance of the engine (Sarmaneav et al. 1980).

The extent of absorption following oral dosing (provided through feeding tubes) of volunteers at a rate of 2 mg/min of toluene for a period of 3 hours was almost 100% (Baelom et al., 1993). However, absorption rate has been reported lower for inhalation exposure. In fact, it has been shown that the retention is higher after a single lower dose exposure (83% - 189 mg/m^3) compared to a single higher dose exposure (50% - 300 mg/m^3) (Benoit et al., 1985; Carlsson et al., 1982). In rats, researchers suggest that absorption of toluene happens, in average, faster after inhalation exposure (1-2 hours) than oral exposure (1.3- 6.3 hours) (Gospe and Al-Bayati, 1994). Overall, absorption from the gastrointestinal route is as important as the respiratory system when exposure lasts for about an hour. On the other hand, during short exposures, 85-90% of toluene is absorbed via the respiratory tract (Faust, 1994).

To find out the skin capacity to absorb toluene, volunteers were exposed to liquid toluene and toluene from aqueous solution (Dutkiewicz and Tyras, 1967). The authors showed that, for a mean concentration of 180-600 mg/l, the absorption rate of liquid toluene was 14-23 mg/cm^2/h compared to 0.16-0.6 mg/cm^2/h for the aqueous solution. They concluded that in both cases absorption rate increases with increases in toluene concentration. Although Gospe and Bayati (1994) mentioned that skin should be considered as an important exposure route, the U.S. EPA (1990) reported that skin absorption accounts for only 1% of the

inhalation route. Therefore, it was concluded that because of the strong volatile character of toluene, skin is not an important absorption route (U.S. EPA, 1990). Liver is the main organ of toluene metabolism (U.S. EPA, 1990). Toluene is metabolized by the family of cytochromes P450, principally by CYP2E1 and CYP2B1/B2 in liver, mostly into hippuric acid (HA) (Nakajima et al., 1991). It is eliminated principally through the urinary system (80%) as HA and as unchanged substance through exhalation (20%) (Nakajima et al.,1997).

1.2.1.2. Toxicity

In a two-year inhalation study, female and male F344/N rats were exposed to 600 to 1200 ppm of toluene. No evidence of carcinogenic activity in either male or female was observed (NTP, 1990). The U.S. EPA and International Agency for Research on Cancer (IARC) have classified it, according to their ranking system, in group D and 3, respectively. The threshold limit value (TLV)[®] for toluene was established at 50 ppm.

The most important effect of inhalation exposure to toluene is on the central nervous system and the brain in both humans and animals (Filley et al., 2004). Chronic exposure in workers at 30 to 150 ppm could result in hearing loss, color vision impairment and lower performance in neurobehavioral tests (ATSDR, 2000). Furthermore, effects on the reproductive system and development in children whose mothers were exposed to high concentrations of toluene during pregnancy have been reported (Bowen et al., 2006). Other effects have been reported on the respiratory, cardiovascular, hematological, hepatic and renal systems (ATSDR, 2000).

1.2.2. n-Hexane (C_6H_{14})

1.2.2.1. Toxicokinetics

n-Hexane is highly volatile, colorless and slightly soluble in water. However, unlike toluene it is not an additive but a natural component of fuel (WHO, 1991). The respiratory tract is the main absorption route of n-hexane, but it can also be absorbed by the gastrointestinal tract and the skin (Bonnard et al., 2008). On the other hand, absorption of n-hexane via the skin is not significant (Stellman, 1998).

n-Hexane is metabolized in the liver by CYP2B1 into 2,5-hexanedione (HD) (Fabioano et al., 2010). It is eliminated either by the respiratory or urinary system (U.S. EPA, 2005). Up to 10% of unchanged n-hexane is eliminated by exhalation with an estimated half-life of 100 minutes. However, it is mostly eliminated in urine as 2-hexanol (60-70%), a urinary metabolite with a half-life of about 12hrs (ATSDR, 1999).

1.2.2.2. Toxicity

n-Hexane affects mostly the peripheral nervous system (Ali and Tardif, 1999). Based on their observation, workers who have been exposed for long periods of time complained about numbness in their arms and feet followed by paralysis. However, they recovered six months to one year after being removed from the contaminated environment. There is no evidence for human or animal carcinogenicity of n-hexane. It has not been classified as a carcinogen by U.S. EPA (group D). TLV$^{®}$ for n-hexane is 50 ppm.

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1.2.3. 2,2,4-Trimethylpentane (Isooctane, C_8H_{18})

1.2.3.1. Toxicokinetics

Isooctane is a colorless liquid, insoluble in water and soluble in toluene and benzene. Its chemical conformation increases the capacity of fuel to burn and reduces the knocking problem of engines (U.S. EPA, 2006). Therefore, by definition isooctane is given the RON of 100. Conversely, with a RON of zero, heptane has the poorest fuel burning capacity, and comparison between these two substances determines the octane number of other hydrocarbons in fuel.

Studies show a high rate of oral absorption (almost 95% of the oral dose) and a lower absorption rate after respiratory exposure (7-12% of the administrated dose) in rats (Kloss et al., 1986). Isooctane is metabolized by P450 mainly into pentanoic acids and 2,4,4-trimethy-2-penthanol (Olson et al., 1985; El-masri et al., 2009). The majority of metabolites are eliminated in urine (67% for males, 50% for females). However, it has been reported that 90% of the absorbed dose after inhalation exposure is eliminated from the respiratory system after about 70hr. It will be as much as 43% in males and 49% in females from exhalation (Kloss et al. 1986).

1.2.3.2. Toxicity

Studies have shown that isooctane induces proximal tubular tumor in male rats (Swann et al., 1974). This happens after its metabolite, 2,4,4-trimethy-2-penthanol, binds to α_{2u}-globulin protein which results in a complex that leads to the development of tumor in this part of kidney. The protein α_{2u}-globulin is specific to male rats (Charbonneau et al. 1987; El-masri et al., 2009) and therefore isooctane nephrotoxicity/carcinogenicity is not considered to be relevant for humans by the U.S. EPA and other scientific/regulatory agencies.

1.2.4. Cyclohexane (C_6H_{12})

1.2.4.1. Toxicokinetics

Cyclohexane is a colorless liquid, slightly soluble in water. It is part of natural chemical substances found in crude oils and also a part of gasoline (U.S. EPA, 2003).

Espinosa-Aguirre et al., (1997) reported that cyclohexane is metabolized into cyclohexanol and to a lesser extent into cyclohexanone by CYP2B4 in the liver. They observed that cyclohexanol induces CYP2E1 and CYP2B1/B2 isozymes that are involved in the metabolism of toluene and n-hexane. Therefore, it is possible to speculate that the effects resulting from exposure to toluene and n-hexane separately can be different if exposure is to a mixture of cyclohexane, toluene, and n-hexane. Elimination of cyclohexane and its metabolites happens primarily via exhalation. Only 20% of elimination occurs via the urinary system (U.S. EPA, 2003).

1.2.4.2. Toxicity

No adequate human epidemiological studies are available on cyclohexane. Most animal studies suggest effects on the central nervous system. However, liver hypertrophy and differentiation in liver enzyme profile have also been mentioned (U.S. EPA, 2003).

1.3. Previous studies on multi-route exposures and interactions among toluene, n-hexane, cyclohexane and isooctane

Until now, there has been no study on interactions between these four chosen chemicals. There have been, however, two studies on binary exposure to toluene and n-hexane. The first study showed that exposure to the mixture of 1000 ppm

toluene and 1000 ppm n-hexane via the respiratory system, resulted in a reduction of the neurotoxic effects of n-hexane, compared to exposure to n-hexane alone (Takeuchi et al., 1981). The authors suggested that toluene reduces the oxidation rate of n-hexane to 2,5-hexanedione (HD). In the second study, Ali and Tardif (1999) also found a significant reduction of urinary HD (75%) after respiratory exposure to the mixture of 300 ppm toluene and 100 ppm hexane. However, these authors reported that amounts of hippuric acid (HA) (ie., toluene metabolite) did not decrease significantly (only by up to 25%).

There is no research about mixture exposure including cyclohexane or isooctane. However, one study reported that cyclohexanol (i.e., metabolite product of cyclohexane) induces CYP2E1 and CYP2B1/B2, indicating a potential for interaction with other chemicals (Espinosa-Aguirre et al., 1997). As these cytochromes are involved in the metabolism of toluene and n-hexane, hypothetically, simultaneous exposure to toluene, n-hexane and cyclohexane, can lead to metabolic interactions between them. However, neither experimental data nor predictive PBPK models are available to evaluate the relevance of occurrence of interactions among these substances for risk assessment considerations.

1.4. Objective and originality of this study

The general objective of this project is to evaluate the importance of both mixture and multi-route exposures in perspective of establishing better guidelines for chemicals in drinking water.

First, this study focused on developing PBPK models and comparing them to experimental data to determine the kinetics of individual chemicals by a single route (i.e., inhalation and ingestion) and multiple routes of exposure (Article 1). Then, the PBPK models were applied to simulate the kinetics of hydrocarbons (toluene, n-hexane, cyclohexane and isooctane) following mixed exposure via

multiple routes, in an effort to compare the outcome with those of individual chemical exposures via single or multiple routes (Article 2).

Chapter 2: Article 1

PBPK modeling of the aggregate exposure of rats to n-hexane or isooctane by inhalation and oral gavage

Nazanin Kaveh, Michelle Gagne, Ginette Charest-Tardif,
Robert Tardif and Kannan Krishnan

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Short abstract

Physiologically based pharmacokinetic (PBPK) models, as well as *in vivo* experiments were used in order to characterize the impact of simultaneous exposure by inhalation and oral gavage (aggregate exposure) on the total internal dose of n-hexane or isooctane in rats. The PBPK models developed in this study are useful tools for simulating kinetics of hydrocarbons, by accounting not only for the saturable metabolism but also the possible kinetic behavior during aggregate exposures.

Abstract

The objective of this study was to compare the results of the aggregate exposure (inhalation and gavage) to n-hexane or isooctane to the simple exposure (inhalation) on the total internal dose of the substances. Physiologically based pharmacokinetic (PBPK) models, as well as *in vivo* experiments were used in order to simulate the kinetics of each chemical for simple and aggregate exposure. First, experimental data were collected from simple exposure of male Sprague-Dawley rats (n=5) exposed to n-hexane or isooctane (2,2,4-Trimethylpentane; TMP) by inhalation (50 ppm or 200 ppm n-hexane, 300 or 1200 ppm isooctane) or oral gavage (5.5 or 22.3 mg/kg n-hexane, 40 or 162 mg/kg isooctane). Next, other groups of rats were exposed to each substance simultaneously by inhalation and oral gavage. Blood concentration was measured between 2-6 hours after inhalation and aggregate exposure, and 0.5-3 hours after oral gavage. The PBPK model could fit the experimental data of different exposure scenarios and also was validated by the data-derived area under the blood concentration *vs* time curve mg/l. min. PBPK models as well as experimental data suggest that the internal dose resulting from aggregate exposure to n-hexane is not additive of the sum of the internal doses resulting from individual exposure routes. However, results from multi-route exposure to isooctane show that it is additive of the sum of the results from simple exposure routes. The PBPK models developed in this study are useful tool for simulating the kinetics of hydrocarbons, by accounting not only for the saturable metabolism but also for the possible kinetic behavior during aggregate exposures.

Keywords: PBPK, multi-route exposure, VOC, n-hexane, isooctane

Introduction

Contamination of ground water with petroleum products (i.e., gasoline) has widely preoccupied the regulatory agencies such as the U.S. EPA (Andelman, 1985). The main reason for this attention is the extended use of petroleum products and therefore the greater possibility of underground water contamination due to the leakage of fuel from containers of gas stations (U.S. EPA, 1990). In this study, n-hexane and isooctane (2, 2, 4-Trimethylpentane; TMP) are chosen to represent two different chemical classes in petroleum products (i.e., gasoline). n-Hexane and isooctane exemplify classes of alkanes and branched chain paraffins, respectively (Chen et al., 1998). n-Hexane is present in as much as 11% of crude gasoline (U.S. EPA, 2005). The neurotoxic effects of n-hexane have been reported in both animals and humans (Takeuchi et al., 1981; Filser et al., 1987; Hamelin et al., 2005). Isooctane is an additive to unleaded gasoline and is used to increase the octane number of fuel and the performance of the engine (Lock et al., 1993, El-Masri et al., 2009). The neurotoxic effects of isooctane have been reported in experimental animals (Swann et al., 1974; El-Masri et al., 2009). However, it is mostly known for its nephrotoxicity in male rats, which is not relevant to humans (Fowlie et al., 1987; Lock et al., 1993). Liver damage due to exposure to isooctane in rats has also been reported (Fowlie et al., 1987).

Both n-hexane and isooctane are volatile organic compounds (VOCs). VOCs possess low water solubility, high vapor pressure and low molecular weight (Liu and Zhou, 2011). These characteristics give them the capacity and mobility to contaminate different layers of soil and therefore underground water (U.S. EPA, 1990). As a result, consumers at home can be exposed to these chemicals from drinking water during bathing and showering or simply by inhaling the indoor air (Mckone, 1999). For years, regulatory agencies had considered the oral route to be the main exposure route to contaminated drinking water (McKone, 1999; reviewed in Krishnan and Carrier, 2008). Later, studies were able to show that

because of the efficient transfer from water to air, the inhalation route is an important exposure route for VOCs (Andelman, 1985; McKone, 1987, 1999). Several studies have shown that dermal exposure also contributes to the total absorbed dose depending upon the dose and physiochemical characteristic of the substance and duration of skin contact (Brown et al., 1984; McKone, 1999; Xu et al., 2002; Krishnan and Carrier, 2008).

Today, the significance of multiple routes of exposure (inhalation, gastrointestinal and dermal) to VOCs is widely recognized. However, conducting experimental studies with multiple chemicals for different scenarios of exposure, doses, and routes seems practically impossible (Haddad et al., 2001). PBPK modeling is a proven tool that makes it possible to evaluate the different pharmacokinetic phases such as absorption, distribution, metabolism and excretion in animals as well as humans regardless of the exposure scenario (Haddad et al., 2001; Dennison et al., 2004).

The objective of this study was to develop PBPK models to simulate the pharmacokinetics of n-hexane and isooctane after multi-route exposures. This approach would help better understand the effects of aggregate exposure on the internal dose of these chemicals in rats and the capacity of PBPK models to simulate multi-route exposure scenarios.

Materials and Methods

1) *In vivo* study

Chemical

n-Hexane (99%) and isooctane (anhydrous 99.8%) were obtained from Aldrich chemicals (Milwaukee, WI).

Animals

Adult male Sprague-Dawley rats (240-260 g) were obtained from Charles-River Canada (St-Constant, Québec). They were housed in temperature-controlled cages in groups of two or three per cage with a 12-h light–dark cycle. A minimum one week acclimatization period was provided. They were given food and water *ad libitum* until the day of experiment. On the day of experiment they were placed in individual cages and fasted during the experiment.

Exposure scheme

Inhalation exposure

Groups of male Sprague-Dawley rats (n=5) were separately exposed to low and high concentrations of each substance by inhalation (50 ppm and 200 ppm of n-hexane; 300 ppm and 1200 ppm of isooctane; 2-hr) in a closed-atmosphere exposure system (Haddad et al., 2000). The concentrations were monitored using automatic injection of 0.25 ml air samples into a gas chromatograph (HP 5890) equipped with a flame ionization detector (FID). Helium was used as a carrier gas, at the rate of 5.4 ml/min.

Oral exposure

Groups of rats (n=5) were given a single low dose or high dose of the substances. Accordingly, 5.5 mg/kg or 22.3 mg/kg of n-hexane, and 40 mg/kg and 162 mg/kg of isooctane were diluted in Alkamuls® (10%). Ten ml of solution was administered per kg body weight.

Dermal route

In this study, the dermal exposure was omitted as a relevant absorption route because at the exposure levels chosen on the basis of the threshold limit values of n-hexane (50 ppm) and isooctane (300 ppm), the dermal route did not appear to contribute significantly to the systemic dose (Bonnard et al., 2008; U.S. EPA 2007; Faust, 1994). Moreover, the preliminary experiments in rats determined that neither n-hexane (1.39 mg/ml) nor isooctane (10.18 mg/ml) is absorbed significantly by the dermal route (results not shown).

Multi-route exposure

A group of rats (n=5) was given a single oral dose of 22.33 mg/kg of n-hexane and then placed within a closed-chamber system for inhalation exposure to 200 ppm during 2 hr. In the case of isooctane, two groups of rats (n=5) were orally administered with either 40 mg/kg or 162 mg/kg and then exposed to an atmosphere of 300 ppm or 1200 ppm for 2 hr.

Analysis of the blood concentration

Blood samples (25-200 µl) were collected by performing a small incision on the tail veins of rats. The concentration of unchanged substances in blood was quantified using a head-space gas chromatography method (Tardif et al., 1993). After single oral exposure, blood samples were taken from animals 0.5, 1, 1.5, 2, 2.5 and 3 hr post-exposure. For the inhalation and multi-route exposure, however, blood samples were taken 2, 2.5, 3, 3.5, 4 and 5 hr post- exposure.

2) PBPK Modeling

Model representation

The four-compartmental PBPK models were developed similar to Haddad et al. (2000) separately for n-hexane and isooctane. The compartments of the PBPK model were: liver (as the metabolizing tissue), fat, richly-perfused tissues and slowly-perfused tissues, all connected by systemic blood circulation and gas exchange in the lung (Figure 1). In order to simulate oral and multi-route exposures, the gastrointestinal tract was included (Haddad et al., 2000). Accordingly, the stomach was described as additional portal of entry. The absorption rate from the stomach and constituted the systemic input of the orally-administered chemicals investigated in this study. The transfer of unabsorbed chemical from the stomach to the intestine has also included, and it was found to be essential to obtain reasonable model fit experimental data on oral kinetics.

Model parameterization

The physiological parameters of the rat as well as physiochemical parameters (i.e., partition coefficients and metabolic constants) for n-hexane were taken from Ali and Tardif (1999) whereas that of isooctane was obtained from El-Masri et al. (2009) (Table 1-2). The rate of metabolism ($Amet/dt$) in this model was described as a saturable process using $Vmax$ (maximal velocity) of 1.35 mg/kg/h and Km (Michaelis-Menten affinity constant) of 0.4 mg/l for n-hexane as obtained from Ali and Tardif (1999). The values of Kx (absorption rate constant for stomach) and Ko (stomach to intestine transfer constant) were obtained by fitting the PBPK model simulations to kinetic data for the oral route, and this yielded estimates of 0.2 min^{-1} and 0.007 min^{-1}, respectively.

For isooctane, the *Vmax* of 0.3 mg/h and *Km*: 1 mg/l were fitted to the inhalation PBPK model. *Kx:* 0.001 min⁻¹ and *Ko*: 0.003 min⁻¹ were obtained by fitting PBPK model simulation to the oral gavage data.

The metabolism was considered to take place exclusively in the liver. The rate of the change in the amount of chemical (*dAt/dt*) for this compartment accounted for the metabolism rate as follow (Haddad et al., 2000):

$$\frac{dAt}{dt} = Ql\,(Ca - Cvl) - \frac{Cvl \times Vmax}{Cvl + Km} \qquad [1]$$

where:

Ql: rate of blood flow through the liver

Ca: concentration of solvent in the arterial blood, and

Cvl: concentration of solvent in the blood leaving the liver

And for oral and multiple exposure routes (Haddad et al., 2000)

$$\frac{dAt}{dt} = Ql\,(Ca - Cvl) - \frac{Cvl \times Vmax}{Cvl + Km} + (A_{ab}) \qquad [2]$$

where:

A_{ab}: rate of chemical being absorbed during the oral exposure (mg/h). It was calculated as:

$$A_{ab} = Kx \times Q_t \qquad [3]$$

where:

Kx: absorption rate constant of a chemical by the stomach (min⁻¹)

Q_t: remaining quantity of chemical in the stomach (mg)

Transfer of the remaining chemical from stomach to intestine was included as:

$$Q_t = Q - (Aab \times ta) - (Ko \times ta \times Aab) \qquad\qquad [4]$$

where

Ko: stomach to intestine transfer constant (min^{-1})

ta: interval of time (min)

Statistical analysis and model evaluation

The statistical analyses were conducted using SPSS for Windows (v15.0.0, Chicago, IL, USA). In order to validate the model, data-derived area under the blood concentration *vs.* time curves (AUC; mg/L x min) were obtained for every exposure scenario (inhalation, gavage and multi-route) and dose (low and high doses), and compared with those obtained using the PBPK model. The results were used to determine the degree of discrepancy between PBPK modeling and experimental data.

