TOXICOKINETICS AND EFFECTS OF 1,2,5,6,9,10-HEXABROMOCYCLODODECANE (HBCD) STEREOISOMERS IN MICE

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Toxicology.

Chapel Hill
2011

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ABSTRACT

DAVID TAYLOR SZABO: Toxicokinetics and Effects of 1,2,5,6,9,10-Hexabromocyclododecane Stereoisomers In Mice
(Under the direction of Linda S. Birnbaum)

Despite its small contribution to global production and usage of hexabromocyclododecane, α-HBCD is the major flame -retardant diastereomer found in environmental samples and human tissue. Limited toxicology studies suggest that the commercially available HBCD mixture (CM-HBCD) is a developmental reproductive and neurotoxicant, as well as an endocrine disrupter. This dissertation investigates the link between exposure and dose of the two major HBCD stereosiomers, α and γ. The thrust and novelty of this research rest on the examination of the different stereoisomers of HBCD. We hypothesized that these isomers differed in pharmacokinetic and toxicological properties and moreover could interconvert, thus conclusions based on testing the commercially-available mixture might not adequately predict the outcome of real-life exposures. To test this hypothesis and provide information essential to the human health risk assessment of HBCD, the basic toxicokinetic parameters of α-HBCD and γ-HBCD were characterized in adult mice, which included: the impact of repeated dosing on the disposition and elimination of α-HBCD and γ-HBCD and the disposition and elimination of α-HBCD and γ-HBCD in infantile mice were also investigated. Pathway analysis was performed which may shed

light on the mechanisms involved in effects leading to cognitive (learning and memory) deficits.

We found that in adult female mice, α-HBCD and γ-HBCD are both well absorbed, 90 and 85% respectively. Distribution is dictated by lipophilicity for α- but not for γ-HBCD. This is due to the rapid elimination of γ-HBCD by metabolism and stereoisomerization in contrast to the much slower elimination of α-HBCD, with a terminal whole -body half life of 4 days for γ-HBCD and 17 days for α-HBCD. Repeated exposure results in higher body burdens than a single exposure alone, demonstrating the potential for bioaccumulation of α-HBCD. This was not observed for γ-HBCD. α-HBCD has toxicokinetic properties similar to other POPs. Tissue distribution in developing animals is similar to adults; however, actual concentrations are higher in younger animals for both α-HBCD and γ-HBCD because of a less developed metabolism and excretion capability. A systems biology approach involving transcriptomics, proteomics, and metabolomics was used to characterize the mechanisms involved in the infantile mouse response to α-HBCD, γ-HBCD and CM-HBCD in liver and brain. Alteration in synaptic long term potentiation is a potential mechanism observed from these studies which may account for the reported developmental neurotoxicity previously observed *in vivo*. Overall, we have identified differences in both, the toxicokinetics and molecular effects associated with the two HBCD stereoisomers.

ACKNOWLEDGEMENTS

I am sincerely grateful to my advisor, Linda S. Birnbaum, for her guidance, support, encouragement, mentorship and friendship throughout my graduate school experience. I have benefited greatly from her experience including that provided by Janet Diliberto; whose open door was invaluable. I would also like to thank the members of my dissertation committee for their direction on my dissertation project.

I would also like to express my gratitude to the following people who were not only collaborators but shared their thoughts and insight along the way: Heldur Hakk and Janice Huwe. I appreciate the educational experience extended by the University of North Carolina, Curriculum in Toxicology as well as the Integrated Systems Toxicology Division, Office of Research and Development, U.S. Environmental Protection Agency.

I am grateful to Vicki Richardson, Chris McPherson and Rachael Turner for their encouragement and friendship during graduate school. Finally, I am infinitely thankful to my family, father Steven Szabo Sr., mother Kasija Szabo and aunt Olga Milkovich, who has been role-models while unconditionally supporting me throughout the years. A special acknowledgement is extended to my twin brother Steven Szabo Jr. who, along with my parents, has been exceptionally inspirational in my life.

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ADME	absorption, distribution, metabolism and excretion
BDE #	brominated diphenyl ether IUPAC #
BFR	brominated flame retardant
BSEF	Bromine Science and Environmental Forum
DNT	developmental neurotoxicity
HBCD	hexabromocyclododecane
LOEL	lowest observable effect level
MDR	multi-drug resistant proteins
NOAEL	no observable adverse effect level
NOEC	no observable effect concentration
OAT	organic anion transporter
PBDE	polybrominated diphenyl ether
PBT	persistent, bioaccumulative, toxic
PentaBDE	pentabrominated diphenyl ether
Pgp	P-glycoprotein
PHDD	polyhalogenated dibenzodioxins
PHDF	polyhalogenated dibenzofurans
POP	persistent organic pollutant
PND	postnatal day
T^4	thyroxine
TBBPA	tetrabromobisphenol A

CHAPTER I

GENERAL INTRODUCTION

What are Flame Retardants?

Every year, fires kill over 3000 people, injures more than 20,000, and results in property damages exceeding an estimated $11 billion in the United States alone (Karter, 2002). Fire incidence has dropped over the past twenty-five years, which are suspected to be due to decreases in the incidence of smokers, increases in fire alarms and smoke detectors, and fire prevention policies requiring the presence of flame retardant chemicals in many industrial products, There are over 175 different types of flame retardants, most of which are generally divided into classes that include the halogenated organics (usually brominated or chlorinated), phosphorus-containing, nitrogen-containing, and inorganic flame retardants. The brominated compounds are currently the largest market group because of their low cost and high performance efficiency.

How do brominated flame retardants inhibit fires?

A flame retardant is added to a variety of products to slow down the burning process or to delay the ignition or combustion of the product. In order for a flame retardant to work, it must interfere with one of the three elements required for combustion; heat, fuel, or oxygen. There are four stages during a fire: ignition; propagation; steady combustion; and termination. A flame retardant can interfere at any of these stages (Kesner et al., 2001). When BFRs absorb the required amount of energy (heat), the carbon-bromine bond breaks leading to the formation of bromine radicals. The bromine radical reacts with the hydrogen atoms in the polymer or captures free hydrogen atoms to form HBr (Kesner et al., 2001).

$$RH + Br\bullet \rightarrow HBr + R\bullet$$, where RH is the polymer

HBr slows down the chain reaction that takes place during the burning process. One example of this is deactivation of hydroxyl radicals, thereby reducing the amount of free oxygen to fuel the fire and forming water (Kesner et al., 2001).

$$\cdot OH + HBr \rightarrow H_2O + \cdot Br$$

The overall effect is that the bromine atom withdraws energy from the combustion-propagation stage of the fire, slowing the burning process (Kesner et al., 2001). A good flame retardant should be inexpensive, stable to heat, colorless, easily blended in with the polymer, should decompose before the polymer, must not change physical and mechanical properties once it is in the final product, must be stable at temperatures at which the product is formed, and does not release toxic substances into the environment (Kesner et al., 2001).

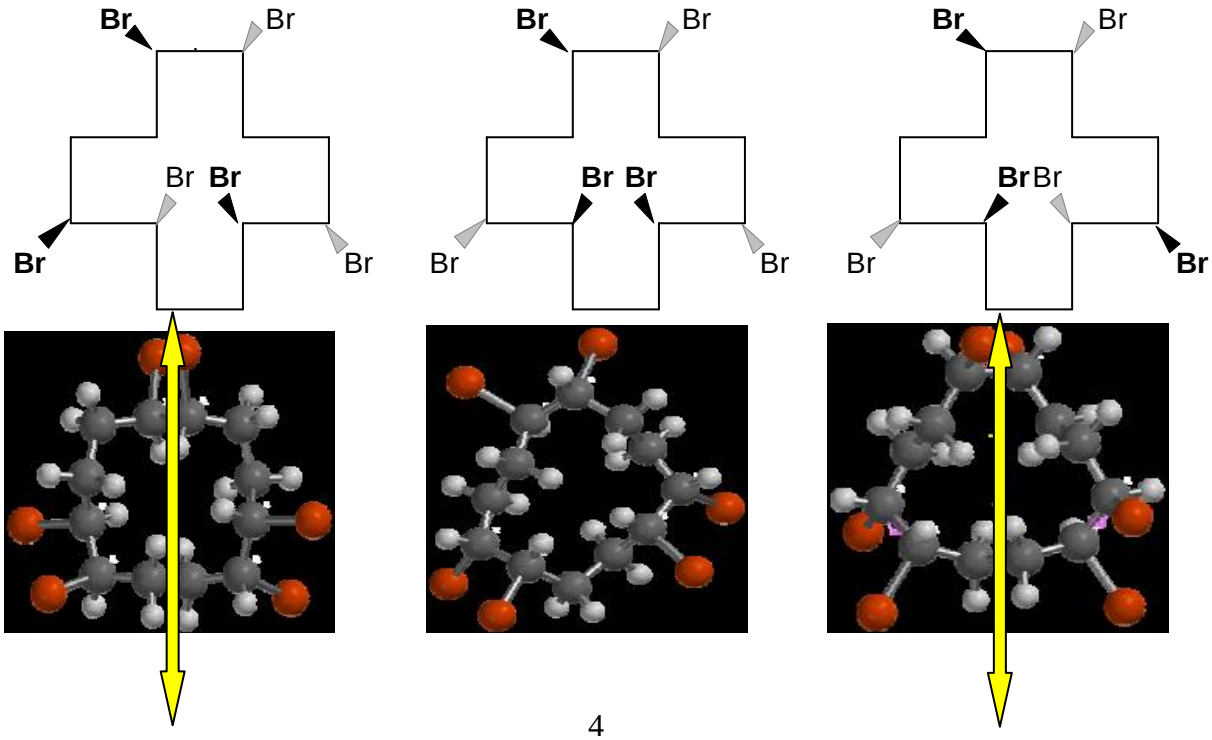
What are Hexabromocyclododecanes?

1,2,5,6,9,10-hexabromocyclododecane (HBCD, $C_{12}H_{18}Br_6$) is the most widely used aliphatic BFR and historically, the third BFR in global production volume following tetrabromobisphenol A and polybrominated diphenyl ethers (PBDEs), before the phase out of PBDEs in this past decade. In 2001, global demands for HBCD (~16.7 kilotons) was almost double that of the pentabromodiphenyl ether mixture (~7.5 kilotons) (Heeb et al 2005; de Wit et al., 2002). There are currently no restrictions on the use of HBCD in the US. There are two types of flame retardants, reactive and additive. Reactive flame retardants are covalently bonded to the polymer and additive flame retardants are added as a component of the

dissolved material. HBCD is an additive flame retardant. Being additive, there is the risk that HBCD can leach into the environment during use or after disposal of the product; other sources of release occur during production and manufacturing of the final product. HBCD is the principal flame retardant in extruded (XEPS) and expanded (EPS) polystyrene foams used as thermal insulation in the building industry. The amount of HBCD in plastics ranges from 0.5 to 7%, depending on the type of plastic (Remberger et al., 2004). Extruded polystyrene contains ~1-2% HBCD (Lassen et al., 1999; Zitco et al., 1993). Secondary uses of HBCD include residential and commercial upholstery textiles and electronics.

HBCD is synthesized by high temperature gas phase bromination of 1Z, 5E, 9E-cyclododecatriene (Peled et al., 1995). The resulting technical HBCD mixture (CM-HBCD) consists primarily of 3 HBCD diasteromers: α, β and γ (Figure 1.1). (The assignment of Greek letters is based on their elution order from a reverse phase HPLC column.)

Figure 1.1. Structures of α– (left), β– (center), and γ– (right) HBCD isomers.



Other isomers exist in lower quantities and attention has focused on the three main HBCD stereoisomers, which themselves are racemic mixtures of enantiomers. The proportions of the isomers in the technical mixture are dependent on the final synthetic process. Industrially, there are four different CM-HBCDs: low melt, medium range, high melt and thermal stabilized (Peled et al., 1995). Although there is a range, the overall *average* approximate proportions of each isomer in each technical mixture are 6, 8, and 80% for HBCD -α, -β and -γ, respectively. What makes each technical mixture different is the percent of γ-HBCD present, which ranges from ~70-90%. The selection of CM-HBCD grade used depends on the on the usage of the end-product. CM-HBCD is a solid, white powder at room temperature (National Academy Press, 2000). Physicochemical properties of CM-HBCD are as follows: molecular weight of 641.7 g/mol; melting point ranges from 185-195^{o}C and depends on the isomer ratio and impurity composition; vapor pressure of 4.7 x 10^{-7}mm Hg (at 25^{o}C) and the log octanol water partition coefficient (log K_{ow}) of approximately 5.6 (National Academy Press, 2000). The fact that HBCD has similar physiochemical properties to other persistent organic pollutants such as polychlorinated biphenyls (PCBs) and PBDEs, suggests that CM-HBCD may be persistent, bioaccumulative, and toxic (Remberger et al., 2004).

HBCD Stereoisomers are detected in the Environment and in Humans

Possible emission sources of HBCD stereoisomers into the environment include; manufacturing, use and disposal (Remberger et al., 2004). Emissions during

manufacturing can be by air, but due to HBCD stereoisomers' low volatility, emissions are more likely to occur with the release of plastic in dust (Remberger et al., 2004). The presence of HBCD stereoisomers in household dust has suggested that dust serves as a major source of exposure (Abdallah et al., 2008). Because it is an additive flame retardant, HBCD stereoisomers can leach from the material it has been impregnated into during the lifetime of the product. This can occur over a long period of time, as some products have long lifetimes. After disposal of a product in a landfill or during recycling it is possible for HBCD stereoisomers to leach into the air (particle bound) and water (surface and ground) (Remberger et al., 2004; Morris et al., 2004). HBCD (either as a total of each stereoisomer or each stereoisomer individually) has been quantified in sewage treatment plant sludge, which suggests that HBCD is associated with suspended particulate material and can be removed during the wastewater treatment process (Morris et al., 2004). E-waste is a source of HBCD to the local population and the local environment (Law et al., 2008). Recent trends in Asia show a leveling in concentrations of PBDEs but increases in concentration of HBCD stereoisomers (Law et al., 2008).

HBCDs has been shown to be bioavailable by measuring levels of HBCDs in pike captured near textile factories along the River Viskan in Sweden (Sellstrom et al., 1998). This suggests that food intake may be another major source of human exposure (Darnerud et al., 1998) coming mostly from fish with the estimated intake being approximately three times greater than certain PBDE congeners . HBCDs have been detected in the few human exposure studies conducted to date. Thomsen et al. (2007) reported that all serum samples tested in workers from an industrial plant occupationally exposed to CM-HBCD tested

positive for the presence of HBCD stereoisomers (ranged 6-856 ng/g lw). Ryan et al. (2006) reported that the mean concentrations in human breast milk of the HBCD stereoisomers collected in Ontario, Canada (2002-2003) and Texas, USA (2002) were 3.8 and 0.5 ng/g lipid weight (lw), respectively. Kakimoto et al. (2008) analyzed breast milk from Japanese women (age 25–29) over the period 1973–2006; total mean HBCD stereoisomer concentration over the period 2000 – 2006 ranged from 1 to 4 ng/kg lw. Higher values were reported in breast milk of women living in Spain between 2006-2007 (Eljarrat et al. 2009) where the mean concentration was 27 ng·kg-1 lw (range 3 to 188 ng/kg lw). Abdallah et al., (2011) detected HBCD in all breast milk samples in a UK population (average ΣHBCDs=5.95 ng/g lw).

Diastereomer shift

Until recently, all HBCD physicochemical property measurements were performed on the technical mixture as individual isomers were not readily available. Table 1.1 displays measured differences in water solubility were for α-, β-, and γ-HBCD stereoisomers (MacGregor and Nixon

Table 1.1. Water solubility for HBCD mixture and the three main stereoisomers.

	Technical Mixture	α–HBCD	β –HBCD	γ –HBCD
Water Solubility(ug/L)	3.4	48.8	14.7	2.08

(2004). Log K_{ow} for α-, β-, and γ-HBCDs have also been shown to have measurable differences from one another (5.65, 6.05 and 6.34, respectively) (Kuramochi et al., 2010). The 3-dimensional views of the isomers (Figure 1.1) show that the α– and γ–isomers both have a C_2 axis of symmetry while β–HBCD does not. High concentrations of HBCD stereoisomers have been detected in some top predators (marine mammals, birds of prey, humans) indicating persistence and biomagnification of HBCDs. At the diastereomer level, environmental samples, such as sediment and dust are shown to be predominately γ-HBCD (Covaci et al., 2006). Interestingly, recent studies have shown that there is a predominance of α-HBCD in wildlife and humans (Law et al., 2008). Abdallah et al., (2011) report in a breast milk biomonitoring study that α-HBCD dominated; comprising of 62-95% of ΣHBCDs while β- and γ-HBCD constituted 2-18% and 3-33% respectively. Law et al., (2006a) observed a sharp increase in α-HBCD levels within the blubber of harbor porpoises beginning in 2001 (within a period of 1994-2003). Another study by Law et al., (2006b) identified that α-HBCD had higher levels than other HBCD stereoisomers measured in Lake Winnepeg food chain. However, it is currently unknown whether this process is due to *in vivo* biotransformation (γ to α) or simply bioaccumulation, where the low levels of α-HBCD released into the environment accumulate in biota and biomagnify up the food chain. Limited evidence in the variation in solubility and partitioning behavior have been thought to explain the predominance of α-HBCD in aquatic and terrestrial organisms however, uptake and in vivo distribution and metabolism (stereoisomerization) of individual diastereoisomers currently remain untested.

Toxicokinetics (absorption, distribution, metabolism and excretion).

Information on the toxicokinetics of CM-HBCD and stereoisomers is limited. There is no human data on the toxicokinetics of HBCD for any route of exposure. Also, there are no peer-reviewed *in vivo* kinetic studies on the CM-HBCD or individual stereoisomers in any mammalian model. One study conducted by a manufacturer showed that the CM-[C^{14}]HBCD commercial mixture was readily absorbed in rats (unknown sex and strain) that received a single oral dose (Yu and Atallah, et al., 1980). The half-life was exceedingly short, i.e. approximately 2h. Within 72h, 16% of the dose was excreted in the urine and 72% in the feces. Four metabolites of unknown structure were found. CM-HBCD derived radioactivity had deposited in every sampled tissue in the rat, and the tissues with the highest levels were the adipose, liver, kidney, lung, and gonads. In another study conducted by a manufacturer, 7-9 mg/kg of the CM-HBCD was given to rats (unknown sex and strain) as a single oral dose, and gastrointestinal absorption was facile (Dean et al., 1977). However, the exact extent of oral absorption is unknown. Approximately 86% was eliminated in 72h, the majority in the feces (70%) and a lesser amount in the urine (16%). The half-life was again estimated to be less than 2h. Conflicting data was obtained in another unpublished study in which CM-[C^{14}]HBCD commercial mixture was administered daily for 5 days at 500 mg/kg (Ryuich et al., 1983). In that study design, there was no detectable urinary excretion over 96h, and the average daily fecal excretion ranged from 29-37%. In yet another unpublished study (NRC-NAP, 2000), CM-HBCD (500 mg/kg-d for 5 d) was found to accumulate exclusively in the adipose tissue with none detected in spleen, pancreas, liver, kidney, or heart of male Wistar rats. No toxicokinetic studies via the dermal or inhalation exposure

routes were reported in any experimental animals for individual diasterreomers. However, female New Zealand white rabbits exposed dermally to a high dose (8 g/kg) of CM-HBCD in saline experienced diarrhea and slight weight loss (Dean et al., 1977). This finding indicates that at least some absorption occurs via the dermal route. A dermal *in vitro* study on human skin has been performed according to OECD Test Guideline 428 in order to assess the rate and extent of dermal absorption of CM=HBCD (Roper et al., 2005). A value of 4% is assumed to be applicable for uptake of CM-HBCD commercial powder by the dermal route (Hughes et al., 2006). With conflicting data regarding the oral route of exposure and limited studies with other routes, these are clear data gaps which need to be filled.

Overview of HBCD Toxicity

The literature available for HBCD toxicity is relatively limited; however, increasing public attention on HBCDs has spurred more research to be conducted. Public health concern has focused primarily on potential hazardous effects resulting from exposure of infants and young children. These concerns are founded based on evidence from rodent studies showing that the CM-HBCD is a developmental neurotoxicant (Eriksson et al., 2006), as well as endocrine disruptor (Chegelis et al., 2001, van der Ven et al., 2006) and that exposure estimates suggest nursing infants and young children have the overall highest exposure to other flame retardants (Jones-Otazo et al., 2005). CM-HBCD has previously demonstrated developmental neurotoxicity (DNT) in mice following a single exposure (Eriksson et al., 2006). In this study, mice exposed on postnatal day 10 (PND 10) to one

single dose of the CM-HBCD exhibited permanent aberrations in spontaneous behavior and habituation capacity behaviors that continued to worsen over time, including changes in learning and memory. Other reports have identified toxicity of CM-HBCD in cerebella cells and CM-HBCD has been shown to alter levels of the neurotransmitters, glutamate and dopamine *in vitro* (Mariussen and Fonnum, 2003). Because evidence is mounting for toxicity in combination with persistent exposure, it is important to understand the toxicokinetics and toxicity of HBCD stereoisomer, α and γ in potentially susceptible populations (i.e., young, developing animals) especially considering there are no stereoisomer toxicity studies in any mammalian system.

A 28 day study was conducted in female and male Wistar rats (van der Ven et al., 2006), using a benchmark model design and oral administration of the CM-HBCD. The study mainly shows effects on the liver, thyroid, and pituitary. A NOAEL/BMD-L of 22.9 mg/kg/day for liver weight increase is calculated from this study. It has been suggested that the liver weight increase is caused by hepatic enzyme induction, as indicated by histopathology (proliferation of SER) and induced hepatic enzyme activities/mRNA/protein. Although there is no consistent difference in sensitivity towards hepatic enzyme induction between females and males, the concentration of HBCDs was higher in females than in males. Enzyme and protein induction is clearly involved, and is likely the most important reason for the liver weight increase; however pathways analysis has yet to be established. With regard to effects on the thyroid system, studies have either shown: no effect, effects *in vitro* effects only in females, or effects in both sexes concluding that the thyroid system

appears to be an inconsistent marker of toxicity after exposure to the CM-HBCD. Considering other toxicity endpoints, CM-HBCD has also been linked to carcinogenesis by inducing intragenic recombination in mammalian cells, similar to PCBs (Helleday et al., 1999).

What are potential mechanisms for HBCD toxicity?

Hepatic weight and thyroid hormone

So far, studies on the toxic potential of CM-HBCD have mainly focused on endocrine disruption. Although an inconsistent measure of toxicity after exposure to the CM-HBCD, adverse effects on the thyroid hormone system have been studied (Ema et al. 2008; Hamers et al. 2006; Palace et al. 2010; van der Ven et al. 2006). Effects on hepatic gene expression and hepatic enzymes have been observed (Cantón et al. 2008; Germer et al. 2006; Ronisz et al. 2004; Zhang et al. 2008). A hepatic mechanism of thyroid hormone disruption has been speculated after exposure to CM-HBCD. This would lead to increased liver weight through induction of XMEs. Induction of CAR and PXR mediated XME would metabolize thyroid hormones, facilitating its elimination. Ultimately, this results in a reduction of circulating T_4 levels. In addition, induction of CAR and PXR mediated XMEs is associated with the hepatic weight increases.

Developmental neurotoxicity

The mechanisms for behavioral and cognitive effects are not currently known for HBCD. It is assumed that a reduction in serum thyroid hormone levels during development *may* causes a reduction in the levels of thyroid hormone in the brain (van der Ven et al., 2009) leading to neurotoxicity. However, brain levels of thyroxine have not been measured and it is difficult to get a reliable measure considering the low levels present. Furthermore, direct effects of HBCD on neuronal cells (independent of thyroid hormone mechanism) have also been observed, but the overall acute *in vivo* toxicity studies of HBCD appears limited (Darnerud 2003). Researchers have suggested that the observed neurotoxic developmental effects in mice may be associated with alterations in cholinergic receptors (Eriksson *et al*. 2001) as well as due to oxidative stress and the cellular antioxidant defense systems in fish brain (Zhang et al. 2008). *In vitro* neurotoxicity data on CM-HBCD or stereoisomers is also limited. So far, only inhibition of dopamine uptake in rat synaptosomes (IC50: 4 μM) and synaptic vesicles (Mariussen and Fonnum 2003) has been observed.

Need for Research

Brominated flame retardants represent major industrial chemicals whose use has increased dramatically over the past few decades. They are produced to decrease flammability and increase the time to flash over and thus can have a direct and obvious benefit. However, concerns are being raised because of their persistence, bioaccumulation,

and potential for toxicity, both in animals and humans. Production and use patterns are different in various parts of the world. There is clearly a need for more systematic environmental and human monitoring to understand how and where these chemicals are being released into the environment, and what is happening to them once they enter the environment. What fate and transport processes are involved in their environmental movement? Are the commercial products stereoisomerizing in the environment or in biota? And if they are degrading, what are the resultant products? How are all of these chemicals getting into people? Is dietary intake the major pathway, as true for many other POPs or are there other potential sources? Once we understand what the exposure levels are in both people and wildlife, what should be our level of concern? Our toxicology database is inadequate to truly understand the risk associated with HBCDs. Many of the studies that do exist involve the commercial mixtures, which do not represent human exposure. Studies are needed that focus on the individual stereoisomers, and potentially their metabolite products, present in people and wildlife in order to understand the risk from exposure to HBCDs.

Classical toxicology is traditionally focused on understanding xenobiotic metabolism, the dose-response relationships between chemicals and adverse health effects, and mode-of-action evaluation for toxicant-induced disease. Novel molecular, biochemical, genetic and genomics approaches are increasingly used to understand the mechanisms of environmental agent-related organ injury. Toxicogenomics, proteomics and metabolomics is a rapidly maturing field which

provides the ability to define in greater detail the underlying molecular events preceding or accompanying toxicity. The incorporation of this new information requires careful validation, and altered gene expression patterns should be corroborated with conventional indices of toxicity (ie. developmental neurotoxicity; learning and memory). The systems biology approach intends to bring together -omics databases obtained from exposed and unexposed cellular or animal models and to establish relevant associations using newly developed computational technologies. The paradigm is to use the system biology based methods for describing molecular pathways involved in the human endpoint of interest. Applying this to the study of HBCDs, the main focus will be on pathway analysis leading to developmental neurotoxicity previously observed *in vivo* (Erikkson et al., 2006).

Rationale and Hypothesis for Project

In predicting human health risks posed by HBCD, it is necessary to accurately predict systemic dosimetry and the fate of these compounds. The dose/response relationship of a chemical must be well characterized in order to accurately describe the associated risk. A major focus for HBCD is to establish a basic knowledge of stereoisomer specific absorption, distribution, metabolism, and elimination (ADME). Currently, studies on the toxicokinetics of HBCDs are very limited and provide no data on specific stereoisomers in any mammalian system. There are no peer reviewed published pharmacokinetic studies performed on any mammalian system to date with HBCD.