Results

Single route exposure: inhalation or oral gavage

n-Hexane

The experimental data and PBPK model simulations after 2-hr inhalation exposure of rats to 50 ppm and 200 ppm n-hexane are presented in Figure 2A. Blood elimination rate (Kel) and half-life of n-hexane based on the experimental data (2-6 hr) were estimated as 1.22 hr^{-1} and 0.56 hr for the low dose and 0.84 hr^{-1} and 0.82 hr for the high dose, respectively. After 2-hr exposure to 50 ppm, maximum blood concentration of unchanged n-hexane (Cmax) was 0.09 ± 0.01 mg/l $vs.$ 0.13 mg/l, respectively, as indicated by the experimental data and the PBPK model. In contrast, Cmax after exposure to 200 ppm were 0.45 ± 0.02 mg/l according to experimental data $vs.$ 0.66 mg/l for PBPK model. The data-derived area under the blood concentration (AUC) after exposure to 50 ppm n-hexane by inhalation was 3.29 mg/l.min $vs.$ 4.5 mg/l.min as simulated by the PBPK model (2- 4 hr). The AUCs of high dose exposure as obtained from experimental data and PBPK model were 20.75 mg/l.min $vs.$ 20.25 mg/l.min, respectively.

Figure 2B compares the experimental data with PBPK model simulations after a single oral dose exposure to n-hexane. The experimental data from single oral exposure to high dose n-hexane could well fit our PBPK model (Figure 2B). The values of Kx (absorption rate constant for stomach) and Ko (stomach to intestine transfer constant) obtained by fitting were 0.2 min^{-1} and 0.007 min^{-1}, respectively. The only measurable concentration after exposure to 5.53 mg/kg dose of n-hexane occurred at 0.5 hr post-exposure (0.016± 0.006 mg/l). After that, n-hexane was not detectable in blood. Following the administration of 22.33 mg/kg, the Cmax observed 10 minutes post-dosing was 0.3 mg/l whereas the AUC based on experimental data was 21.06 mg/l.min The PBPK model-simulated Cmax and AUC for high dose exposure were 0.26 mg/l and 13.01 mg/l.min, respectively.

Isooctane

The PBPK model adapted for isooctane has been compared to the experimental data after 2-hr inhalation exposure to low (300 ppm) and high (1200 ppm) concentrations in Figure 3A. Figure 3B shows a significant correlation between the experimental data and the PBPK model from both low dose (40 mg/kg) and high dose (162 mg/kg) gavage exposure. Kx and Ko were fitted at 0.001 min^{-1} and 0.003 min^{-1}, respectively.

Based on results from experimental data (2-6 hr), the Kel and half-life of isooctane were 0.39 hr^{-1} and 1.76 hr after exposure to 300 ppm, and 0.30 hr^{-1} and 1.15 hr after exposure to 1200 ppm of isooctane, respectively. The Cmax from experimental data was 1.25 ± 0.04 mg/l compared to 2.68 mg/l from the PBPK model after 2-hr exposure to 300 ppm. After exposure to 1200 ppm, Cmax from experimental data was 5.06 ± 0.35 mg/l vs. 11.07 mg/l from the PBPK model. The AUC after 2-hr exposure to 300 ppm (2-6 hr) from experimental data vs PBPK model were 73.36 mg/l.min and 68.48mg/l.min. At 1200 ppm it was 295.58 mg/l.min $vs.$ 287.72 mg/l.min, respectively.

The PBPK model could adequately simulate the kinetics of isooctane after 2 hr exposure of rats to low and high concentrations by inhalation route (Figure 3A).

The ability of PBPK model to evaluate a single oral gavage exposure at low (40 mg/kg) and high (162 mg/kg) concentrations is shown in Figure 3B. Kel and half-life were 0.66 hr^{-1} and 1.04 hr after exposure to low dose, and 0.31 hr^{-1} and 2.2 hr after exposure to high dose, respectively. Cmax of low dose exposure based on the experimental data was 1.26 mg/l vs 1.13 mg/l from the PBPK model. After exposure to high dose, this amount increased to 1.8 mg/l and 3.6 mg/l for experimental data and PBPK model, respectively. The AUCs of PBPK model and experimental data (0.5-6 hr) were at low dose 148.61 mg/l.min and 135.51 mg/l.min, and at high dose 663.66 mg/l.min and 477.47 mg/l.min, respectively.

Multiple routes of exposure by inhalation and oral gavage

n-Hexane

Figure 2C compares PBPK model simulations with experimental data after simultaneous exposure of rats to n-hexane by inhalation and oral gavage. The values of Kx and Ko used in PBPK model of multi-route were the same as those used for simulating single route (oral) exposures (0.007 min^{-1} and 0.2 min^{-1}). Figure 2C also compares results from multi-route exposure to the sum of data obtained for the two single routes (inhalation and oral gavage) in the initial experiments.

Following multi-route exposures to 22.33 mg/kg (oral gavage) and 200 ppm (inhalation), Kel and half-life based on experimental data were 1.025 hr^{-1} and 0.67 hr, respectively. Cmax of n-hexane after multi-route exposures compared to the sum of the two single routes were 0.5 mg/l *vs.* 0.68 mg/l. The AUC from experimental data and PBPK model were 37.59 mg/l.min *vs* 27.25 mg/l.min. The ratio of AUC between multi-route exposure and the sum of single route exposure was 0.64.

Isooctane

Figure 3C depicts the PBPK model simulations of the blood concentration *vs.* time course after multi-route exposure to isooctane in rats at low dose (40 mg/kg + 300 ppm) and at high dose (162 mg/kg + 1200 ppm). The same values of Kx and Ko (0.001 min^{-1} and 0.003 min^{-1}) used for oral exposure of the PBPK model, were used in the PBPK model of multi-route exposure.

The Kel and half-life of isooctane after multi-route exposure were 0.36 hr^{-1} and 0.67 hr after low dose *vs* 0.35 hr^{-1} and 1.97 hr after high dose exposure. The Cmax of unchanged isooctane in the blood stream were 1.7 mg/l and 7.3 mg/l, respectively after low and high concentrations, after multi-route exposures. The AUCs calculated from PBPK model and experimental data were respectively

184.07 mg/l.min *vs* 148.56 mg/l.min after low dose, and 798.26 mg/l.min *vs.* 688.07 mg/l.min after high dose exposure. The ratios of the AUC between experimental data from multi-route and the sum of the single exposure (low and high dose) routes were 1.1 and 0.91, respectively.

Discussion

The PBPK model developed in this study was able to predict the kinetics of n-hexane and isooctane in rats following inhalation exposure and oral gavage. The systemic concentration of each chemical as a function of time after inhalation exposure, oral gavage or multiroute exposures was obtained using PBPK models and compared with experimental data. Previous experimental studies on the kinetics of n-hexane have primarily focused on the inhalation exposure in humans or rats (Bus et al., 1979; Veulemans et al., 1982; Perbellini et al., 1985; Gargas et al., 1989; U.S. EPA 2005). Interestingly, the tissue:air partition coefficients reflecting the volume of distribution in humans, were found to be comparable to that of F344 rats (example: 5.0, 5.2 and 104 for human muscle, liver and fat (Perbellini et al., 1985) *vs.* 2.9, 5.2 and 159 for rat muscle, liver and fat (Gargas et al., 1989)). No study on the kinetics of uptake and distribution of n-hexane after oral administration is yet available (U.S. EPA 2005).

In contrast, the pharmacokinetics of isooctane has been investigated by the oral route. Reviewed in U.S. EPA (2005), the study of Kloss et al. (1986) on the distribution of isooctane in F344 rats after single oral administration demonstrated sex-specific differences in the radioactivity in different organs (i.e., liver, heart, testis, kidney and spleen) 72 hrs after single oral administration of 500 mg/kg of this substance. The majority of isooctane was found in the kidney and its accumulation was 8 to 10-fold greater in male rat kidney than female (1225 nmol equivalent [eq]/g of wet tissue compared to 157 nmol-eq/g wet tissue). Following this study, Charbonneau et al. (1987) demonstrated that in the female rat, the concentration of ^{14}C labeled isooctane in kidney, plasma and liver decreased significantly at 8 hrs after oral administration, whereas it remained fairly constant even after 12 to 24 hrs post-exposure in male rats (U.S. EPA 2005).

There are limited efforts on the development of PBPK models for isooctane (El-Masri et al., 2009) and n-hexane (Ali and Tardif, 1999; Yu et al., 1998; Dennison et al., 2004; Hamelin et al., 2005). All these modeling studies have focused only on inhalation exposure. In the present study, for the first time, not only the PBPK model was used to simulate inhalation and oral exposures separately but also the model was applied to simulate combined exposure via inhalation and oral routes.

n-Hexane

After 2-hr exposure to a lower concentration of 50 ppm or 0.18 mg/l of n-hexane in the rat, the maximum blood concentration decreased by 50% to 0.09 ± 0.01 mg/l in blood *vs*. 0.13 mg/l simulated by the PBPK model. However, at the high concentration of 200 ppm or 0.72 mg/l, it decreased by about 40% in 2 hrs and the resulting blood concentration was 0.45 ± 0.02 mg/l *vs*. 0.66 mg/l as simulated by the PBPK model. Comparable blood concentration (0.45 ± 0.1 mg/l) has been reported by Bus et al. (1979) after 6-hr exposure to 1000 ppm (3.5 mg/l) n-hexane in pregnant rats. Furthermore, Filser et al. (1987) with mathematical analyses showed that, for inhaled concentrations below 300 ppm, the metabolism of n-hexane is directly proportional to its atmospheric concentration, whereas at higher concentrations (i.e., >300 ppm), metabolism is non-linear and saturated.

No previous experimental study or PBPK modeling has focused on the oral absorption kinetics of n-hexane. However, neurotoxic effects of n-hexane following oral dosing of rats have been reported (U.S. EPA, 2005). In the study of Ono et al. (1981), n-hexane was not detectable after a single oral dose (5.53 mg/kg). Bonnard et al. (2008) corroborated these findings on the basis of the marked pulmonary clearance of n-hexane. Similarly, in the present study, no data could be obtained from oral exposure at lower concentration (5.53 mg/kg), because the concentrations were below the limit of quantification. Therefore

multi-route exposure to n-hexane was only performed at higher dose (200 ppm + 22.33 mg/kg) by inhalation and oral routes. The dermal route was ignored in these experiments after the preliminary experiments showed that at the doses used in this study, the dermal route did not significantly contribute to the total internal dose (Gagné, 2008). The PBPK model of multi-route exposures used in this study could fit the results from *in vivo* experiments (Figure 2C). Same values of *Ko*: 0.003 and *Kx*: 0.007 from oral exposure study, were used for simulating multi-route exposures. Results from both single and multi-route exposures were used to determine if simultaneous exposure to n-hexane by inhalation and oral gavage could have an additive effect. A perfect additive effect between single and multi-route exposures was not observed, except during the first 30 min of post-exposure (Figure 2C).

Isooctane

This is the first study of PBPK modeling of isooctane following oral dosing and multi-route exposure. Previous experimental studies in rat reported neurotoxicity following inhalation exposure (Swann et al., 1974; U.S. EPA 2006), or development of a PBPK model of inhalation exposure to isooctane (El-Masri et al., 2009). El-Masri et al. (2009) reported biphasic diminution of isooctane with the rapid phase indicative of absorption, distribution and metabolism of the substance, and the slower phase corresponding to the elimination part. The pharmacokinetic data collected and modeled in the present study reflect the same pattern. Right after 2-hr exposure to 300 ppm (1.42 mg/l), the blood concentration of isooctane was 1.25 mg/l. However, 30 minutes later, it dropped by almost 64% to 0.45 mg/l. The blood concentration at 180, 210, 270 and 360 minutes dropped at a much lower rate in rats, post-exposure, in both the low and high dose groups. Overall, the PBPK model used in this study could simulate well the inhalation kinetics of isooctane in rats (Figure 3A).

Based on the experimental data for inhalation routes, the derived *Kel* values are comparable for both exposure concentrations (i.e., 0.36 *vs.* 0.35 hr^{-1} for 300 ppm or 1200 ppm), indicating that there is no metabolic saturation. Similarly, the *Kel* associated with the high oral dose (162 mg/kg) was 0.31 hr^{-1}, which is quite comparable. However, the *Kel* derived from the kinetic data for low oral dose (40 mg/kg) was indicative of faster elimination. Figure 3C shows additive effect between single and multi-exposures routes at high dose. At lower dose, the perfect additive effect starts about 100 mins post-exposure. Overall, there are good correlations between the PBPK models and the experimental data.

This study as well as that of others (Liao et al., 2007; Gagné, 2008) show that, internal dose of a substance after multiple routes of exposure is not always equal to the sum of the individual routes, although in the case of isooctane a good concordance was obtained. The PBPK model can be used as a reliable tool to simulate the effect of the single and multi-route on the internal doses.

Conclusion

Overall, the PBPK model could demonstrate the effect of the multi-route exposures on the internal doses in the case of two VOCs (n-hexane and isooctane). Investigations of the kinetic behavior of contaminants such as VOCs are important because of their volatile and lipophilic effect. VOCs can easily and abundantly contaminate underground water which will end up in drinking water and therefore human exposure to these chemicals. Having said that, humans are exposed not to a single chemical but to mixtures of chemicals every day (Konemann and Pieters, 1996). Hence, looking at the exposures as a mixture and not as a single chemical seems essential. Previous studies have shown the capacity of the PBPK model to simulate exposure to mixtures (Haddad et al., 2001, 2010). However, there is no study on the capacity of PBPK models to represent an exposure to a mixture by multiple routes. Future research should therefore focus to develop a PBPK model that can simulate both multi-route and multi-chemical exposures.

References

Ali N, Tardif R. 1999. Toxicokinetic modeling of the combined exposure to toluene and n-hexane in rats and humans. *J. Occup. Health* **41**: 95- 103.

Andelman J. 1985. Human exposures to volatile halogenated organic chemicals in indoor and outdoor air. *Environ. Health Perspect.* **62**:313- 318.

Bonnard N, Brondeau M T, Falcy M, Jargot D, Schneider O. 2008. CAS No. 110-54-3 n-hexane fiche toxicologique. http://www.inrs.fr/inrs-pub/inrs01.nsf/IntranetObject-accesParReference/INRS-FR/$FILE/fset.html/ [10 January 2011]

Brown H S, Bishop D R, Rowan C A. 1984. The role of skin absorption as a route of exposure for Volatile Organic Compounds (VOCs) in drinking water. *Am J Public Health* **74**: 479- 484.

Charbonneau M, Lock E A, Strasser J, Cox M G, Turner M J, Bus J S. 1987. 2,2,4-trimethylpentane-induced nephrotoxicity : I. metabolic disposition of TMP in male and female Fischer 344 rats. *Tox. Appl. Pharm.* **91**: 171-181.

Chen J S, Litzinger T A, Curran H J. 1998. The lean oxidation of iso-octane at elevated pressures. Edited by western states section combustion institute. https://e-reports-ext.llnl.gov/pdf/235217.pdf

Dennison J E, Andersen M E, Clewell H J, Yang R S H. 2004. Development of a physiologically based pharmacokinetic model for volatile fractions of gasoline using chemical lumping analysis. *Environ. Sci. Technol.* **38**: 5674–5681.

El-Masri H A, Dowd S, Pegram R A, Harrison R, Yavanhxay S J, Simmons J E, Evans A. 2009. Development of an inhalation physiologically based

pharmacokinetic (PBPK) model for 2, 2, 4-trimethylpentane (TMP) in male Long-Evans rats using gas uptake experiments. *Inhal. Toxicol.* **21**: 1176–1185.

EPA, Unites States Environmental Protection Agency. 1990. Citizen's guide to ground-water protection. Washington, DC.
http://www.epa.gov/ogwdw/sourcewater/pubs/guide_citguidegwp_1990.pdf

EPA, Unites States Environmental Protection Agency. 2007. Toxicological review of 2,2,4-trimethylpentane. Washington, DC.
http://www.epa.gov/iris/toxreviews/0614tr.pdf

EPA, Unites States Environmental Protection Agency. 2005. Toxicological Review of n-Hexane (CAS No. 110-54-3). Washington, DC.
http://www.epa.gov/iris/toxreviews/0486tr.pdf

Filser J G, Peter H, Bolt H M, Fedtke N. 1987. Pharmacokinetics of the neurotoxin n-hexane in rat and man. *Arch. Toxicol.* **60**: 77- 80.

Fowlie A J, Grasso P, Bridges J W. 1987. Renal and hepatic-lesions induced by 2,2,4-trimethylpentane. *J. Appl. Toxicol.* **7**: 335- 341.

Gagné M. 2008. *Caractérisation de la pharmacocinétique suite à l'exposition multivoie au toluène, au n-hexane et au cyclohexane chez le rat*. M.Sc. theses. Université de Montréal. Montreal, Canada.

Gargas M L, Burgess R J, Voisard D E, Cason G H, Andersen M E. 1989. Partition coefficients of low-molecular-weight volatile chemicals in various liquids and tissues. *Toxicol. Appl. Pharm.* **98**, 87- 99.

Haddad S, Charest-Tardif G, Tardif R, Krishnan K. 2000. Validation of a physiological modeling framework for simulating the toxicokinetics of chemicals in mixtures. *Toxicol Appl Pharmacol*.**167**:199-209.

Haddad S, Beliveau M, Tardif R, Krishnan K. 2001. A PBPK modeling based approach to account for interactions in the health risk assessment of chemical mixtures. *J. Toxicol. Sci.***63**: 125- 131.

Haddad S, Tardif R, Boyd J, Krishnan K. 2010. Physiologically-based modeling of pharmacokinetic interactions in chemical mixtures. *In quantitative modeling in toxicology*, Krishnan K and Andersen M E (eds). Wiley and Sons. 83- 105.

Hamelin G, Charest-Tardif G, Truchon G, Tardif R. 2005. Physiologically based modeling of n-hexane kinetics in humans following inhalation exposure at rest and under physical exertion: impact on free 2,5-hexanedione in urine and on n-hexane in alveolar air. *J. Occup. Environ. Hyg.* **2**: 86-97.

Konemann W H, Pieters M N. 1996. Confusion of concepts in mixture toxicology. *Food and Chem. Toxicol.* **34**: 1025-1031.

Krishnan K, Carrier R. 2008. Approaches for evaluating the relevance of multiroute exposures in establishing guideline values for drinking water contaminants. *J.Environ. Sci. Health Part C* **26**: 300-316.

Liao K H, Tan Y M, Clewell H J. 2007. Development of a screening approach to interpret human biomonitoring data on volatile organic compounds: reverse dosimetry on biomonitoring data for trichloroethylene. *Risk Anal.* **27**: 1223-1236.

Liu L, Zhou H. 2011. Investigation and assessment of volatile organic compounds in water sources in China. *Environ. Monit. Assess.* **17**: 825-836.

Lock E A, Strasser J Jr, Bus J S, Charbonneau M. 1993. Studies on the renal transport of trimethylpentanoic acid metabolites of 2,2,4- trimethylpentane in rat renal cortical slices. *J. Appl. Toxicol.* **13**: 291-296.

McKone T E. 1987. Human exposure to volatile organic compounds in household tap water: The indoor Inhalation pathway. *Environ. Sci. Technol.* **21**: 1194-1201.

McKone T E. 1999. The rise of exposure assessment among the risk sciences: an evaluation through case studies. *Inh. Toxicol.* **11**: 611-622.

Ono Y, Takeuchi Y, Hisanaga N. 1981. A comparative study on the toxicity of n-hexane and its isomers on the peripheral nerve. *Int. Arch. Occup. Environ. Health* **48**: 289-294.

Swann H E J, Kwon B K, Hogan G K, Snellings W M. 1974. Acute inhalation toxicology of volatile hydrocarbons. *Am. Ind. Hyg. Assoc. J.* **35**: 511-518.