Therefore, It is important to characterize the stereoisomers individually as differences in isomer properties (i.e. structure, log K_{ow}, and water solubility) can lead to over (or under) estimation of the overall toxicity. The differences in the source of exposure from either the mixture or from biota may be important as the composition of the HBCD stereoisomers which constitutes such matrices may dictate differential effects. Based on the limited toxicity profile and the need for basic distribution and elimination kinetics, the major commercial stereoisomer, γ-HBCD as well as the major biotic isomer α-HBCD will be investigated in adult female mice. In addition, we will examine the toxicokinetic and effects of a single oral dose of γ-HBCD and α-HBCD in infantile animals to assess if there are differential kinetic behaviors from adults which could at least in part explain the window of sensitivity for DNT. Furthermore, we will also investigate the possible neuronal and hepatic effects after exposure to CM-HBCD by using a systems biology approach with high throughput 'omics technologies (transcriptomics, proteomics and metabolomics) to help explain the DNT previously observed. These studies will provide data for the development of a PBPK model and aid in extrapolation of animal results to humans in order to assess the human health risk of HBCD.

The thrust and novelty of this research rest on the examination of the different stereoisomers of HBCD. We hypothesized that these isomers differ in pharmacokinetic and toxicological properties and moreover could interconvert, thus conclusions based on testing the commercially-available mixture might not adequately predict the outcome of real-life exposures. To test this hypothesis and provide information essential to elucidating the

diastereomer shift while adding valuable information for the human health risk assessment of HBCD, specific aims included: (1) characterize basic toxicokinetics in adult female mice; (2) investigate the impact of repeated dosing on the distribution and elimination of α-HBCD and γ-HBCD in adult female mice; (3) investigate the distribution and elimination of α-HBCD and γ-HBCD in infantile mice, a developmental model; and (4) identify mechanisms leading to the reported developmental neurotoxicity after exposure to the CM-HBCD and further determine whether that toxicity is driven by α-HBCD and/or γ-HBCD.

We hope to gain a much better understanding of the pharmacokinetic properties of individual diasteriomers of HBCD in mice as preliminary research suggests that the primary health outcomes involve endocrine and neurotoxic developmental effects. In addition, α-HBCD has been hypothesized to be produced from γ-HBCD in animals, resulting in increased lipophilicity and bioaccumulation. Neurobehavioral alterations and thyroid hormone disruption have been shown to occur in animals exposed to CM-HBCD. In order to understand the health effects it is essential to understand the pharmacokinetic as well as the phamacodynamic properties of this commonly-used flame retardant.

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Chapter II

TOXICOKINETICS OF THE FLAME RETARDANT HEXABROMOCYCLODODECANE GAMMA: EFFECT OF DOSE, TIMING, ROUTE, REPEATED EXPOSURE AND METABOLISM

2010
Szabo DT, Diliberto JJ, Hakk H, Huwe J and Birnbaum LS
Toxicological Sciences 117, 282-93

Title: Toxicokinetics of the flame retardant hexabromocyclododecane gamma: effect of dose, timing, route, repeated exposure and metabolism.

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1) Running Title: Toxicokinetics of the flame retardant hexabromocyclododecane gamma: effect of dose, timing, route, repeated exposure and metabolism.

2) Key words: brominated flame retardants (BFRs), HBCD, stereoisomer, toxicokinetics

3) Acknowledgements:

The information in this document has been subjected to review by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The research presented in this document was funded in part by the U.S. Environmental Protection Agency. The research was partially supported by the EPA/UNC Toxicology Research Program Training Agreement; NHEERL-DESE Cooperative Training in Environmental Sciences Research, EPA CT826513, with the Curriculum in Toxicology, University of North Carolina at Chapel Hill. A conflict of interest was not reported.

ABSTRACT

1,2,5,6,9,10-Hexabromocyclododecane-gamma (γ-HBCD) is the predominate diastereoisomer in the commercial HBCD mixture used as a flame retardant in a wide variety of consumer products. Three main diastereoisomers, alpha (α), beta (β) and gamma (γ) comprise the mixture. Despite the γ-diastereoisomer being the major diastereoisomer in the mixture and environmental samples, the α-diastereoisomer predominates human tissue and wildlife. This study was conducted to characterize absorption, distribution, metabolism and excretion parameters of γ-HBCD with respect to dose and time following a single acute exposure and repeated exposure in adult female C57BL/6 mice. Results suggest that 85% of the administered dose (3mg/kg) was absorbed after oral exposure. Disposition was dose-independent and didn't significantly change after 10 days of exposure. Liver was the major depot (<0.3% of dose) four-days after treatment followed by blood, fat and then brain. γ-HBCD was rapidly metabolized and eliminated in the urine and feces. For the first time, *in vivo* stereoisomerization was observed of the γ-diastereoisomer to the β-diastereoisomer in liver and brain tissues, and to the α- and β-diastereoisomer in feces. Polar metabolites in the blood and urine were a major factor in determining the initial whole-body half-life (1 day) after a single oral exposure. Elimination, both whole-body and from individual tissues, was biphasic. Initial half-lives were approximately 1 day, whereas terminal half-lives were up to 4 days, suggesting limited potential for γ-diastereoisomer bioaccumulation. The toxicokinetic behavior reported here has important implications for the extrapolation of toxicological studies of the commercial HBCD mixture to the assessment of risk.

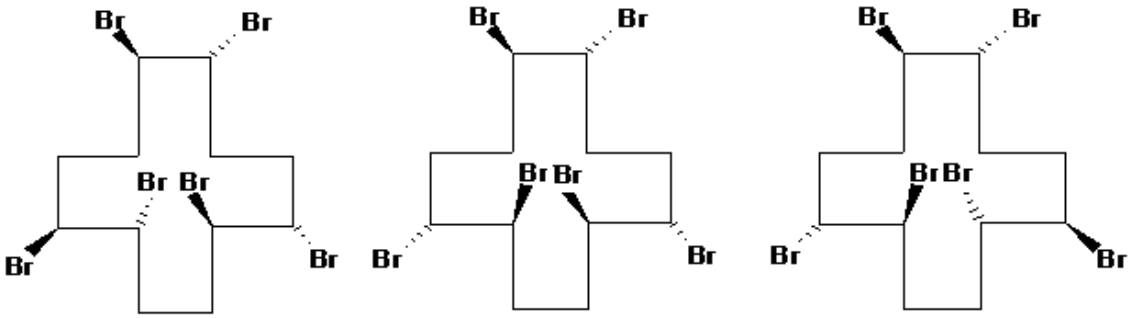
INTRODUCTION

Flame retardants are added to a variety of consumer goods in an effort to reduce flammability. With the ban of all polybrominated diphenyl ether (PBDE) commercial mixtures in the European Union and North America's increased awareness of safer alternatives, hexabromocyclododecanes (HBCD) has increased in production and use. HBCD is a brominated aliphatic cyclic hydrocarbon and a high production volume chemical used as a flame-retardant for plastics and textiles. In 2001 the total market demand for HBCD was estimated to be over 16700 tons per year, of which 2800 tons were from the United States, 9500 tons from Europe, 3900 tons from Asia, and 500 tons from the rest of the world (Law et al., 2006; Hale et al., 2006). HBCD is used as an additive flame retardant allowing release from products into our environment. HBCD is now a ubiquitous contaminant in environmental media and biota (Law et al., 2008). Currently, there are no restrictions on HBCD production and use. It has been shown in laboratory animals to be a hepatic enzyme inducer (Hamers et al., 2006), developmental neurotoxicant (Eriksson et al., 2006), and endocrine disruptor (van den Ven et al., 2006). Recent studies have suggested HBCD is highly bioaccumulative (Law et al., 2006).

Measurable HBCD concentrations have been reported in few human exposure studies conducted to date. HBCD can be considered an emerging contaminant with median blood values varying in humans between 0.35 and 1.1ng/g lipid (Covaci et al. 2006). Occupationally exposed workers in Norway showed higher concentrations in serum ranging

from 6 to 856 ng/g lipid (Thomsen et al., 2007). Dermal and inhalation routes of HBCD exposure may be quantitatively important for human uptake (Thomsen et al., 2007). However, diet is considered a more important source for HBCD exposure (Schecter et al., 2009), especially for humans consuming large quantities of fish, which reportedly contains relatively high HBCD levels from 9 to 1110ng/g lipid (Janak et al., 2005; Xian et al., 2007). Besides diet, house dust is likely another important pathway of human exposure to HBCD due to high levels present indoors (Roosens et al., 2009). Non-dietary ingestion of dust and soil may especially represent an important route of exposure for toddlers and young children (Lioy et al., 2000; Wilford et al., 2005; Wu et al., 2007).

Commercial HBCD is a mixture of different 1,2,5,6,9,10-hexabromocyclododecane diastereoisomers (Figure 1) with previous research focusing on the three diastereoisomers in the commercial mixture, denoted as alpha (α), beta (β), and gamma (γ) with the γ-diastereoisomer predominating (>70%) (Heeb et al., 2005).



α-HBCD β-HBCD γ-HBCD

Figure 2.1. Chemical structures of 1,2,5,6,9,10-Hexabromocyclododecane alpha, (α-HBCD), beta (β-HBCD) and gamma (γ-HBCD).

High concentrations of HBCD in some top predators (marine mammals, birds of prey, humans) indicate persistence and biomagnification of HBCD. However most of these early studies did not examine individual HBCD diastereoisomers. Recent studies have shown that there is a predominance of alpha in biota (Law et al., 2008). Furthermore, γ-HBCD is susceptible to thermal rearrangement at temperatures above 160°C (Peled *et al.*, 1995), resulting in predominant conversion to the α-diastereoisomer (Baratoni et al., 2001). Differences in water solubility are found for α-, β- and γ-HBCDs (48.8, 14.7 and 2.1 ug/L, respectively) (MacGregor and Nixon 2004). Variations in the solubility and partitioning behavior, as well as uptake and metabolism of individual diastereoisomers, are thought to explain the predominance of α-HBCD in aquatic and terrestrial organisms. These physico-chemical differences of HBCD diastereoisomers in wildlife and laboratory studies may also result in different biological effects.

Aside from thermal rearrangement observed during the manufacturing processes of residential and commercial foam insulation, *in vivo* metabolism may also convert the γ-diastereoisomer in the technical mixture to a prevalence of the α-diastereoisomer in wildlife and humans (Covaci et al., 2006). This phenomenon can be explained by either a difference in pharmacokinetic rates, where the γ-diastereoisomer is metabolized and eliminated at a more rapid rate than the α-diastereoisomer, and/or stereoisomerization of the γ-diastereoisomer to α. Selective metabolism is supported by *in vitro* assays with phenobarbital induced hepatic rat and non-phenobarbital-induced seal microsomes, where the γ- and β- diastereoisomers were significantly metabolized, while the α-diastereoisomer was

not (Zegers et al., 2005). Results from this study further suggest the occurrence of cytochrome P450 (CYP) mediated metabolism. Thus, in order to address the biological fate, biotransformation and effects of HBCD, individual diastereoisomers should be studied.

In predicting human health risks posed by HBCD, it is necessary to accurately predict internal dose and the biological fate of these compounds. Therefore, more detailed information about the kinetics, toxicology, pathways of exposure, and bioavailability of HBCD is needed. The objective of this study is to describe absorption, distribution, metabolism, and excretion (ADME) of γ-HBCD following an acute exposure in adult female mice at several doses, after repeated treatment, and up to 14 days.

MATERIALS AND METHODS

Chemicals: [^{14}C]1,2,5,6,9,10-hexabromocyclododecane (HBCD) (2mCi/mmol) was purchased from American Radiochemicals Corporation (ARC) (St Louis, MO) as a mixture of ß- and γ-diastereoisomer and [^{14}C]1,2,5,6,9,10-hexabromocyclododecane gamma (γ-HBCD) was purified at the USDA Agricultural Research Service (ARS) laboratory (Fargo, ND) by flash chromatography on a silica gel column eluting with hexane containing increasing amounts of methylene chloride (0–50 %). The elution order from the silica gel column was γ followed by ß. The γ-[^{14}C]HBCD was 98% radiochemically and diasteromerically pure as determined by thin-layer chromatography (TLC) using silica gel plates (250 mm; Whatman Lab. Div., Clinton, NJ) and a 50:50 methylene chloride:hexane

mobile phase with radiochemical detection by a System 2000 Imaging Scanner (Bioscan, Washington, DC) and by liquid chromatography-mass spectrometer (LC-MS) on a Symmetry C_{18} column (2.1x100 mm) (Waters, Beverly, MA) (details below). Unlabeled γ-HBCD was generously provided by Wellington Labs (98% purity). Other chemicals used were of the highest grade commercially available.

Dosing Solutions: Doses were selected based on published toxicity studies, environmental relevance, and specific activity of γ-[^{14}C]HBCD (van der Ven et al., 2006 and Law et al., 2008). A stock solution of γ-[^{14}C]HBCD was made by dissolving 19.23 mg of γ-[^{14}C]HBCD (3.12 μCi/mg) in toluene (400ul). Aliquots were used directly from this solution for all dosing regimens. All dosing solutions were subjected to pre- and post- dosing radioactivity examination to ensure proper delivery of dose. All solutions were designed to deliver approximately 0.2μCi to each mouse. Unlabeled γ-HBCD was added to the γ-[^{14}C]HBCD to achieve desired mass (all doses except low dose of 3 mg/kg), and was added directly to the dosing solution vial and dissolved in acetone. Corn oil by weight was then added to the vials followed by the evaporation of toluene and acetone under vacuum (Speed Vac, Savant Instruments, Inc. Farmingdale, NY). For intravenous (iv) treatment, 10 ml of stock γ-[^{14}C]HBCD was allowed to evaporate in an amber vial, and the γ-HBCD was resuspended in 95% ethanol followed by Emulphor®. De-ionized water was slowly added to a final volume of 4.3 ml with an ethanol:Emulphor:water ratio of 1:1:8.

Animals. Female C57BL/6 mice (~20 grams) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Mice were chosen for these experiments because 1) of the limited amount of radiolabeled chemical and 2) neurotoxicity was observed in mice (Eriksson et al., 2006). Mice were maintained on a 12 h light/dark cycle at ambient temperature (22°C) with relative humidity (56 ± 5%), and were provided with Purina 5001 Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water *ad libitum*. Prior to the commencement of the study, mice were adapted (3 mice/cage) for 1 week to Nalgene metabolism cages (Nalgene, Rochester, NY). Mice were then assigned randomly to treatment groups (n= 4-6) and housed individually for the remainder of the study. All mice were 60 days old at time of treatment.

Route of Exposure: *Oral treatment* (n=4-8). A single dose (0, 3, 10, 30 and 100 mg/kg) was administered directly by oral gavage into the stomach of each mouse using a teflon animal feeding needle. Dose volume was 10ml/kg. *Intravenous treatment* (n=6-8). A single dose (3 mg/kg) was administered intravenously via the tail vein at a dosing volume of 2 ml/kg.

Treatment: *Dose/Response:* A single dose (3, 10, 30, or 100 mg/kg at 10ml/kg) was administered by oral gavage. After dosing, mice were held in metabolism cages for 4 days and urine and feces were collected daily. *Time course:* Mice were treated by gavage at a single dose of 3 mg/kg and held for 14 days where urine and feces were collected daily.

Repeated: Mice were dosed for 9 days with 3 mg cold γ-HBCD/kg and on day 10 with 3 mg γ-[^{14}C]HBCD/kg and held for 4 more days (total of 14 days) in metabolism cages for collection of urine and feces. Animals were euthanized by CO_2 asphyxiation followed by exsanguination via cardiac puncture. Tissues were collected and weighed: blood, bile, liver, lung, kidneys, skin, adrenal glands, urinary bladder, spleen, thymus, adipose (abdominal), muscle (abdominal), and brain. Bile was removed directly from the gallbladder using the BDUltra-fine Insulin syringe.

Sample Analysis: Radioactivity in the tissues was determined by combustion to $^{14}CO_2$ (Packard 307 Biological Oxidizer, Downers Grove, IL) of triplicate samples when available (~100mg/sample) followed by liquid scintillation spectrometry (LSS; Beckman, Beckman Instruments, Fullerton, CA). Tissue data are reported based on wet weight. Feces were air dried, weighed, and analyzed for radioactivity by combustion and LSS. Daily urine volume was recorded, and 100 µl aliquots (triplicate) were analyzed by direct addition into scintillant for radioactivity determination by LSS.

Tissue Extraction and Analysis: Liver samples were weighed and then homogenized in 3 volumes of 0.9% sodium chloride solution. Feces were dried and ground into a very fine powder with a mortar and pestle. Serum, liver, bile, urine and feces samples were extracted sequentially with 3 volumes of hexane, ethyl acetate, and methanol. Samples were vortexed for 5 minutes and the top organic layer removed. Brains were homogenized by hand in 2ml

of hexane:acetone (1:3.5) while the adipose tissue. Two milliliters of hexane: ether (9:1) was added, vortexed, and centrifuged to allow layer separation. The upper layer was decanted and transferred to a vial. Extractions were performed twice and pooled. Several analytical procedures were used to determine the concentrations of γ-[^{14}C]HBCD and its metabolites in the experiments. Liquid scintillation counting was used to analyze for total ^{14}C while thin-layer chromatography (TLC) was used to differentiate parent from metabolites of γ-[^{14}C]HBCD based on polarity. Gel permeation chromatography (GPC) was used to remove large macromolecules which can interfere with further analysis of low level of target molecules found in brain samples. Liquid chromatography/mass spectrometry (LC-MS) was also used to analyze selected samples. Liquid scintillation counting was done using a 1900 CA Scintillation Counter (Packard, Downers Grove, IL), and TLC analysis was done using a System 2000 Imaging Scanner (Bioscan, Washington, DC). TLC was conducted using silica gel plates (250 mm; Whatman Lab. Div., Clinton, NJ) and a (50:50) methylene chloride:methanol mobile phase. Brain sample extracts were applied to a GPC column (Bio-Beads S-X3 Beads, 200-400 mesh; Bio-Rad Laboratories Inc., Hercules, CA; Catalog #152-2750). Column (40cm height by 1cm diameter) was packed with Bio-Beads, after swelling in methylene chloride, and rinsed several times before applying sample. Sample was applied and the column was eluted with methylene chloride, at a flow rate of 0.5ml/min. Fractions were individually assayed by LSC (Packard 1900CA Liquid Scintillation Counter). Fractions (peaks) were collected and submitted for LC-MS. Unextractable ^{14}C was also determined by combustion from the liver and feces. The liquid chromatograph system of the LC-MS was an Alliance 2695 Separation Model (Waters, Beverly, MA) equipped with a Symmetry C_{18}

column (2.1 mm x 100 mm) and guard column (2.1 by 10 mm), and a quadrupole-time of flight mass spectrometer (Waters Q-TOF Ultima API-US; Waters, Beverly, MA). Isocratic elution conditions were used which consisted of 15% aqueous 10 mM NH_4OH and 85% methanol:acetonitrile (80:20 v/v), 10 mM NH_4OH. The flow rate of the mobile phase was 0.3 mL min^{-1}. The mass spectrometer analysis was performed in negative ion mode (ES-) using a 634 m/z filter.

Data Analysis: Mouse body composition estimates for blood, fat, skin, and muscle were 8,8,12 and 35% respectively (ILSI, 1994). In the route of exposure study, the oral tissue disposition data refers to the mean of all data collected from the several dose and time phases in which a) animals were exposed orally with a 3mg/kg dose, and b) 4 day time points were available. Intergroup comparisons for dose and tissue levels were performed by a two-way ANOVA followed by Bonferroni post tests significant when $p<0.05$. All data are presented as mean ± standard deviation (Prism 5.0 is distributed by GraphPad Software, Inc, La Jolla, CA). Hearn Scientific Software (Melbourne, Australia) was used for half-life calculations and to calculate the % dose and statistical analysis.

RESULTS

Dose Response

Tissue distribution results as a percent of γ-[^{14}C]HBCD derived radioactivity in female C57BL/6 mice are presented in Table 1. The top table (A) represents the dose response

(where response is a function of disposition) after administration of a single and repeated oral dose of γ-[^{14}C]HBCD at a concentration of 3, 10, 30 or 100 mg/kg. Tissue distribution was analyzed four days after the administration of γ-HBCD. All tissues examined had low but measurable levels four days after dosing. We found tissue disposition to be independent of dose for the 3, 10, 30, 100 mg/kg doses. The largest percentage of the dose in the mice was localized in the liver and ranged between 0.21 to 0.29%. This was followed by skin (0.14% to 0.17%) and muscle (0.09% to 0.10%). Low levels were detected in the blood (0.06% to 0.09%), brain (0.01%) and then fat (0.003% to 0.005%). These results also demonstrate the absence of tissue-specific sequestration.

Table 2.1. Disposition of γ-HBCD in Mice

(A)

Dose	Adrenals	Skin	Liver	Lung	Muscle	Kidney	Blood	Adipose	Brain
mg/kg	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)
3	0.008 ± 0.001 (492 ± 62)	0.140 ± 0.020 (248 ± 35)	0.240 ± 0.023 (160 ± 15)	0.018 ± 0.001 (79 ± 4.3)	0.090 ± 0.011 (68 ± 8.3)	0.013 ± 0.001 (60 ± 4.6)	0.081 ± 0.005 (24 ± 1.5)	0.005 ± 0.001 (27 ± 5.4)	0.008 ± 0.001 (24 ± 3.0)
10	0.008 ± 0.001 (1745 ± 358)	0.149 ± 0.031 (744 ± 89)	0.207 ± 0.039 (440 ± 86)	0.018 ± 0.002 (258 ± 23)	0.100 ± 0.010 (203 ± 7.2)	0.012 ± 0.001 (193 ± 12)	0.086 ± 0.009 (90 ± 9.5)	0.003 ± 0.001 (146 ± 73)	0.007 ± 0.001 (73 ± 10)
30	0.009 ± 0.002 (5537 ± 893)	0.165 ± 0.069 (2480 ± 301)	0.289 ± 0.029 (2015 ± 213)	0.014 ± 0.001 (607 ± 406)	0.101 ± 0.043 (678 ± 80)	0.014 ± 0.001 (664 ± 72)	0.082 ± 0.008 (270 ± 26)	0.003 ± 0.001 (625 ± 262)	0.009 ± 0.001 (248 ± 28)
100	0.007 ± 0.001 (18354 ± 426)	0.154 ± 0.037 (7688 ± 809)	0.241 ± 0.061 (6811 ± 1233)	0.017 ± 0.002 (2502 ± 227)	0.094 ± 0.021 (2101 ± 87)	0.013 ± 0.002 (2124 ± 290)	0.061 ± 0.008 (867 ± 93)	0.004 ± 0.001 (2580 ± 890)	0.008 ± 0.001 (808 ± 101)
3 Repeated	0.009 ± 0.002 (504 ± 38)	0.190 ± 0.020 (302 ± 70)	0.260 ± 0.034 (178 ± 22)	0.019 ± 0.005 (80 ± 21)	0.110 ± 0.020 (77 ± 14)	0.015 ± 0.001 (70 ± 5.0)	*0.134 ± 0.02 (40 ± 8.1)	*0.008 ± 0.001 (39 ± 4.9)	0.010 ± 0.002 (27 ± 5.4)

(B)

Time	Adrenals	Skin	Liver	Lung	Muscle	Kidney	Blood	Adipose	Brain
1 hour	0.008 ± 0.001 (492 ± 1.7)	0.042 ± 0.002 (76 ± 7)	3.33 ± 0.76 (2309 ± 220)	0.018 ± 0.002 (79 ± 6.0)	0.249 ± 0.050 (148 ± 3.1)	0.013 ± 0.001 (63 ± 3.8)	1.23 ± 0.30 (340 ± 85)	0.002 ± 0.000 (15 ± 2.5)	0.013 ± 0.001 (35 ± 5.2)
3 hours	0.024 ± 0.002 (1627 ± 37)	0.165 ± 0.010 (248 ± 12)	2.63 ± 0.24 (1862 ± 86)	0.083 ± 0.010 (377 ± 23)	0.519 ± 0.051 (311 ± 9.2)	0.11 ± 0.001 (560 ± 20)	0.303 ± 0.011 (113 ± 19)	0.008 ± 0.001 (40 ± 8.0)	0.035 ± 0.001 (101 ± 2.8)
8 hours	0.018 ± 0.002 (1145 ± 54)	0.280 ± 0.026 (420 ± 37)	1.82 ± 0.51 (1335 ± 59)	0.065 ± 0.008 (270 ± 11)	0.436 ± 0.020 (256 ± 14)	0.11 ± 0.001 (522 ± 19)	0.278 ± 0.002 (105 ± 8.7)	0.022 ± 0.001 (96 ± 7.2)	0.018 ± 0.001 (55 ± 10)
1 day	0.014 ± 0.001 (850 ± 40)	0.173 ± 0.018 (260 ± 21)	0.886 ± 0.03 (631 ± 26)	0.032 ± 0.002 (172 ± 14)	0.259 ± 0.022 (155 ± 13)	0.035 ± 0.002 (163 ± 11)	0.153 ± 0.001 (65 ± 9.3)	0.030 ± 0.001 (108 ± 11)	0.010 ± 0.001 (29 ± 175)
2 days	0.009 ± 0.002 (498 ± 5.9)	0.144 ± 0.011 (251 ± 19)	0.382 ± 0.09 (233 ± 15)	0.023 ± 0.002 (98 ± 11)	0.105 ± 0.021 (84 ± 9.2)	0.015 ± 0.001 (74 ± 5.0)	0.100 ± 0.010 (32 ± 3.1)	0.006 ± 0.05 (29 ± 3.0)	0.008 ± 0.002 (28 ± 3.7)
4 days	0.008 ±0.001 (492 ± 62)	0.140 ± 0.020 (248 ± 35)	0.240 ± 0.023 (160 ± 15)	0.018 ± 0.001 (79 ± 4.3)	0.090 ± 0.011 (68 ± 8.3)	0.013 ± 0.001 (60 ± 4.6)	0.081 ± 0.005 (24 ± 1.5)	0.005 ± 0.001 (27 ± 5.4)	0.008 ± 0.001 (24 ± 3.0)
7 days	0.007 ± 0.001 (450 ± 20)	0.130 ± 0.012 (192 ± 15)	0.171 ± 0.09 (139 ± 9.3)	0.014 ± 0.001 (61 ± 12)	0.085 ± 0.010 (60 ± 10)	0.010 ± 0.002 (44 ± 4.1)	0.069 ± 0.002 (21 ± 2.4)	0.003 ± 0.001 (20 ± 1.9)	0.007 ± 0.001 (23 ± 1.7)
14 days	0.006 ± 0.002 (430 ± 38)	0.123 ± 0.011 (184 ± 12)	0.099 ± 0.05 (76 ± 2.4)	0.012 ± 0.001 (50 ± 2.1)	0.072 ± 0.002 (47 ± 3.4)	0.009 ± 0.001 (38 ± 3.4)	0.052 ± 0.015 (16 ± 2.1)	0.0021 ± 0.001 (15 ± 2.0)	0.007 ± 0.002 (22 ± 3.7)

(C)

Route	Adrenals	Skin	Liver	Lung	Muscle	Kidney	Blood	Adipose	Brain
i.v.	0.011 ± 0.002 (677 ± 123)	0.162 ± 0.025 (287 ±.44)	*0.289 ± 0.021 (193 ± 18)	0.021 ± 0.002 *(96 ± 9.1)	*0.103 ± 0.020 (75 ± 7.3)	*0.016 ± 0.001 *(72 ± 5.2)	*0.095 ± 0.007 *(29 ± 2.1)	0.006 ± 0.001 (32 ± 5.1)	0.008 ± 0.001 (24 ± 3.0)
oral	0.008 ± 0.001 (492 ± 62)	0.140 ± 0.020 (248 ± 35)	0.240 ± 0.023 (160 ± 15)	0.018 ± 0.001 (79 ± 4.3)	0.090 ± 0.01 (68 ± 8.3)	0.013 ± 0.001 (60 ± 4.6)	0.081 ± 0.005 (24 ± 1.5)	0.005 ± 0.001 (27 ± 5.4)	0.008 ± 0.001 (24 ± 3.0)

Disposition of γ-HBCD derived radioactivity (A) four days following a single (3, 10, 30, 100 mg/kg) and 10 day repeated (3 mg/kg) oral dose, (B) four days following a 3 mg/kg dose through i.v. or oral and (C) at multiple time points following a single 3 mg/kg oral dose. All

data are mean ± standard deviation; represented as percent dose (top value) or concentration of nanogram of administered dose/gram of tissue (ng/g; bottom value in parenthesis).
*Indicates significance as compared to lowest dose ($p<0.05$).

Repeated Exposure

Tissue disposition was not altered after a 10 day repeated exposure in all tissues except for adipose and blood (Table 1. A). Disposition was significantly increased between a single and repeated oral exposure of 3mg/kg γ-[^{14}C]HBCD in the adipose tissue (0.005 ± 0.002% vs. 0.010 ± 0.001% respectively) and in the blood (0.081 ± 0.005 vs. 0.134 ± 0.02).

Time-Course

From the dose/response study, the lowest dose was chosen (3mg/kg) for the kinetic study because its behavior was in the linear range and it is the most environmentally relevant dose examined from that study. By observing tissue disposition over time, low but detectable concentrations were present in tissues at all time points investigated (Table 1.B). This 14 day time course study shows a biphasic profile with an initial steep decline on average from 1 hours to 2 days and a less steep decline between 2 days and 14 days for liver, kidney, blood and brain (Figure 2).

Tissue Specific Two Component Exponential Decay Curve after Oral Exposure to γ-HBCD.

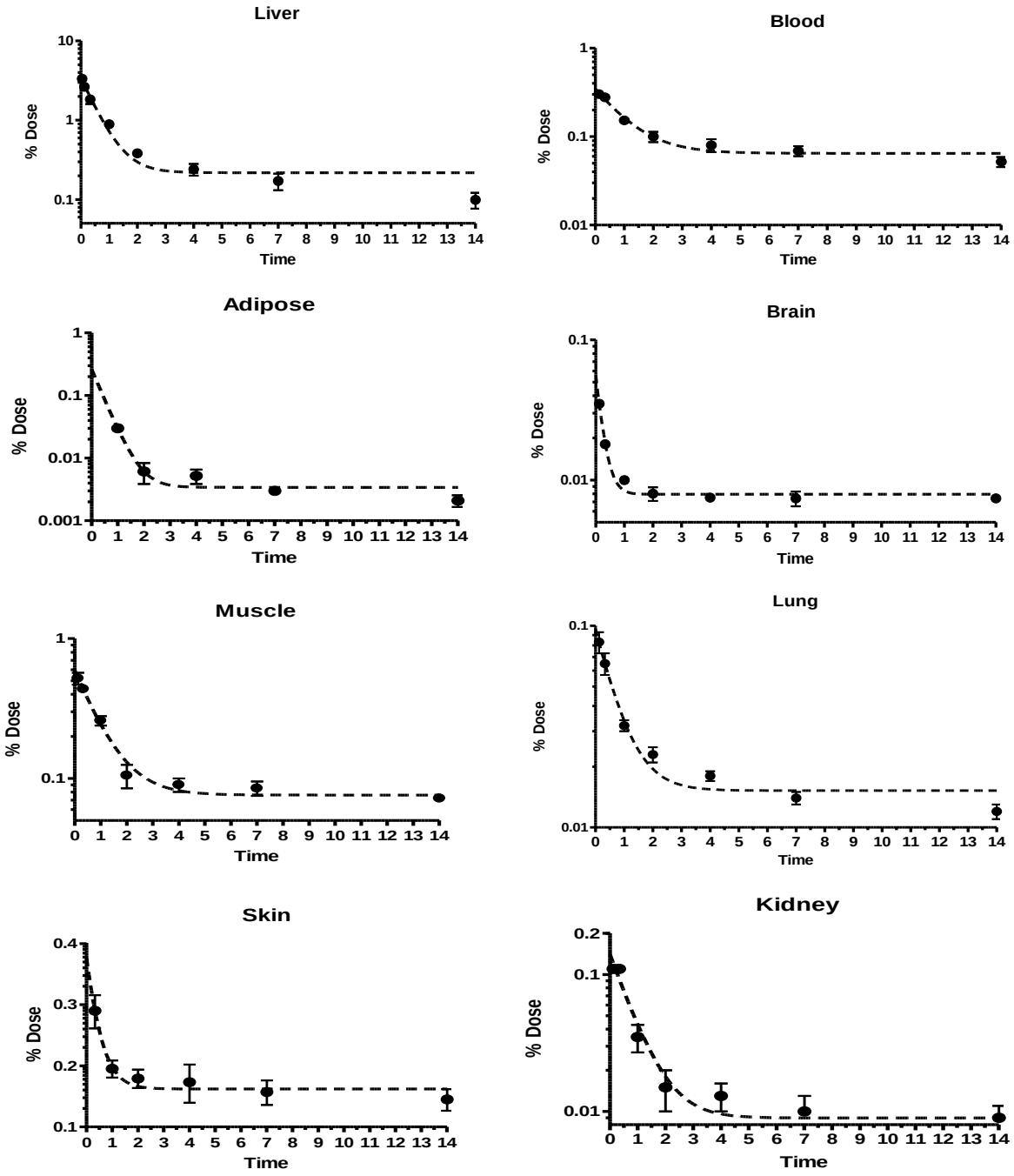


Figure 2.2. Percentage of total γ-HBCD derived radioactivity in tissues vs. time (in days) after a single oral administration of 3mg/kg γ-[^{14}C]HBCD. Each point represents the average

value ± standard deviation obtained from 4-6 animals. The dashed line represents a nonlinear regression fit line for a two component exponential decay curve from the *maximum* concentration in each tissue and through day 14. The y-intercept at time = 0 is back extrapolated from peak concentration in each tissue.

Liver tissue and blood had the highest observable levels where maximum concentrations were measured at 1 hour. While most tissues had highest measured levels at 3 hours, γ-[^{14}C]HBCD derived radioactivity had maximum measured concentration at 1 day in fat. The delay in maximum measured concentration in the adipose tissue is a function of a slow rate of perfusion and possibly transient partitioning based on this compound's lipophilicity. Times to maximum observed concentrations were plotted in Figure 2.

After oral exposure to γ-[^{14}C]HBCD, the distribution of radioactivity as a function of time was examined in the major tissue depots: liver, blood, fat, skin and muscle. In all cases, the loss of radioactivity from the tissue could be described by an exponential decay curve, consisting of two components. As can be seen in Figure 2, γ-HBCD derived radioactivity is rapidly cleared from the blood, so that by 1 hour after oral administration, less than 1.3% of the dose remained in the blood. From this point on, a two component exponential decay curve is used to fit the clearance of γ-HBCD derived radioactivity from tissues. The half lives and pool sizes of these two components are shown in Table 2.

Table 2.2. Estimated Tissue Half-lives of γ-HBCD in Mice

	Half-life (days)		Pool size (% dose)	
Tissue	α½	β ½	α phase	β phase
Liver	0.3 ± 0.0	2.3 ± 0.2	2.47 ± 0.42	0.61 ± 0.04
Blood	0.6 ± 0.1	3.5 ± 0.3	0.21 ± 0.01	0.08 ± 0.00
Lung	0.4 ± 0.1	2.3 ± 0.2	0.07 ± 0.01	0.03 ± 0.00
Kidney	0.2 ± 0.0	2.8 ± 0.2	0.12 ± 0.01	0.02 ± 0.00
Muscle	1.0 ± 0.1	3.6 ± 0.3	0.57 ± 0.03	0.45 ± 0.02
Skin	0.4 ± 0.0	5.2 ± 0.3	0.24 ± 0.02	0.15 ± 0.01
Brain	0.1 ± 0.0	0.8 ± 0.1	0.04 ± 0.0	0.02 ± 0.00
Fat	0.9 ± 0.1	3.6 ± 0.2	--	--

Tissue specific, biphasic half-lives were calculated from female mice given a single oral dose (3mg/kg) of γ-[^{14}C]HBCD. Calculations are derived from % of administered dose. α and β phase time points are individually based on peak tissue concentrations. Pool size was calculated by determining the y-intercept of each phase. Back extrapolation from adipose tissue peak concentration over predicted the pool size due to increased time to reach maximal peak concentration based on perfusion coefficient and therefore not reported here. All data presented in days.

IV vs. Oral: Tissue Disposition

Environmental chemicals are rarely encountered by an intravenous route. However, by comparing the tissue disposition between iv and oral routes we are able to determine the

percentage absorbed through the gut. To address gut absorption, a dose identical to the oral dose was administered to mice by iv, and tissue disposition was compared. A comparison of the major tissue depots 4 days after treatment with the same oral and iv dose is shown in Table 1.C. The percent dose and concentration (ng/g) was similar between both routes, but slight differences were observed. Although every tissue measured had higher levels after iv as compared to the oral route of exposure (same values for brain), not all were statistically significant. There were slight increases in the iv as compared to the oral route of exposure. Statistically significant differences are listed here as a percent of the oral to iv levels: liver (83%), muscle (82%), kidney (83%) and blood (85%). The higher levels found in these tissues after iv administration implies an oral absorption efficiency of 83 ± 2%. For this compound a four day post-exposure turned out to be not the most ideal time point for this calculation as only trace levels are present in the tissues during this terminal phase of tissue elimination. This was not known prior to the study. However, as absorption is not a static but kinetic process; this analysis is simply a snapshot in absorption and *implies* a high level of absorption.

Elimination

The elimination of γ-HBCD derived radioactivity was analyzed by daily collection of urine and feces from individual animals held from 1-14 days post-treatment. Total cumulative elimination after oral administration in urine and feces is shown in Figure 3 for the four treatment levels.

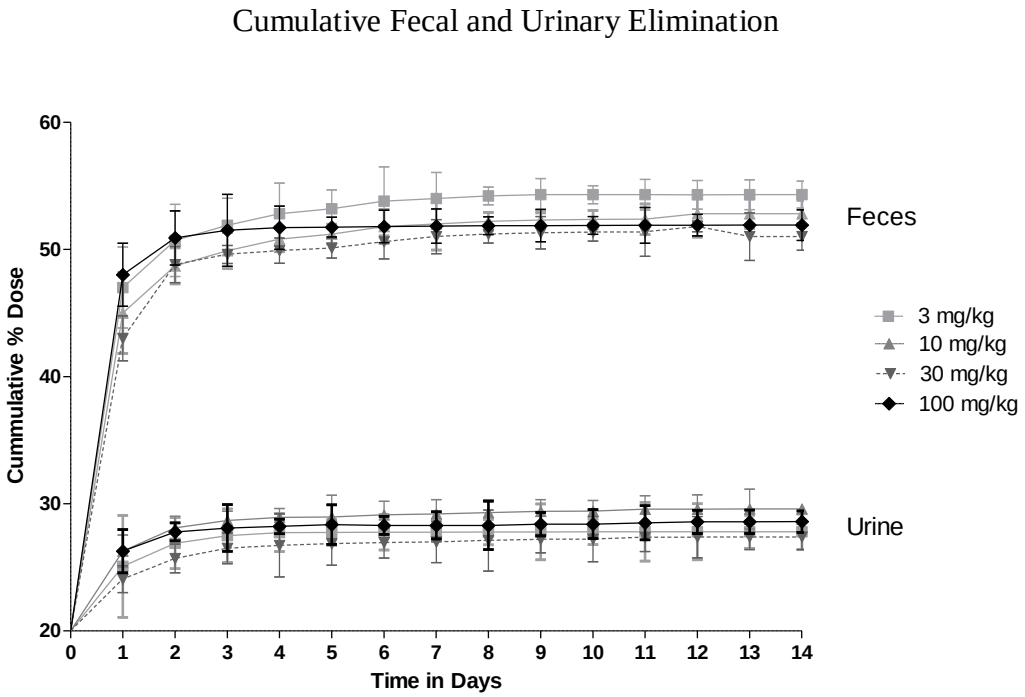


Figure 2.3. Cumulative elimination of γ-HBCD derived radioactivity following a single, oral dose (3, 10, 30, 100 mg/kg) in feces and urine. Data presented as mean percent administered dose eliminated ± standard deviation (n=4-6/group).

This data clearly demonstrate that the major route for elimination of this compound is via the feces. Approximately 80% of the administered dose had been eliminated in the urine and feces, collectively, by the fourth day. In all groups, 25% of the dose was eliminated in the urine on the first day. By the end of the collection period, up to 30% was eliminated in the urine. Expressed as percent dose, the data demonstrate a lack of dose dependency in urinary excretion. Essentially no radioactivity was detected (<1.5 times background) in the urine past Day 5. Average recovery of ^{14}C, including that recovered from urine and feces was 80.1 ± 4.2%. Elimination of ^{14}C derived radioactivity into feces was also rapid. As with urinary elimination, there was no dose dependency in fecal elimination of γ-HBCD. The percent of the dose eliminated in feces was consistent between dose groups on all days. In every dose group, approximately 45% was eliminated on the first day (Figure 3). Significantly less was eliminated on the second day, averaging 5% of the dose across all dose groups. By day five, a total of 50% had been eliminated in the feces.

IV vs. Oral: Elimination

Figure 4 compares the percent of dose in the urine and feces over four days. Excreta profiles are consistent between iv and oral routes of administration. Forty-five percent of the dose was eliminated in the feces of the iv administered mice on the first day whereas 52% was eliminated in the orally treated animals. After 4 days, 55% percent of the dose was eliminated in the feces following oral exposure and 51% following iv, an approximate 8% difference in the two routes.

Cumulative Fecal and Urinary Elimination between IV and Oral Routes

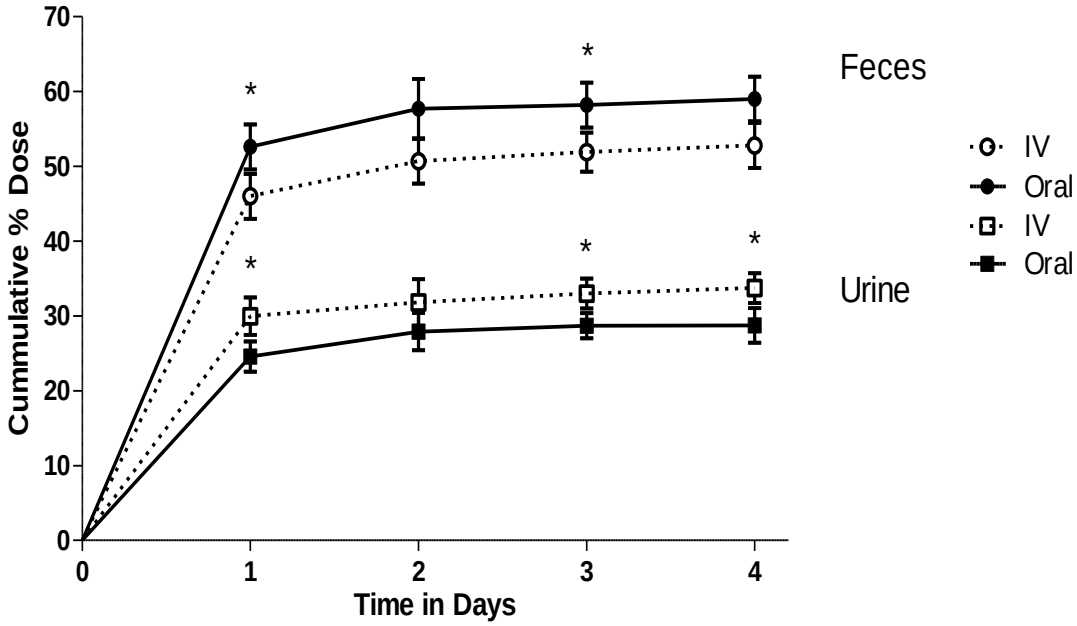


Figure 2.4. Cumulative elimination of γ-HBCD derived radioactivity following a single dose (3mg/kg) via i.v and oral route in urine and feces. Data presented as mean percent administered dose eliminated ± standard deviation (n=4-6/group). Presence of a * data point indicates significance of daily percent eliminated (*p <0.05 as compared to other route at same time point).

The amount of ^{14}C derived radioactivity in the urine eliminated on the first day is relatively constant between the iv and oral routes of administrations. This implies that γ-[^{14}C]HBCD derived radioactivity was absorbed and eliminated similarly after exposure by these two routes. The iv route of exposure had significantly higher levels of ^{14}C derived radioactivity in the urine than oral at Day 1, 3 and 4. This change reflects slightly decreased oral absorption. The large variance at Day 2 may have prevented this time point from also

being significantly increased. Twenty eight percent of the dose was eliminated in the urine of the iv administered mice on the first day, whereas 23% was eliminated in the oral dosed animals. Cumulatively 31% of the dose was eliminated in the urine following exposure following iv treatment, while 26% was eliminated after the oral route in four days. Based on fecal and urinary elimination patterns, comparison between the iv and oral routes indicate that γ-[^{14}C]HBCD may be well absorbed (85 ± 3%) orally. This agrees with the difference found in tissues deposition when comparing the iv and oral routes of exposure (83 ± 2%). The remaining amount [(85 and 83% of absorbed material) – 100% total absorption] is a small portion (an estimated 15-17%) which is most likely unabsorbed material. Ideally to calculate absorption, the concentration of radioactivity in the plasma over time is the gold standard. However, the data points in this study were too limited during this time resulting in the inability to analyze the absorption this way.

TLC

The nature of the γ-[^{14}C]HBCD derived radioactivity in the urine and feces was examined by thin-layer chromatography (Figure 5). No parent compound was detectable in the urine at 1 or 2 days after treatment. TLC consistently revealed one major peak (Rf = 0.0) which contains several polar metabolites. A similar metabolite pattern was seen in the blood and bile (data not shown).

Thin Layer Chromatography of Tissues and Excreta after Oral Administration of γ-HBCD.

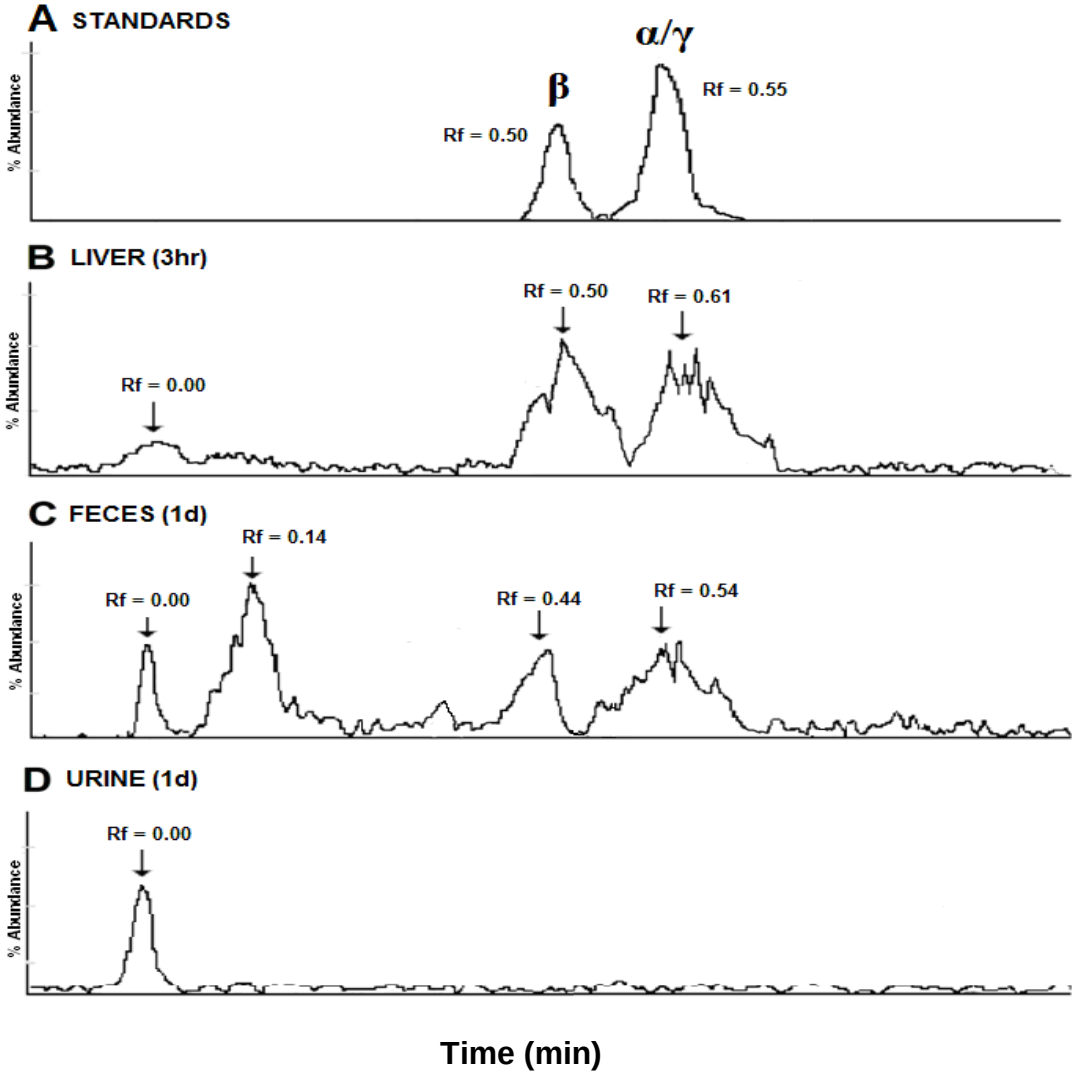


Figure 2.5. ^{14}C derived radioactivity was determined based on retention time using TLC. Once resolved, peaks were scraped from the silica plate, eluted with acetone, and characterized on LC-MS. Chromatographs of A) α-, β-, and γ-HBCD standards B) liver homogenate C) feces and D) urine. A representative sample and extract were chosen for presentation. Blood, bile and urine all contained similar chromatographic profiles.

This is in contrast to fecal elimination where low levels of the parent compound are present. It should be pointed out that only 48% of the γ-[^{14}C]HBCD derived radioactivity was extractable from the excreted feces. This contrasts with essentially complete extraction of γ-[^{14}C]HBCD following its addition to control feces. This suggests that nonextractable radioactivity was not parent γ-HBCD, but metabolites. Our results indicate that greater than 95% of the radioactivity that was excreted from the body was no longer γ-HBCD, but several metabolites of this compound. To verify this, standards for the three main diastereoisomers, α-[^{14}C]HBCD, β- and γ- were spotted on TLC plates and two bands with baseline resolution formed. In this system, β-[^{14}C]HBCD band migrated on a TLC with a shorter retention (Rf = 0.50) value than α- and γ-[^{14}C]HBCD which co-migrated as a second band with a Rf = 0.61. There were several bands detected in the fecal extracts with reference values at 0.0 and 0.14. Interestingly, two bands in the fecal extract (Rf = 0.44 and 0.54) migrated the same as the two standard bands (Rf = 0.50 and 0.55). The slight differences in Rf values can be explained as a function of fecal matrix purity and expected. Fecal co-extractables slightly impede migration on the TLC plate. Liver extracts were analyzed near the time of peak tissue concentration, 3 hours. In the liver tissue, several bands were detected with Rf values at 0.0, 0.46, 0.50, and 0.62. Similar to the fecal extracts, two bands in the liver extracts (Rf = 0.50 and 0.62) had similar values to the two standard peaks (Rf = 0.50 and 0.55).

GPC

The nature of the γ-[^{14}C]HBCD derived radioactivity in the brain and fat was initially examined by gel permeation chromatography (GPC). GPC separates based on size and was used as an initial separation and clean up step. Unlike TLC, GPC is not useful for the identification of individual stereoisomers as all HBCD stereoisomers migration at the same rate. GPC chromatograms revealed one major peak (data not shown) which was further analyzed using LC-MS.

LC-MS

Due to the inability of TLC to resolve α- or γ-HBCD and GPC to resolve any HBCD stereoisomer, the peaks were further characterized by analyzing them with LC-MS. This was also performed to determine whether 1) the parent γ-HBCD was present, and 2) if it had been converted to α- or β-HBCD. This was performed by comparing the TLC bands or GPC fraction to LC-MS standards; α-, β-, and γ-HBCD with retention times of 3.40, 4.06, and 4.89 minutes, respectively (Figure 6).

Title: LC-MS chromatograms of Tissues and Excreta after Oral Administration of γ-HBCD.

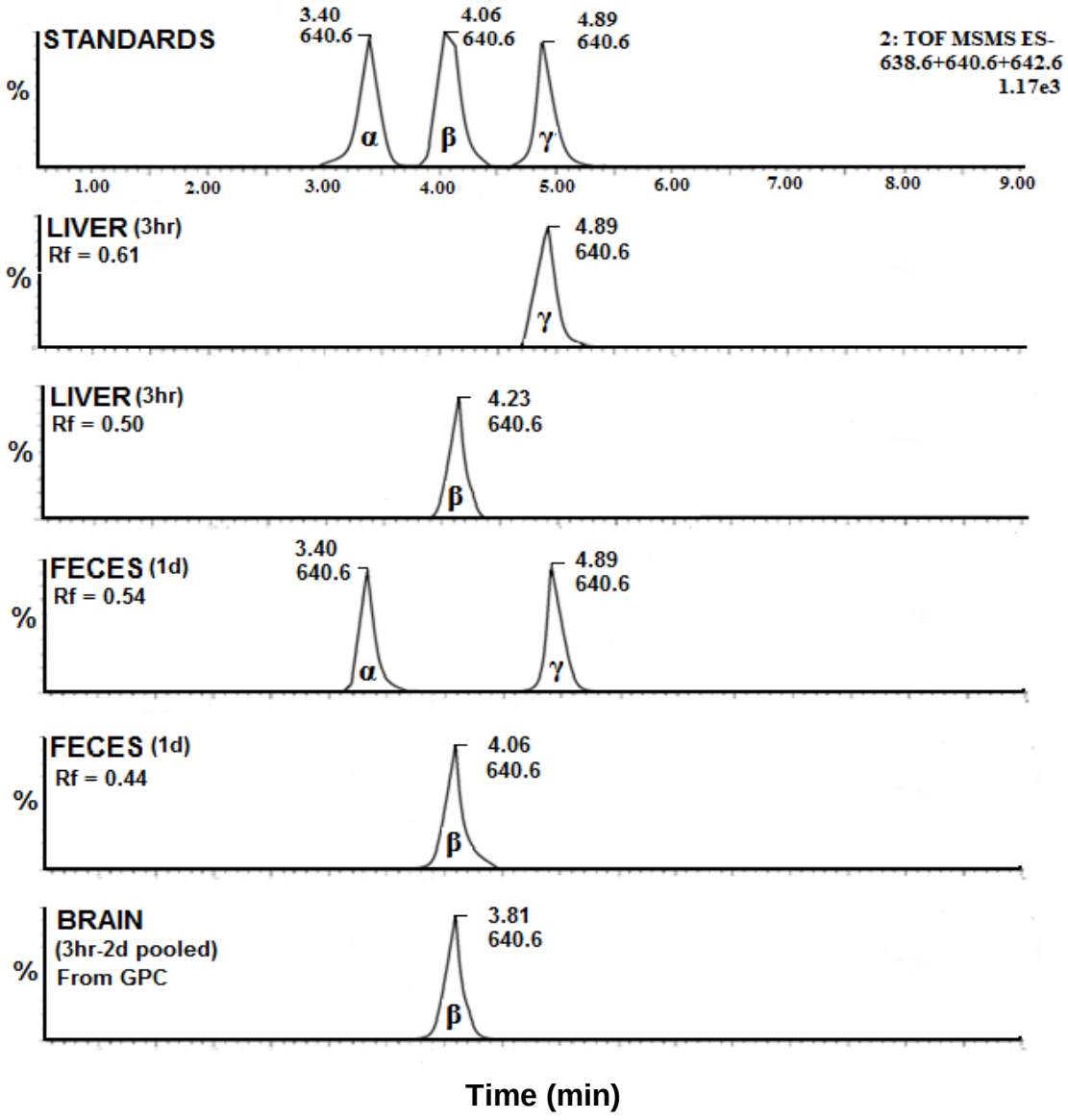


Figure 2.6. ^{14}C derived radioactivity was determined based on retention time and molecular weight using LC/MS. Chromatograms of α-HBCD, β-, and γ- standards, liver, and feces peaks from TLC were analyzed. Standards are a representative sample and retention times

varied slightly from day to day. To conserve low levels of radioactivity, brain tissue were pooled and Gel Permeation Chromatography (GPC) was used prior to LC/MS analysis.