Takeuchi Y, Ono Y, Hisanaga N. 1981. An experimental study on the combined effects of n-hexane and toluene on the peripheral nerve of the rat. *Brit. J. Ind. Med.* **38**:14-19.

Tardif R, Lapare S, Krishnan K, Brodeur J. 1993. A descriptive and mechanistic study of the interaction between toluene and xylene in humans. *Int. Arch. Occup. Environ. Health* **65**: S135- S137.

TESORO. *Material Safety data Sheet*. http://firstfuelbank.com/msds/Tesoro.pdf [1 February 2011]

Xu X, Mariano T M, Laskin J D, Weisel C P. 2002. Percutaneous absorption of trihalomethanes, haloacetic acids, and haloketones. *Toxicol. Appl. Pharmacol.* **184**: 19-26.

Table 1: Physiological parameters in rats used in PBPK models for n-hexane and isooctane.

Parameters	Values[1]
Alveolar ventilation rate (L/h/kg)	15
Cardiac output (L/h/kg)	15
Blood flow rate (fraction of cardiac output)	
Liver	0.25
Fat	0.09
Richly-perfused tissues	0.51
Slowly-perfused tissues	0.15
Volume (fraction of body weight)	
Liver	0.049
Fat	0.075
Richly-perfused tissues	0.05
Slowly-perfused tissues	0.72

[1] Ali and Tardif (1999)

Table 2: Partition coefficients and metabolic constants of n-hexane and isooctane in rats

Partition Coefficients	n-Hexane[1]	Isooctane[2]
Blood:air	2.29	2.7
Liver:air	5.2	10.7
Fat:air	159	320
Richly-perfused tissues:air	5.2	11.14
Slowly-perfused tissues:air	2.9	4.3
Metabolic Constants		
Vmax (mg/h/kg)	1.35	0.01[3]
Km (mg/L)	0.55[3]	1[3]

[1] Ali and Tardif (1999)

[2] El-Masri et al (2008)

[3] Fitted values

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[adipose tissue; FAT; richly-perfused tissues; RPT, poorly-perfused tissues; PPT; arterial blood (ART); venous blood (VEN)].

Figure 2A: Inhalation exposure: comparison of experimental data (symbols) and PBPK model simulation (black lines) of venous blood concentration (mg/l) versus time (min) following 2-hr exposure to n-hexane (50 ppm and 200 ppm) in rats.

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Figure 2C: Multiple routes of exposure: comparison of experimental data (filed squares) and PBPK model simulations (black lines) of venous blood concentration (mg/l) versus time (min) of single exposure by oral gavage (22.33 mg/kg) followed by 2-hr inhalation exposure (200 ppm) to n-hexane. The 'x' symbol indicates the sum of the simple inhalation and oral gavage.

Figure 3A: Inhalation exposure: comparison of experimental data (symbols) and PBPK model simulations (black lines) of venous blood concentration (mg/l) versus time (min) following 2-hr exposure to isooctane (300 ppm, 1200 ppm) in rats.

Figure 3B: Oral gavage exposure: comparison of experimental data (symbols) and PBPK model simulations (black lines) of venous blood concentration (mg/l)

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Figure 3C: Multiple routes of exposure: comparison of experimental data (symbols) and PBPK model simulations (black lines) of venous blood concentration (mg/l) versus time (min) of single exposure by oral gavage (40 mg/kg, 162 mg/kg) followed by 2-hr inhalation exposure (300 ppm, 1200 ppm) to isooctane. The 'x' and '+' symbols indicate the sum of the simple inhalation and oral gavage for the low dose and high dose, respectively.

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Figure 1:

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Figure 2B:

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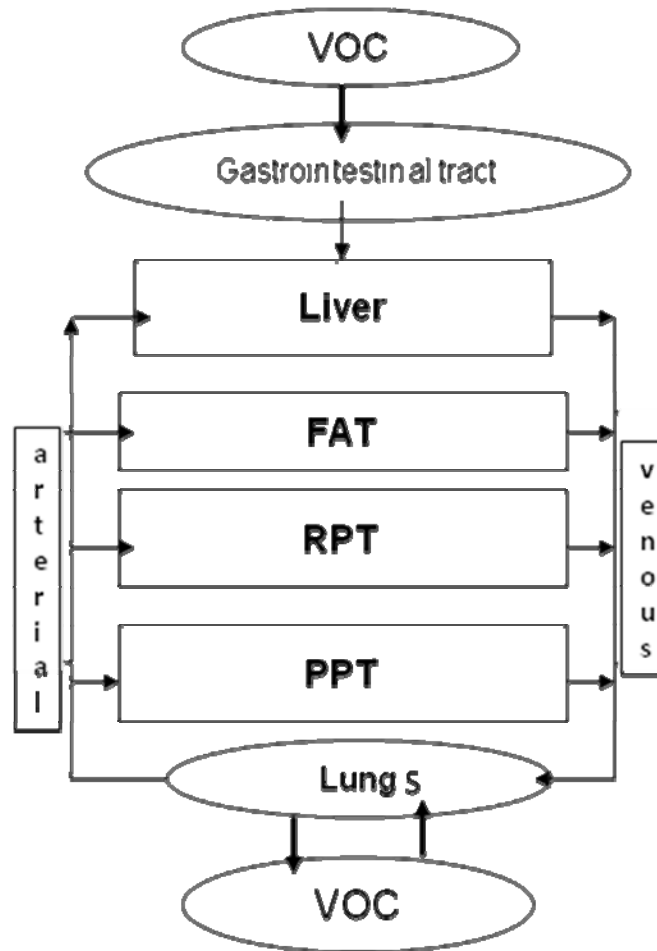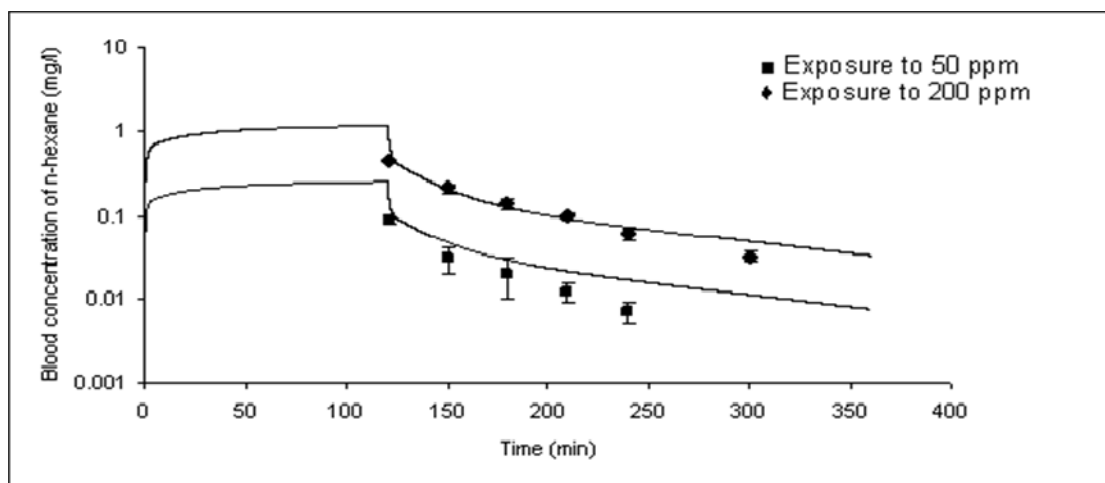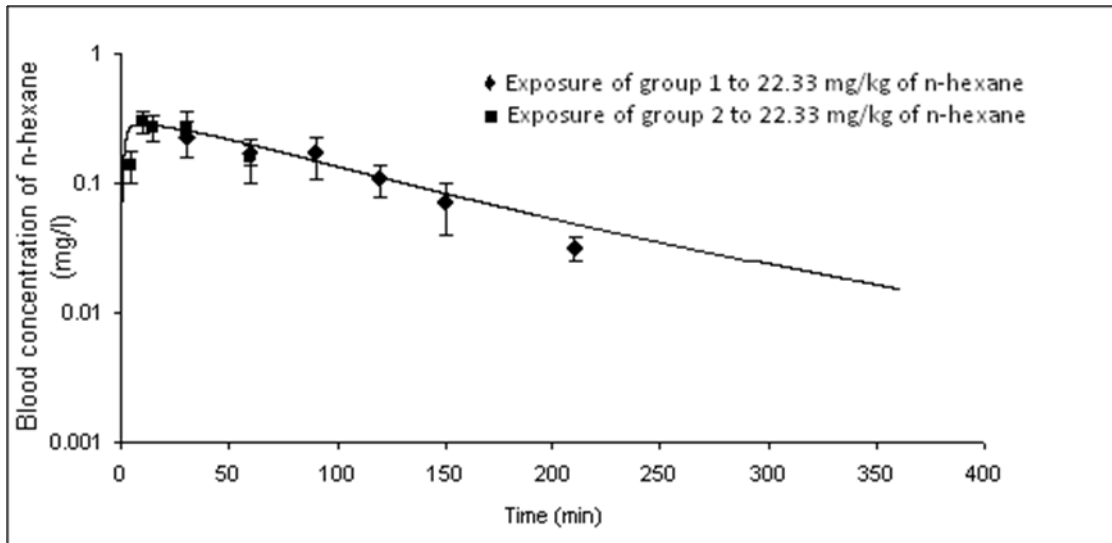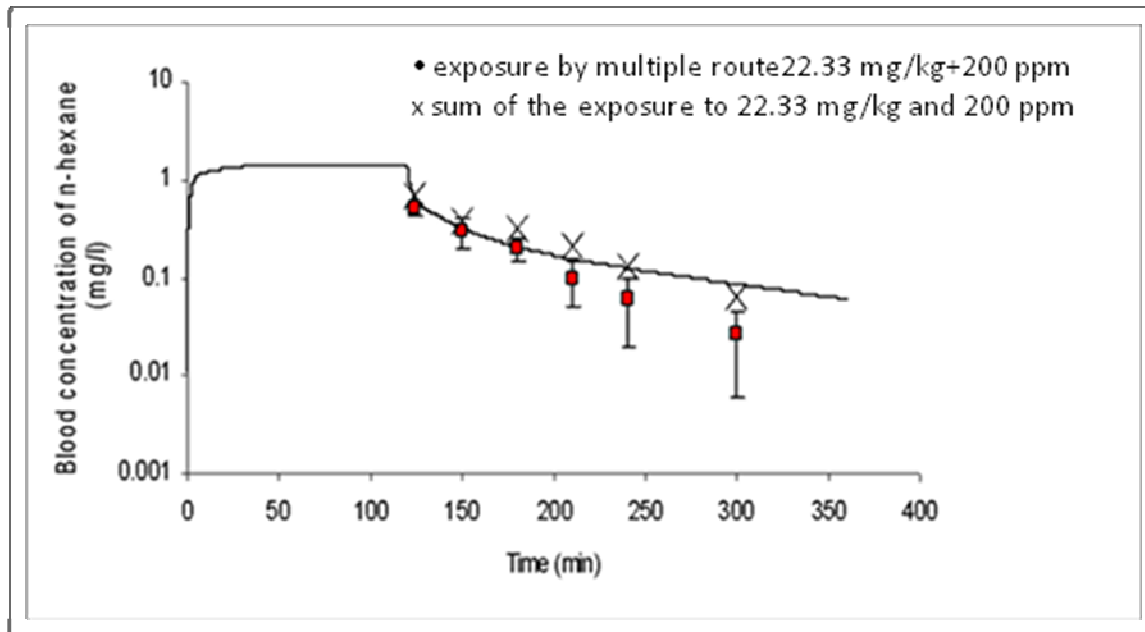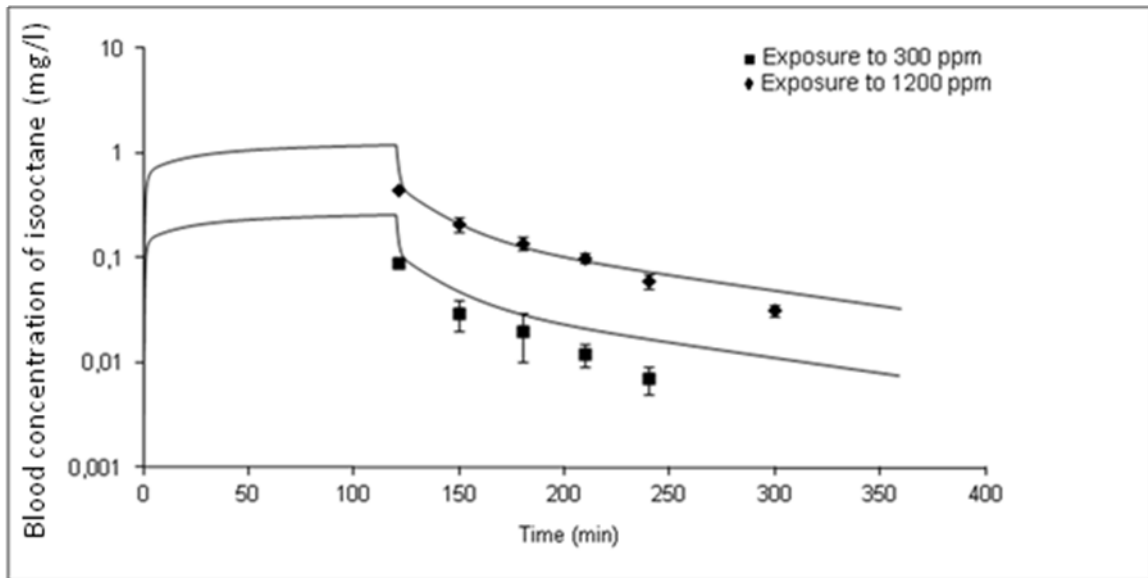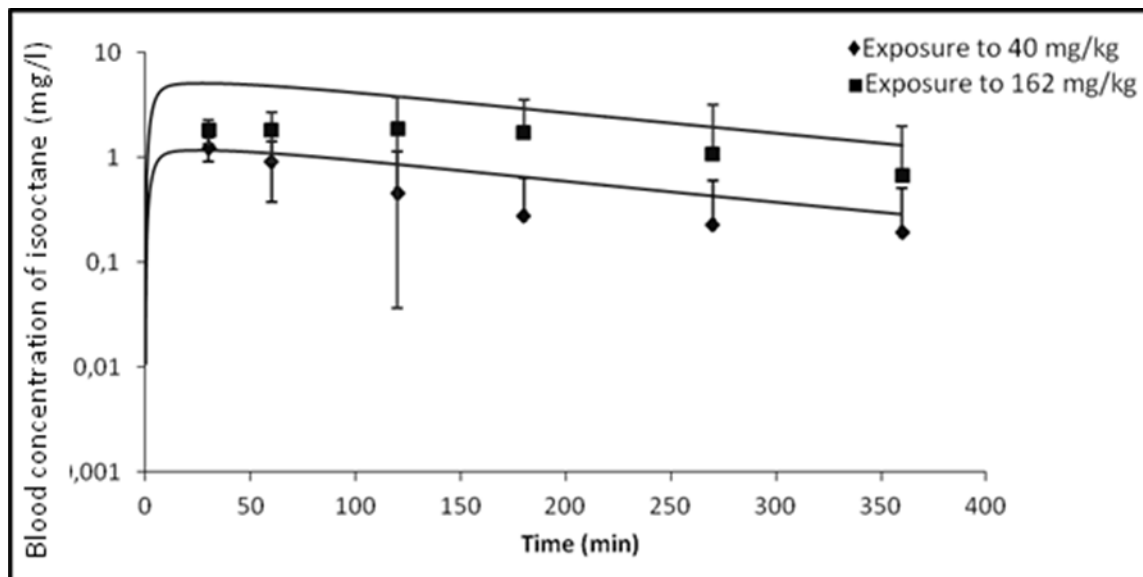
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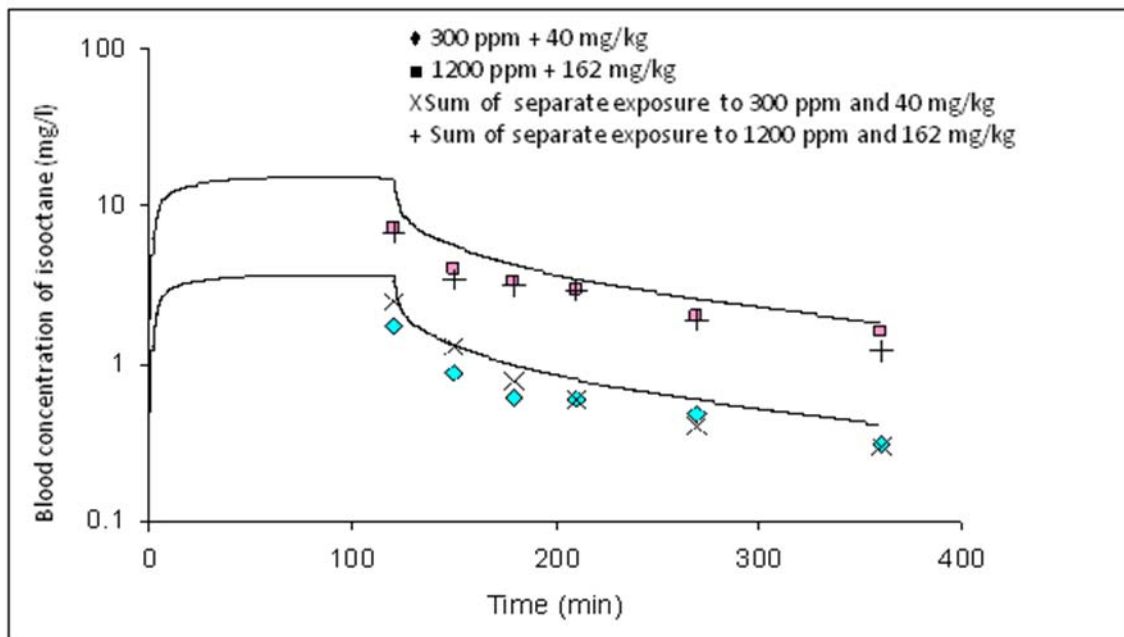
Manuscript prepared for submission to: "Journal of Toxicology and Environmental Health, Part A"

PBPK modeling of the aggregate and cumulative exposures of rats to toluene, n-hexane, cyclohexane and isooctane

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Running Title: PBPK model of aggregate exposure to VOC mixture

Abstract:

Mixture risk assessments focusing on both aggregate and cumulative exposures can be improved with an understanding of the change in internal dose. The modeling of the internal dose and pharmacokinetics of hydrocarbons following cumulative and aggregate exposures has not yet been undertaken. The objective of this study was to develop a PBPK model to simulate the mixed exposure to toluene, n-hexane, cyclohexane and isooctane (chosen to represent chemical classes in large volume petroleum products) by inhalation and oral routes in rats. PBPK models of individual chemicals were first developed, and were then interconnected using the metabolic interaction terms. The capacity of the PBPK model was evaluated by comparing it with experimental data. These data were collected in groups of male Sprague-Dawley rats (n=5) exposed to each substance by inhalation (50 ppm of toluene and n-hexane; 300 ppm of cyclohexane and isooctane) or oral gavage (8.3, 5.5, 27.9, and 41.27 mg/kg, respectively, for toluene, n-hexane, cyclohexane and isooctane) or to multi-products given by both routes together at similar concentrations. The AUCs of experimental data were 157.25, 18.77, 117.57 and 176.54 mg/L.min whereas the AUC values derived from the mixture and multi-route PBPK model were: 121.73, 21.91, 19.65 and 170.54 mg/L.min respectively, for toluene, n-hexane, cyclohexane and isooctane. Overall, the PBPK model developed in this study is a useful tool for simulating kinetics of hydrocarbon mixture, by accounting for the saturable metabolism and the interactive effects during aggregate and cumulative exposures.

Keywords: Mixtures, PBPK modeling, hydrocarbons

Introduction

Risk assessment methods for chemical mixtures in contaminated water continue to evolve (U.S. EPA, 2005; Simmons, 1995; Teuschler and Hertzberg, 1995; Yang et al., 1995). Underground water is the source of drinking water for more than 75% of cities in the United States (U.S. EPA, 1991). The release of petroleum products from damaged underground gas station containers is an important cause of underground water contamination (U.S. EPA, 1990; El-Kholy et al., 2009). The severity of the problem depends, first, upon the frequency and quantity of petroleum products released from these sources. In fact, it has been estimated that about 25% of five to six million storage tanks in the United States that contain fuel oil or gasoline are leaking (Shehata, 1985; U.S. EPA, 1990; Harris et al., 2008). Thus, the leakage as little as one drop of fuel per second could result in a total of 400 gallons of fuel released per year potentially contaminating underground water (Harris et al., 2008). Furthermore, the nature and the severity of the health consequences depend upon the hazardous nature of these petroleum products.