The TLC peaks in the liver (Rf = 0.50 and 0.61) and fecal extracts (Rf = 0.44 and 0.54), were scraped, eluted with acetone and injected into the LC-MS. Results indicate that all peaks had a molecular weight of ~641 suggesting that they are HBCD stereoisomers. In the liver, TLC peaks of Rf = 0.50 and 0.61 had retention times of 4.89 and 4.23 minutes, similar to γ-HBCD and β-HBCD, respectively. In the fecal extracts, TLC peak 0.44 had a retention time of 4.06 minutes, which corresponds to HBCD-β. Rf =0.54 resolved into two LC-MS peaks with retention times of 3.40 and 4.89 minutes, which corresponding to α-HBCD and γ-HBCD, respectively. All three diastereoisomers, α-, β- and γ-HBCD, were detected in the feces 1 day post oral administration of γ-[^{14}C]HBCD (Figure 6). However, in the liver we were only able to detect the presence of γ-HBCD and β-HBCD, 3 hours post oral administration of γ-[^{14}C]HBCD. The GPC fraction of the adipose tissue (fat) on the LC-MS had molecular weight of 641 and retention times that matched γ-HBCD, β-HBCD and α-HBCD. However, the brain extract had a molecular weight of 641 and a retention time which matched that of the β-HBCD standard.

Summary of the parent, stereoisomer and metabolite profiles in tissues, biological matrices and excreta after exposure to γ-[^{14}C]HBCD can found in Table 3. Most of what has been detected in the liver and feces are metabolites, 79 and 85% respectively. However, 15 and 11% of the radioactivity present in the liver and feces were stereoisomerized HBCD products.

Table 2.3. Summary of Metabolite Profiles in Female Mouse Tissue and Excreta at 14 days after Oral Exposure to γ-HBCD

Matrix	Time	Parent	Isomerization	Metabolites
Liver	3 hours	**6%** Rf = 0.61; HBCD-γ	**15%** Rf = 0.50; HBCD-β	**79%** Rf = 0.00; Unknown (62%) Unextractable (17%)
Fat	24 hours	**84%** HBCD-γ	**16%** HBCD- β (11%) HBCD- α (5%)	**0%**
Brain	3 - 48 hours	**0%**	**100%** HBCD- β	**0%**
Feces	24 hours	**4%** Rf = 0.54; HBCD-γ	**11%** Rf =0.44; HBCD- β (4%) Rf = 0.54; HBCD- α (7%)	**85%** Rf = 0.00; Unknown (29%) Rf = 0.14; Unknown (4%) Unextractable (52%)
Urine **Blood** **Bile**	24 hours	**0%**	**0%**	**100%** Rf = 0.00; Unknown

Stereoisomerized products were also detected in the fat, 16%, and brain, 100%. Low levels of the parent γ-HBCD were found in the liver and feces, 6 and 4%, respectively. The bile, serum and urine contained 100% metabolites. This data in conjunction with the kinetic data support the rapid metabolism and elimination of γ-HBCD.

DISCUSSION

The presence and rapid increase in environmental and human concentrations of HBCD diastereomers has heightened interest in toxicological consequences of these chemicals. The toxicity of HBCD commercial mixtures has been investigated recently; however it raises questions as to whether diastereoisomeric specific effects are seen. The diastereoisomers of HBCD differ in their structure, physical orientation and chemistry which may result in differences in pharmacokinetics, metabolism and biological response (Hunzinker et al., 2004; Covaci et al., 2006). It is therefore essential to understand the factors involved in human health risk for individual diastereoisomers of HBCD. γ-HBCD accounts for a large proportion of HBCD global production and usage and is consistently the dominant diastereoisomer found in the environment. However, it is usually at lower levels in human and wildlife tissues, where α-HBCD predominates (Covaci et al., 2006). Furthermore, there is a large variation in observed human body burden concentrations of HBCD stereoisomers that are not explained by exposure data.

The objectives of this investigation were to determine the absorption, distribution, metabolism and excretion of γ-HBCD in female mice to better evaluate the toxicity and behavior of this compound. This study suggests that approximately 85% of an oral dose of γ-HBCD was absorbed from the gut and was dose independent. This was based on the oral and iv routes of administration having similar tissue and excreta concentrations of γ-HBCD derived radioactivity. Although, absorption typically is calculated using plasma concentration over time, further time points are needed. This compound shows similar behavior as other large halogenated molecules (Kedderis et al., 1994, Staskal et al., 2005). Distribution of γ-HBCD was initially to the liver and muscle, followed by some redistribution to skin and lungs, with very little to adipose tissue. This is in contrast to other persistent organic pollutants, such as TCDD, which accumulates in the liver and fat (Rose et al., 1976), and PBDEs, which accumulates in the fat (Staskal et al., 2005). The pattern seen here was not dependant on dose or route of γ-HBCD exposure.

The primary route of γ-HBCD elimination was through the feces (50% of dose). However, almost 30% of the administered dose was eliminated via the urine. Elimination, as tissue disposition, was also not affected by the dose within the range studied or by the two routes of administration tested. The rapid rate of elimination of γ-HBCD derived radioactivity was in contrast to that observed for TCDD, where a whole-body half-life in mice of 3-5 weeks has been reported (Rose et al., 1976), or with BDE-47, which had a whole-body half-life of approximately 3 weeks (Staskal et al., 2005).

Of the γ-HBCD derived radioactivity eliminated in the urine, 100% were metabolites. The same was true for the radioactivity detected in the bile and blood. Feces and liver consisted of both, i.e., parent γ-HBCD and metabolites. The radioactivity patterns present in the liver and feces were similar with the majority 94-96%, being detected as metabolites, and only 4-6% was parent γ-HBCD. Of the metabolites present, 11-15% constituted stereoisomerization products. β-HBCD and parent γ-HBCD were detected in the liver, while the three major diastereoisomers, i.e. α-, β-, and γ-HBCD were present in the feces. These preliminary results suggest that intestinal flora may alter the biliary metabolites, since the chromatographic results for extracts of bile and feces were quite distinct. The action of intestinal microorganisms on HBCD remains unknown. In a recent study, bacteria isolated from soil were shown to metabolize γ-HBCD; however, the same bacterial strain failed to degrade α-HBCD (Yamada et al., 2009). Since the HBCD derived radioactivity eliminated in the urine, bile and blood was in the form of polar metabolite(s) and storage of HBCD metabolites in the tissues was negligible, the more rapid clearance of HBCD when compared to TCDD or PBDEs can be attributed to rapid metabolism of γ-HBCD in the mouse.

It is unclear in the case for γ-HBCD whether the parent compound or its metabolites is the toxic agent. Metabolism often results in detoxification. Using this assumption, we might predict that animals with higher rates of metabolism of γ-HBCD will be more resistant to its toxic actions. Such a correlation, in fact, may exist in the mouse. Differential metabolism capacities of γ-HBCD and α-HBCD have been observed *in vitro* (Zegers et al., 2005). α-HBCD was more slowly metabolized than γ-HBCD, and because of this was

hypothesized to be several times more toxic. However, for PBDEs and PCBs it is well known that hydroxyl or methylsulfonyl metabolites can have a significant endocrine modulating effect. At present we simply know very little about the mode of action of HBCD and individual diastereomers. This emphasizes the need for toxicity studies on individual HBCD diastereoisomers.

Metabolites have been detected here for the first time in mice after oral exposure of γ-HBCD. These metabolites were found in the liver, blood, bile, urine and feces between 3-24 hours post-exposure. Although, identification of these metabolites is beyond the scope of this report, previous reports have identified metabolites after oral administration of the HBCD commercial mixture. One study identified four metabolites of HBCD of unknown structure in rats (Yu et al., 2003). Another *in vitro* study showed three HBCD metabolites were detected with LC-MS, where two metabolites were identified as monohydroxy-HBCD (Zegers et al., 2005). Identification of a monohydroxy-HBCD was also observed after an *in vitro* study using rainbow trout liver microsomes (Huthala et al. 2006). Degradation/metabolite products have been observed in office dust samples, i.e. pentabromocyclododecene (PBCDe) and two isomers of tetrabromocyclododecene TBCDe (Abdallah et al. 2008). In chicken eggs and whitefish PBCDe was identified using GC (Hiebl and Vetter, 2007). Therefore, the presence of HBCD metabolites has been previously noted; however, it remains unclear whether these metabolites are the product of α-, β-, or γ-HBCD. The present *in vivo* study reported here suggests that γ-HBCD can be rapidly

metabolized, and may serve as a source of the metabolites identified in these previous studies. In the case of HBCD, it is unclear whether the more highly brominated parent or lower brominated metabolites are more or less toxic.

Our *in vivo* data indicates two factors may be responsible for the shift observed from the predominance of γ-HBCD in the commercial mixture and environment relative to α-HBCD in biota. First, γ-HBCD is rapidly metabolized and eliminated. Second, *in vivo* stereoisomerization of γ-HBCD to α- and β- is occurring. Furthermore, the stereoisomerization seen is rapid. Stereoiosmerization is also supported by work previously reported in non-mammalian species such as fish (Law et al., 2006). *In vitro* experiments with rat and harbor seal microsomes have showed biotransformation of β-HBCD and γ-HBCD was faster than that of α-HBCD (Zegers et al., 2005). Although we have detected rapid elimination of γ-HBCD in this mammalian mouse model, future *in vivo* kinetic studies using α-HBCD are needed to determine whether *in vivo* bioaccumulation, not seen here with γ-HBCD, may further explain the diastereoisomeric shift.

We conclude the biological persistence of γ-HBCD in mice is low and may explain low levels of γ in biota. This ADME data would support the hypothesis that metabolism and stereoisomerization, in addition to differential exposure, plays a role in the observed stereoisomer profiles in biota.

ACKNOWLEDGEMENT AND DISCLAIMER

The authors would like to thank Frances McQuaid, Brenda Edwards, Colleen Pfaff and Barbara Magelky for technical assistance. This work is funded in part by a cooperative agreement between the University of North Carolina in Chapel Hill and the United States Environmental Protection Agency CR 833237 predoctoral training grants. This abstract does not reflect USEPA, NIH or USDA policy. The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

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Chapter III

TOXICOKINETICS OF THE FLAME RETARDANT HEXABROMOCYCLODODECANE ALPHA: EFFECT OF DOSE, TIMING, ROUTE, REPEATED EXPOSURE AND METABOLISM

2010
Szabo DT, Diliberto JJ, Hakk H, Huwe J and Birnbaum LS
Toxicological Sciences. 121, 234-44.

Title: Toxicokinetics of the flame retardant hexabromocyclododecane alpha: effect of dose, timing, route, repeated exposure and metabolism.

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1) Running Title: Toxicokinetics of the flame retardant hexabromocyclododecane alpha: effect of dose, timing, route, repeated exposure and metabolism.

2) Key words: brominated flame retardants (BFRs), HBCD, stereoisomer, toxicokinetics

3) Acknowledgements:

The information in this document has been subjected to review by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The research presented in this document was funded in part by the U.S. Environmental Protection Agency. The research was partially supported by the EPA/UNC Toxicology Research Program Training Agreement; NHEERL-DESE Cooperative Training in Environmental Sciences Research, EPA CT826513, with the Curriculum in Toxicology, University of North Carolina at Chapel Hill. A conflict of interest was not reported.

ABSTRACT

Alpha-hexabromocyclododecane (α-HBCD) is an emerging persistent organic pollutant present in the hexabromocyclododecane (HBCD) commercial mixture. HBCD is used as an additive flame retardant in a wide variety of household consumer products. Three main stereoisomers, alpha (α), beta (β), and gamma (γ), comprise roughly 10%, 10%, and 80% of the mixture, respectively. Despite its small contribution to HBCD global production and usage, α-HBCD is the major stereoisomer found in wildlife and human tissues including breast milk and blood in North America, European Union and Asia. No mammalian or human data are currently available regarding the toxicokinetics of α-HBCD. This study was conducted in an effort to fully characterize the absorption, distribution, metabolism and elimination of α-HBCD following a single and repeated exposure with respect to dose, time, and route of administration in female C57BL/6 mice. Results indicate that approximately 90% of the administered dose (3mg/kg) was absorbed after oral exposure. Disposition was 1) dictated by lipophilicity as adipose, liver, muscle and skin were major depots and 2) was dose-dependent with non-linear accumulation at higher doses. Elimination, both whole-body and from individual tissues, was biphasic. α-HBCD derived radioactivity was excreted in the feces as parent and metabolites whereas, urine only contained metabolites. Presence of polar metabolites in the blood and urine were a major factor in determining the rapid initial whole-body half-life after a single oral exposure. Initial half-lives were ~1-3 days and much longer terminal half-lives of 17 days were observed, suggesting the potential for α-HBCD bioaccumulation. A 10 day repeated study supports α-HBCD bioaccumulation potential.

Stereoisomerization previously observed after exposure to γ–HBCD was not seen after exposure of α-HBCD. The toxicokinetic behavior reported here has important implications for the extrapolation of toxicological studies of the commercial HBCD mixture to the assessment of risk of α-HBCD which is the major stereoisomer found in wildlife and people.

INTRODUCTION

1,2,5,6,9,10-hexabromocyclododecane (HBCD) is a brominated aliphatic cyclic hydrocarbon and a high production volume chemical used as a flame-retardant primarily in polystyrene foams used for insulation, with secondary uses in upholstered furniture, automobile textiles/cushions, packaging material, and electronic equipment. Concern for HBCDs has risen as it has been detected not only in wildlife (Tomy et al., 2004; Covaci et al., 2006) but also human breast milk and serum (Weiss et al., 2004; Thomsen et al., 2005; Meijer et al., 2008). Time trend studies indicate that concentrations of HBCD in the environment, humans and wildlife are increasing (Sellström et al., 2003; Kakimoto et al., 2008; Hermanson et al., 2010). The discovery of HBCD in Arctic marine ecosystems provides compelling evidence of long-range transport of this compound (de Wit et al, 2010). Field studies in marine mammals and aquatic wildlife have suggested HBCD is also highly bioaccumulative (Law et al., 2006).

Diet is likely a major source of HBCD exposure. Intake of HBCD was estimated at 16 ng/day, primarily from meat; however fish and vegetables also had significant levels (Schecter et al., 2010). Dust, in addition to the diet, is likely another important pathway of human exposure to HBCD due to levels present indoors (Roosens et al., 2009). Non-dietary ingestion of dust may represent an important route of exposure especially for toddlers and young children (Lioy et al., 2000; Wilford et al., 2005; Wu et al., 2007).

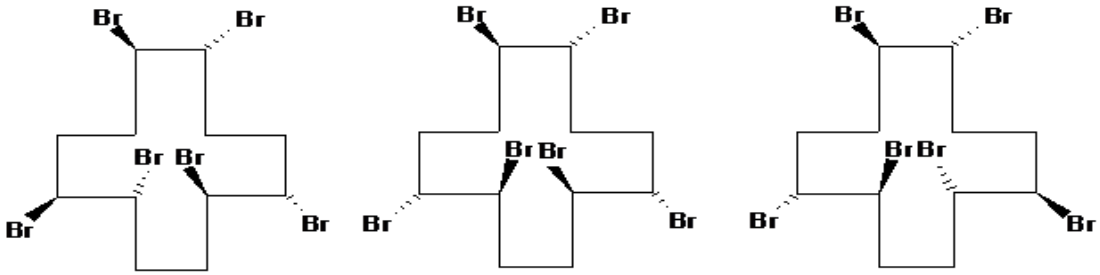

Figure 3.1. Chemical structures of 1,2,5,6,9,10-Hexabromocyclododecane alpha, (α-HBCD), beta (β-HBCD) and gamma (γ-HBCD).

Previous literature has focused on the three main diastereomers present in the commercial mixture, denoted as alpha (α), beta (β), and gamma (γ); with the γ-diastereomer predominating (>70%) (Heeb et al., 2005). It is well documented that there is a shift in the relative diastereomer contribution with increasing trophic species in aquatic food webs (Covaci et al., 2006, Law et al., 2006). In general lower level organisms contain mostly γ-HBCD (closely mimicking the commercial mixture and certain environmental levels) while

α-HBCD dominates the diastereomer pattern in apex predators. In fish and aquatic mammals, HBCD has been observed to accumulate in lipid rich organs including liver, gonads, muscle, and adipose tissue (Janák et al.; 2005, Peck et al., 2008; Xian et al., 2008) and, where investigated, α-HBCD predominated. Presence of high concentrations of α-HBCD in marine mammals and birds of prey suggests persistence and biomagnifications up the food chain. Despite α-HBCD's small contribution to HBCD global production and usage (±10% of commercial mixtures), α-HBCD is the major congener found in biota.

HBCD has been detected in the few human exposure studies conducted to date and, where investigated, α-HBCD predominates. Thomsen et al. (2007) reported that all serum samples tested in workers from an industrial plant occupationally exposed to commercial HBCD had 60% α-HBCD and 39% gamma (ranged 6-856 ng/g lw). Ryan et al. (2006) reported that the mean concentrations of α-HBCD in human breast milk collected in Ontario, Canada (2002-2003) and Texas, USA (2002) were 3.8 and 0.5 ng/g lipid weight (lw), respectively. Kakimoto et al. (2008) analyzed breast milk from Japanese women (age 25–29) over the period 1973–2006; total mean HBCD concentrations over the period 2000 – 2006 ranged from 1 to 4 $ng \cdot kg^{-1}$ lw where α-HBCD predominated in nearly 90% of samples tested. Higher total HBCD (α, β, and γ) values were reported in breast milk of women living in Spain between 2006-2007 (Eljarrat et al. 2009) where the mean concentration was 27 $ng \cdot kg^{-1}$ lw (range 3 to 188 $ng \cdot kg^{-1}$ lw). α-HBCD dominated a smaller subset of the samples tested with γ-HBCD predominating in others. Thomsen et al., (2007) suggested that the elevated

levels of γ-HBCD in some samples are an indication of recent exposure to the commercial mixture, and with enough time; γ-HBCD will either be cleared from the system and/or converted to α-HBCD.

The field observations described in the current human and wildlife studies are unfortunately unable to fully characterize the exposure pathway, dose, and duration of exposure. The current hypothesis which may explain of the elevated levels of the α-HBCD found in biota is thought to be due to either diastereomer-selective uptake, diastereomer-specific metabolic rates (Law et al., 2006; Zegers et al., 2005), and/or *in vivo* stereoisomerization (Szabo et al., 2010). Stereoisomerization of purified γ-HBCD to α and/or β was reported in mice after a single oral exposure by Szabo et al.(2010) who demonstrated that *in vivo* stereoisomerization of γ-HBCD is rapid, 3-48 hours post-oral exposure, and is tissue specific in the liver, fat and brain. However, more detailed information about the kinetics, toxicology, pathways of exposure, and bioavailability of the HBCD diastereomers are needed to help explain observations seen in the environment, wildlife and humans.

In predicting human health risks posed by HBCD, it is necessary to accurately predict internal dose and the fate of these compounds. There are currently no toxicokinetic or toxicity data on α-HBCD in any mammalian species. This study is a companion paper to our previous work with γ-HBCD (Szabo et al., 2011) where the same methodology was used allowing a direct comparison of the kinetics between the two diastereomers. The dose

concentrations selected for this toxicokinetic study were based on previous effect studies after exposure to the commercial mixture of HBCD. Developmental neurotoxicity was observed in mice after a single (acute) exposure with a LOAEL = 0.9 mg/kg bw/day (Erikkson et al., 2006). A 28 day repeated dose study in rats observed increased thyroid, liver, pituitary weight at 3.4, 29.9, and 50.6 mg/kg-bw per day (BMDL of 1.6, 22.9, and 29.9 mg/kg-bw per day), respectively (Van der ven et al., 2006). In a one-generation rat study, decreased concentration of apolar retinoids in the liver, increased immune response, and mineral bone density in F1 females were reported at 0.18, 1.45, and 5.1 mg-kg-bw per day, respectively (Lilienthal et al., 2009). Increased liver weight was observed in a 90 day rat study with a LOAEL of 100 mg/kg-bw per day (Chegelis et al., 2001). The choices of dose in the current study were also driven by limited availability of the α-[^{14}C]HBCD and the appropriate comparison to studies of γ-HBCD in mice.

The objective of this study is to characterize the fate of α-HBCD up to 14 days following an acute exposure across a range of doses and after repeated administration of a low dose to adult female mice.

MATERIALS AND METHODS

Chemicals: [^{14}C]1,2,5,6,9,10-hexabromocyclododecane ([^{14}C]HBCD) (2mCi/mmol) was purchased from American Radiochemicals Corporation (ARC) (St Louis, MO) as a mixture of ß-[^{14}C]HBCD and γ-[^{14}C]HBCD diastereomers as determined by liquid

chromatography-mass spectrometer (LC-MS) retention time comparisons with authentic standards (α-, ß-, δ-, γ-, and ε-HBCD, Wellington Laboratories, Guelph, ON). The two diastereomers were separated by flash chromatography on a silica gel column eluting with hexane containing increasing amounts of methylene chloride (0–50 %) (Szabo et al., 2010). A fraction containing predominantly (>90%) γ-[^{14}C]HBCD but some ß-[^{14}C]HBCD was thermally converted to α-[^{14}C]HBCD by heating at 170°C for up to 3 hr (Heeb et al., 2008). α-[^{14}C]HBCD was purified by flash chromatography on a silica gel column as above, followed by preparative reverse phase HPLC on two Delta-Pak C_{18} cartridges (25 x 100 mm) (Waters Corp., Milford, MA) in series and isocratic elution with acetonitrile:water (80:20) at 20 mL/min. The radiochemical and diasteromeric purities (98% each) were respectively determined by thin-layer chromatography (TLC) using silica gel plates (250 mm; Whatman Lab. Div., Clinton, NJ) and a 50:50 hexane:methylene chloride mobile phase and by LC-MS retention time comparison to authentic standards of α-, ß-, δ-, γ-, and ε-HBCD (Wellington Laboratories, Guelph, ON). The impurities present in the α-[^{14}C]HBCD were detected to be ≤1% each of the other two main stereoisomer β- and γ-HBCD. Radiochemical detection was performed on a System 2000 Imaging Scanner (Bioscan, Washington, DC). Retention times comparisons by LC-MS were made on a Symmetry C_{18} column (2.1x100 mm) (Waters, Beverly, MA) using an isocratic program which separated all five HBCD diastereomers (details below). Additionally, ^{1}H-NMR of the α-[^{14}C]HBCD performed on a Bruker AM 400 spectrometer (Bruker, Billerica, MA) matched the previously published spectrum of this

diastereomer (Arsenault et al., 2007). Unlabeled α-HBCD was generously provided by Wellington Labs (98% purity). Other chemicals used were of the highest grade commercially available.

Dosing Solutions: Doses were selected based on published toxicity studies on the HBCD commercial mixture due to lack of any available α-HBCD whole animal studies. Furthermore, the low specific activity of the radiolabeled compound was a contributing factor in choosing 3mg/kg as the low dose ensuring proper detection. A stock solution of α-[^{14}C]HBCD was made by dissolving 19.23 mg of α-[^{14}C]HBCD (3.12 μCi/mg) in toluene (400ul) . Aliquots were used directly from this solution for the dosing regimens. Dosing solutions were subjected to pre- and post- dosing radioactivity examinations to ensure proper delivery of dose. All solutions were designed to deliver approximately 0.2μCi to each mouse. Unlabeled α-HBCD was added to the α-[^{14}C]HBCD to achieve desired mass (except the 3mg/kg low dose), and was added directly to the dosing solution vial and dissolved in acetone. Corn oil by weight was then added to the vials followed by the evaporation of toluene and acetone under vacuum (Speed Vac, Savant Instruments, Inc. Farmingdale, NY). For intravenous (iv) treatment, the α-[^{14}C]HBCD solvent from the stock solution, toluene, was allowed to evaporate in an amber vial under the flow of nitrogen, and the α-HBCD was resuspended in 95% ethanol followed by Emulphor®. De-ionized water was slowly added to a final volume to achieve a ratio of (ethanol:Emulphor®:water ratio was 1:1:8). Where applicable, glass was used in the handling, containment, transfer, and storage of the

compound; no loss of radioactivity occurred with glass. Concentration of the dosing solution was verified by liquid scintillation chromatography (LSC).

Animals. Young adult female mice were used in this study as limited supply of purified α-[^{14}C]HBCD was available and to compare results to recent toxicokinetic mice studies on other related environmental chemicals (Szabo et al., 2010; Staskal et al., 2006; Diliberto et al., 1999) including toxicity studies where mice were exposed to the commercial mixture of HBCD (Erikkson et al., 2006; Kurokawa et al., 1984). Future mechanistic studies on disposition can be more easily tested in mice as transgenics are also readily available (Hakk et al., 2009).

Female C57BL/6 mice (~20 grams) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Females were chosen as they appear to elicit a more sensitive response after exposure to the commercial mixture of HBCD in several toxicity studies (Lilienthan et al., 200; Van den ven 2006). Animals were maintained on a 12 h light/dark cycle at ambient temperature (22°C) with relative humidity (56 ± 5%), and were provided Purina 5001 Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water *ad libitum*. Prior to the commencement of the study, mice were adapted (3 mice/cage) for 1 week to Nalgene metabolism cages (Nalgene, Rochester, NY). Mice were then assigned randomly to treatment groups (n= 4-8) and housed individually for the remainder of the study. All mice were 60 days old at time of treatment.

Route of Exposure: *Oral treatment* (n=4-8). A single dose (0, 3, 10, 30 and 100 mg/kg) was administered directly by oral gavage into the stomach of each mouse using a PTFE animal feeding needle. Dose volume was 10ml/kg. *Intravenous treatment* (n=6-8). A single dose (3 mg/kg) was administered intravenously via the tail vein at a dosing volume of 2 mL/kg.

Treatment: *Dose/Response:* A single dose was administered by gavage of either 3, 10, 30, or 100 mg/kg at a volume of 10ml/kg. The mice were held in metabolism cages for 4 days where urine and feces were collected daily. *Time course:* Mice were treated by gavage at a single dose of 3 mg/kg and held for 14 days while urine and feces were collected daily. *Repeated*: Mice were dosed for 9 days with 3 mg unlabeled α-HBCD/kg; and only on day 10 the mice were exposed to a single gavage of 3 mg α-[^{14}C]HBCD/kg, and then held for 4 more days (total of 14 days). This repeated protocol allows comparison of "naïve" radioactive exposures to mice pretreated with unlabeled compound.

Animals were euthanized by CO_2 asphyxiation followed by exsanguination via cardiac puncture at which point blood was collected. Liver, lung, kidneys, skin (ears), adrenal glands, urinary bladder, spleen, thymus, adipose (abdominal), muscle (abdominal), and brain were collected and weighed. Bile was removed directly from the gallbladder using a 0.2 millimeter bore needle and syringe.

Sample Analysis: Radioactivity in the tissues were determined by combustion with a tissue oxidizer (Packard 307 Biological Oxidizer, Downers Grove, IL) of triplicate samples when

available (~100mg/sample) followed by LSS (Beckman, Beckman 6000IC, Fullerton, CA) with limits of detection (LODs) of 50 dpm (3 x background) or 6.7 ng HBCD for the 3mg/kg dose. Tissue data are reported based on wet weight and as percent of administered dose. Feces were air dried, weighed, and analyzed for radioactivity by combustion and LSS. Daily urine volume was recorded, and 100 μL aliquots (triplicate) were analyzed for [^{14}C] by direct addition into scintillation cocktail.

Tissue Extraction and Analysis: Livers were weighed, pulverized and then homogenized in 3 volumes of 0.9% sodium chloride. Liver samples were extracted sequentially with 3 volumes of hexane, ethyl acetate, and methanol. The organic layers were pooled for each solvent and assayed by LSC. The liver sample extracts were applied to an acid silica gel column (0.5 x 7.0 mm, 40% concentrated sulfuric acid by weight) and eluted with hexane, followed by 50:50 hexane:methylene chloride. The collected fractions were analyzed by LC/MS (see below).

Feces were dried and ground into a fine powder with a mortar and pestle, and then extracted for 24 h stirring sequentially with 15 mL of hexane, ethyl acetate, and methanol. Fecal residues from the extractions were combusted to yield the non-extractable fraction. The extractable fractions were reduced in volume to ~1 mL with nitrogen. Each extract was applied to a silica gel column (silica gel 60, EM Reagents, Cincinnati, OH; particle size <0.063μm), preconditioned with hexane and eluted with a gradient from 100% hexane to 80:20 hexane/toluene. The gradient was increased in 2% increments, and fractions were

assayed by LSC. Peaks of [^{14}C] were collected, reduced in volume and analyzed by LC/MS. Urine was injected unto RP-HPLC and [^{14}C] peaks were collected. HPLC fractions were acidified to pH 2 with 0.01 M HCl, and applied to a C18 SepPak cartridge (Waters) pre-conditioned with methanol and water. Each sample was eluted with water followed by methanol. The methanol SepPak fractions were applied to a Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) column and eluted with water followed by methanol, and each eluted fraction was analyzed by LC/MS (see below).

Serum was extracted with hexane followed by ethyl acetate. Each extract was applied to a silica gel TLC plate, and developed in 50:50hexane:methylene chloride. Bile was extracted with methanol and applied to silica gel TLC plate, using a 50:50hexane:methylene chloride as a developing solvent. TLC had limits of detection (LODs) of 200 dpm (S/N =3) or 0.03 µg HBCD for the 3 mg/kg dose. Gel permeation chromatography (GPC) was used to remove large macromolecules which can interfere with further analysis of low level of target molecules found in brain and adipose samples. Fat samples were pooled (n=4) and homogenized in water, and 2 mL of hexane:acetone (1:3.5) was added and sonicated for 15 minutes. An additional 2 mL of hexane:acetone was added, sonicated and combined with the previous organic layer. Four milliliters of hexane:ether (9:1) was added, vortexed, centrifuged, and decanted; this was repeated twice and organic layers were combined with the first extracts. The fat extract was assayed by LSC, evaporated with nitrogen, applied to a GPC column (Biobeads SX-3; BioRad), and eluted with methylene chloride. Fractions were

collected, reduced in volume, applied to an acid silica column, eluted with hexane followed by 50:50 hexane:methylene chloride, and analyzed by LC/MS (see below).

In LC/MS was used to analyze selected samples and consisted of an Alliance 2695 Separation Model (Waters, Beverly, MA) equipped with a Symmetry C_{18} column (2.1 mm x 100 mm) and guard column (2.1 by 10 mm), and a quadrupole-time of flight mass spectrometer (Waters Q-TOF Ultima API-US; Waters, Beverly, MA). Isocratic elution conditions were used which consisted of 15% aqueous 10 mM NH_4OH and 85% methanol:acetonitrile (80:20 v/v), 10 mM NH_4OH. The flow rate of the mobile phase was 0.3 ml/min^{-1}. The mass spectrometer analysis was performed in negative ion mode (ES-) using a 634 m/z filter. LC-MS had an estimated LOD of 0.3 ng (S/N =3 calculated for a tissue matrix).

Data Analysis: To calculate the percent of dose in each compartment, mouse body composition estimates were used for blood, fat, skin, and muscle - 8, 8, 12, and 35%, respectively (ILSI, 1994). Estimates were used for tissues for which it was not possible to obtain total weights from actual animals. The oral tissue disposition data refer to the mean of all data collected in which a) animals were exposed orally with a 3mg/kg dose and b) 4 day time points where available. Intergroup comparisons were performed by a two-way ANOVA followed by Bonferroni post-hoc tests were significant when $p < 0.05$. All data are presented as mean ± standard deviation. GraphPad Prism 5.0, Hearn Scientific Software (Melbourne, Australia) was used to calculate half-life, the percent of dose, and statistical analysis.

RESULTS

Tissue Disposition

Dose Dependency

Tissue distribution, as a percent of α-[^{14}C]HBCD administered dose in female C57BL/6 mice, are presented in Table 1. The top table (A) shows the disposition after administration of single and repeated oral doses of α-[^{14}C]HBCD at concentrations of 3, 10, 30 or 100 mg/kg or of 10 d repeated exposure to 3 mg/kg.

Table 3.1. Disposition of α-HBCD in Mice

(A)

Dose	Skin	Liver	Lung	Muscle	Kidney	Blood	Adipose	Brain
mg/kg	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)	% dose (ng/g)
3	2.5 ± 0.23 (9000 ± 828)	2.70 ± 1.20 (9720 ± 4320)	0.4 ± 0.08 (1440 ± 287)	1.3 ± 0.21 (4680 ± 756)	0.04 ± 0.01 (144 ± 36)	1.3 ± 0.29 (4680 ± 1044)	3.70 ± 0.42 (13320 ± 1512)	0.40 ± 0.23 (1440 ± 828)
10	2.5 ± 0.51 (26700 ± 6114)	3.2 ± 0.31 (38361 ± 3716)	0.2 ± 0.01 (2397 ± 120)	1.6 ± 0.16 (19181 ± 1918)	0.06 ± 0.01 (719 ± 120)	1.01 ± 0.46 (12107 ± 5514)	3.93 ± 0.25 (47112 ± 2997)	0.52 ± 0.45 (6234 ± 5394)
30	2.3 ± 1.41 (88911 ± 50760)	*4.16 ± 0.50 (149760 ± 1800)	0.42 ± 0.02 (15120 ± 720)	1.7 ± 0.13 (61200 ± 4680)	0.06 ± 0.01 (2161 ± 360	1.32 ± 0.72 (47520 ± 25921)	*6.36 ± 0.66 (22896 ± 23760)	0.51 ± 0.07 (18336 ± 2520)
100	*3.92 ± 1.20 (373426 ± 14398	*5.52 ± 1.11 (662334 ± 133186)	0.43 ± 0.02 (51594 ± 2400)	*2.1 ± 0.31 (251748 ± 37196)	0.06 ± 0.02 (7199 ± 2397)	1.83 ± 0.39 (43876 ± 930)	*8.91 ± 1.13 (106909 ± 40630)	0.47 ± 0.03 (56394 ± 3600)
3 Repeated	2.52 ± 0.51 (9072 ± 1836)	*3.75 ± 0.75 (13500 ± 2700)	0.61 ± 0.03 (2196 ± 108)	*1.9 ± 0.21 (17100 ± 756)	0.07 ± 0.03 (252 ± 108)	*2.4. ± 0.36 (8640 ± 1296)	*6.50 ± 0.93 (23400 ± 3348)	0.48 ± 0.05 (1728 ± 180)

(B)

Time	Skin	Liver	Lung	Muscle	Kidney	Blood	Adipose	Brain
1 hour	0.0001 ± 0.000 (13 ± 7)	13.23 ± 4.6 (47628 ± 16560)	0.8 ± 0.02 (2880 ± 72)	0.80 ± 0.050 (2880 ± 180)	7.0 ± 0.001 (25200 ± 3.4)	11.31 ± 1.30 (40716 ± 4680)	0.002 ± 0.001 (7.2 ± 3.6)	3.5 ± 0.1 (12600 ± 360)
3 hours	0.0002 ± 0.010 (30 ± 12)	12.24 ± 2.4 (44028 ± 8640)	3.0 ± 0.51 (10800 ± 1836)	1.0 ± 0.051 (3600 ± 184)	5.2 ± 0.31 (18720 ± 1116)	7.03 ± 1.01 (25308 ± 3636)	0.006 ± 0.001 (22 ± 3.6)	1.9 ± 0.01 (6840 ± 36)
8 hours	0.013 ± 0.26 (420 ± 37)	9.82 ± 0.51 (35352 ± 1836)	2.1 ± 0.08 (7560 ± 288)	3.5 ± 0.02 (13320 ± 72)	3.6 ± 0.11 (12960 ± 396)	4.68 ± 1.21 (16848 ± 4356)	0.072 ± 0.001 (259 ± 3.6)	1.0 ± 0.01 (3600 ± 36)
1 day	1.53 ± 1.80 (2600 ± 2110)	6.23 ± 1.3 (22428 ± 4680)	0.62 ± 0.02 (2232 ± 72)	3.69 ± 0.22 (13284 ± 792)	0.8 ± 0.2 (2880 ± 720)	2.0 ± 1.24 (7200 ± 4464)	2.30 ± 0.91 (8280 ± 3276)	0.85 ± 0.01 (3060 ± 36)
2 days	4.1 ± 1.21 (14760 ± 4356)	4.21 ± 0.91 (13321 ± 3276)	0.51 ± 0.01 (1836 ± 36)	1.8 ± 1.10 (6480 ± 3960)	0.075 ± 0.01 (270 ± 36)	1.60 ± 0.10 (5760 ± 360)	5.25 ± 0.5 (18900 ± 1800)	0.66 ± 0.02 (2376 ± 72)
4 days	2.5 ± 0.23 (9000 ± 828)	2.7 ± 1.2 (9720 ± 4320)	0.4 ± 0.08 (1440 ± 287)	1.3 ± 1.21 (4680 ± 4320)	0.04 ± 0.01 (144 ± 36)	1.3 ± 0.29 (4680 ± 1044)	3.70 ± 0.42 (13320 ± 1512)	0.40 ± 0.23 (1440 ± 828)
7 days	2.1 ± 0.21 (7560 ± 756)	2.1 ± 0.9 (7560 ± 3240)	0.2 ± 0.01 (720 ± 36)	0.9 ± 0.10 (3240 ± 360)	0.02 ± 0.02 (72 ± 72)	0.71 ± 0.02 (2556 ± 72)	3.10 ± 0.01 (11160 ± 36)	0.21 ± 0.31 (756 ± 1116)
14 days	1.4 ± 0.11 (5040 ± 396)	1.2 ± 0.2 (4680 ± 720)	0.09 ± 0.001 (324 ± 3.6)	0.29 ± 0.02 (1044 ± 72)	0.004 ± 0.001 (14 ± 3.4)	0.33 ± 0.015 (1152 ± 54)	2.2. ± 0.001 (7920 ± 3.6)	0.04 ± 0.02 (144 ± 72)

(C)

Route	Skin	Liver	Lung	Muscle	Kidney	Blood	Adipose	Brain
iv	2.8 ± 0.24 (10080 ±.864)	*3.7 ± 1.12 (13320 ± 4032)	0.5 ± 0.09 *(1800 ± 324)	*1.7. ± 0.63 (6120 ± 2268)	*0.06 ± 0.003 *(216 ± 11)	*1.51 ± 0.24 (5436 ± 864)	4.1 ± 0.9 (14760 ± 3240)	0.45 ± 0.08 (1620 ± 288)
po	2.5 ± 0.23 (9000 ± 828)	2.70 ± 1.20 (9720 ± 4320)	0.4 ± 0.08 (1440 ± 287)	1.3 ± 0.21 (4680 ± 756)	0.04 ± 0.009 (144 ± 32)	1.3 ± 0.29 (4680 ± 1044)	3.70 ± 0.42 (13320 ± 1512)	0.40 ± 0.23 (1440 ± 828)

Note. Disposition of α-HBCD derived radioactivity (A) four days following a single (3, 10, 30, and 100 mg/kg) and 10-day repeated (3 mg/kg) po dose, (B) at multiple time points

following a single 3 mg/kg po dose and (C) four days following a 3 mg/kg dose through iv or po. All data are mean ± SD; represented as percent dose (top value) or concentration of nanogram of administered dose per gram of tissue (ng/g; bottom value in parenthesis). *Indicates significance as compared with lowest dose ($p < 0.05$).

Tissue distribution was analyzed four days after the administration of α-[^{14}C]HBCD. Data is represented in tabular form as it is useful for investigators who wish to model the data. All tissues examined had measurable levels four days after dosing. We found that the relative amount of α-[^{14}C]HBCD derived radioactivity was increased in tissues as a function of dose. The increases occurred at the 30 and/or 100mg/kg exposures in tissues which contained higher lipid content - adipose, muscle and skin, as well as the highly perfused liver tissue. Figure 2 represents this in a bar-graph format.

Tissue Distribution after a single increasing dose and repeated exposure

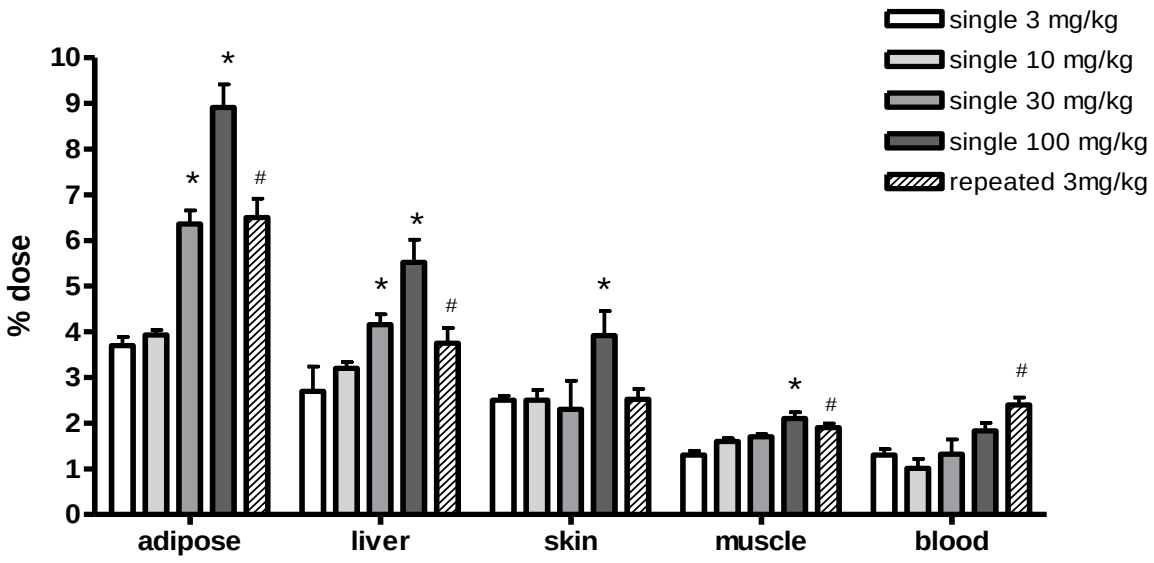

Figure 3.2. Tissue levels of α-[^{14}C]HBCD four days following a single oral dose (3, 10, 30, or 100 mg/kg) or 10 day repeated dose of 3mg/kg. Presence of a * data point indicates significance of daily percent of dose measured in the tissue (*p <0.05 as compared with the lower dose.) While the presence of a "#" data point indicates significance of daily percent of dose measured in the tissue from the 10 day repeated 3mg/kg dose (#p <0.05 as compared with a single 3mg/kg dose).

Repeated Exposure

Tissue concentrations were altered after a 10 day repeated exposure. Levels in the adipose, liver, muscle and blood were significantly increased between a single and 9 days of repeated oral exposure followed by α-[^{14}C]HBCD at 3mg/kg on the tenth day (Table 1.A and Figure 2). In fact, 4d after a 9 d unlabeled and 10th day α-[^{14}C]HBCD with 3 mg/kg, the % dose in the tissues are more similar to the 30mg/kg as opposed to the 3 mg/kg single dose (Figure 2).

Time-Course and Half-life

From the dose/response study, the lowest dose was chosen (3 mg/kg) to conduct the time course (kinetic) study because it is within the range where effects have been seen (Erikkson et al., 2006), the specific activity of the starting radiolabeled material, and to

compare the data to previous published kinetic data on γ-HBCD in mice at the same dose (Szabo et al., 2010). Only tissue distribution and elimination over time was performed after the 3mg/kg administered dose. By observing tissue distribution over time, detectable concentrations were present at all time points investigated (Table 1B). This 14 d time course study showed a biphasic profile with an initial steep decline from approximately 3 h to 2 d and a markedly slower decline between 2 d and 14 d for liver, kidney, blood and brain. Liver tissue had the highest observable level at 1 h. While most tissues peaked in concentration at 1 day, α-[^{14}C]HBCD derived radioactivity was found to peak at 2 d in fat. Basically, the highly perfused tissues reached their highest observable peak at earlier time points (1 or 3 hrs), while the poorly perfused organs such as skin, fat, thymus, and muscle peaked later (at 2 days). As can be seen in Figure 2, α-[^{14}C]HBCD derived radioactivity is initially cleared rapidly from the blood, so that by 1 hour post-gavage, only 11.3% of the dose remained. By 1, 2, and 14 days, 2.0, 1.6, and 0.33% of the α-[^{14}C]HBCD derived radioactivity was detected in the blood, respectively. At all time points measured, liver tissue contained higher levels of α-[^{14}C]HBCD derived radioactivity than that found in blood. This was also observed in adipose tissue between 2-14 days, where higher levels were measured than those found circulating. A non-linear regression two phase decay curve was used to fit the α-[^{14}C]HBCD derived radioactivity elimination from tissues. Half-lives and pool sizes, were analyzed for each elimination phase (initial and terminal) separately where appropriate (Table 2).

Table 3.2. Estimated Tissue Half-Lives of α-HBCD derived radioactivity in Mice

	Half-life (days)		Pool size (% dose)	
Tissue	α½	β ½	α phase	β phase
Liver	0.4 ± 0.1	3.0 ± 0.7	13.9 ± 0.91	4.18 ± 0.31
Blood	0.1 ± 0.0	0.5 ± 0.2	16.6 ± 2.59	3.56 ± 0.41
Lung	0.3 ± 0.1	15 ± 9.1	3.97 ± 0.22	0.93 ± 0.00
Kidney	0.2 ± 0.0	2.1 ± 0.4	7.06 ± 0.14	0.15 ± 0.02
Muscle	0.3 ± 0.1	8.0 ± 4.0	22.4 ± 200	1.59 ± 0.21
Brain	0.1 ± 0.6	3.0 ± 2.0	5.20 ± 0.21	0.99 ± 0.08
Fat	---	17 ± 4.9	---	17.8 ± 3.00

Note. Tissue-specific, biphasic half-lives were calculated from female mice given a single po dose (3 mg/kg) of α-[^{14}C]HBCD. Calculations are derived from percent of administered dose; α and β phase time points are individually based on peak tissue concentrations. Where appropriate, the pool size was calculated by determining the y-intercept of each phase. All data presented in days.

Although there is uncertainty for those tissues in which the calculated half-life exceeds the study's data points (ie adipose tissue) we report its value for comparison to other tissues and the γ-HBCD stereoisomer (Szabo et al., 2010). Increased variance around the estimate is another factor contributing to this uncertainty.

IV vs Oral: Absorption

By comparing disposition between iv and oral routes of exposure it is possible to estimate the percentage of α-[^{14}C]HBCD that was absorbed into the systemic circulation and delivered to the tissues. A comparison of the major tissue depots 4 d after treatment with the same oral and iv dose (3mg/kg) is shown in Table 1C. The percent dose and concentration of the α-[^{14}C]HBCD derived radioactivity in the tissues were similar for both routes. Every tissue measured had slightly higher levels after iv as compared with the oral route of exposure, but not all were statistically significant. Statistically significant differences are listed here as a percent of the oral to iv levels: liver (93%), muscle (89%), kidney (88%) and blood (92%). By comparing the calculated differences for each tissue between the two routes of administration (po vs iv) for the four major tissues, the higher levels found in these tissues after iv administration indicate an approximate average oral absorption of 90 ± 3% for α-[^{14}C]HBCD.

Elimination

Dose/Response and Repeated Exposure

Total cumulative elimination of the α-[^{14}C]HBCD derived radioactivity in the urine and feces after oral administration is shown in Figure 3 for the four increasing dose groups; 3, 10, 30 and 100 mg/kg/day.

Cumulative Fecal and Urinary Elimination

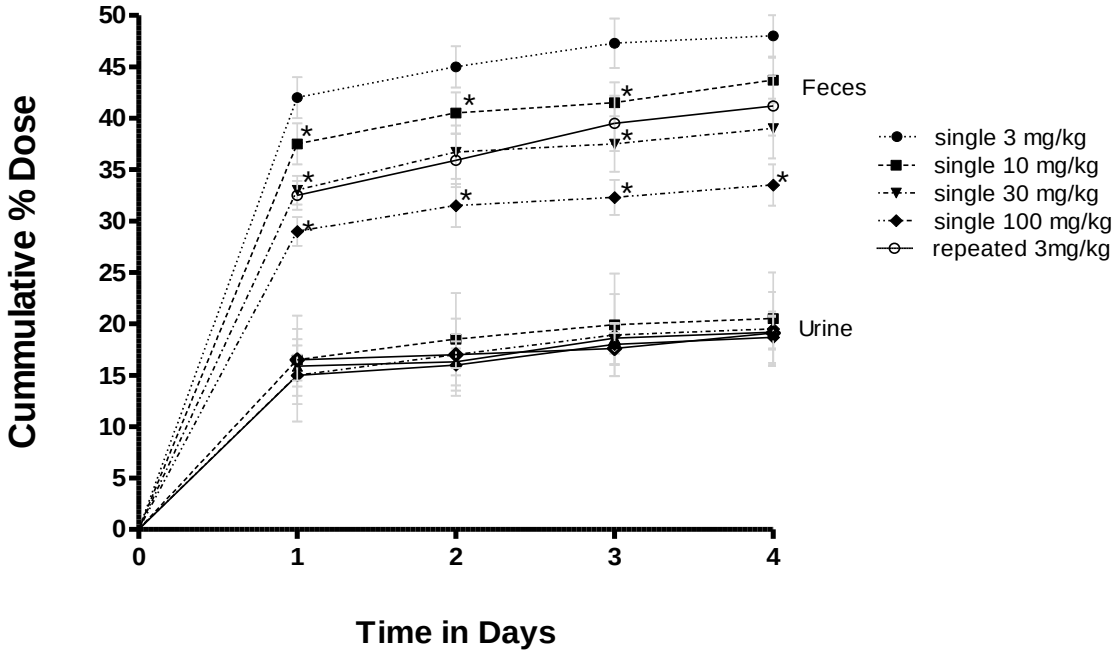

Figure 3.3. Cumulative elimination of α-HBCD derived radioactivity following a single, oral dose (3, 10, 30, 100 mg/kg) in feces and urine. Data presented as mean percent administered dose eliminated ± standard deviation (n=4-6/group). Presence of a ‘‘*’’ data point indicates significance of daily percent eliminated (*$p < 0.05$ as compared with previous dose).

This figure shows clear dose dependence on elimination; i.e. as dose increases, the amount of elimination decreases via the fecal route but not the urinary. After exposure to increasing dose levels, total recovery of α-[^{14}C]HBCD derived radioactivity is similar between all dose groups. At the 3, 10, 30 and 100 mg/kg doses, approximately 84, 82, 80 and

81% total radioactivity was recovered, respectively. Average recovery of ^{14}C, including that recovered from the tissues, urine and feces, was 82 ± 4%.

After 10 daily doses at 3mg/kg, the fecal elimination profile more closely resembles that of a single exposure of 30mg/kg; as compared to a single exposure to 3 mg/kg dose (Figure 3). In the urinary elimination profile, a single exposure of 3mg/kg is not different than the 10 day repeated exposure.

IV vs Oral: Absorption

Figure 4 compares the cumulative percent of dose in the urine and feces over four days after either iv or oral exposure. Similar elimination profiles are observed between the iv and oral routes of administration.

Cumulative Fecal and Urinary Elimination between IV and Oral Routes

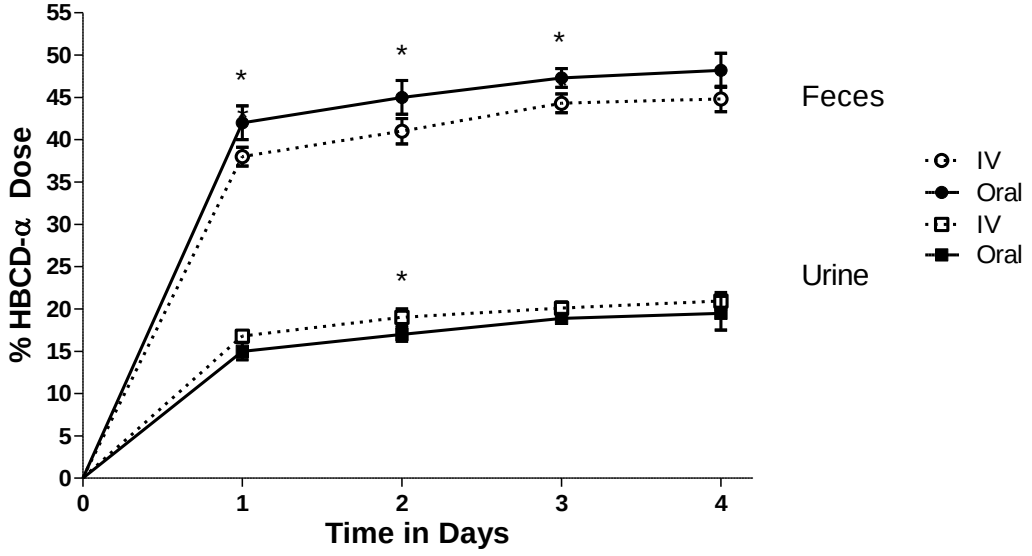

Figure 3.4. Cumulative elimination of α-HBCD derived radioactivity following a single dose (3mg/kg) via i.v and oral route in urine and feces. Data presented as mean percent administered dose eliminated ± standard deviation (n=4-6/group). Presence of a * data point indicates significance of daily percent eliminated (*$p < 0.05$ as compared to other route at same time point).