Petroleum products contain large amounts of volatile organic compounds (VOC) (Labranche and Collins, 1996), some of which are added to gasoline in order to increase the burning efficiency of automobile engines (Pérez-Pavón et al., 2007). VOCs have low water solubility and high vapor pressure (Liu and Zhou, 2011) and therefore possess a marked capacity to be released from water to air (McKone, 1999). As a result, human exposure to these chemicals occurs via inhalation during and after bathing and showering simply by inhaling the indoor air (Mckone, 1999).

For many years, the oral route of exposure to contaminated drinking water was considered alone in the risk assessment process (McKone, 1999; reviewed in Krishnan and Carrier, 2008). Additional studies demonstrated the importance of the inhalation route to the total exposure of VOCs during household use of

drinking water (Andelman, 1985; McKone, 1987; Weisel and Jo, 1996). The importance of the skin as an additional exposure route in the case of water contamination with VOCs has also been documented (Brown et al., 1984; McKone, 1999; Xu et al., 2002; Krishnan and Carrier, 2008). Therefore, there is an increasing focus on the consideration for aggregate or multiple exposures to these contaminants in the environment (Newill, 1989; FSA, 2002; Rider et al., 2010).

The presence of a great variety of components and different blends of them, and the numerous different possible interactions between them, that can modify toxic effects, renders toxicity studies of petroleum extremely challenging (Dennison et al., 2004) or unrealistic for animal studies, and unethical for human studies. An alternative approach is to use mathematical modeling to predict the interaction in various species, scenarios and doses (Haddad et al., 2010). Physiologically based pharmacokinetic (PBPK) modeling is a well-established tool that can be used to predict the pharmacokinetics of chemical compounds for different exposure scenarios and therefore assess the risk of exposure to these chemicals. PBPK models have been used for predicting kinetics in different species (i.e., animal or human), exposure routes, acute and chronic effects, and doses (Haddad et al., 2010). Haddad et al. (2007) used PBPK modeling to evaluate the importance of multi-route exposure (i.e., inhalation, gavage and dermal) in risk assessment of VOCs in the case of water contamination.

In the current study, four VOCs were chosen to represent different families of hydrocarbons found in petroleum products. Toluene, n-hexane, cyclohexane and isooctane represent the aromatic hydrocarbon family, alkane class, cycloalkane class and branched-chain paraffin class, respectively (Chen et al., 1998). The toxic effects of each of these chemicals have already been documented in the literature. Toluene affects the central nervous system (CNS), whereas the effects of n-hexane are on the peripheral nervous system (Ali and Tardif, 1999; ATSDR, 1993). Isooctane is known primarily as a nephrotoxin in male rats (Fowlie et al.,

1987; Lock et al., 1993) and has also been shown to damage the liver in both male and female rats (Fowlie et al., 1987). The neurotoxicity of isooctane has also been reported in experimental animals (Swann et al., 1974; El-Masri et al., 2009). Cyclohexane is a hydrocarbon that rather affects the CNS (Hissink et al., 2009).

The pharmacokinetics of each of these VOCs have already been studied separately using PBPK models: toluene and n-hexane by Ali and Tardif (1999); cyclohexane by Perico et al. (1999) and Hissink et al. (2009); and isooctane by El-Masri et al. (2009). These studies have evaluated the effect of exposure to one of these substances or a mixture of two by a single exposure route (i.e., inhalation, oral or dermal). However, no study has so far assessed the ability of the PBPK model to simulate exposure to a mixture of these hydrocarbons by multiple routes (i.e., inhalation + oral).

The objective of this study was therefore to develop a PBPK model aimed at simulating the kinetics during simultaneous exposure to a mixture of toluene, n-hexane, cyclohexane and isooctane by inhalation and gavage.

Material and Methods

The experimental data on the time courses of toluene, n-hexane or isooctane following single and multiple routes exposure have already been studied (Gagné, 2008). Therefore, in the current study only the pharmacokinetics of cyclohexane was evaluated before proceeding with the construction of the multi-chemical PBPK models. Accordingly, this study involved the conduct of *in vivo* experiments in conjunction with PBPK modeling. During the *in vivo* experiments, groups of five male Sprague-Dawley rats were exposed to separate high dose and low dose of cyclohexane by single route and multiple routes of exposure (inhalation and gavage). Another group was exposed to a mixture of the four substances by multiple routes. Subsequently, PBPK models of the individual

chemicals were interconnected using the metabolic interaction terms in order to simulate the kinetics of cumulative exposure to both single product and multiple products. Finally, the comparison of the models for single products and mixtures of chemicals by multiple routes of exposure was used to determine the impact of mixed exposure scenario on the internal dose of each substance.

Chemicals

Toluene 99.8%, n-hexane 99%, cyclohexane (anhydrous 99.5%) and isooctane (anhydrous 99.8%) were obtained from Aldrich chemicals (Milwaukee, WI).

Animals

Adult male Sprague-Dawley rats (240-260 g) were obtained from Charles-River Canada (St-Constant, Québec). They were housed in temperature-controlled cages in groups of two or three per cage with a 12-h light–dark cycle. A minimum of one week acclimatization period was provided. They were given food and water *ad libitum* until the day of experiment. On the day of experiment they were placed in individual cages and fasted during the experiment.

Exposure scheme

Inhalation exposure

Groups of male Sprague-Dawley rats (n=5) were separately exposed to low and high concentrations of cyclohexane by inhalation (300 ppm and 1200 ppm; 2-hr) in a closed-atmosphere exposure system (Haddad et al., 2000). The concentrations were monitored using automatic injection of 0.25 ml air samples into a gas chromatograph (HP 5890) equipped with a flame ionization detector (FID). Helium was used as a carrier gas, at a rate of 5.4 ml/min.

Oral exposure

Groups of rats (n=5) were given a single low dose (27.9 mg/kg) and high dose (108.9 mg/kg) of cyclohexane, diluted in Alkamuls[®] (10%). Tem ml of the solution was administrated by gavage.

Multi-route exposure

Multi-route exposure to cyclohexane was conducted by exposing groups of five rats simultaneously by inhalation and oral routes. It was performed at low dose (300 ppm and 27.9 mg/kg) and high dose (1200 ppm and 108.9 mg/kg). The inhalation exposure was performed right after oral exposure in a closed-chamber system for 2 hr.

Mixture exposure

Exposure to the mixture (toluene + n-hexane + cyclohexane + isooctane) by multi-route was performed only at the lower doses. Animals were exposed to the mixture of toluene n-hexane, cyclohexane and isooctane first by oral gavage to a single dose of 8.30, 5.53, 27.9 and 41.27 mg/kg, respectively, and immediately followed by inhalation (50, 50, 300 and 300 ppm, respectively) for two hours.

Analysis of blood concentration

Blood samples (25-200 µl) were collected by performing a small incision on tail veins of rats. The concentration of unchanged substances in blood was quantified using a head-space gas chromatography method (Tardif et al., 1993). After single oral exposure, blood samples were taken from animals at 0.5, 1, 1.5, 2, 2.5 and 3 hr post-exposure. For the inhalation and multi-route exposure, blood samples were taken at 2, 2.5, 3, 3.5, 4 and 5 hr post- exposure.

PBPK modeling

Model representation

The four compartmental PBPK models to simulate the kinetics of each of the four VOCs consist of: liver (the metabolizing tissue), fat, richly-perfused tissues and slowly-perfused tissues, all connected by systemic blood circulation and gas exchange in the lung. The PBPK models were based on the Haddad et al. (2001). The new model was developed to simulate the exposure to cyclohexane for either inhalation or oral exposure. In the model for oral gavage and mixture exposure (toluene, n-hexane, cyclohexane and isooctane), the rate of chemicals absorbed by the gastro-intestinal tract (A_{ab}) were added to the liver. In addition, in the case of exposure to the mixture (Figure 1), any interactions or inhibitions of the metabolism between the four chemicals were assumed to happen in the liver (Haddad et al., 2001). Ki_{ab} was considered as the competitive inhibition constant of the metabolism of the chemical *a* by chemical *b*.

The metabolism was assumed to be a saturable process and was described by *Vmax* (maximum velocity) and *Km* (Michaelis-Menten affinity constant) (Krishnan and Peyret, 2009). The rate of change in the amount of chemical (*dAt/dt*) was estimated as:

$$\frac{dAt}{dt} = Ql\ (Ca - Cv) - \frac{Cv\ x\ V\max}{Cv + Km} \qquad [1]$$

where:

Ql: rate of blood flow through the liver

Ca: concentration of solvent in the arterial blood

Cv: concentration of solvent in the blood leaving the liver

For simulating oral and multi-route exposures, the amount absorbed per unit time was added to the above equation as follow (Haddad et al., 2001):

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$$\frac{dAt}{dt} = Ql\,(Ca\text{-}Cv) - \frac{Cv\;x\;V\max}{Cv + Km} + (Aab)$$ [2]

where

A_{ab}: rate of chemical being absorbed during the oral exposure (mg/h), calculated as:

$A_{ab} = Kx \times Q_t$ [3]

Where

Kx: absorption constant of a chemical by the stomach (min^{-1})

$Q_{t:}$ remaining quantity of chemical in the stomach (mg)

Therefore,

$Q_t = Q - (Aab \times ta) - (Ko \times ta \times Aab)$ [4]

where

Ko: stomach to intestine transfer constant (min^{-1})

ta: interval of time (min)

The rate of metabolism (*ram*) for each chemical after exposure to the mixture was calculated as for Haddad et al. (2000). For example, for toluene, *ram* was calculated as follows:

$$ramT = \frac{V\max T\;x\;CvT}{\left[KmT(1 + \dfrac{CvH}{KiHT} + \dfrac{CvC}{KiCT} + \dfrac{CvI}{KiIT})\right] + CvT}$$ [5]

where:

T (toluene), H (n-hexane), C (cyclohexane) and I (isooctane)

In general:

ramA: rate of the metabolized quantity of chemical *A*

$Ki_{AB:}$ Competitive inhibition constant of the metabolism of the chemical *A* by chemical *B*

Model parameterization

The physiological parameters of the rat were obtained from the literature (Tardif et al., 1997). Physicochemical (i.e., partition coefficients) and biochemical parameters of toluene and n-hexane were taken from Ali and Tardif (1999), of cyclohexane from Hissink et al. (2009) and of isooctane from El-Masri et al. (2009). The PBPK model of inhalation was used to determine the values of Vmax= 0.005 mg/min and Km = 1 mg/l for isooctane which were then used in the mixture model. The metabolism was considered competitive; therefore, the amount of Ki_{ab} of each chemical was equal to the *Km* of the same chemical. The absorption rate by the stomach (*Kx*) and the rate of substance transfer from the stomach to the intestine (*Ko*) of each chemical were fitted first in the oral gavage model and later those values were used in the mixture PBPK model.

Statistical analysis and model evaluation

The statistical analyses were performed with SPSS for Windows (v15.0.0, Chicago, IL, USA). In order to validate the models, data-derived area under the blood concentration *vs.* time curve (AUC; mg/L x min) was calculated for every exposure scenario (inhalation, gavage and multi-route) and dose (low and high doses) and compared with those obtained using the PBPK model. The results were used to determine the degree of discrepancy between PBPK models and experimental data.

Results

A: Cyclohexane

1. Inhalation and oral exposure

The PBPK model simulations of inhalation kinetics of cyclohexane was in agreement with the experimental data and estimated well the maximum blood concentrations (Cmax) for both low exposure (300 ppm; 2 hr) and high exposure (1200 ppm; 2 hr) groups (Figure 2A). However, it seemed that after reaching the Cmax, the model underestimated the experimental data for both inhalation and oral exposure conditions.

Results from the cyclohexane PBPK model after a single low dose (27.9 mg/kg) and high dose (108.9 mg/kg) via oral route concurred with the experimental data (Figure 2B). The values of Kx and Ko obtained by fitting to the model were 0.5 min^{-1} and 0.3 min^{-1}, respectively. The area under the curve (AUC) from 0.5 hr to 5 hr following low oral dose was 21.4 mg/L.min for the PBPK model vs 24.6 mg/L.min for the experimental data. For high oral dose of 108.9 mg/kg, the AUCs were 308.3 mg/L.min and 112.2 mg/L.min, respectively, for the PBPK model and experimental data.

2. Multi-route exposure

Although the PBPK model of cyclohexane predicted the Cmax for both low dose (27.9 mg/kg and 300 ppm) and high dose (108.9 mg/kg and 1200 ppm), after this point, model predictions underestimated the experimental data. The model-simulated AUC for low dose multi-route exposure to cyclohexane was 16.63 mg/l.min whereas the data-derived AUC was 112.04 mg/l.min. However, no modification of the physiological and physiochemical parameters of the PBPK model of cyclohexane was performed to improve the fit, because cyclohexane did not significantly interfere or alter the kinetics of other substances in the

mixture. Further work to improve the predictive capacity of the cyclohexane model was beyond the goals of the present study.

B: Mixture/multi-route exposure

The capacity of the PBPK model to simulate multi-route exposure to a mixture in rats was evaluated by comparing the unchanged blood concentrations of each chemical obtained with the PBPK model to the respective experimental data. Toluene, n-hexane, and isooctane showed a reasonable fit (Figures 3A to 3C). However, in the case of cyclohexane, only the Cmax could fit (Figure 3D). The AUC (2-6 hr) of cyclohexane based on the experimental data (159.58 mg/l.min) differed from simulated value (19.55 mg/l.min), by the same factor (~7) during multiple chemicals and simple chemical exposures. Table 2 shows comparisons between the AUCs of the PBPK model for each substance and experimental data after exposure to the mixture by multiple routes (low doses).

Another important result is the demonstration of the effects of mixture exposure by multi-route on the internal dose for each chemical compared to the effects produced by single product multi-route exposures. The results show that blood concentration of toluene and hexane increases during mixture multi-route exposure compared to the single multi-route exposure. However, the extent of change in kinetics for isooctane was not marked. For cyclohexane, examining the experimental data of exposure to either single product or mixture, no change was observed.

C: Sensitivity analysis

Results for cylcohexane showed that whenever inhalation exposure was involved (i.e., simple inhalation, multiple routes), the existing PBPK model did not correspond well with the experimental data. Therefore, a sensitivity test was performed (i.e., change of each parameter by 1% while the rest was kept

constant) (Table 3). These analyses showed that the blood: air partition coefficient (Pb) was the most sensitive parameter. By changing Pb from 1.3 (Hissink et al. 2009) to 7, the model correlated well with the experimental data in all three cases (i.e., cyclohexane inhalation, multi-route exposure to cyclohexane alone, and multi-route exposure to a mixture including cyclohexane) (Figure 7A-D). but due to (i) lack of a mechanistic basis to change Pb of cyclohexane and (ii) its inability to alter kinetics of other hydrocarbons evaluated in this study, no change to the cyclohexane model or its parameters were attempted.

Discussion

This is the first study to show the capacity of a PBPK model to simulate the exposure to a mixture of hydrocarbons simultaneously by inhalation and oral gavage. Although dermal route was not considered in the multi-route experiments, it did not seem to have an important impact on the outcome since at the chosen doses it was not one of the principal absorption routes (Table 1). In fact, preliminary experiments on rats showed that under the experimental conditions of this study, dermal route did not significantly contribute to the absorption of any of the four substances.

The PBPK models, as well as the experimental data, show that exposure of rats to the mixture compared to the single chemical (both by multi-route exposures) results in the inhibition of the metabolism of toluene and n-hexane but no difference in kinetics of isooctane. The PBPK model of cyclohexane could not fit the experimental data (except for Cmax). However, experimental data from cyclohexane did not show significant impact or interaction towards the other components of the mixture.

A brief summary of the metabolism pathway for each of the substances can help a better understanding of the possible interactions between them. The cytochromes (CYP) mammalian hepatics microsomal are responsible for the biotransformation of a large number of xenobiotic substances. CYP enzymes need two electrons and two protons to catalyze the oxidation of the substance. Therefore after the substance binds to CYP, the first electron is transferred by NADPH-CYP450 reductase (CPR). However, the second electron can be delivered from either CPR or CYTb_5 (Denisov et al. 2005). Toluene is metabolized by CYP2E1 and CYP2B1/B2 in the liver (Nakajima et al. 1997).

n-Hexane is also mainly metabolized by CYP2B1 in the liver (Nakajima et al. 1997; Fabioano et al. 2010). Therefore toluene and n-hexane are likely to

compete for the same cytochrome CYP2B1. In fact, it has been shown that respiratory exposure to the mixture of toluene and n-hexane results in a reduced toxic effect compared to an exposure to n-hexane alone (Takeuchi et al. 1981). Takeuchi et al. (1981) showed that exposure to a mixture of 1000 ppm of toluene and 1000 ppm n-hexane in rats, 12 hours a day for 16 weeks, resulted in a reduction of the peripheral neurotoxic effects caused by an exposure to 1000 ppm of n-hexane alone. The interaction in the metabolism has also been shown at lower doses and shorter exposure durations. Exposure to the mixture of 300 ppm toluene and 100 ppm n-hexane, 12 hours a day for four weeks, showed a reduction by up to 75% of the urinary 2,5-hexanedione (n-hexane metabolite) and a 28% reduction of the urinary hippuric acid (toluene metabolite) (Ali and Tardif, 1999).

Consistent with the available information, the current study also shows inhibition of the metabolism of both chemicals regardless of the presence of two other chemicals (isooctane and cyclohexane) and multi-route exposure (Figures 4A-B and 5A-B). However, the effects of cyclohexane and isooctane cannot be underestimated. Cyclohexane is metabolized into cyclohexanol in the liver by CYP2B4 (Zhang et al. 2007). Evidence suggests that cyclohexanol induces CYP2E1, CYP2B1/B2 (Espinosa-Aguirre et al. 1997). Nonetheless, the experimental data in this study showed that metabolism of cyclohexane after two hours of exposure in both simple and multi-route exposure was not considerable. Suspecting that cyclohexane could not have had an important effect on the inhibition of the metabolism of either n-hexane or toluene. In order to prove that, the PBPK model of exposure to the mixture of four substances was compared to exposure to a mixture without cyclohexane. The result showed that after removing cyclohexane from the mixture the metabolism rate and kinetics of both n-hexane and toluene were unchanged (Figures 4A-B).

Furthermore, in order to show the importance of isooctane in the mixture, the PBPK model was adapted to simulate the exposure to a mixture without it. By

comparing the PBPK models, a slight change in the internal dose of toluene was noticed (Figure 5A). These results, together with evidence from toluene and n-hexane mixture studies (Ali and Tardif, 1999), suggest that the inhibition of toluene after a cumulative exposure to a mixture is not caused by cyclohexane or isooctane, but by n-hexane. These assertions are supported by the sensitivity analyses that showed that changes in the input parameters of cyclohexane or isooctane have no important effect on either toluene or n-hexane (Table 4).

The PBPK model adapted in this study to cyclohexane could reasonably fit experimental data from oral gavage but not experiments related to inhalation exposures (i.e., simple and multi-route). The sensitivity test showed that among all the parameters used in the PBPK model of cyclohexane, blood:air partition coefficient is the most sensitive one (Table 3). In fact, by changing this parameter from 1.3 to 7, the PBPK model could fit the experimental data each time (Figures 7A-D). Interestingly, this change made no difference in the PBPK simulation of the kinetics of other chemicals in the mixture (i.e., toluene, n-hexane and isooctane).

In a different attempt to show the reliability of the adapted PBPK model of cyclohexane, the experimental data from a recent study (Hissink al. 2009) were compared to the model. In fact, our PBPK model could fit the data from the other study (Figure 6). The difference between the two studies was in exposure times (2 hr in our study *vs* 8 hr in the study by Hissink et al. (2009)). In fact other studies have shown that, because of the chair conformation of cyclohexane, the activation energy required to break the C-H bond is relatively high (418 Kj.mole^{-1}) and this would explain the slow metabolism rate of cyclohexane in mammalians compared to bacterial CYT P450 system (Lewis, 2001). Interestingly, in an *in-vitro* experiment, Zhang et al. (2007) could determine the possibility of a competition on the binding site of CYP2B4 between CPR and CYTb_5. They reported that increase of the molar ratio of CYTb_5 in a 3-substrate experiment (i.e., CYP2B4 : CPR : CYTb_5 ; 1 : 1 < 1) will cause a decrease of oxidation of

cyclohexane. The authors explained this outcome by replacement of CPR by CYTb_5 (due to competition between them), which would result in the inhibition of the first step in the catalytic cycle of oxidation of cyclohexane.

Isooctane on the other hand, demonstrated no difference in metabolism between aggregate and cumulative exposures, as shown both by the PBPK model and experimental data. There is no study to support the effect of isooctane on the metabolism of other substances.

Conclusion

The importance of exposure to a mixture by multiple routes has been widely accepted by scientists and governmental regulatory agencies. However, it is very difficult to consider the many possible combinations of chemicals and exposure routes in the risk assessment process. Our study shows, for the first time, that PBPK models are reliable tools to overcome, at least in part, the challenges involved in studying multi-route mixture exposure. It also demonstrates that there are not necessarily chemical interactions between all VOCs. Therefore, the study of mixture and possible interactions between substances while considering the multiple routes of exposure is important in regulatory evaluations. This study shows that PBPK models can be used to assess such complex scenarios in rats. Further studies are required to elucidate the possibility of extrapolating these animal models to humans, which could then be used in mixture risk assessment.

References

American Conference of Industrial Hygienists, ACGIH. 1999. TLVs and BEIs. In: *Threshold Limit Values for chemical substances and physical agents and biological exposure indices*. Cincinnati, OH.

The Agency for Toxic Substances and Disease Registry, ATSDR. 1993. http://www.atsdr.cdc.gov/csem/toluene/docs/toluene.pdf. (10 January 2011).

Ali, N., and Tardif, R. 1999. Toxicokinetic modeling of the combined exposure to toluene and n-hexane in rats and humans. *J. Occup. Health* 41: 95-103.

Andelman, J. 1985. Human exposures to volatile halogenated organic chemicals in indoor and outdoor air. *Environ. Health Perspect.* 62: 313-18.

Bonnard, N., Falcy, M., Jargot, D., and Schneider, O. 2008. CAS No. 110-54-3 n-Hexane Fiche Toxicologique. http://www.inrs.fr/inrs-pub/inrs01.nsf/IntranetObject-accesParReference/INRS-FR/$FILE/fset.html. (10 January 2011).

Brown, H. S., Bishop, D. R., and Rowan, C. A. 1984. The role of skin absorption as a route of exposure for Volatile Organic Compounds (VOCs) in drinking water. *Am. J. Public Health* 74: 479-84.

Charbonneau, M., Lock, E. A., Strasser, J., Cox, M. G., Turner, M. J., and Bus, J. S. 1987. 2,2,4-trimethylpentane-Induced nephrotoxicity : I. metabolic disposition of TMP in male and female Fischer 344 rats. *Toxicol. Appl. Pharmacol.* 91: 171-81.

Chen, J. S., Litzinger, T. A., and Curran, H. J. 1998. The lean oxidation of iso-octane at elevated pressures. https://e-reports-ext.llnl.gov/pdf/235217.pdf. (14 February 2011).

Dahl, A. 1989. The fate of inhaled octane and the nephrotoxicant, isooctane, in rats. *Toxicol. Appl. Pharmacol.* 100: 334-41.

Denisov, I. G., Makris, T. M., Sligar, S. G., and Schlichting, I. 2005. Structure and chemistry of cytochrome P450. *Chem. Rev.* 105: 2253-77.

Dennison, J. E., Andersen, M. E., Clewell, H. J., and Yang, R. S. H. 2004. Development of a physiologically based pharmacokinetic model for volatile fractions of gasoline using chemical lumping analysis. *Environ. Sci. Technol.* 38: 5674–81.

El-Kholy, S., Sabry, T. and Alsalamh, I. 2009. Environmental contamination risks due to Leaking Underground Fuel Tanks (LUFT) of gas stations in ALQassim region, Saudi Arabia. Second international conference on environmental and computer science. Dubai, UAE. http://ieeexplore.ieee.org/stamp/stamp.jsp?tp=&arnumber=5383565. (28 March 2011).

El-Masri, H. A., Dowd, S., Pegram, R. A., Harrison, R., Yavanhxay, S. J., Simmons, J. E., and Evans, A. 2009. Development of an inhalation physiologically based pharmacokinetic (PBPK) Model for 2,2,4-trimethylpentane (TMP) in male Long-Evans rats using gas uptake experiments. *Inhal. Toxicol.* 21: 1176–85.

Espinosa-Aguirre, J.J., Rubio, J., Lopez, I., Nosti, R., and Asteinza, J. 1997. Characterization of the CYP isozyme profile induced by cyclohexanol. *Mutagen.* 12: 159-62.

Fabioano H. M., Lepera, J. S., Marquws, M. P., Borrali, V. B., and Lanchote, V. L. 2010. Influence of n-hexane inhalation on the enantioselective pharmacokinetics and metabolism of verapamil in rats. *Chirality* 22: 29–34.

Faust, R. A. 1994. "Toxicity summary for toluene." http://cira.ornl.gov/documents/TOLUENE.pdf (7January 2011).

Fowlie, A. J., Grasso, P., and Bridges, J. W. 1987. Renal and hepatic-lesions induced by 2,2,4-trimethylpentane. *J. Appl. Toxicol.* 7: 335-41.

Gagné, M. 2008. Caractérisation de la pharmacocinétique suite à l'exposition multivoie au toluène, au n-hexane et au cyclohexane chez le rat. M.Sc. thesis. Université de Montréal. Montreal, Canada

Gargas, M. L., Burgess, R. J., Voisard, D. E., Cason, G. H., and Andersen, M. E. 1989. Partition coefficients of low-molecular-weight volatile chemicals in various liquids and tissues. *Toxicol. Appl. Pharmacol.* 98: 87–99.

Haddad S, Charest-Tardif G, Tardif R, Krishnan K. 2000. Validation of a physiological modeling framework for simulating the toxicokinetics of chemicals in mixtures. *Toxicol Appl Pharmacol*. 167:199-209.

Haddad, S., Beliveau, M., Tardif, R., and Krishnan, K. 2001. A PBPK modeling based approach to account for interactions in the health risk assessment of chemical mixtures. *Toxicol. Sci.* 63: 125- 31.

Haddad, S., Charest-Tardif, G., and Tardif, R. 2007. Development of physiologically based toxicokinetic models for improving the human indoor exposure assessment to water contaminants: trichloroethylene and trihalomethanes. *J. Toxicol. Environ. Health, Part A* 69: 2095–136.

Haddad, S., Tardif, R., Boyd, J., and Krishnan, K. 2010. Physiologically based pharmacokinetic interactions in chemical mixtures. In: *Quantitive Modeling in Toxicology*. Edited by Krishnan, K. and Andersen, M. E. Chichester, West Sussex, UK: John Wiley & Sons Ltd.

Harris, B. L., Hoffman, D. W., and Mazac, D. W. Jr. 2008. Reducing the risk of ground water contamination by improving petroleum product storage. http://texashelp.tamu.edu/005-agriculture/pdf/005-22143058-B6027.pdf

Hissink, A. M., Kulig, B. M., Kruse, J., Freidig, A. P., Verwei, M., Muijser, H., Lammers, J. H., Mckee, R.H., Owen, D.E., Sweeney, L.M., and Salmon, F. 2009. Physiologically based pharmacokinetic modeling of cyclohexane as a tool for integrating animal and human data sets. *Int. J. Toxicol.* 28: 498-502.

Kenyon, E. M., Benignus, V., Eklund, C., Highfill, J. W., Oshiro, W. M., Samsam, T. E., and Bushnell, P. J. 2008. Modeling the toxicokinetics of inhaled toluene in rats: influence of physical activity and feeding status. *J. Toxicol. Environ. Health, Part A,* 71 : 249- 65.

Könemenn, W. H., and Pieters, M. N. 1996. Confusion of concepts in mixture toxicology. *Food Chem. Toxicol.* 34: 1025-31.

Krishnan, K., and Carrier, R. 2008. Approaches for evaluating the relevance of multiroute exposures in establishing guideline values for drinking water contaminants. *J. Environ. Sci. Health C. Environ Carcinog. Ecotoxicol. Rev.* 26: 300-16.

Krishnan, K., and Peyret, T. 2009. Physiologically based toxicokinetic (PBTK) modeling in ecotoxicology. In: *Ecotoxicology modeling*. (ed) Devillers, J., Springer, Dordrecht; pp: 145-175.

Labranche, D. F., and Collins, M. R. 1996. Stripping volatile organic compounds and petroleum hydrocarbons from water. *Water Environ. Res.* 68: 348-58.

Lewis, D. F. V. 2001. *Guide to cytochromes P450 structure and function*. CRC press LLC. Boca Raton, Florida, USA, Page 60.

Liu, L., and Zhou, H. 2011. Investigation and assessment of volatile organic compounds in water sources in China. *Environ. Monit. Assess.* 173: 825-36.

Lock, E. A., Strasser, J. Jr., Bus, J. S., and Charbonneau, M. 1993. Studies on the renal transport of trimethylpentanoic acid metabolites of 2,2,4-trimethylpentane in rat renal cortical slices. *J. Appl. Toxicol.* 13: 291-96.

Malley, L. A., Bamberger, J. R., Stadler, J. C., Elliott, G. S., Hansen, J. F., Chiu, T., Grabowski, J. S., and Pavkov, K. L. 2000. Subchronic toxicity of cyclohexane in rats and mice by inhalation exposure. *Drug and Chem. Toxicol. 23*: 513-37.

McKone, T.E. 1987. Human exposure to volatile organic compounds in household tap water: The indoor inhalation pathway. *Environ. Sci. Technol.* 21: 1194-201.

McKone, T. E. 1999. The rise of exposure assessment among the risk sciences: An evaluation through case studies. *Inhal. Toxicol.* 11: 611-22.

Mráz, J., Gálová, E., Nohová, H., and Vitková, D. 1998. 1,2- and 1,4-cyclohexanediol: major urinary metabolites and biomarkers of exposure to cyclohexane, cyclohexanone, and cyclohexanol in humans. *Int. Arch. Occup. Environ. Health* 71: 560-65.

Mutti, A., Falzoi, M., Lucertini, S., Cavatorta, A., and Franchini, I. 1981. Absorption and alveolar excretion of cyclohexane in workers in a shoe factory. *J. Appl.Toxicol.* 1: 220-23.

Mutti, A., Falzoi, M., Lucertini, S., Arfini, G., Zignani, M., Lombardi, S.,and Franchini, I. 1984. n-hexane metabolism in occupationally exposed workers. *Br. J. Ind. Med.* 41: 533-8.

Nakajima, T., Wang, R. S., Elovaara, E., Gonzalez, F. J., Gelboin, H. V., Raunio, H., Pelkonen, O., Vainiot, H., and Aoyama, T. 1997. Toluene metabolism by cDNA-expressed human hepatic cytochrome 450. *Biochem. Pharmacol.* 53: 271-77.

Newill, V. A. 1989. Significance of risk assessment in the management of environmental exposures of chemical mixtures. *Toxicol. Ind. Health* 5: 635-46.

OPPT, Office of Pollution Preventtion and Toxics. 1994. Chemicals in the environment: cyclohexane (CAS NO. 110-82-7). http://www.epa.gov/chemfact/f_cycloh.txt . (Jan 26, 2011).

Perbellini, L., Brugnone, F., Silvestri, R., and Gaffuri, E. 1981. Measurement of the urinary metabolites of n-hexane, cyclohexane and their isomers by gas chromatography. *Int.* Arch. *Occup. Environ. Health* 48: 99-106.

Perbellini, L., Mozzo, P., Brugnone, F., and Zedde, A. 1986. Physiologicomathematical model for studying human exposure to organic solvents: kinetics of blood/tissue n-hexane concentrations and of 2,5-hexanedione in urine. *Br. J. Ind. Med.* 43: 760- 68.

Pérez-Pavón, J. L., Sánchez, M., Del Nogal, M. E., Laespada, F.,and Cordero, B. M. 2007. Simultaneous determination of gasoline oxygenates and benzene,

toluene, ethylbenzene and xylene in water samples using headspace-programmed temperature vaporization-fast gas chromatography–mass spectrometry. *J. Chrom. A* 1175: 106- 11.

Perico, A., Cassinelli, C., Brugnone, F., Bavazzano, P., and Perbellini, L. 1999. Biological monitoring of occupational exposure to cyclohexane by urinary 1,2- and 1,4-cyclohexanediol determination. *Int.* Arch. *Occup. Environ. Health* 72: 115-20.

Rider, C., Furr, J. R., Wilson, V. S., and Gray, L. E. Jr. 2010. Cumulative effects of in-utero administration of mixtures of reproductive toxicants that disrupt common target tissues via diverse mechanisms of toxicity. *Int. J. Androl.* 33: 443-62.

Rosenberg, N. L., Kleinschmidt-DeMasters, B. K., Davis, K. A., Dreisbach, J. N., Hormes, J. T., and Filley, C. M. 1988. Toluene abuse causes diffuse central nervous system white matter changes. *Ann. Neurol.* 23: 611-14.

Senler, T. I., Dean, W. L., Murray, L. F., and Wittliff, J. L. 1985. Quantification of cytochrome P-450-dependent cyclohexane hydroxylase activity in normal and neoplastic reproductive tissues. *Biochem. J.* 227: 379-87.

FSA, Food Standard Agency. "Risk assessment of mixture of pesticides and similar substances. Committee on toxicity of chemicals in food, consumer products and the environment." FSA/0691/0902. Sep 2002.

Shehata, A. T. 1985. A multiple-route exposure assessment of chemically contaminated drinking water. *Toxicol. Ind. Health* 1: 277-98.

Simmons, J. E. 1995. Chemical mixtures: challenge for toxicology and risk assessment. *Toxicol.* 105: 111-9.

Swann, H. E. Jr., Kwon, B. K., Hogan, G. K., and Snellings. W. M. 1974. Acute inhalation toxicology of volatile hydrocarbons. *Am. Ind. Hyg. Assoc. J.* 35: 511-8.

Takeuchi, Y., Ono, Y. and Hisanaga, N. 1981. An experimental study on the combined effects of n-hexane and toluene on the peripheral nerve of the rat. *Br. J. Ind. Med.* 38: 14-19.

Tardif, R., Lapare, S., Krishnan, K., and Brodeur, J. 1993. A descriptive and mechanistic study of the interaction between toluene and xylene in humans. *Int.* Arch. *Occup. Environ. Health* 65: S135- S137.

Tardif, R., Charest-Tardif, G., Brodeur, J., and Krishnan, K. 1997. Physiologically based pharmacokinetic modeling of a ternary mixture of alkyl benzenes in rats and humans. *Toxicol. Appl. Pharmacol.* 144: 120- 34.

TESORO. *Material Safety Data Sheet*. http://firstfuelbank.com/msds/Tesoro.pdf . (1 February 2011)

Teuschler, L. K., and Hertzberg, R. C. 1995. Current and future risk assessment guidelines, policy, and methods development for chemical mixtures. *Toxicol.* 105: 137- 44.

Thrall, K. D., Weitz, K. K., and Woodstock, A. D. 2002. Use of real time breath analysis and PBPK modeling to evaluate dermal absorption of aqueous toluene in human volunteers*. Toxicol. Sci.* 68: 280-287.

U.S. EPA. United States Environmental Protection Agency. 1990. Citizen's guide to ground water protection. Washington, DC, USA. EPA 440/6-90-004.

U.S. EPA. United States Environmental Protection Agency. 1991. Restricting service station wastes in shallow injection wells. Office of water; Washington, DC, USA. EPA 570/9-91-UICA.

U.S. EPA. United States Environmental Protection Agency. 2007. Toxicological review of 2,2, 4 trimethylpentane, CAS no 540-84-1, 2007.Washington, DC. USA. EPA/635/R-07/003.

Xu, X., Mariano, T. M., Laskin, J. D., and Weisel, C. P. 2002. Percutaneous absorption of trihalomethanes, haloacetic acids, and haloketones. *Toxicol. Appl. Pharmacol.* 184: 19-26.

Veulemans, H., Van Vlem, E., Janssens, H., Masschelein, R., and Leplat, A. 1982. Experimental human exposure to n-hexane. *Int. Arch. Occup. Environ. Health* 49 (1982): 251-263.

Weisel, C. P., and Jo, W. K. 1996. Ingestion, inhalation, and dermal exposures to chloroform and trichloroethene from tap water. *Environ. Health Perspect.* 104: 48-51.

Yang, R. S.H., El-Masri, H. A., Thomas, R. S., and Constan, A. A. 1995. The use of physiologically-based pharmacokinetic / pharmacodynamic dosimetry models for Chemical Mixtures. *Toxicol. Let.* 82/83: 497- 504.

Yasugi, T., Kawai, T., Mizunuma, K., Kishi, R., Harabuchi, I., Yuasa, J., Eguchi, T., Sugimoto, R., Seiji, S., and Ikeda, M. 1994. Exposure monitoring and health effect studies of occupationally exposed to cyclohexane Vapor. *Int. Arch. Occup. Environ. Health* 65: 343- 50.

Zhang, H., Im, S., and Waskell, L. 2007. Cytochrome b5 increases the rate of the product formation by cytochrome P450 2b4 and competes with cytochrome P450 reductase for a binding site on cytochrome P450 2b4.
J. Biol. Chem. 282: 29766-76.

Table 1: Characterizations of toluene, n-hexane, cyclohexane and isooctane

	Toluene	n-Hexane	Cyclohexane	Isooctane
TLV[®]* [1] (PPM)	50	50	300	300
Principal toxicity	Central Nervous System (CNS) [13]	Peripheral Nervous System [11]	CNS [9]	nephrotoxin in male rat [8] respiratory arrest at concentration>32000ppm [14]
Route-specific absorption efficiency	Short exposure: Respiratory (85-90) Long exposure> 1h: Respiratory & oral (50/50) [7] Dermal exposure 1% (vapor), less in liquid phase [7]	Principal route: Respiratory tract. At Higher dose gastrointestinal tract and skin [3] Dermal absorption; Not significant (0.82 µg.cm²/hr)[3]	Principal route: Respiratory and digestive system: 23% [10] Dermal Route: 23% (vapor), 5% liquid phase[10]	oral exposure; to 500mg/kg: 95% [6] respiratory exposure; to 0.79 ppm (low): 7% 385 ppm (high): 12%[5]
Metabolic product	Hippuric acid [2]	2,5-hexanedione (HD)[11]	Cyclohexanol [12]	2,4,4-trimethyl-2-pentanol [4]

* (TLV) [®]: Threshold limit value
** Alveolar retention: difference between inhaled and alveolar concentrations of volatile
1. ACGIH (1999)
2. ATSDR (1993)
3. Bonnard et al. (2008)
4. Charbonneau et al. (1987)
5. Dahl (1989)
6. U.S. EPA (2007)
7. Faust (1994)
8. Lock et al. (1993)
9. Malley et al. (2000)
10. Mutti et al. (1981)
11. Ali and Tardif, (1999)
12. Perbellini et al. (1981)
13. Rosenberg et al. (1988)
14. Swann et al. (1974)

Table 2. Comparison of the area under the blood concentration (AUCs) vs time course mg/l.min obtained from PBPK model and experimental data after exposure to mixture by multiple routes (inhalation and oral) in rats.

Chemical	AUC (mg/L.min) (120- 360 min)	
	PBPK	Experimental data
Toluene	121.73	157.25
n-Hexane	21.91	18.77
Cyclohexane	19.55	159.58
Isooctane	170.54	176.54

Table 3: Normalized sensitivity coefficients for input parameters of the PBPK model for cyclohexane. (*Pbc, Plc, Pfc, Prc, Psc*, *Vmaxc* and *Kmc* refer to blood: air partition coefficient, liver : air partition coefficient, richly-perfused : air partition coefficient and slowly-perfused : air partition coefficient, maximal velocity of metabolism and Michaelis-Menten constant).