Comparison of the amount of [^{14}C] derived radioactivity eliminated in the urine and feces between the oral and iv routes of administration allows another approach to estimate absorption. 36% of the dose was eliminated in the feces of the iv administered mice on the first day whereas 41% was eliminated in the oral treated animals. After 4 d, 45% percent of the dose was eliminated in the feces following iv exposure and 49% following oral. A measurable 4% difference is calculated between the two routes of exposure. This data supports the conclusion that α-[^{14}C]HBCD is well absorbed orally.

The iv route of exposure to α-[^{14}C]-HBCD resulted in significantly higher levels of [^{14}C] derived radioactivity in the feces at days 1, 2 and 3. However, this difference suggested only slightly decreased oral absorption between the two routes. Urinary elimination was similar between iv and oral exposure with 15% of the dose eliminated in the urine following oral exposure vs. 17% after the iv route in four days. Based on fecal and urinary elimination patterns, comparison between the iv route and oral route further indicates that α-[^{14}C]HBCD is well absorbed orally (89 ± 2%). This agrees with the estimate derived from the difference found in tissue distribution when comparing the iv and oral routes of exposure (90 ± 3%).

TLC

The nature of the α-[^{14}C]HBCD derived radioactivity in the urine and feces was examined by thin-layer chromatography (Figure 5).

Thin Layer Chromatograms of Tissues and Excreta after Oral Administration of α-HBCD.

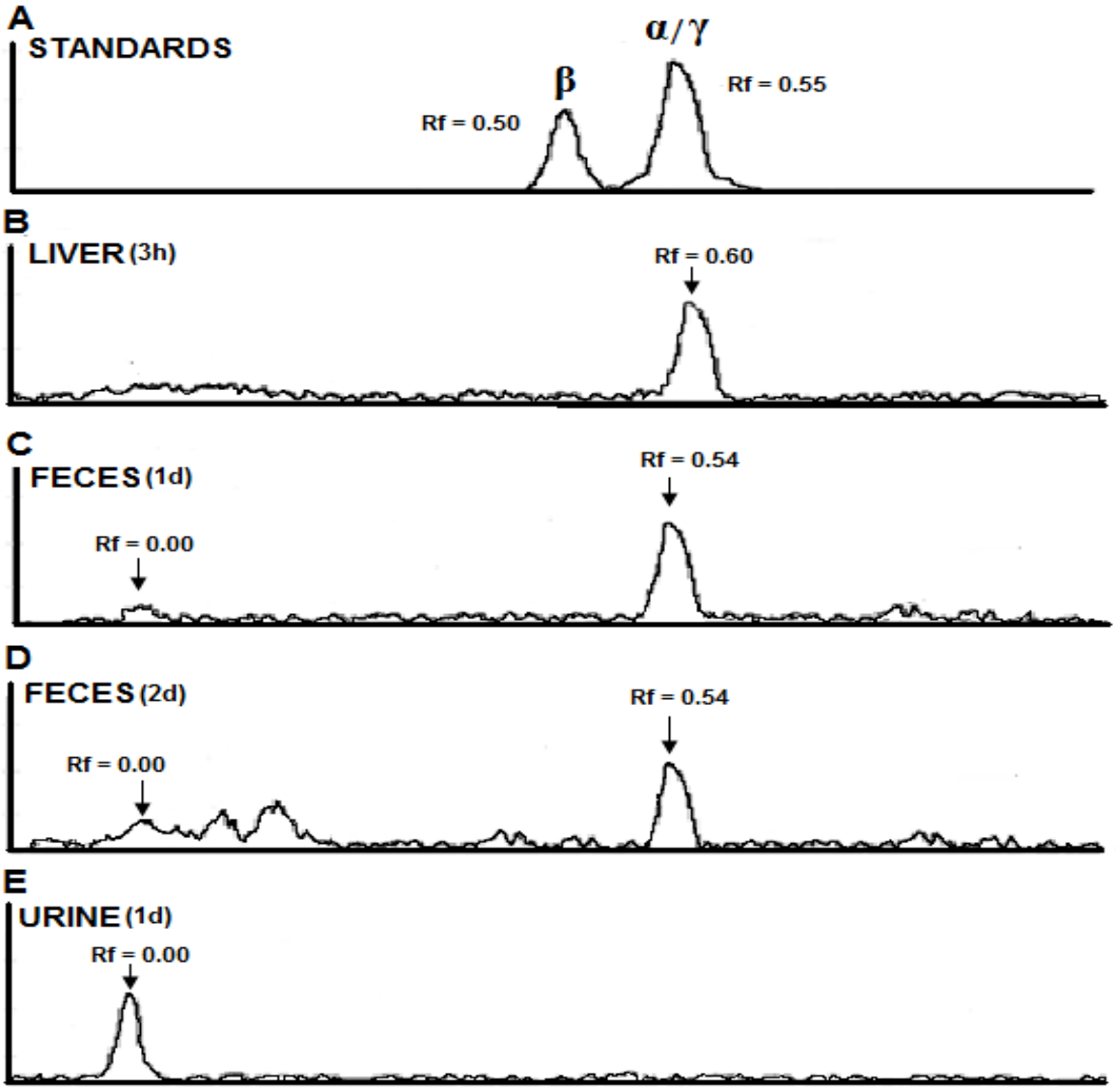

Figure 3.5. TLC radiochromatograms determined for A) α-, β-, and γ-[^{14}C]HBCD standards, B) liver at 3 hours, C) feces at 1 day, D) feces at 2 days, and E) urine . A representative sample and extract was chosen for presentation. Blood, bile and urine all contained similar chromatographic profiles. Axes; x = time in minutes, y = percent abundance.

No parent compound was detected in the urine at 1 or 2 days after treatment. TLC consistently revealed one major peak (Rf = 0.0) which contains polar metabolites. A similar metabolite(s) was seen in the blood and bile, with no parent compound detected

This is in contrast to fecal elimination where the parent compound predominates in the extractable fraction. Preliminary studies using feces spiked with α-[^{14}C]HBCD demonstrated complete extraction. 34% of the α-[^{14}C]HBCD derived radioactivity which was extracted from the feces was parent. Given that approximately 90% of α-[^{14}C]HBCD is orally absorbed (see above), at most 10% is unabsorbed α-[^{14}C]HBCD. The remaining 24% was absorbed but eliminated unchanged in the feces. 66% of the extracted radioactivity was metabolite. The nonextractable radioactivity is assumed to be bound metabolites of α-[^{14}C]HBCD. Our results indicated that by 1d, greater than 66% of the radioactivity that was eliminated from the mice was no longer α-HBCD, but several metabolites. To examine the identity of the extracted α-[^{14}C]HBCD derived radioactivity; the extracts, as well as standards of the three main diastereomers (α-, β-and γ-[^{14}C]HBCD) were assayed by TLC (Figure 5). In this system, β-[^{14}C]HBCD migrated with a shorter retardation factor (Rf = 0.50) than α-

and γ-[^{14}C]HBCD which co-migrated at Rf = 0.61. One day post-exposure, only one band was detected in the fecal extracts with a Rf = 0.54. Interestingly, by 2 days post-exposure, four bands were present in the fecal extract (Rf = 0.00, 0.12 and 0.14 and 0.54), demonstrating polar metabolites as well as parent compound.

Liver extracts were analyzed at 3h, shortly after the time of peak tissue concentration, 1h. In the liver tissue only one clear TLC band was detected with Rf = 0.60, similar to the fecal extracts (Rf = 0.54) and the α- and γ-HBCD standard peaks (Rf = 0.61). Migration differences between the standard α- and γ-[^{14}C]HBCD peak with the α- and γ-[^{14}C]HBCD peak in the feces and liver were attributed to the tissue matrix effects.

The GPC

The nature of the α-[^{14}C]HBCD–derived radioactivity in the brain and fat was initially examined by gel permeation chromatography (GPC). GPC separation is based on size but, unlike TLC, GPC is not useful for the identification of individual stereoisomers since all HBCD stereoisomers have the same molecular weight. GPC chromatograms revealed one major peak (data not shown) that was further analyzed using LC-MS.

LC-MS

Due to the inability of TLC to differentiate α- from γ-HBCD, and the inability for GPC to resolve any HBCD stereoisomers, the [^{14}C]-containing peaks from TLC and GPC

were further characterized by LC-MS, which can distinguish individual HBCD stereoisomers. The TLC peaks in the liver (Rf = 0.60) and fecal extracts (Rf = 0.54), were scraped from the plates, eluted with acetone, and analyzed by LC-MS for comparison to authentic standards, α-, β-, and γ-HBCD, which had retention times of 3.40, 4.06, and 4.89 minutes, respectively. The results indicated that all peaks had an M-H of ~640.6 suggesting that they were HBCD stereoisomers. Both the liver and the fecal extract peaks had retention times of 3.40 minutes, which corresponds to α-HBCD. Furthermore, only α-HBCD was detected in the feces, fat, and brain at 1 d, and at the liver at 3 h post oral administration of α-[^{14}C]HBCD (Figure 6).

LC-MS chromatograms of Tissues and Excreta after Oral Administration of α-HBCD.

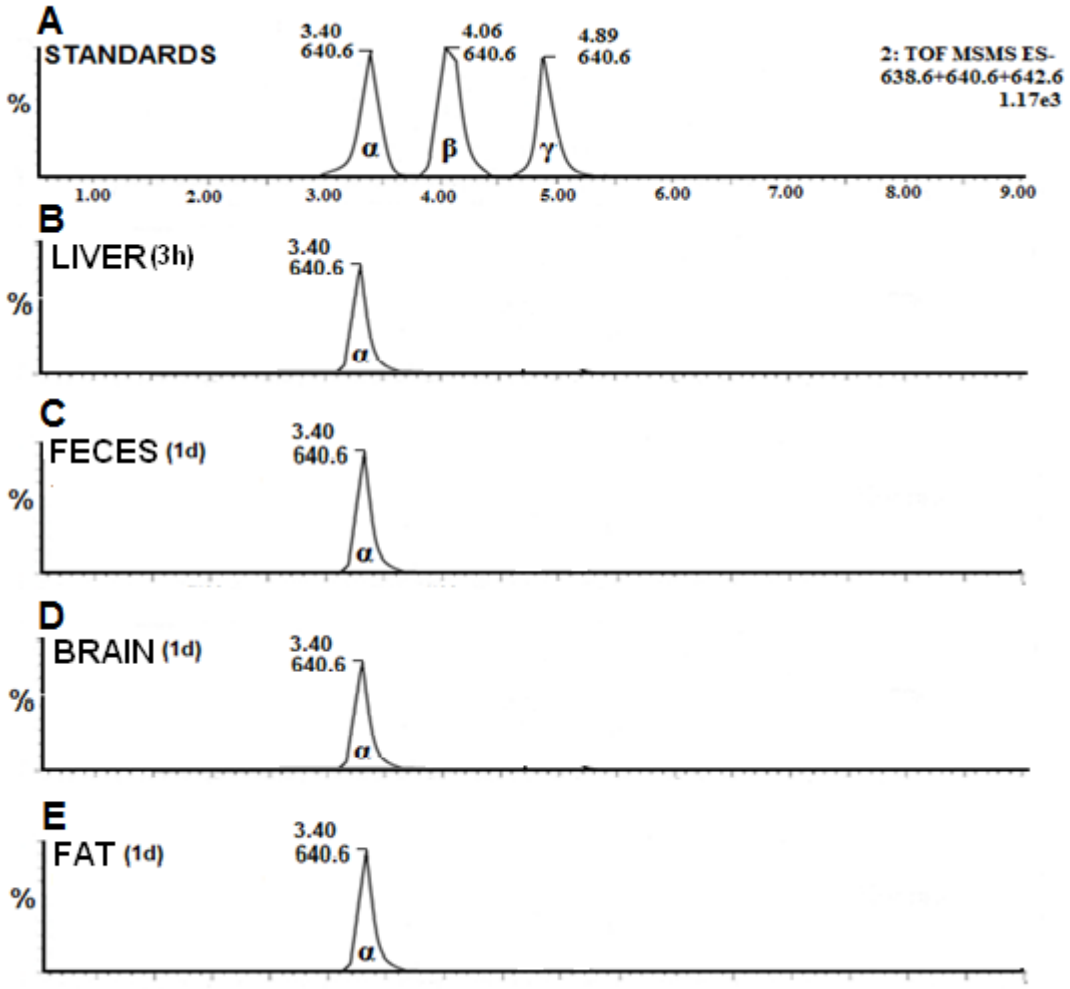

Figure 3.6. LC/MS chromatograms determined for A) α-, β-, and γ-HBCD standards, B) liver at 3 h, C) feces at 1 day, D) brain at 1 day, and E) fat at 1 day. . Standards are a representative sample and retention times varied slightly from day to day.

A summary of the parent, stereoisomer, and metabolite profiles in tissues and excreta after exposure to α-[^{14}C]HBCD can be found in Table 3.

Table 3.3. Summary of Metabolite Profiles in Female Mouse Tissue and Excreta after Oral Exposure to 3mg/kg α-[^{14}C]HBCD

Matrix	Time	Parent	Isomerization	Metabolites
Liver	3 hours	**62%**	**0%**	**38%** Unextractable (38%)
Feces	24 hours	**34%**	**0%**	**66%** Rf = 0.00; Unknown (2%) Unextractable (64%)
Fat	24 hours	**100%**	**0%**	**0%**
Brain	24 hours	**100%**	**0%**	**0%**
Bile **Blood** **Urine**	24 hours	**0%**	**0%**	**100%** Rf = 0.00; Unknown

Fat and brain contained only parent α-[^{14}C]HBCD. Liver and feces both contained large amounts of parent compounds, 62 and 34% respectively. No stereoisomerization products were detected in excreta and tissues. Polar metabolites were found at high levels in the liver and feces, 38 and 66%, respectively. The bile, serum and urine contained

exclusively polar metabolites. Metabolites in serum, tissues and excreta are in the process of being characterized but beyond the scope of this report.

Mass Balance

Approximately 82% of the α-HBCD derived radioactivity could be accounted for by measuring 1) the major mouse tissues and 2) excreta after oral and intravenous routes of exposure. This recovery is similar to recent publications on related lipophilic compounds in mice: γ-HBCD (Szabo et al., 2010); BDE-154 (Hakk et al., 2009); and BDE-47 (Staskal et al., 2005). Lack of total mass balance after 4 days post exposure in this study could be attributed to a number of common factors. Approximate tissue masses (on a percent basis) for blood, skin, fat, and muscle from the literature (ILSI, 1994) were used for the calculation. Although, the [^{14}C] in most major tissues were measured in this study; the carcasses were not and may have contained residual levels not included in the estimates of mass balance. In addition, any exhaled [^{14}C] was not trapped.

DISCUSSION

The objectives of this study were to determine the absorption, distribution, metabolism and excretion of α-HBCD in female mice to better evaluate its behavior. This study demonstrated that approximately 90% of an oral dose of α-HBCD was absorbed.

Distribution of α-HBCD was initially to the highly perfused organs including liver, kidney, and lung, followed by redistribution to skin, muscle, and adipose tissues. Of the major tissues, adipose contained the highest levels detected across all doses measured. Tissue disposition of α-HBCD was dependant on dose at the higher dose levels (30 and 100 mg/kg) in the single (acute) dosing paradigm and also the 10 day repeated exposure paradigm at 3 mg/kg/d.

Fat was found to be a major tissue depot for α-HBCD derived radioactivity after exposure to increasing α-HBCD concentrations. This is in contrast to γ-HBCD which failed to concentrate in fat due to its rapid metabolism and excretion (Szabo et al., 2010), but similar to another lipophilic BFR, PBDE-47 which accumulated in the fat (Staskal et al., 2005). In an industry report, the HBCD commercial mixture was found to partition to adipose tissue in rats (Yu and Atallah, 1980). HBCD was detected in fish oil supplements with a maximum concentration of 5.8 ng/g for total HBCD (UK Food Standards Agency, 2006). Human adipose tissue obtained by liposuction in the Czech Republic had total HBCD concentrations ranging from <0.5 to 7.5 ng kg^{-1} lw (Pulkrabova et al., 2009).

Elimination of α-HBCD either after oral or iv administration was primarily in the feces and to a lesser extent in the urine. Fecal elimination of α-HBCD derived radioactivity decreased as dose increased; this was not observed for urinary elimination. An opposite trend in the liver, fat, muscle, and skin occurred, as the tissue concentration increased non-linearly with increasing administered doses. Tissue concentrations for α-HBCD are in contrast to

what was observed after oral exposure to γ-HBCD where the relative amount of γ-HBCD inexcreta or tissues was independent of dose. Van der Ven (2009) reported similar findings in the liver of rats exposed daily to the HBCD commercial mixture (up to 175 days) where a dose dependent hepatic increase in α-HBCD was observed. This rate of increase for α-HBCD was greater than for other HBCD stereoisomers measured. Similar to the distribution observed with α-HBCD; an industry report on the kinetics and distribution of the radiolabeled HBCD commercial mixture in rats detected higher levels of HBCD in the liver than the blood (Yu and Atallah, 1980). These finding suggests that there is a HBCD stereoisomer specific difference in hepatic function and clearance with increasing concentration. Although the mechanism for the non-linear behavior of α-HBCD is not known, it is possible that inhibition, and/or saturation of metabolism, including binding to hepatic enzymes and transporters, may occur at higher concentrations.

Of the α-HBCD derived radioactivity detected in the urine, bile and blood; 100% consisted of polar metabolites after 24 h. Feces (24-48 h) and liver (3 h) contained both parent α-HBCD and polar metabolites. Differential metabolic capacities for γ-HBCD and α-HBCD have been observed *in vitro* with rat and harbor seal liver microsomes (Zegers et al., 2005) and are supported by the contrast between this present study of α-HBCD and our recently published report on γ-HBCD in mice (Szabo et al., 2010). α-HBCD was more slowly eliminated in female mice than γ-HBCD and therefore is believed to have a greater opportunity to bioaccumulate. Characterizing the toxicity of each stereoisomer remains to be determined.

The *in vivo* mouse data from this as well as the previous study with γ-HBCD (Szabo et al., 2010) suggest that two factors may be responsible for the shift observed from the predominance of γ-HBCD in the commercial mixture to α-HBCD in biota. First, γ-HBCD was more rapidly metabolized and eliminated with a terminal half-life of 4 days (Szabo et al. 2010), while α-HBCD was more biologically persistent due to a greater resistance to metabolism with a terminal half-life of 17 days. Bioaccumulation may be even more pronounced after chronic exposure, as we observed over 20% of α-HBCD administered dose remained in the mice in the 10 day repeated dose study, but <1% was found in the mice exposed to γ-HBCD at the same paradigm (Szabo et al. 2010). In addition, *in vivo* stereoisomerization (11-15%) of γ-HBCD to α- and β- was observed in female mice treated with γ-HBCD (Szabo et al., 2010); however, the stereoisomer shift was not seen for α-HBCD.

Thus, α-HBCD's persistence and bioaccumulation in mice is relatively high and may explain the observed predominance of α in biota. These data lend support to a theory that the reason α-HBCD is the dominant HBCD stereoisomer in biota is its relatively slower metabolism, biological persistence, bioaccumulation potential, and absence of stereoisomerization.

ACKNOWLEDGEMENT AND DISCLAIMER

The authors would like to thank Francis McQuaid, Brenda Edwards, Colleen Pfaff and Barbara Magelky for technical assistance. This work is funded in part by a cooperative agreement between the University of North Carolina in Chapel Hill and the Environmental Protection Agency CR 833237 predoctoral training grants. This abstract does not reflect USEPA, NIH or USDA policy. The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

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Chapter IV

DIFFERENCES IN TISSUE DISTRIBUTION OF HBCD ALPHA AND GAMMA BETWEEN ADULT AND DEVELOPING MICE

2011
Szabo DT, Diliberto JJ, Huwe J and Birnbaum LS
Toxicological Science; doi 10.1093/toxsci/kfr161

Title: Differences in tissue distribution of HBCD alpha and gamma between adult and developing mice.

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1) Running Title: Differences in tissue distribution of HBCD alpha and gamma between adult and developing mice.

2) Key words: development, brominated flame retardants (BFRs), HBCD, stereoisomer, toxicokinetics, age susceptibility

3) Acknowledgements:

The information in this document has been subjected to review by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The research presented in this document was funded in part by the U.S. Environmental Protection Agency. The research was partially supported by the EPA/UNC Toxicology Research Program Training Agreement; NHEERL-DESE Cooperative Training in Environmental Sciences Research, EPA CT826513, with the Curriculum in Toxicology, University of North Carolina at Chapel Hill. A conflict of interest was not reported.

ABSTRACT

Hexabromocyclododecane (HBCD) is a mixture of three stereoisomers alpha (α), beta (β) and gamma (γ). γ-HBCD dominates the mixture (~70%) and despite α-HBCD's minor contribution to global HBCD production and usage (~10%), it is the dominant congener found in most biotic samples worldwide. Evidence of toxicity and lack of stereoisomer studies drives the importance of understanding HBCD toxicokinetics in potentially susceptible populations. The majority of public health concern has focused on hazardous effects resulting from exposure of infants and young children to HBCD due to reports on adverse developmental effects in rodent studies, in combination with human exposure estimates suggesting nursing infants and young children have the highest exposure to HBCD. This study was designed to investigate differences in the disposition of both γ-HBCD and α-HBCD in infantile mice reported to be susceptible to the HBCD commercial mixture. The tissue distribution of α-[^{14}C]HBCD and γ-[^{14}C]HBCD derived radioactivity was monitored in C57BL/6 mice following a single oral dose of either compound (3 mg/kg) after direct gavage at postnatal day 10 (PND 10). Mice were held up to 7 days in shoebox cages after which pups were sacrificed, tissue collected, and internal dosimetry was measured. Developing mice exposed to α-HBCD had an overall higher body burden than γ-HBCD at every time point measured; at 4 days post exposure they retained 22% of the α-HBCD administered dose while pups exposed to γ-HBCD retained 10%. Total body burden in infantile mice after exposure to γ-HBCD was increased 10 fold as compared to adults.

Similarly, after exposure to α-HBCD, infantile mice contained 2.5 fold higher levels than adult. These differences lead to higher concentrations of the HBCD diastereomers at target tissues during critical windows of development. The results indicate that the toxicokinetics of the two HBCD diastereomers differ between developing and adult mice; whereas distribution patterns are similar, concentrations of each HBCD diastereomer's derived radioactivity are higher in the pup's liver, fat, kidney, brain, blood, muscle and lungs than in the adult's. This study suggests that developmental stage may be a risk factor for the harmful effects of both HBCD diastereomers when developing animals may be more sensitive to effects and increases in body burden.

INTRODUCTION

Brominated flame retardants (BFRs) are chemicals incorporated into plastics, electrical and electronic products, textiles, and other materials to reduce flammability. Currently, tetrabromobisphenol A (TBBPA), polybrominated diphenylethers (PBDEs), and hexabromocyclododecane (HBCD) account for the largest volume of BFRs. HBCD is a lipophilic additive flame retardant mixture extensively used since the 1970s, primarily added to polystyrene insulations and building materials to reduce combustibility (de Wit, 2002). Annual demand for HBCD ten years ago was about 17,000 metric tons (Covaci et al., 2006), and approximately half of this quantity was consumed in Europe (Hale et al., 2006). HBCD is considered a ubiquitous and global environmental contaminant and undergoes long range

transport with detectable levels found in abiotic and biotic samples including human blood and breast milk (de Wit, 2002, Law et al., 2005, Law et al., 2008).

Toxicity studies suggest that the HBCD commercial mixture is an enzyme inducer, endocrine disruptor, and developmental neurotoxicant. The commercial mixture has been shown to induce both phase I and II metabolic enzyme systems, specifically CYP2B and CYP3A, by interacting with the constitutive androgen receptor (CAR) and/or the pregnane-X-receptor (PXR) receptor, respectively (Germer et al., 2006). Van der Ven et al. (2006) reports a decrease in circulating total thyroxin (T4) levels, increased pituitary weight, thyroid weight, and immunostaining of thyroid stimulating hormone (TSH) in the pituitary and thyroid follicular cell activation in adult female rats exposed to the commercial mixture of HBCD. Hypothyroid conditions especially during the period of brain growth can cause abnormal brain development with severe physical and/or mental retardation in the offspring (Dobbing et al., 1973; Koibushi et al., 2000; Yen et al., 2001), and can cause decreases in IQ (Haddow et al., 1999). Exposure to neonatal mice at postnatal day 10 (PND10) with the commercial mixture of HBCD have been shown to cause impairment in learning, memory and aberrant spontaneous behavior (Eriksson et al., 2006). HBCD has been shown to directly inhibit the uptake of neurotransmitters, dopamine and glutamate into synaptosomes in the rodent brain (Mariussen et al., 2003). Therefore, neurodevelopmental effects may be due to either a direct or indirect result of HBCD during periods of growth.

The commercial mixture is composed of three diastereoisomers, denoted as alpha (α), beta (β) and gamma (γ) with the γ -diastereoisomer predominating (>70%) (Heeb *et al.*, 2005). High concentrations of HBCD in some top predators indicate persistence and biomagnification. However, recent studies shown there is a selective predominance of α-HBCD in biota (Law *et al.*, 2006). Due to the different physical, chemical and biological properties of the diastereomers, there is a growing need to characterize the individual diastereomers in the commercial mixtures.

To better understand the biological behavior of a chemical requires examinination of its toxicokinetic properties. Toxicokinetic information on HBCD is limited. Unfortunately, early toxicokinetic and toxicity studies contain study design flaws in which animals were administered HBCD *suspensions* in oil (Yu and Atallah, 1980, Arita et al, 1983, Chengelis et al., 2001). Undissolved particles of HBCD in oil, in addition to adsorption to laboratory glass equipment, may result in decreased bioavailability and decreased internal absorption. These factors create further uncertainties and inconsistencies when comparing dose and effects across studies.

To address the growing need for a toxicokinetic evaluation of HBCD at the stereoisomer level, our laboratory conducted two studies (Szabo *et al.* 2010 and Szabo et al., 2011) to characterize the absorption, distribution, metabolism and excretion of HBCD diastereomers, γ-HBCD and α-HBCD, the predominant diastereomers in the commercial mixture and in biota, respectively. The results from these studies demonstrate that the

stereoisomers have different toxicokinetic behaviors in adult (PND 60) mice. Both are readily absorbed from the GI tract (85-90%). However, γ-HBCD is rapidly metabolized and eliminated with a terminal half-life of 4 days (Szabo et al. 2010), while α-HBCD is more biologically persistent with a terminal half-life of 17 days (Szabo et al., 2011). Bioaccumulation of α-HBCD, but not of γ-HBCD derived radioactivity was observed in adipose tissue. Greater than 20% of the α-HBCD administered dose remained in the mouse after a 10 day repeated dose study, but <1% was measured in the mice after a similar exposure to γ-HBCD. *In vivo* stereoisomerization (11-15%) of γ-HBCD to α- and β-HBCD was observed in female mice treated with γ-HBCD (Szabo et al., 2010); however, the stereoisomer shift was not seen after exposure to α-HBCD. It was concluded that the biological persistence of γ-HBCD in mice was low and may explain low levels of γ-HBCD in biota. Overall this data lends support to a hypothesis that the reason α-HBCD is the dominant HBCD stereoisomer in biota is its slower metabolism leading to its biological persistence and bioaccumulation potential in addition to the absence of α-HBCD stereoisomerization.