Parameters	Cyclohexane
Pbc	-0.831
Plc	0.000
Pfc	0.256
Prc	-0.075
Psc	-0.416
Vmaxc	0.115
Kmc	-0.049

Table 4: Normalized sensitivity coefficients for input parameters of cyclohexane (c) and isooctane (i) in the mixture PBPK model. (*Pb, Pl, Pf, Pr, Ps, Vmax, Km, Ki, Ko, and Kx* refer to blood : air partition coefficient, liver : air partition coefficient, richly-perfused : air partition coefficient, slowly-perfused : air partition coefficient, maximal velocity of metabolism, Michaelis-Menten constant, inhibition constant, absorption rate constant for stomach and stomach to intestine transfer rate).

Paremeters	toluene	n-hexane	cyclohexane	isooctane
Pl_c	-0.008	-0.004	-0.005	-0.006
Pf_c	0.008	0.000	0.320	0.003
Pr_c	-0.002	0.000	-0.076	-0.001
Ps_c	0.000	0.000	0.000	0.000
Pb_c	-0.045	-0.013	-1.038	-0.033
$Vmax_c$	0.044	0.013	0.148	0.031
Km_c	-0.044	-0.013	-0.142	-0.032
Ki_c	0.071	0.021	0.010	0.051
Ko_c	-0.003	0.000	-0.056	-0.003
Kx_c	0.005	0.000	0.061	0.003
Pl_i	-0.005	0.000	0.000	-0.015
Pf_i	0.005	0.000	0.005	0.062
Pr_i	0.000	0.000	0.000	-0.008
Ps_i	-0.008	-0.004	0.127	-0.060
Pb_i	-0.022	-0.009	0.259	-0.060
$Vmax_i$	0.000	0.000	0.000	0.000
Km_i	-0.007	-0.004	0.051	-0.026
Ki_i	0.180	0.060	0.051	0.118
Ko_i	0.000	0.000	0.000	0.000
Kx_i	0.000	0.000	0.000	0.000

List of Figures:

Figure 1: PBPK model of multi-route exposure to the mixture of four chemicals (tolune (t), n-hexane (h), cyclohexane(c) and isooctane (i)) by inhalation and oral gavage. The toxicokinetic interactions among the four substances occur in the liver. *RAM*; rate of the metabolism of each substance, arterial blood (Arterial), venous blood (Venous), richly-perfused tissues; RPT, poorly-perfused tissues (PPT), GI tract; gastrointestinal tract.

Figure 2A: Comparison of PBPK model simulations (solid lines) with the experimental data (symbols) of inhalation exposure to 300 ppm or 1200 ppm (2 hr) of cyclohexane in rats.

Figure 2B: Comparison of the PBPK model simulations (solid lines) to the experimental data (symbols) of single oral gavage exposure to 27.9 mg/kg 108.9 mg/kg of cyclohexane in rats.

Figure 2C: Comparison of the PBPK model simulations (solid lines) with the experimental data (symbols) of aggregate exposure to 27.9 mg/kg + 300 ppm or 108.9 mg/kg + 1200 ppm of cyclohexane (single oral dose followed by 2 hr inhalation).

Figure 3A: PBPK model simulations (solid lines) and experimental data on venous blood concentration in rats exposed to toluene alone (♦) or as a mixture (■) by multiple routes.

Figure 3B: PBPK model simulations (solid lines) and experimental data on venous blood concentration in rats exposed to n-hexane alone (♦) or as a mixture (■) by multiple routes.

Figure 3C: PBPK model simulations (solid lines) and experimental data on venous blood concentration in rats exposed to isooctane alone (♦) or as a mixture (■) by multiple routes.

Figure 3D: PBPK models simulation (solid lines) and experimental data on venous blood concentration in rats exposed to cyclohexane alone (♦) or as a mixture (■) by multiple routes.

Figure 4A: Comparison of the simulations of PBPK models of toluene in a mixture in presence or absence of cyclohexane. Gray line indicates simulation without cyclohexane.

Figure 4B: Comparison of the simulations of PBPK models of n-hexane in a mixture in presence or absence of cyclohexane. Gray line indicates simulation without cyclohexane.

Figure 5A: Comparison of the simulations of PBPK model of toluene in a mixture in presence or absence of isooctane. Gray line indicates simulation without isooctane.

Figure 5B: Comparison of the simulations of PBPK model of n-hexane in a mixture in presence or absence of isooctane. Gray line indicates simulation without isooctane.

Figure 6: PBPK model simulations of the kinetics of cyclohexane and comparison with the experimental data (points) from Hissink et al. (2009) (400 ppm and 2000 ppm).

Figure 7A: PBPK model simulations of the inhalation kinetics of cyclohexane in rats to 300 ppm and 1200 ppm. To obtain these simulations, blood: air partition

coefficient (Pb) was set to 7 instead of 1.3 based on the result of sensitivity analysis.

Figure 7B: PBPK model simulations of the oral kinetics of cyclohexane in rats administrated 27.9 mg/kg and 108.9 mg/kg. To obtain these simulations, blood: air partition coefficient (Pb) was set to 7 instead of 1.3 based on the result of sensitivity analysis.

Figure 7C: PBPK model simulations of multi-route exposure to cyclohexane. Blood: air partition coefficient (Pb) was set to 7 instead of 1.3 based on the result of sensitivity analysis (High dose (1200 ppm + 108.9 mg/kg); low dose (300 ppm + 27.9 mg/kg)).

Figure 7D: impact of the blood : air partition coefficient (pb) on the simulations of PBPK model of cyclohexane kinetics during multi-route exposure (300 ppm + 27.9 mg/kg).
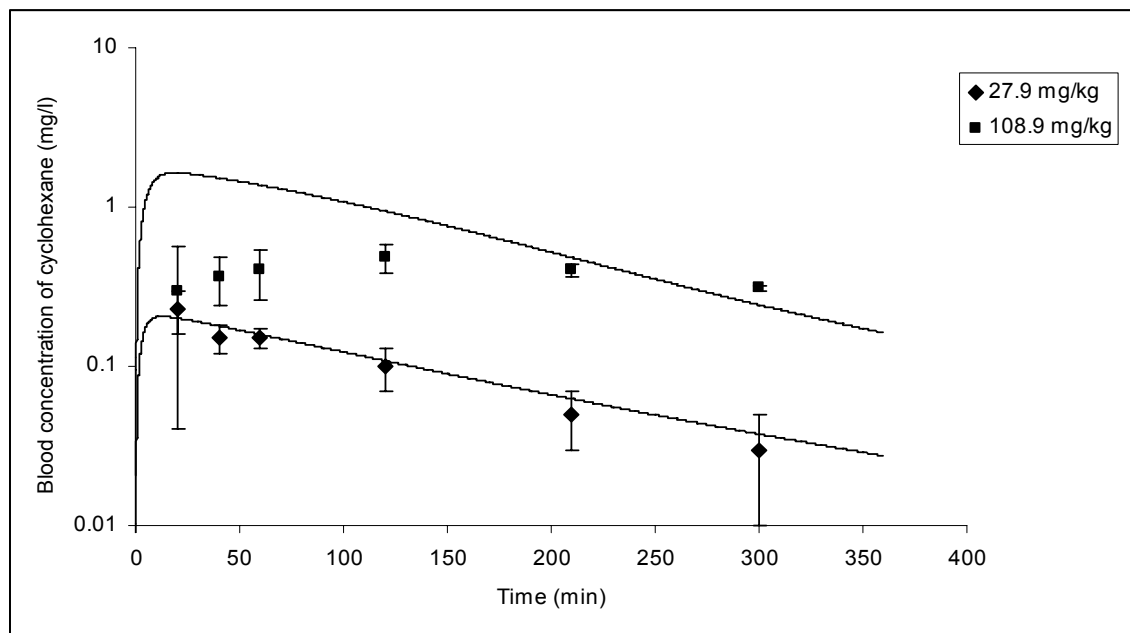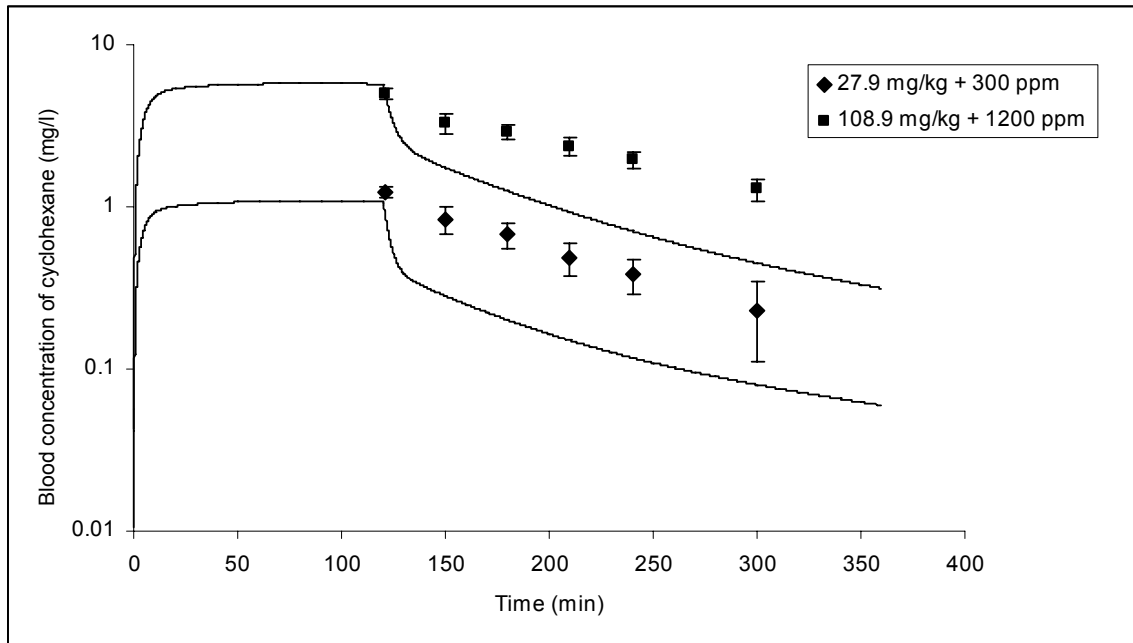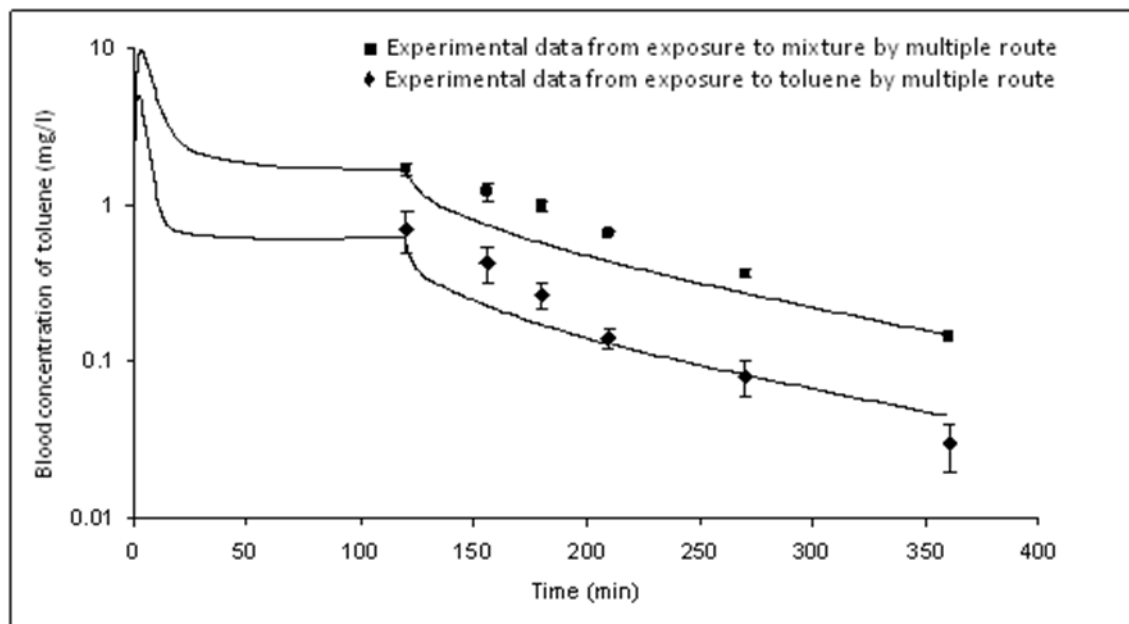
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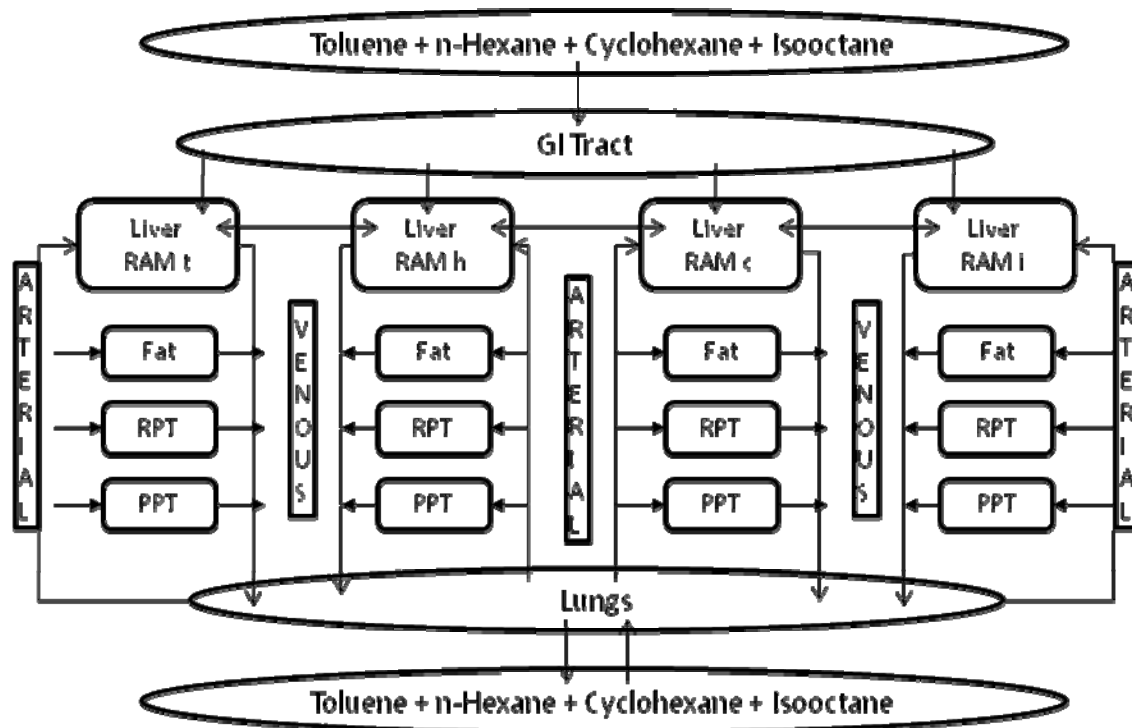
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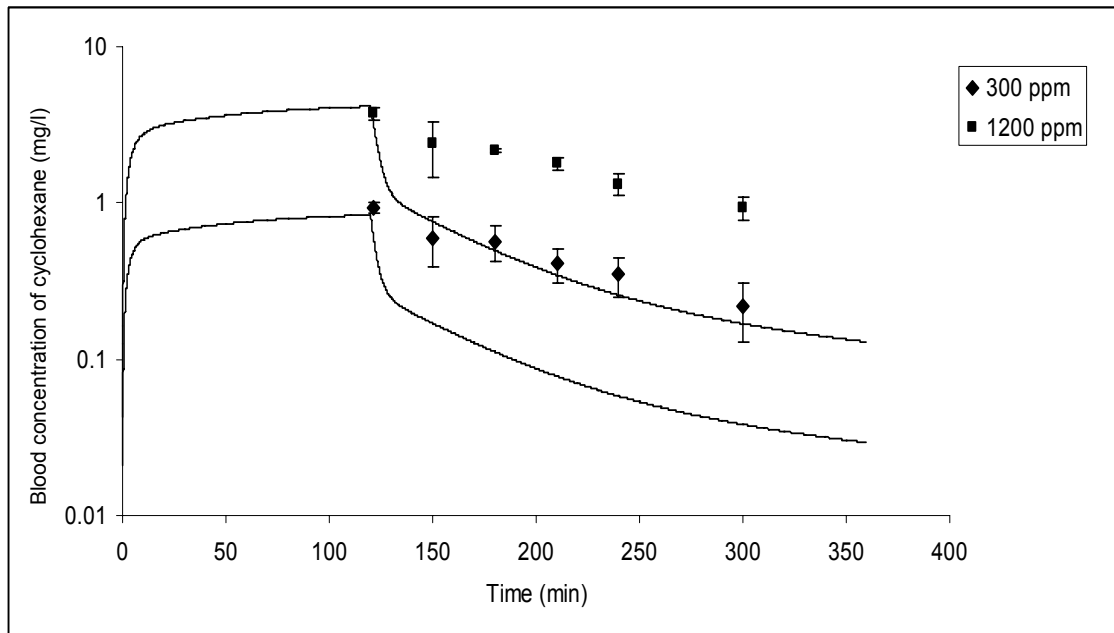
Figure 2B

Figure 2C

Figure 3A

Figure 3B
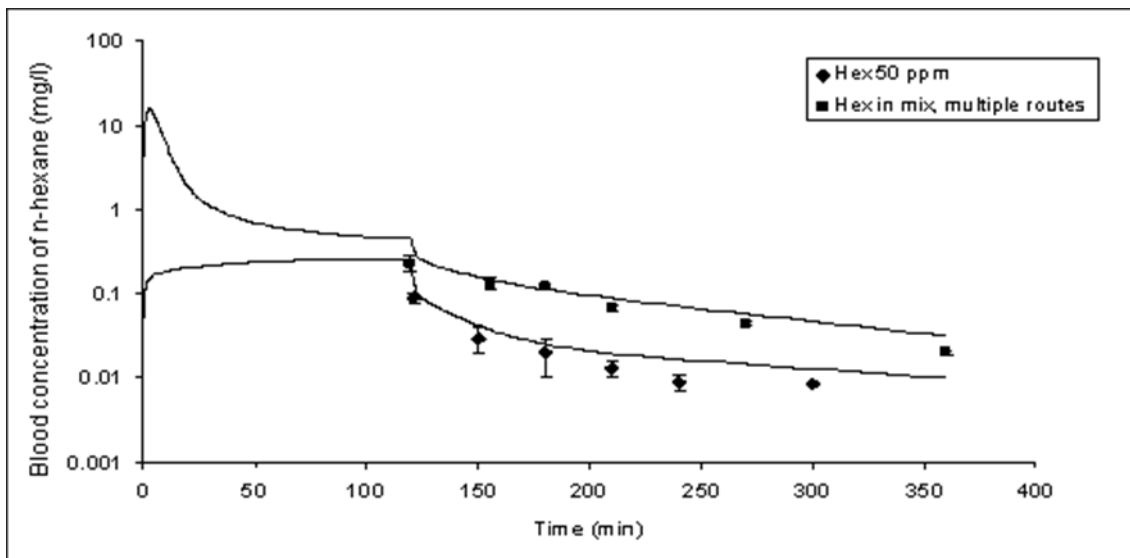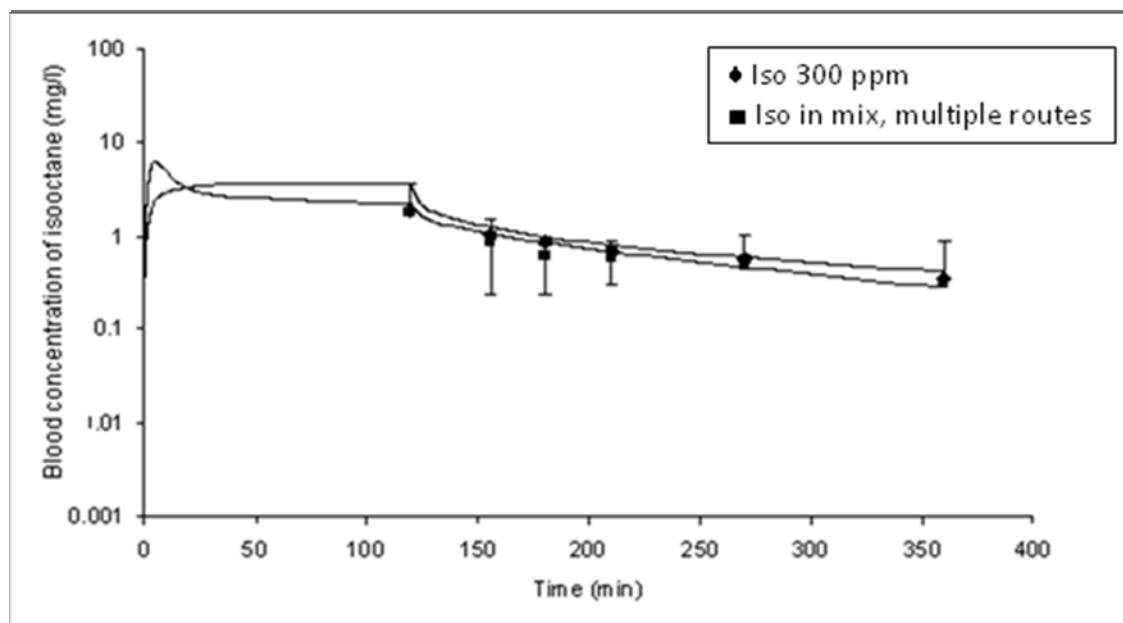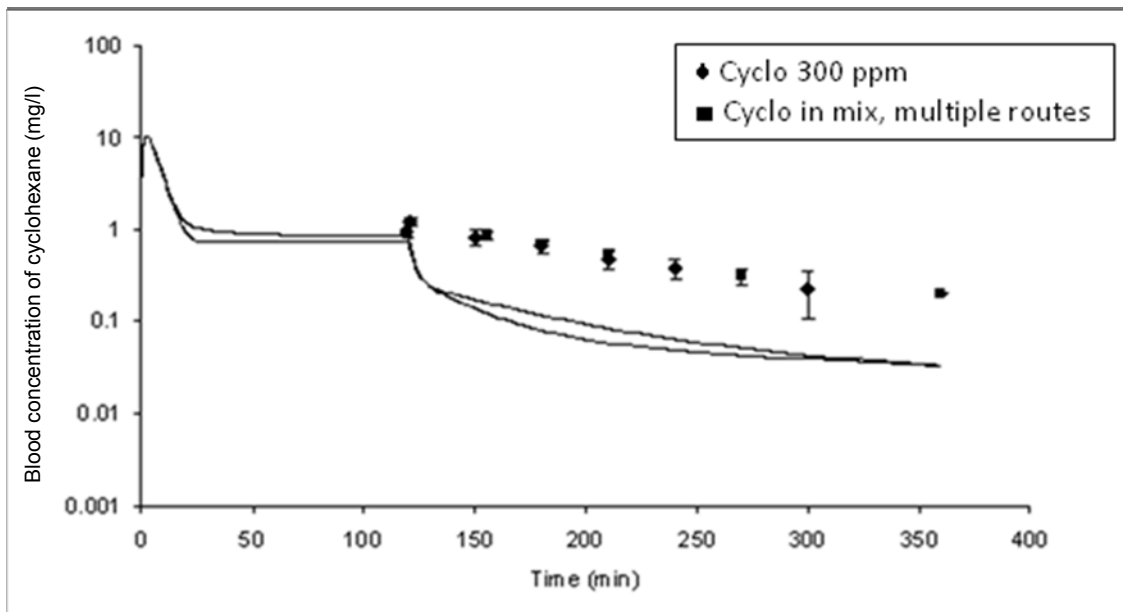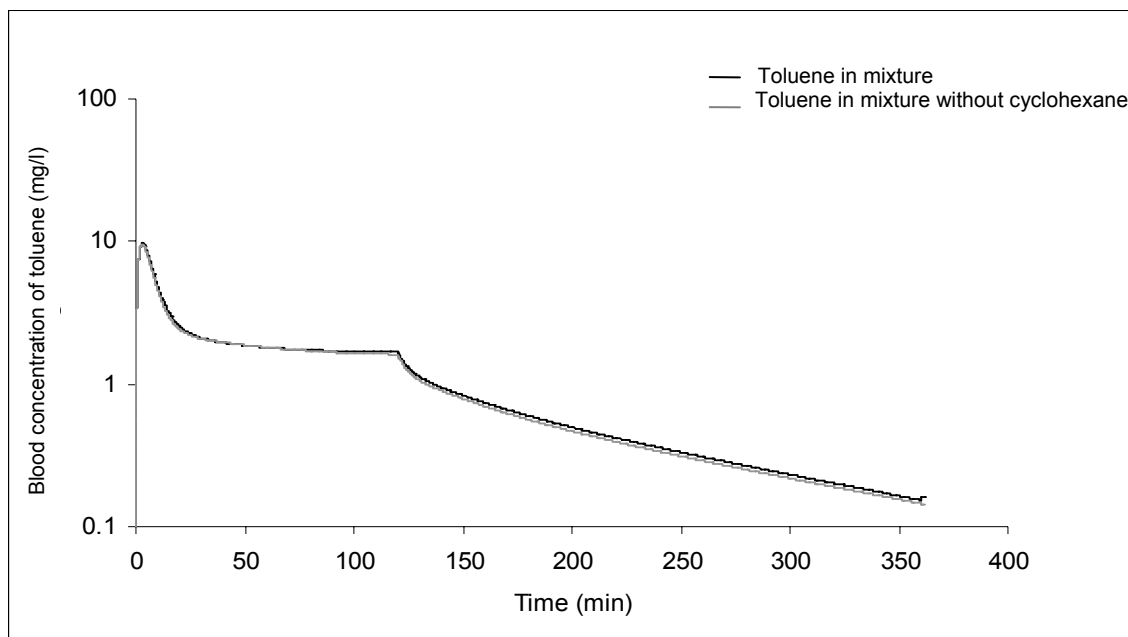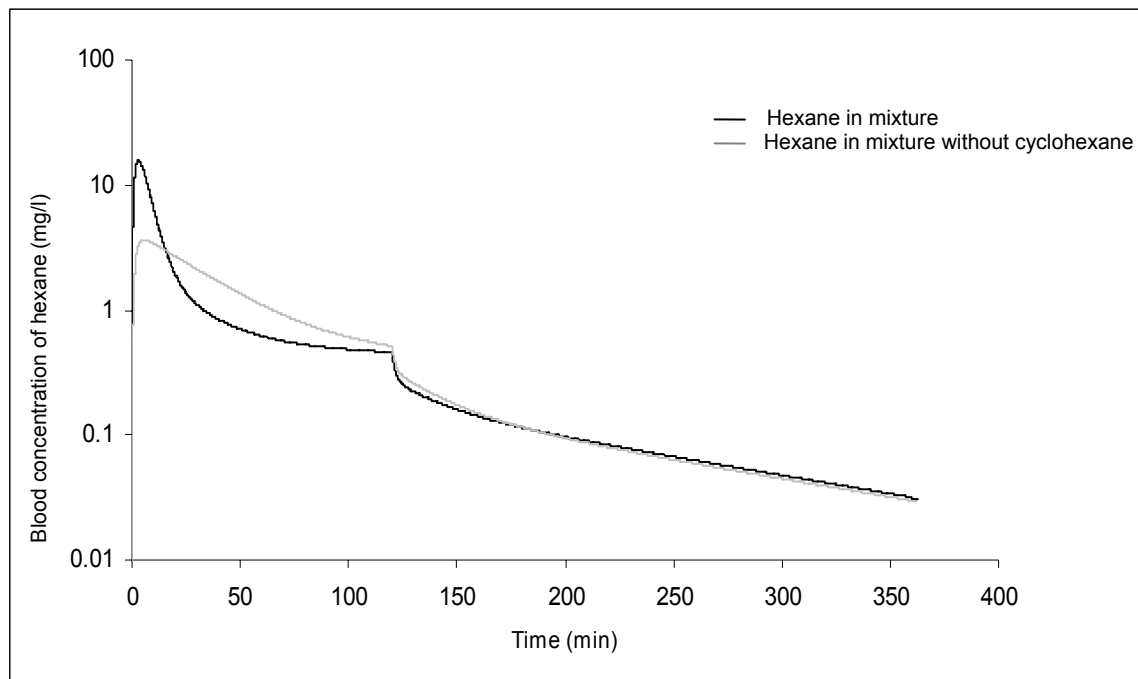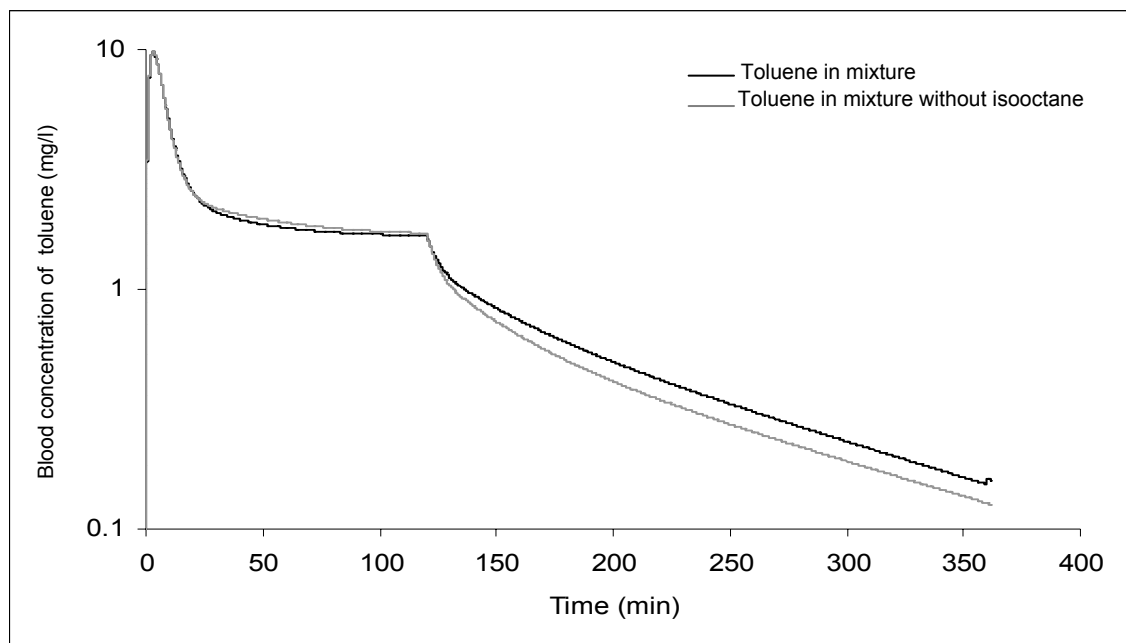
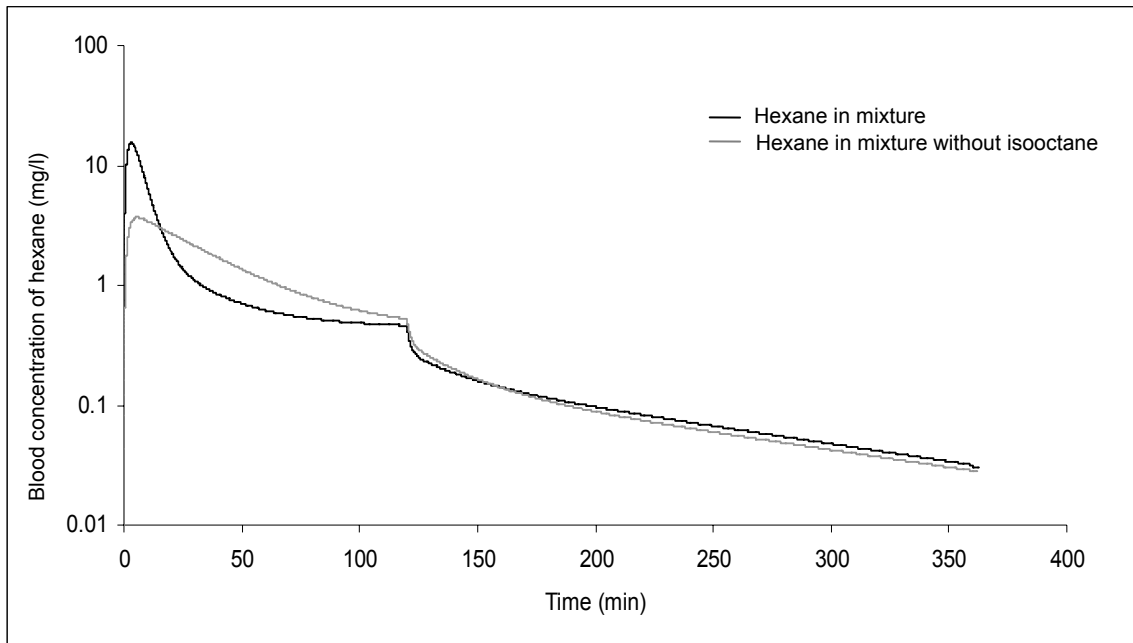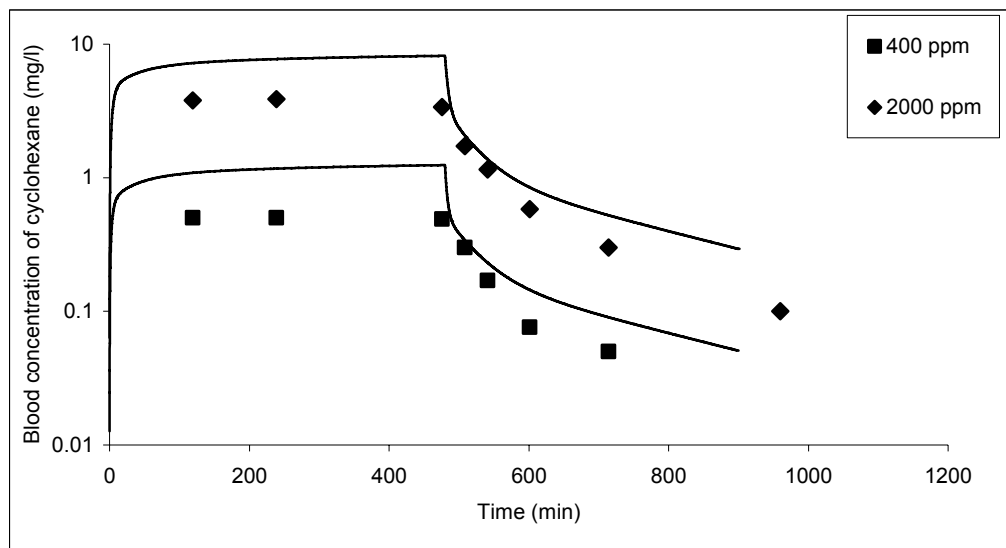Figure 3C

95

Figure 3D

Figure 4A

Figure 4B

Figure 5A

Figure 5B

Figure 6

Figure 7A

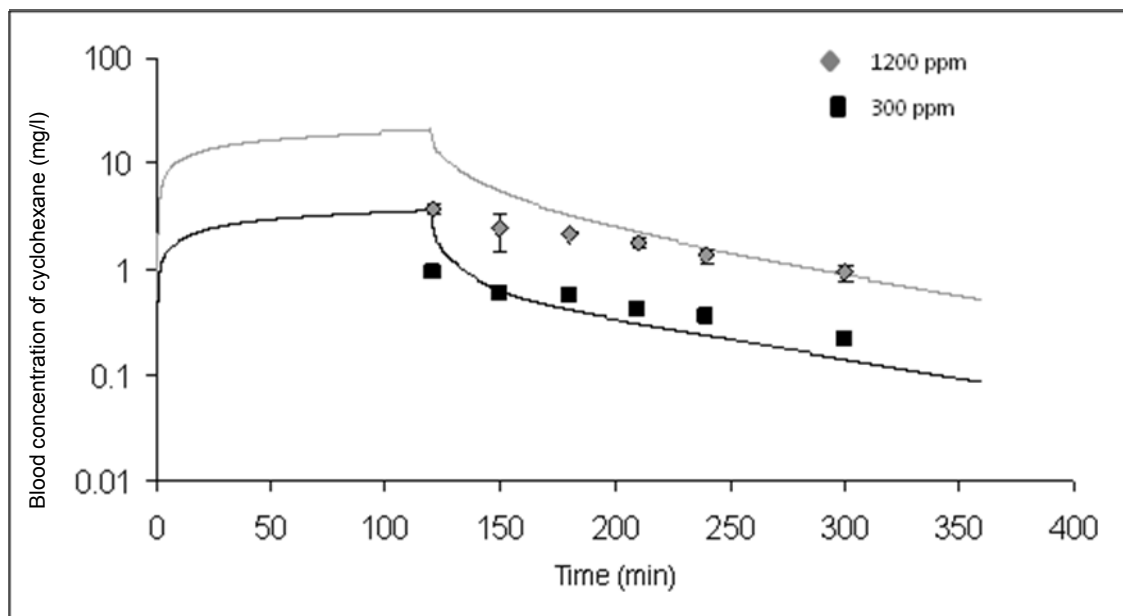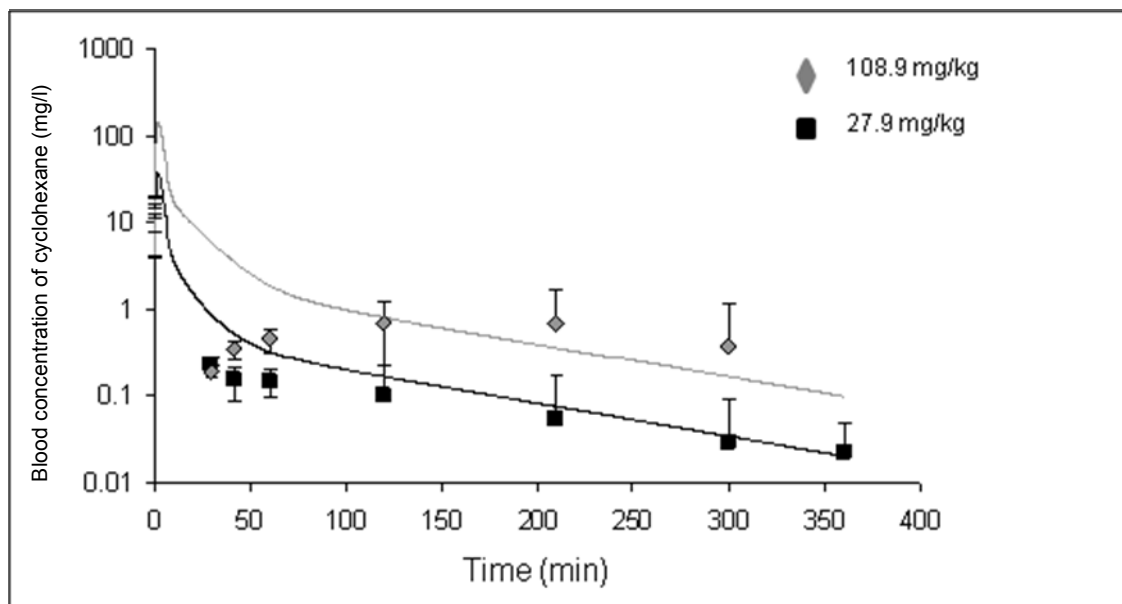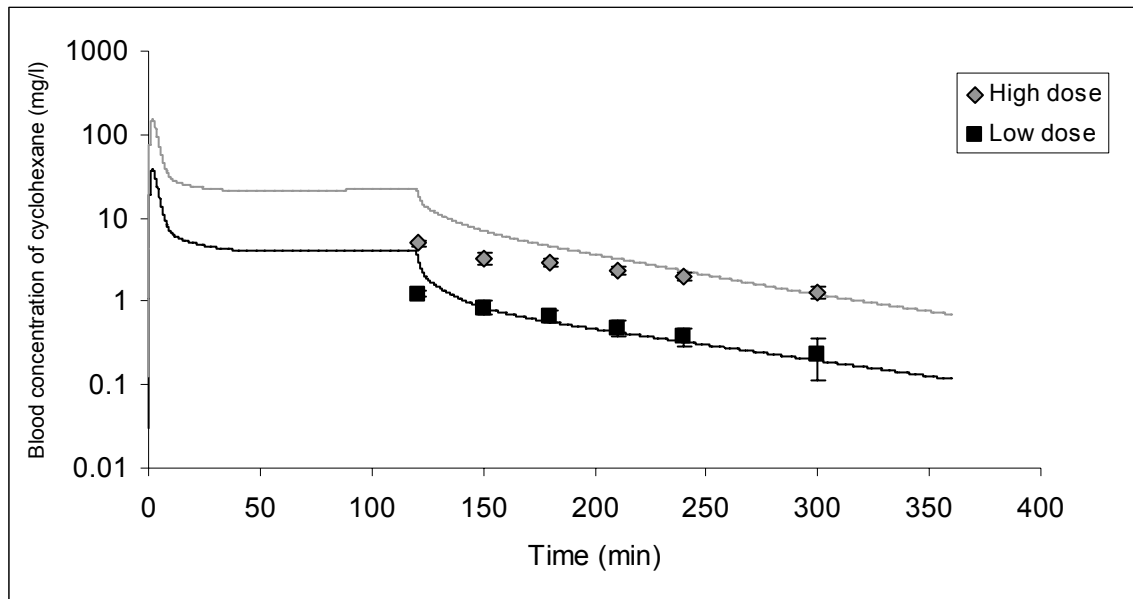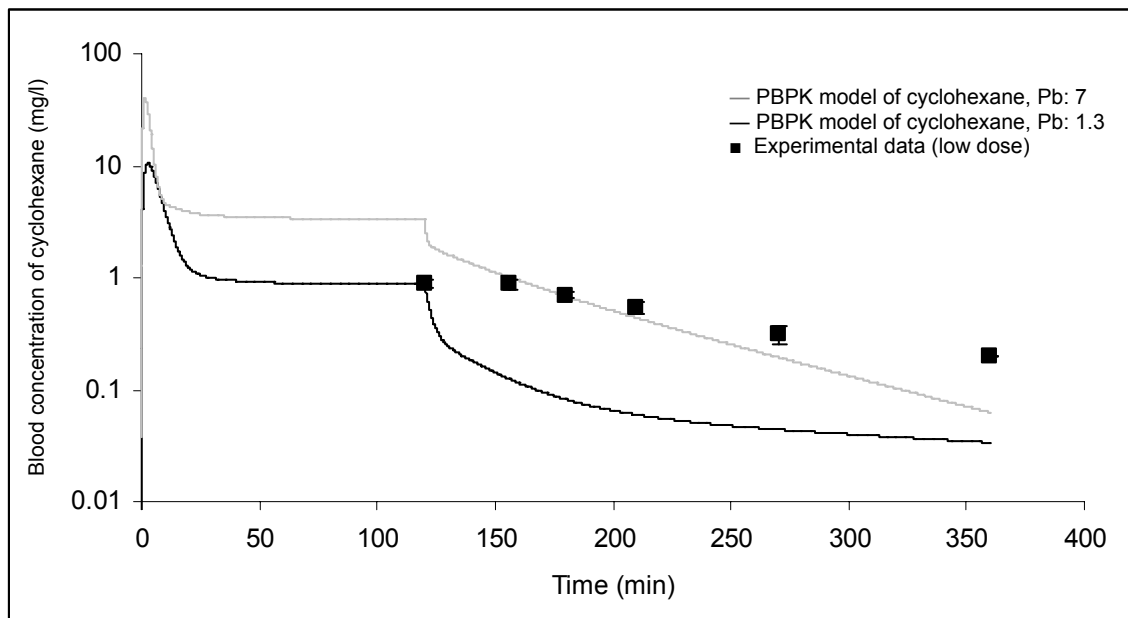Figure 7B