While there is evidence of developmental neurotoxicity in PND 10 mice after exposure to the commercial mixture of HBCD (Eriksson et al., 2006), there are currently no toxicokinetic studies in developing animals. Since differences in the two main HBCD stereoisomers have been observed in adult mice (Szabo et al., 2010; Szabo et al., 2011), we asked whether a difference in the tissue distribution and body burden of alpha and gamma in developing mice exists and whether tissue distribution patterns differ between infants and adults.

The present study was designed to investigate the tissue distribution and body burden of the HBCD stereoisomers α-HBCD and γ-HBCD in 10-day-old mice reported to be susceptible to the HBCD commercial mixture. C57BL/6 mice were exposed to a single oral dose of either α-[^{14}C]HBCD or γ-[^{14}C]HBCD (3 mg/kg) on postnatal day 10. Tissue distribution was monitored at multiple time points up to 7 days post-dose, and tissue concentrations were compared to previously reported adult tissue concentrations using the same exposure scenario (Szabo et al., 2010; Szabo et al., 2011).

MATERIALS AND METHODS

Chemicals: [^{14}C]1,2,5,6,9,10-hexabromocyclododecane ([^{14}C]HBCD) (2mCi/mmol) was purchased from American Radiochemicals Corporation (ARC); (St Louis, MO) as a mixture of ß-[^{14}C]HBCD and γ-[^{14}C]HBCD. Methods for separating the diastereomers and thermal conversion of γ-[^{14}C]HBCD to α-[^{14}C]HBCD were previously published (Szabo et al., 2010, 2011). Other chemicals used were of the highest grade commercially available.

Dosing Solutions: Doses were selected based on published toxicity and toxicokinetic studies, environmental relevance, and specific activity of α-[^{14}C]HBCD and γ-[^{14}C]HBCD. A stock solution of each was made by dissolving 19.23 mg of α-[^{14}C]HBCD or γ-[^{14}C]HBCD (3.12 μCi/mg) in toluene (400 uL) . Aliquots were used directly from this solution for all dosing regimens. All dosing solutions were subjected to pre- and post- dosing radioactivity examination to ensure proper delivery of dose. All solutions were designed to deliver

approximately 0.2 μCi to each mouse. Corn oil by weight was then added to the vials followed by the evaporation of toluene under vacuum (Speed Vac, Savant Instruments, Inc. Farmingdale, NY).

Animals and Treatment: Dams (n=6/diastereomer) and female C57BL/6 mice (PND 9, ~ 7 grams) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Dams and 6 pups per litter were acclimated for 24 hours in shoebox cages. PND 10 pups were exposed by gavaged with a single dose of α-[^{14}C]HBCD and γ-[^{14}C]HBCD at 3 mg/kg. Animals were maintained on a 12 h light/dark cycle at ambient temperature (22°C) with relative humidity (56 ± 5%), and were provided with Purina 5001 Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water *ad libitum*. One pup/litter (total of 6 litters/diastereomer) was randomly selected at 3h, 8h, 1, 2, 4 or 7 days following exposure when tissues were harvested (Figure 1).

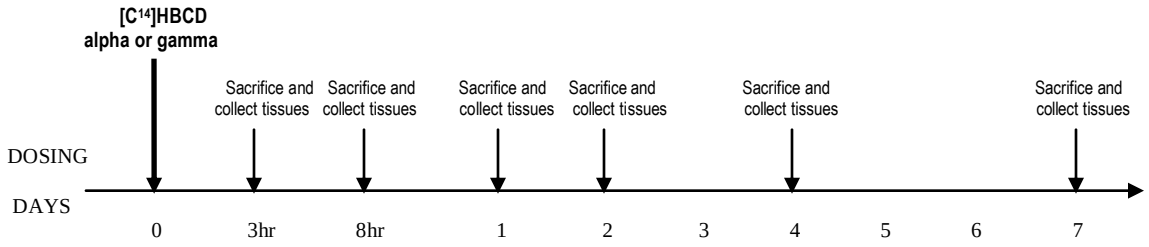

Figure 4.1. Dosing paradigm. C57BL/6 female mouse pups were gavaged at PND10 or PND60 (Szabo et al., 2010; Szabo et al., 2011) with a single exposure of α-HBCD or γ-HBCD at 3mg/kg. Pups were sacrificed 3hr, 8hr, 1 day, 2 day, 4 day and 7 day post exposure where major tissues were harvested.

Exposure: A single dose (3 mg/kg) of each diastereomer was administered directly by gavage into the stomach of each mouse using a curved ball-tipped animal feeding needle. Dose volume was 10 mL/kg.

Sample Analysis: Radioactivity in the tissues was determined by combustion to $^{14}CO_2$ (Packard 307 Biological Oxidizer, Downers Grove, IL) of triplicate samples when available (~100 mg/sample) followed by liquid scintillation spectrometry (LSS; Beckman, Beckman Instruments, Fullerton, CA) with limits of detection (LOD) of 50 dpm (3 x background) or 6.7 ng HBCD. Tissue data are reported based on wet weight.

Data Analysis: Tissue concentrations are presented as nanograms of α-HBCD or γ-HBCD derived radioactivity/gram of tissue wet weight and percent administered dose of tissue. Intergroup comparisons were performed by a two-way ANOVA followed by Bonferroni post tests significant when $p < 0.05$. All data are presented as mean ± standard deviation. GraphPad Prism 5.0, Hearn Scientific Software (Melbourne, Australia) was used for statistical analysis. Excretion was estimated using whole-body analyses of residual radioactivity.

RESULTS

The distribution of the two high profile HBCD diastereomers, α-[^{14}C]HBCD or γ-[^{14}C]HBCD, were monitored from PND 10 for 7 days following a single oral dose and compared to patterns observed in adult animals (Szabo et al., 2010; Szabo et al., 2011). Administration of either α-[^{14}C]HBCD or γ-[^{14}C]HBCD in corn oil did not impact pup development (as monitored by body weight of pups treated with vehicle only). No overt toxicity was observed in response to oral exposure to either diastereomer.

Tissue Distribution of α-HBCD and γ-HBCD

One pup/litter (total of 6 pups) was randomly selected at 3 hr, 8 hr, 1, 2, 4 or 7 days following exposure to either α-[^{14}C]HBCD or γ-[^{14}C]HBCD. Overall trends resulted in α-[^{14}C]HBCD, and to a lesser extent γ-[^{14}C]HBCD, being distributed to lipophilic tissues (Tables 1a and 1b).

Time	Skin	Liver	Lung	Muscle	Kidney	Blood	Adipose	Brain
3 hours	1.1 ± 0.1	19 ± 2.2	1.2 ± 0.1	3.1± 0.8	6.0 ± 0.3	7.3 ± 0.2	1.0 ± 0.1	1.3 ± 0.1
8 hours	2.7 ± 0.2	1.4 ± 2.4	2.4 ± 0.3	4.8 ± 0.5	2.1± 0.4	3.4 ± 0.2	2.4 ± 0.2	1.0 ± 0.1
1 day	1.3 ± 0.2	9.0 ± 0.5	0.9 ± 0.2	2.1 ± 0.2	1.1 ± 0.2	2.8 ± 0.1	5.2± 0.6	0.9 ± 0.1
2 days	0.7 ± 0.2	5.0 ± 0.9	0.5 ± 0.0	1.0 ± 0.1	0.4 ± 0.0	1.9 ± 0.0	3.1 ± 0.5	0.5 ± 0.0
4 days	0.4 ± 0.1	2.8 ± 0.3	0.3 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	1.1 ± 0.2	1.8 ± 0.2	0.3 ± 0.0
7 days	0.2 ± 0.0	1.3 ± 0.2	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	0.8 ± 0.2	0.1 ± 0.0

Table 4.1a. Disposition of γ – [^{14}C] HBCD derived radioactivity at multiple time points following a single 3 mg/kg oral dose at PND 10. All data are mean ± standard deviation; percent dose.

Time	Skin	Liver	Lung	Muscle	Kidney	Blood	Adipose	Brain
3 hours	2.2 ± 0.1	34 ± 3.2	1.6 ± 0.1	2.1 ± 0.8	9.5 ± 0.3	11 ± 0.3	2.5 ± 0.2	2.5 ± 0.2
8 hours	3.7 ± 0.2	19 ± 0.9	2.4 ± 0.3	3.8 ± 0.5	3.8 ± 0.4	3.9 ± 0.5	5.3 ± 0.2	1.4 ± 0.2
1 day	4.9 ± 0.3	13 ± 0.9	1.8 ± 0.2	5.1 ± 0.2	1.9 ± 0.2	2.9 ± 0.1	6.1 ± 0.1	1.2 ± 0.2
2 days	2.4 ± 0.3	8.1 ± 0.9	1.3 ± 0.2	4.2 ± 0.1	1.6 ± 0.1	2.0 ± 0.1	7.0 ± 0.5	1.0 ± 0.2
4 days	3.6 ± 0.3	3.9 ± 0.3	0.9 ± 0.0	3.9 ± 0.3	1.3 ± 0.0	1.6 ± 0.2	5.2 ± 0.4	0.8 ± 0.0
7 days	2.9 ± 0.2	3.2 ± 0.3	0.8± 0.0	3.2 ± 0.0	0.9 ± 0.0	1.1 ± 0.2	4.9 ± 0.4	0.6 ± 0.0

Table 4.1b. Disposition of α-[^{14}C] HBCD derived radioactivity at multiple time points following a single 3 mg/kg oral dose at PND 10. All data are mean ± standard deviation; percent dose.

Highly perfused tissues, such as liver, kidney and brain had peak concentrations within 3 to 8 hrs of administration for both stereoisomers examined. However, peak concentrations in slowly perfused tissues, such as muscle and skin, were observed at 24 h post dose while lipophilic tissues such as adipose peaked at 2 days after exposure with α-[^{14}C]HBCD. γ-[^{14}C]HBCD had a similar distribution pattern as α-[^{14}C]HBCD, however, lower levels of γ-[^{14}C]HBCD were present in each tissue examined, and its peak concentrations occurred at earlier time points.

Body Burden of α-HBCD vs. γ-HBCD

Total radioactivity in tissues was summed and used here as a measure of total body burden. Since pups were kept with dams in shoebox cages, excreta were not collected. The amount of α-[^{14}C]HBCD and γ-[^{14}C]HBCD remaining in the body over time in pup tissues is shown in Figure 4.2.

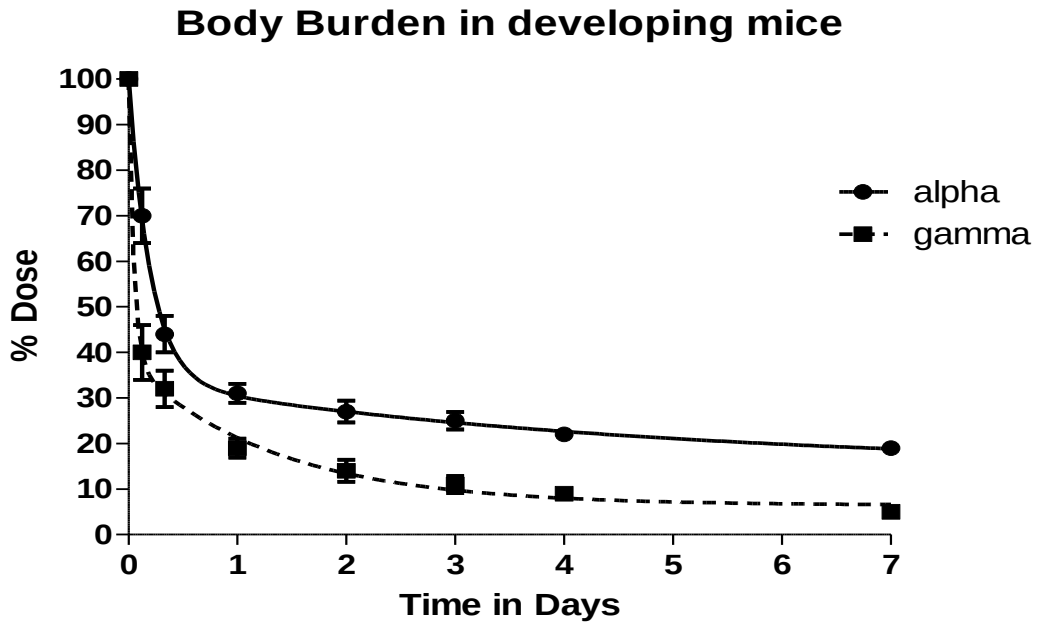

Figure 4.2. Body burden is the sum of all the tissues measured in developing mice. Body burden was compared as a function of stereoisomer derived radioactivity (γ-[^{14}C]HBCD vs. α-[^{14}C]HBCD) over time.

After oral exposure to α-[^{14}C]HBCD, higher levels of total radioactivity are observed at every time point measured than were observed from exposure to γ-[^{14}C]HBCD. Over

time, the % dose difference in body burden between the two stereoisomers slightly increased. At 24 hours, the body burden difference was 1.5- fold higher in α-[^{14}C]HBCD exposed mice than γ-[^{14}C]HBCD exposed mice and by 7 days post-dose, the difference had increased to 2.2-fold.

Adults vs Developing Animals- Tissue Levels

While pup disposition trends paralleled those observed in adults, actual tissue concentrations of both α-[^{14}C]HBCD and γ-[^{14}C]HBCD were generally higher in pups than levels found in adults previously reported (Szabo et al., 2010; Szabo et al., 2011) (Figure 3). The majority of comparisons in this section are based on the concentration of stereoisomer-derived radioactivity per gram of tissue (ng/g), which normalizes for differences in body composition during development and allows for a more direct comparison across age groups. When using this dose-metric, concentrations in adipose, blood, brain, kidney, muscle, and skin were higher in pups at respective time points after exposure to γ-[^{14}C]HBCD with similar trends but higher levels measured after exposure to α-[^{14}C]HBCD at tissues measured.

Tissue Levels between PND 10 vs PND 60 mice after exposure to

α-[^{14}C]HBCD and γ-[^{14}C]HBCD

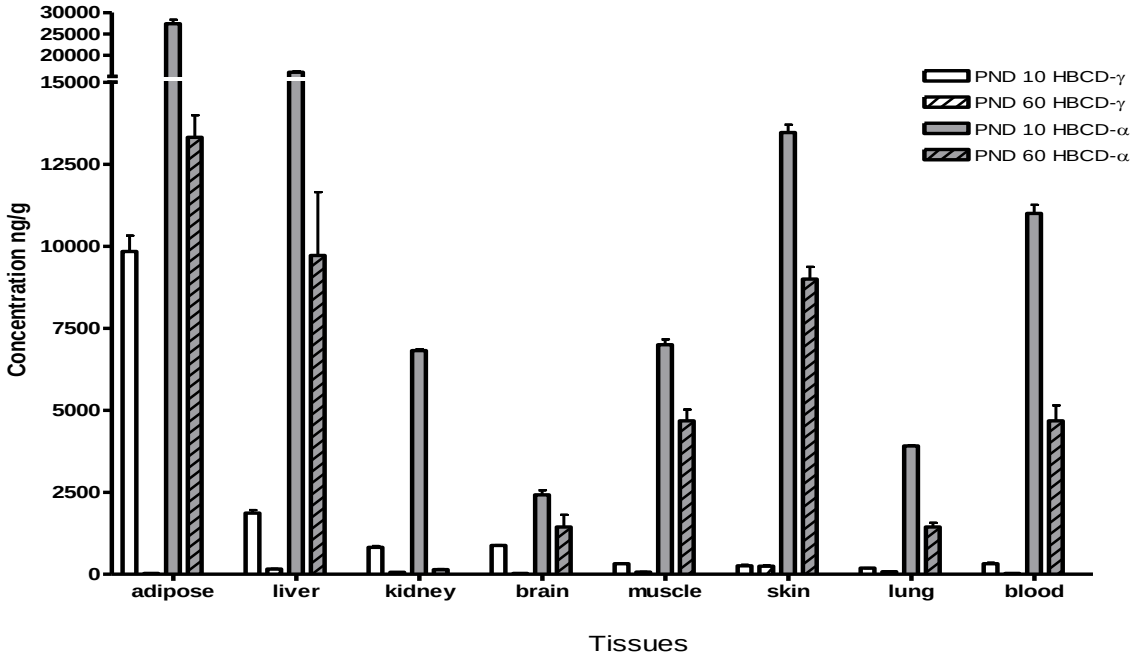

Figure 4.3. Tissue disposition of γ-[^{14}C] HBCD and α-[^{14}C]HBCD derived radioactivity 4 after oral administration of a single 3 mg/kg dose in juvenile mice at PND 10 and compared to previously published levels in adult mice at PND 60 (Szabo et al., 2010, 2011). Data are represented as concentration in the tissue (ng/g wet weight).

Adults vs Developing Animals - Body Burden

Figures 4a and 4b show the amount of α-[^{14}C]HBCD and γ-[^{14}C]HBCD remaining in the body over time, directly comparing total tissue levels measured in pups to those previously reported in adult mice (Szabo et al., 2010,2011). Within 24 h after oral exposure to α-[^{14}C]HBCD, only 25% of the dose remained in the body of the adult mice versus 33% in the pups (Figure 4a).

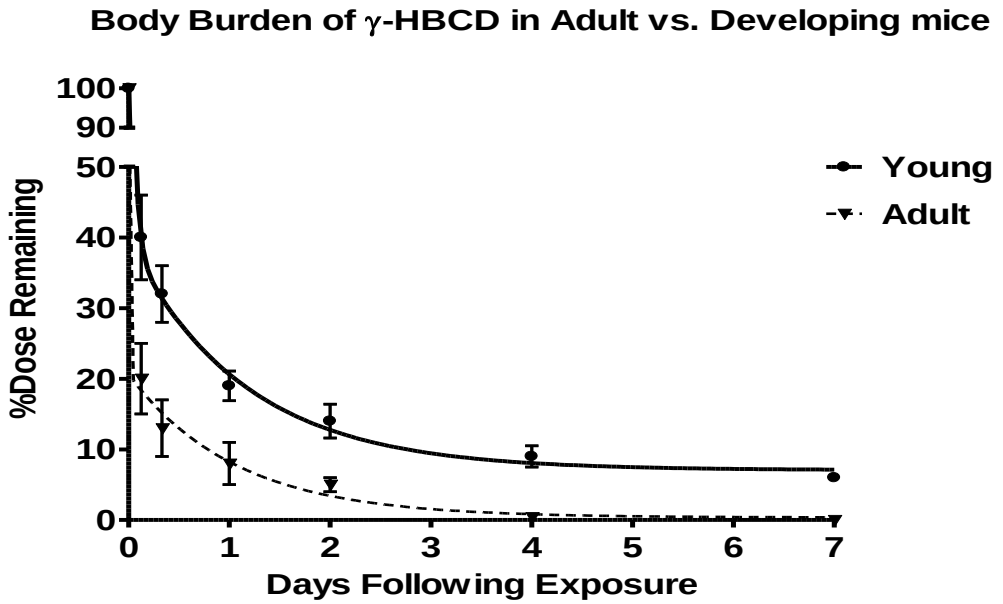

Figure 4.4a. Body burden is the sum of all the tissues measured for γ- ^{14}C HBCD derived radioactivity. Body burden was compared as a function of age (PND 10 vs PND 60) over time.

By four day post-dose, 17% remained in adults, while 23% of the α-[^{14}C]HBCD derived radioactivity remained in the pups. After seven days, 9 and 20% were detected in the adults and pups respectively (Figure 4a). Lower levels of γ-[^{14}C]HBCD derived radioactivity were measured in both the adults and pups as compared to α-[^{14}C]HBCD (Figure 4b). Within 24 h, only 2% of the dose remained in the body of the adult mice versus 16% in the pups. By the fourth day, less than 1% of γ-[^{14}C]HBCD derived radioactivity was detected in the adults while 7% remained in the pups (Figure 4b).

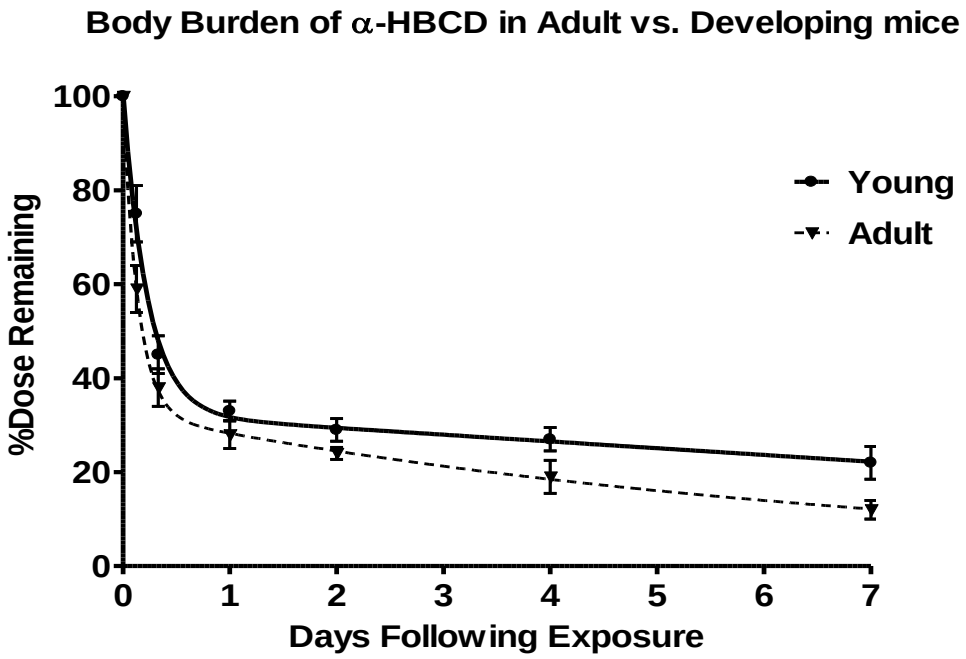

Figure 4.4b. Body burden is the sum of all the tissues measured for α-^{14}C HBCD derived radioactivity. Body burden was compared as a function of age (PND 10 vs PND 60) over time.

DISCUSSION

Neonatal mice have been previously reported to be susceptible to the commercial mixture of HBCD (Eriksson et al., 2006). The objectives of this study were to investigate the disposition of the α- and γ-HBCD stereoisomers in 10-day-old mice and to compare these neonatal levels to adult levels previously reported in Szabo et al., (2010) and Szabo et al., (2011). Overall disposition trends in infant mice were similar to those observed in adults, α-[^{14}C]HBCD distributes to lipophilic tissues to a greater degree than γ-[^{14}C]HBCD. Because

it has been previously demonstrated that α-HBCD is more water soluble than γ-HBCD, 48.8 and 2.1 ug/l, respectively (MacGregor and Nixon 2004), it is paradoxical that α-HBCD is more biologically persistent and detected to a higher degree than γ-HBCD in adipose tissue. Reasons for this may be due to the lack of rapid metabolism of α-HBCD, demonstrated in rat and seal microsomal *in vitro* assays (Zegers et al., 2006) and in a mouse *in vivo* study (Szabo et al., 2011). This in part, would permit α-HBCD to partition into fat stores more readily than other HBCD stereoisomers, such as γ-HBCD, which have been shown to be rapidly metabolized *in vitro* (Zegers et al., 2006) and rapid metabolized and eliminated *in vivo* (Szabo et al., 2010).

Tissue concentrations were generally higher in the infant mice exposed to either HBCD stereoisomer relative to adults. The changing physiology of developing animals can cause tissue disposition and overall toxicokinetics to differ from those observed in adult animals. Immaturity in the gastrointestinal (GI) tract of neonatal animals can lead to increased chemical absorption (Ginsberg et al., 2004). This may be a function of greater pinocytic activity of intestinal epithelium (NRC, 1993; Teichberg et al., 1990), higher stomach pH, differences in blood flow and absorptive surface area before maturation (NRC, 1993). Evidence also suggests that milk in the GI tract enhances absorption of certain xenobiotics and may contribute to the age differences in bioavailability (Kostial et al., 1978 and NRC, 1993). Increased GI absorption in developing animals have been observed after exposure to lead (USEPA, 1994a; USEPA, 1994b; Bowers and Cohen, 1998; NRC, 1993)

polonium nitrate (Haines et al., 1993), mercuric chloride (Walsh, 1982), and for select pharmaceuticals (Hoffman, 1982; NRC, 1993).

Age differences in the composition of tissues can also contribute to altered chemical disposition. The brain constitutes a higher percentage of body weight in the young, and the immaturity of the blood–brain barrier (BBB) may lead to a significant increase in chemical partitioning (Renwick et al., 2000), resulting in potential for increased neurotoxic effects (Saunders et al., 2000). Tissues such as liver, kidney, and lung undergo rapid growth through infancy (NRC, 1993). Liver mass per body weight is greatest in early life (Blanco et al., 2000 Gibbs et al., 1997), but hepatic elimination of rapidly metabolized chemicals may be limited by blood flow (Kedderis, 1997). Renal glomerular filtration rates are reduced in neonates; therefore, a reduced rate of excretion and thus greater body burden may in part explain increased levels during development seen after HBCD exposure. In addition, neonates and infants have a higher percentage of body water and lower lipid content than adults (Clewell et al., 2002, Kearns and Reed, 1989; Morselli, 1989); this can cause an increased volume of distribution for water-soluble chemicals, including secondary polar HBCD metabolites which could possibly increase half-lives during early stages of life.

Considerable differences in the ontogenic gene expression can contribute to variations in xenobiotic metabolism between neonates and adults. Less efficient drug metabolism and transport in neonates may result in increased susceptibility to toxic effects. The cytochrome

P450 superfamily is responsible for approximately 75% of xenobiotic metabolism however; these levels are generally not mature at birth (Fouts and Adamson, 1959). Hydroxylated metabolites of HBCD have been detected in an *in vivo* rat study (Brandsma et al., 2009) and polar metabolites have been detected after an *in vivo* exposure to γ-HBCD in mice (Szabo et al., 2010). Zegers et al., (2005) determined that individual stereoisomer differences within rat and seal microsomes results in rapid biotransformation of gamma and beta, but biotransformation of alpha was slower. HBCD has been shown to be an agonist for the CAR and PXR nuclear receptors, which regulate the Cyp2b and Cyp3a family of enzymes (Crump et al., 2010, Fery et al., 2010, Crump et al., 2008, Germer et al., 2006). The ontogeny of Cyp2b and Cyp3a gene transcripts from gestation to adulthood has been investigated in C57BL/6 mice (Hart et al., 2009). In female and male mice, Cyp2b10, the ortholog for the human CYP2B6, was low at all ages but increased steadily over the first 30 days of life followed by a constant plateau to adult levels. Cyp3a11 and Cyp3a41b both code for the human CYP3A4 and are either at similar or slightly lower levels at PND 10-15 than PND 60 in female mice, respectively. This could indicate that endogenous levels of Cyp2b10, and to a lesser extent Cyp3a11 or Cyp3a41b, may be driving the differences of HBCD disposition between age groups observed in this study.

Active transporters are also expressed at lower levels during development. In the liver, Mdr2 gradually increases over time from birth. Mdr2 expression levels in female C57BL/6 at PND 10 are approximately 50% of the adult levels (Cui et al., 2009). The BBB

protects the adult brain with the expression of Mdr1a and 1b against xenobiotic insults but the expression of both are low in the fetal and neonatal brain. In C57BL/6 mice, the expression levels increased after the first 10 days of age and low expression of these transporters in the brain correlates with the high vulnerability of the newborn to certain xenobiotics.