Figure 7C

Figure 7D

CHAPTER 4: General discussion

Discussion

In the process of deriving the guideline values of chemical contaminants in drinking water, there has not been any attempt to take into account mixed exposures and multiple routes of exposure. The main reason is that the number of experiments using individual and mixture of chemicals by single and multiple routes of exposure required would be enormous, and that would be impractical, expensive and unethical in humans. The present study showed the capacity of PBPK models to support the interpretation of results from limited but focused experiments for multi-route and multi-chemical exposures.

To accomplish this objective, four VOCs (toluene, n-hexane, cyclohexane and isooctane), present in the petroleum products that have great potential to contaminate the drinking water, were chosen. PBPK models of toluene for both single and multi-routes of exposure were already available (Gagné, 2008). Therefore, PBPK models of n-hexane, isooctane (first article) and isooctane (second article) were developed to simulate separate chemical exposure by both single and multiple routes (inhalation and oral) in rats. The models were compared with the experimental data collected for the same scenarios of exposure in rats. The results showed the capacity of the PBPK models to simulate the kinetics of individual chemicals for simple or multiple routes of exposure. Then, the PBPK models were applied to analyze simultaneous exposure to the mixture of toluene, n-hexane, cyclohexane and isooctane by multiple routes (second article). Experimental data and PBPK modeling showed that after a 2-hr exposure to lower doses, there were interactions between toluene and n-hexane. These interactions result from a likely inhibition of the metabolism of n-hexane and toluene due to their competition for CYP2B1 (Nakajima et al. 1997; Fabioano et al. 2010), leading to higher blood concentrations of the parental compounds. However, no change in the metabolism of isooctane was observed. The role of cyclohexane for the possible interaction with other chemicals was omitted, as supported by the sensitivity

analyses. This emphasizes the fact that one or more of the components of a mixture may not interact with other components, and may just act independently.

In exploring the results obtained in the present study, the logical next step would be to extrapolate the PBPK model to humans and use it in the mixture risk assessment process. The advantage of PBPK modeling approaches is the ability to conduct calculations for untested/selected exposure scenarios. In this regard, the PBPK model of multiple routes of exposure to a mixture of four chemicals was compared to the result of the PBPK model of individual chemicals. The skin compartment was added to the mixture PBPK model described in Article 2 of this thesis. The integrated multiple route PBPK model was then used to simulate a subject (body weight: 70 kg, body surface area: 18000 cm^2) taking a 10-min shower (exposure to VOCs through dermal and inhalation) and spending the rest of the day indoors and consuming a total of 1.5 L of drinking water (as per Health Canada). For conducting simulations, in addition to the physiological parameters of humans from Haddad et al. (2007), chemical-specific parameters (i.e., partition coefficients and biochemical) were obtained from Ali and Tardif (1999; toluene and n-hexane), Hissink et al. (2009; cyclohexane) and El-Masri et al. (2009; isooctane). Parameters such as Kp (dermal permeability constant to a chemical), Psw (skin: water partition coefficient) and Psa (skin: air partition coefficient) were calculated using the following formulas:

Log Kp = - 0.812 - 0.00104 x MW + 0.616 x logKow   (Bogen, 1994)

Psw: 1.2771 + 0.1208 Kow   (Robert et al. 1975)

Psa: Psw / Kaw

Kow: Koa / Kwa

Where:

Koa (oil: air partition coefficient): toluene, 1539; n-hexane, 157; cyclohexane, 331 and isooctane, 366) are from Meulenberg and Vijverberg (2000).

Kwa (saline: air partition coefficient): toluene, 2.19; n-hexane, 0.015; cyclohexane, 0.15 and isooctane, 0.0014 are from Meulenberg and Vijverberg (2000).

Total amounts absorbed during multiple routes of exposure was compared to the quantity absorbed via the oral route (or 1.5 L) to derive the liter equivalent (L-eq) for each substance as per Krishnan and Carrier (2008):

$$\text{L-eq multiple routes} = \frac{\text{quantity of chemical absorbed by multiple routes } (\mu g / L)}{\text{quantity absorbed by oral route } (\mu g / L)}$$

L-eq total = 1.5 + (1.5 x L-eq of multiple routes)

Therefore, the guideline value would equal: $\dfrac{(\text{TDI x BW X SAF})}{\text{L-eq total}}$

The following Tables 3 and 4 present L-eq obtained using the PBPK models developed in the present study and scaled to humans.

Table 3: Comparison of the L-eq of each substance resulting from multi-routes exposure to mixture between different concentrations: 100 µg/L and 1 µg/L.

	L-eq of Mixture 100 µg/L	L-eq of Mixture 1 µg/L
Toluene	4.93	4.55
n-Hexane	2.20	2.18
Cyclohexane	3.89	3.47
Isooctane	1.62	1.62

Table 4: Comparison of the L-eq of each substance resulting from multi-routes exposure of separate substances between different concentrations: 100 µg/L and 1 µg/L.

	L-eq of single substance 100 µg/L	L-eq of single substance 1 µg/L
Toluene	3.77	3.77
n-Hexane	2.13	2.13
Cyclohexane	4.46	4.46
Isooctane	1.61	1.61

The use of PBPK models indicate that under the single chemical exposure situations, the calculated L-eq is similar for various water concentrations that essentially stay in the 'linear' range of metabolic enzymes (Table 4). However, in case of mixture exposure via multiple routes, as it was expected, there is an increase in the amount of L-eqs of toluene and n-hexane, more likely as a result of competition among chemicals (Table 3).

The PBPK models can be used to simulate various scenarios to determine the combination of water concentrations of VOCs that would or not result in significant metabolic inhibition among them. Using this predictive simulation approach, we can then potentially improve the standard-setting process for drinking water chemicals that occur as mixtures.

Chapter 5: References

References:

Ali, N., and R. Tardif. "Toxicokinetic modeling of the combined exposure to toluene and n-hexane in rats and humans." J. *Occup. Health* 41 (1999): 95-103.

Andelman, J. "Human exposures to volatile halogenated organic chemicals in indoor and outdoor air." *Environ. Health Perspect.* 62 (1985): 313-18.

ATSDR. Toxicological profile for n-hexane, 1999.

Baelum, J., L. Molhave, S. "Honore Hansen, and M. Dossing. Hepatic metabolism of toluene after gastrointestinal uptake in humans." *Scand. J. Work Environ. Health* 19, (1993): 55-62.

Benoit, F. M., W. R. Davidson, A. M. Lovett, S. Nacson, and A. Ngo. "Breath analysis by ApI/MS-Human exposure to volatile organic solvents." *Int. Arch. Occup. Environ. Health* 55, (1985): 113-20.

Bogen, K. T., and L. C. Hall. "Pharmacokinetics for regulatory risk analysis: The case of 1,1,1-trichloroethane (Methyl Chloroform). " *Regul. Toxicol. Pharmacol.* 10, (1988): 26-50.

Bowen, S. E., and J. H. Hannigan. "Developmental toxicity of prenatal exposure to toluene." *AAPS J.* 8, (2006): E419-E24.

Bonnard, N., M. Falcy, D. Jargot, and O. Schneider. "CAS No. 110-54-3 n-Hexane fiche toxicologique." 2008. http://www.inrs.fr/inrs-pub/inrs01.nsf/IntranetObject-accesParReference/INRS-FR/$FILE/fset.html (Jan 10 2011).

Brown, H. S., D. R. Bishop, and C. A. Rowan. "The role of skin absorption as a route of exposure for Volatile Organic Compounds (VoCs) in drinking water." *Am. J. Public Health* 74, (1984): 479-84.

Carlsson, A. Exposure to toluene: "uptake, distribution and elimination in man." *Scand. J. Work Environ. Health* 8, (Mar 1982): 43-55.

Carpenter, D. O., K. Arcaro, and D. C. Spink. "Understanding the human health effects of chemical mixtures."*Environ. Health Perspect.* 110, (Feb 2002): 25-42.

Charbonneau, M., E. A. Lock, J. Strasser, M. G. Cox, M. J. Turner, J. S. Bus. "2,2,4-trimethylpentane-induced nephrotoxicity : I. metabolic disposition of TMP in male and female Fischer 344 rats." *Tox. Appl. Pharm.* 91 (1987) : 171-181.

Chen, J. S., T. A. Litzinger, and H. J. Curran. "The lean oxidation of iso-octane at elevated pressures." In edited by Western States Section Combustion Institute, 1998. https://e-reports-ext.llnl.gov/pdf/235217.pdf (Fev 14 2011).

De Godoy, L. A. F., M. P. Pedroso, E. C. Ferreira, F. Augusto, and R. J. Poppi. "Prediction of the physicochemical properties of gasoline by comprehensive two-dimensional gas chromatography and multivariate data processing." *J. Chromatogr. A* 1218 (2011): 1663-67.

Deshpande, S. S. 2005. "*Handbook of food toxicology.*" Marcel Dekker, New York, NY, USA. Page: 16.

Dutkiewcz, T., and H. Tyras. "The quantitative estimation of toluene skin absorption in man." *Int. Archiv fur Gewerbepathologie und Gewerbehygiene* 24 (1968): 253-57.

El-Masri, H. A., S. Dowd, R. A. Pegram, R. Harrison, S. J. Yavanhxay, J. E. Simmons, and A. Evans. "Development of an inhalation physiologically based pharmacokinetic (PBPK) model for 2,2,4-trimethylpentane (TMP) in male Long-Evans rats using gas uptake experiments. " *Inh. Toxicol.* 21, (2009): 1176–85.

Environment Canada. Comprehensive approach to clean water. 2012. http://www.ec.gc.ca/eau-water/default.asp?lang=En&n=B1128A3D-1. February 6, 2012.

Espinosa-Aguirre, J.J., J. Rubio, I. Lopez, R. Nosti, and J. Asteinza. "Characterization of the CYP isozyme profile induced by cyclohexanol." *Mutagenesis,* 12, no. 3 (1997): 159-62.

Fabioano H. M., J. S. Lepera, M. P. Marquws, V. B. Borrali, and V. L. Lanchote. "Influence of n-hexane inhalation on the enantioselective pharmacokinetics and metabolism of verapamil in rats." *Chirality* 22 (2010): 29–34.

Faust, R. A. 1994. "Toxicity summary for toluene."

http://cira.ornl.gov/documents/TOLUENE.pdf (7January 2011).

Filley, C. M., W. Halliday, and B. K. Kleinschmidt-DeMasters. "The effects of toluene on the central nervous system." *J. Neuropath. Exp. Neur.* 63, no. 1 (Jan 2004): 1-12.

FSA, Food Standard Agency. "Risk assessment of mixture of pesticides and similar substances. Committee on toxicity of chemicals in food, consumer products and the environment." FSA/0691/0902. Sep 2002.

Gospe, S. M., and M. A. S. Al-Bayati. "Comparison of oral and inhalation exposures to toluene." *Int. J. Toxicol.* 13, no. 1 (Feb 1994): 21-32.

Haddad, S., G. Charest-Tardif, and R. Tardif. "Development of physiologically based toxicokinetic models for improving the human indoor exposure assessment to water contaminants: trichloroethylene and trihalomethanes." *J. Toxicol. Environ. Health Part A* 69 (2007): 2095–136.

Huff, J. "NTP technical report on the toxicology and carcinogenesis studies of toluene, (CAS No. 108-88-3), in F344/N rats and B6C3F1 mice (inhalation studies)." NIH publication No. 90-2826. 1990.

Kim, E., J.C. Little, N. Chiu, and A. Chiu. "Inhalation exposure to volatile chemicals in drinking water." *Environ. Carcin. Eco. R. Part C* 2 (2001): 387-413.

Krishnan, K., and R. Carrier. "Approaches for evaluating the relevance of multiroute exposures in establishing guideline values for drinking water contaminants." *J. Environ. Sci. Health Part C* 26 (2008): 300-16.

Lee, S.C., M.Y. Chiu, K. F. Ho, S.C. Zou, and Xinming. Wang. "Volatile Organic Compounds (Vocs) in urban atmosphere of Hong Kong." *Chemosphere* 48 (2002): 375-82.

Lin, C., S. Chiang, and S. Lu. "Investigation of MTBE and aromatic compound concentrations at a gas service station." *Environ. Monit. Assess.* 105 (2005): 327-39.

Liu, L., and H. Zhou. "Investigation and assessment of volatile organic compounds in water sources in China." *Environ. Monit. Assess.* 173 (2011): 825-36.

Marchetti, A. A., J. I. Daniels, and D. W. Laton. 1994. Volume 4: "Potential ground and surface water impacts." Chapter 6: Environmental transport and fate of fuel hydrocarbon alkylates. edited by U. S. Department of Energy. University of California. Livermore. 1999.

Mateus, F. H., J. S. Lepera, M. P. Marques, V. B. Boralli, and V. L. Lanchote. "Influence of n-hexane inhalation on the enantioselective pharmacokinetics and metabolism of verapamil in rats." *Chirality* 22 (2010): 29-34.

Meulenberg, C.J.W, and H.P.M. Vijverberg, "Empirical relations predicting human and rat tissue:air partition coefficients of volatile organic compounds." *Toxicol. Appl. Pharmacol.* 165 (2000): 206-216.

McKone, T. E. "Human exposure to volatile organic compounds in household tap water: The indoor inhalation pathway." *Environ. Sci. Technol.* 21 (1987): 1194-201.

McKone, T. E. "The rise of exposure assessment among the risk sciences: an evaluation through case studies." *Inh. Toxicol.* 11 (1999): 611-22.

Nakajima, T., R.S. Wang, E. Elovaara, S.S. Park, H. V. Gelboin, E. Hietanen, and H. Vainio. "Monoclonal antibody-directed characterization of cytochrome P450 isozymes responsible for toluene metabolism in rat liver." *Biochem. Pharmacol.* 41, no. 3 (Feb 1991): 395-404.

Nakajima, T., R. S. Wang, E. Elovaara, Frank. J Gonzalez, H. V. Gelboin, H. Raunio, O. Pelkonen, H. Vainiot, and T. Aoyama. "Toluene Metabolism by CcDNA -expressed Human Hepatic Cytochrome P450. *Biochem. Pharmacol.* 53 (1997): 271-77.

Newill, V. A. "Significance of risk assessment in the management of environmental exposures of chemical mixtures." *Toxicol. Ind. Health* 5, no. 5 (1989): 635-46.

Pohl, H. R., and F. Scinicariello. "The impact of CYP 2e1 genetic variability on risk assessment of VOCs mixtures." *Regul. Toxicol. Pharm.* 59 (2001): 364-74.

RAMAS. "Methods and guidance for health risk assessment of chemical mixtures". 2003. http://www.ramas.com/mixture.htm June 23, 2011.

Rider, C., J. R. Furr, V. S. Wilson, and L. E. Jr. Gray. "Cumulative effects of in utero administration of mixtures of reproductive toxicants that disrupt common target tissues via diverse mechanisms of toxicity." *Int. J. Androl.* 33, no. 2 (2010): 443-62.

Saéz, P. B., and B. E. Rittmann. "Biodegradation kinetics of a mixture containing a primary substrate (Phenol) and an inhibitory co-metabolite (4-chlorophenol)." *Biodegradation* 4 (1993): 3-21.

Sexton, K., and D. Hattis. "Assessing cumulative health risks from exposure to environmental mixtures, three fundamental questions." *Environ. Health Persp.* 115, no. 5 (May 2007): 825-32.

Stellman, J. M. "*Hydrocarbons, Saturated and Alicyclic*." Encyclopaedia of Occupational Health and Safety. Vol. 4, Genova international labour office.1998.

Swann, H. E. Jr., B. K. Kwon, G. K. Hogan, and W. M. Snellings. "Acute inhalation toxicology of volatile hydrocarbons." *Am. Ind. Hyg. Assoc. J.* 35, no. 9 (Sep 1974): 511-8.

Takeuchi, Y., Y. Ono, and N. Hisanaga. "An experimental study on the combined effects of n-hexane and toluene on the peripheral nerve of the rat." *Brit. J. Ind. Med.* 38 (1981): 14-19.

ACGIH. 1999. "Threshold Limit Values for chemical substances and physical agents and Biological Exposure Indices." TLVs and BEIs, Cincinnati, OH.

U.S. EPA. "Citizen's guide to ground-water protection." 1990. Washington, DC. http://www.epa.gov/ogwdw/sourcewater/pubs/guide_citguidegwp_1990.pdf

U.S. EPA. Unites States Environmental Protection Agency. "Toxicological review of cyclohexane (CAS No. 110-82-7)." 2003. Washington, DC.

U.S. EPA. Unites States Environmental Protection Agency. "Toxicological review of n-hexane (CAS No. 110-54-3)." 2005. Washington, DC.

U.S. EPA. Unites States Environmental Protection Agency. "Toxicological Review of 2,2,4-Trimethylpentane (CAS No. 540-84-1)." 2007. Washington, DC.

WHO. 1991. "n-Hexane." Environmental health criteria 122. International programme on chemical safety, World Health Organization, Geneva. http://www.inchem.org/documents/ehc/ehc/ehc122.htm