α-[^{14}C]HBCD and γ-[^{14}C]HBCD disposition trends in pup brains were of particular interest in light of previous developmental neurotoxicity studies (Eriksson et al., 2006) which demonstrated a single oral dose at PND 10 elicited altered spontaneous behavior, learning and memory deficits in adulthood. In our study, the concentrations we measured in the brains 4 days post-exposure were different as a function of age and stereoisomer. At the 4 day time point, γ-HBCD is not measurable in the adult brain (Szabo et al., 2010) but has a concentration of nearly 1000 ng/g four days post-exposure in PND 10 mice. Similarly but to a lesser degree, α-HBCD exposure distributed nearly 25% more to the developing animal than in the brains of adults with approximately 2500 ng and 1000ng of derived radioactivity per gram of brain tissue, respectfully. We also observe that higher levels of total radioactivity measured in the blood did not lead to significantly increased partitioning into the brain after exposure to α-[^{14}C]HBCD or γ-[^{14}C]HBCD. As previously discussed, it is well documented that many active transport proteins along the BBB barrier are responsible for controlling xenobiotic access and removal from the tissue and that these transporters may not be fully developed in infantile mice (Cui et al., 2009, Schiengold et al., 2001). Since

HBCD has been shown to be an agonist for the nuclear receptors CAR and PXR (Fery et al., 2010) whereby controlling expression of Phase III efflux transporters (pumping out of the brain), it is plausible that this may be a mechanism leading to increased levels of α-HBCD and γ-HBCD stereoisomer derived radioactivity in developing mice brains as compared to adults. Future studies designed to examine HBCD diastereomers as a substrate for membrane transporters may in part support the observed toxicokinetics observed here.

In addition to HBCD, other persistent organic pollutants (POPs) such as perfluorooctane sulfonate (PFOS), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and PBDEs have shown higher body burdens in developing animals than adult animals. After exposure to PFOS, similar tissue distribution patterns were observed between rat dams and fetuses but higher levels were measured in the serum and brains at gestational day 21 (Lau et al., 2003). The rate of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) distribution to the fetal brains of rats was ≥100 times higher than that in adults (Ishida et al., 2010). In a similarly performed study from our laboratory using the PBDE congener BDE-47, disposition patterns in tissues were similar across ages, but concentrations of BDE-47 were higher after exposure to PND 10 pups (Staskal et al., 2006). Comparing the body burden differences (between PND 10 and PND 60 mice), the three brominated flame retardants (BDE-47, γ-HBCD and α-HBCD) resulted in all having higher levels in pups than adults with 3, 8 and 2-fold increases, seven days after exposure, respectively. Although γ-HBCD exposed pups contained the highest fold change between age groups, body burden levels (on a % dose basis) were lowest after

exposure to γ-HBCD (9% of dose) followed by α-HBCD (20% of dose) and highest for BDE-47 (40% of dose). Along with differences in α-HBCD and γ-HBCD metabolism and stereoisomerization in adult mice (Szabo et al., 2010; 2011) the higher overall body burden levels seen with BDE-47 may also due to differences in elimination by mouse urinary protein (MUP); (Staskal et al., 2005), not observed to occur for either α-HBCD or γ-HBCD (data not shown).

BDE-47, PFOS and TCDD toxicity have been well examined in developing animals, however, data on the toxicity of HBCD commercial mixture in developing animals is growing, but still remains limited (Saegusa et al., 2009; van der Ven et al., 2009; Eriksson et al., 2006; Deng et al., 2009) with *no toxicity data* in developing animals after exposure to any individual HBCD stereoisomer. With larger age-related body burden differences observed for γ-HBCD than for BDE-47 and similar differences between α-HBCD and BDE-47, the need for HBCD stereoisomer toxicity testing in developing animals should be considered a priority.

There is compelling evidence that the HBCD commercial mixture has the potential to disrupt reproductive (Ema et al., 2008), developmental (Saegusa et al., 2009), and neurological (Eriksson et al., 2006) processes and may also have epigenetic (Aniagu et al., 2008), and endocrine-disrupting effects (van der Ven et al., 2009). Many individuals throughout the world have already accrued a measurable body burden, making exposure to HBCD a potential health risk (Arnot et al., 2011). α-HBCD appears to be persistent and bioaccumulative in adult animals (Szabo et al., 2011) with a greater potential for the developing animal. With evidence that γ-HBCD can stereoisomerize to α-HBCD in

mammals (Szabo et al., 2010) and higher body burden levels of both stereoisomers are found in developing mice as compared to adults, concerns from exposure to both stereoisomers should be taken into consideration when assessing the risk of HBCD to the potentially susceptible young population.

The results of this study demonstrate that the toxicokinetics of the two HBCD stereoisomers, α and γ, are different in developing mice than in adult mice. These differences may lead to higher concentrations of HBCD at target tissues during critical windows of development. Because HBCD have demonstrated toxicity during development, it is essential to understand the kinetic parameters in order to accurately describe the dose available to target tissues after exposure, which may more accurately assess risk to human health. This age group should remain a target population for defining susceptibility factors in future toxicokinetic and toxicodynamic research.

ACKNOWLEDGEMENT AND DISCLAIMER

The authors would like to thank Francis McQuaid and Brenda Edwards for technical assistance. This work is funded in part by a cooperative agreement between the University of North Carolina in Chapel Hill and the Environmental Protection Agency CR 833237 predoctoral training grants. This abstract does not reflect USEPA, NIH or USDA policy. The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval

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Chapter V

EFFECTS OF HBCD IN DEVELOPING MICE: A SYSTEMS BIOLOGY APPROACH TO DEVELOPMENTAL NEUROTOXICITY

Brominated flame retardants (BFRs) are a structurally diverse class of compounds added to consumer products for decreased flammability and increased time to burn. Increasing concentrations of these compounds in the environment, human food chain, and in human tissues raise concern about possible neurotoxic effects, particularly for the developing nervous system (Costa and Giordano 2007). This concern has led to voluntary action and legislative measures to eliminate the penta- and octabrominated diphenyl ethers from the market in Europe and North America. After their use was stopped in consumer products, a sharp rise in environmental levels of the BFR hexabromocyclododecane (HBCD) was observed (Law et al. 2008). This is possibly due to an increased use of HBCD as a replacement of the banned polybrominated diphenyl ethers (PBDEs), mainly in polystyrene products and textile backings. The technical mixture of HBCD consists of three stereoisomers, denoted α-, β-, and γ-HBCD. HBCD has been shown to biomagnify in marine food chains (Jenssen et al. 2007). Interestingly, biotic samples are in general dominated by

the α-stereoisomer although the technical mixture consists for 70 - 90% γ-HBCD (Covaci et al. 2006). This is probably due to isomer composition changes during heating of the technical mixture during industrial application (Heeb et al. 2008), and/or biotransformation of α- to β- and γ-HBCD in biota (Szabo et al. 2010).

Fetal and neonatal exposures to neurotoxicants have adverse effects on neurodevelopment. Direct effects of xenobiotics on the developing brain could cause these effects, during a critical window for neurotoxicity. However, the underlying mechanisms are not well understood (Szpir et al., 2006). Of concern is that children, during the infantile period, may be more highly exposed than adults to HBCD because of their diet (breast-feeding/relatively large intake relative to body weight) and behavior (contact with house-dust) (Jones-Otazu et al. 2005). Disposition studies show that developing mice have higher tissue concentrations of HBCD stereoisomers α and γ, compared with adult mice after identical dosing regimens (Szabo et al.; 2011b). Behavioral studies have demonstrated adverse neurodevelopmental effects on learning and memory after neonatal HBCD exposure (Eriksson et al., 2009)

A proteomics approach was used to investigate the effect of a single oral dose of 12 mg/kg body weight (bw) of BDE-99 on brain protein levels in mice, 24 hr after exposure. Levels of striatal proteins associated with neurodegeneration and neuroplasticity and of hippocampal proteins associated with metabolism and energy production were found to be

changed (Alm et al. 2006). BDE-99 causes similar development neurotoxic behavioral effects as observed for HBCD (Eriksson et al., 2001; Eriksson et al., 2006). It is unclear whether such molecular changes occur after exposure to HBCD and whether these effects are stereoisomer dependent.

An adverse effect on learning and behavior in mice (0.9 and 13.6 mg/kg bw) of HBCD (Eriksson et al. 2006), as well as on oxidative stress and the cellular antioxidant defense systems in fish brain after 28 days exposure to 500 μg/l of HBCD (Zhang et al. 2008) have been observed. *In vitro* neurotoxicity data on HBCD is limited. So far, only inhibition of neurotransmitter uptake in rat synaptosomes (IC50: 4 μM) and synaptic vesicles (IC50: 3 μM) (Mariussen and Fonnum 2003) and necrotic cell death in cerebellar granule cells after 24 h exposure (LC50: 3 μM) Reistad et al. 2006) have been reported.

Alterations in intracellular Ca2+ homeostasis, as observed for PBDEs and *ortho*-substituted (non-coplanar) PCBs (Mariussen and Fonnum 2006), may play a role in the observed cytotoxicity and oxidative stress (Orrenius et al. 2003). In hippocampal neurons, the main excitatory neurotransmitters are glutamatergic. Depolarization occurs with regulating the influx of Ca^{2+} by voltage gated calcium channels (VGCCs) as well as NMDA and glutamatergic receptors, located in the cell membrane which opens, depolarizing the neuron. The subsequent rapid influx of Ca^{2+} can trigger various intracellular processes including signal transmission and neurotransmitter release (Barclay et al. 2005; Clapham et al., 2007).

The main objectives of our study are to 1) to gain insight into the mechanisms underlying the observed effects of HBCD on learning and memory (Eriksson et al. 2006). We propose that HBCD alters molecular mechanisms responsible for learning and memory as seen previously for BDEs and PCBs. This objective investigates the effects of the technical HBCD mixture on gene transcription in the hippocampus. The second objective 2) investigates the individual HBCD stereoisomers, α-HBCD and/or γ-HBCD, and which is responsible for the neurotoxicity observed after exposure to the HBCD commercial mixture. Overall, there is little data on stereoisomer specific metabolism in the literature and there is currently no *in vivo* data for effects or gene expression in any mammalian system in any organ. In predicting human health risks posed by HBCD, it is therefore necessary to accurately predict diastereomer specific effects. Based on their toxicokinetic differences in adult (Szabo et al., 2010; Szabo et al., 2011a) and developing mice (Szabo et al., 2011b) we hypothesize that HBCD-α and -γ will have different transcriptome and proteome profiles in the hippocampus of infantile mice, previously shown to be susceptible to HBCD commercial mixture effects (Eriksson et al., 2006).

In the present work, we examined the gene expression of the HBCD commercial mixture and the gene transcription and protein expression profiles of α-HBCD and γ-HBCD in hippocampus from female mice exposure to a single oral dose on PND 10. Our main objective was to elucidate the mechanisms underlying the adverse effect (developmental neurotoxicity) previously observed *in vivo*. Mice were administered 0, 3, 10, and 30mg/kg

dose of α-HBCD, 0, 3 and 30mg/kg of γ-HBCD, and 0 and 30 mg/kg of the CM-HBCD. However, this chapter will focus on the effects observed in mice administered the high dose for each condition (30mg/kg). The main target tissue of interest and motivation for choosing the neonatal dosing paradigm was to report on effects observed in the hippocampal tissue and to determine the molecular mechanisms involved after exposure to the commercial mixture and whether α-HBCD or γ-HBCD elicit similar or different effects.

Additional data on the metabolomics of the serum, and transcriptomics and proteomics of the liver will be presented in this chapter, where applicable, but only to support the similarities or difference observed in the hippocampus and briefly discussed. Attention in this chapter is focused on the hippocampal effects.

MATERIALS AND METHODS

Chemicals: 1,2,5,6,9,10-hexabromocyclododecane ([HBCD) (2mCi/mmol) was purchased from Sigma-Aldrich; (St Louis, MO) as a mixture of α-HBCD, ß-HBCD and γ-HBCD. Methods for separating the diastereomers from the commercial mixture (CM-HBCD) and thermal conversion of γ-HBCD to α-HBCD were previously described for [^{14}C] labeled stereoisomers (Szabo et al., 2010; Szabo et al., 2011a). Other chemicals used were of the highest grade commercially available.

Dosing Solutions: Doses were selected based on environmental relevance and published toxicity (Eriksson et al., 2006) and toxicokinetic studies (Szabo et al., 2010; Szabo et al., 2011a, and Szabo et al., 2011b), percent of stereoisomers in the commercial mixture and limited supply of purified compounds. All solutions were designed to deliver approximately 3, 10, and 30mg/kg doses of α-HBCD, 3 and 30mg/kg of γ-HBCD, and 30 mg/kg of the CM-HBCD to each mouse. Corn oil by weight was then added to the vials followed by the evaporation of toluene (dissolving agent) under vacuum (Speed Vac, Savant Instruments, Inc. Farmingdale, NY).

Animals and Treatment: Mouse dams (n=6) and their infantile female C57BL/6 pups (PND 9, ~ 7 grams) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Dams and 4 pups per litter were acclimated for 24 hours in shoebox cages. PND 10 pups were gavaged with a single dose of vehicle, α-HBCD, γ-HBCD, or CM at 30 mg/kg at 10 ml/kg. Animals were maintained on a 12 h light/dark cycle at ambient temperature (22°C) with relative humidity (56 ± 5%). Dams were provided with Purina 5001 Rodent Chow (Ralston Purina Co., St. Louis, MO) and tap water *ad libitum*. Total of 6 pups/treatment group (one pup/litter) was sacrificed four days following exposure when tissues were harvested. Mice were housed in an AAALAC-accredited facility and the EPA/NHEERL IACUC approved the procedures.

Sample Analysis: Tissues harvested from PND 14 mice were the liver, hippocampus, and serum. The liver was rapidly diced and separated into two equal tubes: one for transcriptomic

and the other for proteomic analysis. The hippocampus from each brain hemisphere was harvested, one for each analysis. Tissues were flash frozen in liquid nitrogen and then stored in -80 °C until they could be processed for either RNA isolation (transcriptomics) or protein isolation (proteomics). Serum samples were frozen at -80C until preparation for the metabolomic analysis.

Transcriptomics

RNA Extraction. RNA was extracted using Qiagen RNeasy Mini and Midi Kits (Valencia, CA) in accordance with the manufacturer's protocol. Approximately 5 mg of hippocampus and 100 mg of liver tissue was used for each RNA extraction (Mini Kit). After extraction, the RNAs were resuspended in RNAse-free water and concentrations were measured using a spectrophotometer. Integrity of RNA samples from both studies was determined by the size distribution of 18 S and 28 S ribosomal RNA using an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). In both studies all samples passed quality control and showed no signs of degradation.

Microarray Hybridization. 500 ng of total RNA was converted into labeled cRNA with nucleotides coupled to fluorescent dye Cy3 using the Low RNA Input Linear Amplification Kit (Agilent Technologies, Palo Alto, CA) following the manufacturer's protocol. The A260/280 ratio and yield of each of the cRNAs were determined and cRNA integrity was

evaluated using an Agilent Bioanalyzer. nCy3-labeled cRNA (1.65 ng) from each sample was hybridized to Agilent Mouse Whole Genome oligonucleotide Microarrays in 4X44K format. The hybridized array was then washed and scanned and data were extracted from the scanned image using Feature Extraction version 9.5 (Agilent Technologies). All microarrays passed QC standards set forth in the Agilent Feature Extraction Software (V9.5) reference guide.

Microarray Data Normalization and Quality Control. Raw gene expression intensity values were background subtracted and normalized (within array normalization) using the Agilent Feature Extractor software. The across array normalization was performed using the Quantile normalization method (Bolstad et al., 2003). The normalized data for all samples was used to generate Box plots and Density plots as implemented in Bio-conductor (www.bioconductor.org). The normalized data was also used for Principal Component Analysis (PCA) to investigate the effect of known experimental variables such as treatment type (alpha, gamma or mixture) at 30 mg/kg; as well as technical variables such as sample hybridizations performed at different times.

Identification of differentially expressed probes. To identify probes that were differentially expressed in treated samples compared to controls, we employed Welch's t-test which is an adaptation of Student's t-test for comparing two independent samples in the presence of heterogeneity of variance. This test does not utilize pooled variance estimate and computes

degrees of freedom associated with variance estimate using the Welch-Satterthwaite equation (Welch et al., 1947). The resulting p-values were corrected for multiple testing using Benjamini Hochberg's method (Benjamini et al., 1995). The fold change values for each probe was computed by taking a ratio of expression intensity in treated over control samples.

For each treatment type vs. control comparison, we selected a list of probes that satisfy p-values < 0.05 and absolute fold change greater than 1.5 (up-regulation or down-regulation) as criteria for further analyses.

Ingenuity Pathway Analysis (IPA). We employed IPA to identify over represented biological pathways in probe lists selected for each comparison type. For each treatment vs. control comparison, probe lists were selected using the aforementioned criteria (p-value < 0.05 and Absolute Fold > 1.5) and IPA was used to find enriched canonical pathways that displayed p-value <0.05 (right-tailed Fisher's exact test as implemented in IPA) directly correlated with the transcript level changes measured via the Agilent microarray platform.

Proteomics

Antibody Array Slide. The Phospho Explorer Antibody Array (Full Moon Biosystems; Sunnyvale, California), a discovery tool, is a glass microarray slide used to identify protein changes after exposure to HBCD. This microarray consists of 1318 well-characterized specific antibodies. It is broad in scope and allows for the detection and analysis of over a

thousand proteins simultaneously on a single slide. The antibodies are printed on standard-size coated glass microscope slides and can be scanned on all microarray scanners that are compatible with 76 x 25 x 1 mm (3 in. x 1 in. x 1mm) slides. To validate the transcriptomic data and support our findings, we performed a pilot study using the antibody array. Therefore, mouse hippocampal and liver tissues administered 30mg/kg (high dose) of α-HBCD and γ-HBCD were processed for proteomic analysis only. Direct comparison between the two platforms will be analyzed and discussed for hippocampal effects after administration of an oral dose of α-HBCD and γ-HBCD at 30mg/kg.

Tissue Preparation. Tissues were washed with cold 1X PBS (4°C) 3 to 5 times with vortexing to remove excess blood. One tube of lysis beads was added to each 10 – 40 mg of tissues. Extraction Buffer was added at 100 ul of Extraction Buffer to 10 – 20 mg of tissue and mixed rigorously by vortexing for 30 second. The mixture was then incubated on ice for 10 minutes. This procedure was repeated for 10-minute intervals for up to 60 minutes where it was then centrifuged at 10,000 x g (14000 rpm) for 15 to 20 minutes at 4°C and the clear supernatant was transferred to a clean tube.

Buffer Exchange/Protein Purification. This step ensures the removal of unwanted buffer from the protein extract and replaces it with the Labeling Buffer provided in the Antibody Array Assay Kit. 100uL of protein extract was transferred from the tissues to the top of the gel of each column. The column was placed in a collection tube and centrifuged at 750 x g for 2 minutes, collecting the purified protein.

Protein quantification and Quality Control. Protein was measured by UV absorption. All absorbance values were greater than 4 OD to ensure proper protein concentration. Two separate peaks were observed at 200-230nm and 240-280nm and were well separated, signifying the lysate is clear.

Protein Labeling – Biotinylation of Protein Samples. 100 ul of DMF (*N*,*N*-dimethylformamide) was added to 1 mg of Biotin Reagent to give a concentration of 10 ug/ul. Labeling Buffer was added to the protein sample to bring the volume to 75 µl. The solution should contain 80 – 150 OD of protein (or 40 – 100 ug with a concentration of 2 – 10 ug/ul). 3 ul of the Biotin/DMF solution was added to the protein sample, incubated at room temperature for 1 – 2 hours with mixing. 35 ul of Stop Reagent was then added to the protein samples and incubated for 30 minutes at room temperature.

Blocking. The Antibody Microarray was removed from the refrigerator and allowed to warm up to room temperature (30 to 45 minutes). Packages were opened to expose the slides and allowed to dry for 10 to 15 minutes. 30 ml of Blocking Solution was added in a 100x15 mm Petri dish and each antibody microarray slide was placed in the Blocking Solution and incubated on an orbital shaker rotating at 55 rpm for 30 to 45 minutes at room temperature. The slides were then rinsed 10 times with Milli-Q grade water.

Coupling. One tube of biotin labeled proteins (80 – 150 OD or 40 – 100 ug) was vortexed and briefly mixed and then the slide was placed in a coupling chamber. 6 ml of Protein

Coupling Mix was poured over the slide and then incubated on an orbital shaker rotating at 35 rpm for 1 – 2 hours at room temperature. The slide was then transferred to a 100x15 mm Petri dish containing 30 ml of 1X Wash Solution where it was incubated on an orbital shaker rotating at 55 rpm for 10 minutes at room temperature. The wash solution was then discarded. The slides were then rinsed 10 times with Milli-Q grade water.

Detection. 60 ul of Cy3-Streptavidin (0.5 mg/ml) was added to 60 ml of Detection Buffer and 30 ml of Cy3-Streptavidn Solution in a 100x15 mm Petri dish. The slide was submerged in the Cy3-Streptavidin solution and incubated on an orbital shaker rotating at 55 rpm for 20 minutes at room temperature in the dark. The antibody slide was transferred to a new 100x15 mm Petri dish containing 30 ml of 1X Wash Solution and incubated on an orbital shaker set at 55 rpm for 10 minutes at room temperature. The wash was repeated twice. The slide was then rinsed 10 times with Milli-Q grade water. It is critical to rinse the slide extensively to completely remove solution from the slide surface. The slide is now ready for scanning.

Scanning of Antibody Slides. Scanning of the slides was performed at Full Moon systems (Sunnyvale, California) http://www.fullmoonbiosystems.com/Services/ArrayScanning.html.

Antibody Array Data Normalization and Quality Control. Raw protein expression intensity values were background subtracted and normalized (within array normalization) using the Agilent Feature Extractor software. The across array normalization was performed using the Quantile normalization method (bioDBnet: http://biodbnet.abcc.ncifcrf.gov/db/db2db.php).

Identification of differentially expressed probes. To identify probes that were differentially expressed in treated samples compared to controls, we employed Welch's t-test which is an adaptation of Student's t-test for comparing two independent samples in the presence of heterogeneity of variances. This test does not utilize pooled variance estimate and computes degrees of freedom associated with variance estimate using the Welch-Satterthwaite equation; for details refer to (Welch et al., 1947). The resulting p-values were corrected for multiple testing using Benjamini Hochberg's method (Benjamini et al., 1995). The fold change values for each probe was computed by taking the ratio of expression intensity in treated over control samples.

For each treatment type vs. control comparison, we selected a list of probes that a) p-value $^{*}< 0.05$ AND fold > 1.2; b) p-value $^{*}< 0.05$ AND fold > 1.5, and; c) p-value $^{*}< 0.01$ AND fold > 1.5 (up-regulation or down-regulation greater than 1.2 or 1.5 fold) as criteria for further analyses.

Comparison of Transcriptomics with Proteomics. To be able to compare the treatment specific changes in transcription to changes in the levels of corresponding proteins, we first created a mapping between the gene expression probes present on the Agilent mouse chip with the antibodies utilized by the Proteomics chip using the *db2db* utility from (bioDBnet; :http://biodbnet.abcc.ncifcrf.gov/db/db2db.php). It mapped the human Uniprot identifier to

Agilent mouse identifiers in the following three steps:

- Human Uniprot identifiers to Mouse analogue ensemble gene identifiers.
- Ensemble gene identifiers to gene symbols.
- Gene symbols to Agilent probe identifiers.

Once the protein probes (antibodies) on the proteomics platform were mapped to their corresponding Agilent microarray probes, we employed Spearman's correlation test to assess whether the changes observed in protein level measurements arising from Proteomics platforms correlated to Agilent microarray data.

Metabolomics

Sample Preparation, data acquisition, and processing. Serum samples were prepared for NMR analysis by mixing an aliquot (60 µL) of serum with 80 mL of a solution containing 5mM Formate and 0.2% NaN3 in D_2O and by addition of 260 µL of saline.

NMR spectra were acquired on a Bruker Avance III 950 MHz instrument located in the NMR facility at David H. Murdock Research Institute, Kannapolis, NC, USA. ^{1}H NMR spectra were acquired using a CPMG pulse sequence (Nicholson et al., 2003) with water suppression (cpmgpr1d) and 256 scans. All spectra are acquired at 25°C with 32k data points, and the quality of each NMR spectrum was assessed for the level of noise and alignment of identified markers. Spectra were accessed for missing data and underwent quality checks. Spectra were referenced to a formate signal at 8.44 ppm.

NMR data was processed using a traditional binning approach and by a quantitative approach (Sumner et al. 2010, Sumner et al. 2009, Pathmasiri et al. 2010). Binning was performed by automated integration (increments of 0.04 ppm) over the spectral window (after excluding the region of water suppression), and normalized to the total spectral intensity. Metabolites in serum samples were identified and concentrations were determined by using the Chenomx NMR Suite 5.1 Professional software. The formate internal standard was used as the reference for performing the library matching. This software contains an internal library adjustment for increments in chemical shift based on pH variations (Weljie et al., 2006). A concentration determination for each metabolite was made by relative integration of the analyte to the internal standard, where the library of concentrations was developed to account for differences in integral values as related to the relaxation time of the signal (Weljie et al., 2006). This quantitative method has some advantages over binning in two major ways. First, small increments in pH can result in portions of metabolite signals aligning with different bins when using the binning approach, while deconvolution circumvents this problem. A second advantage is that this quantitative analysis depends on the concentration of each metabolite, while the binning approach results in situations where each metabolite has multiple signals that fall within separate bins. For subsequent data reduction of the data derived from library matching, the concentration of each metabolite was normalized to formate concentration.

Data Reduction and Visualization**.** Data captured by NMR (metabolite id and concentration; or bin region and integral value) were transferred to SIMCA-P+ 12.0 software (Umetrics) for

data reduction and visualization. Several approaches were taken to analyze the NMR data to provide the best set of analytes that could distinguish the study groups (e.g., by specific chemical or by dose group). For analysis with binned data, the integral was normalized to the total integral for each spectrum. Principal component analysis and partial least squares projection to latent structures discriminant analysis (PLS-DA) was conducted using SIMCA-P+ 12.0 for the binned and metabolite concentration data. Loadings, variable importance, and contribution plots were examined to determine the bins or the metabolites that best define the separation of study groups.

RESULTS

Transcriptomics of Hippocampus

To identify altered gene expression after exposure to α-HBCD, γ-HBCD or CM-HBCD, a Welch-Test (analysis of variance) was performed for the liver and hippocampus (Table 1). Three separate analyses were performed and the selection criteria were based on a p-value and fold gene expression change.

Organ	Comparison	Number of Probes with p-value *< 0.01 AND fold > 1.5	Number of Probes with p-value *< 0.05 AND fold > 1.5	Number of Probes with p-value *< 0.05 AND fold > 1.2
Liver	Alpha 30 Vs Control	18	186	547
Liver	Gamma 30 Vs Control	115	582	1812
Liver	Alpha 30 Vs Gamma 30	115	371	1585
Liver	Alpha 30 Vs Mixture	35	210	622
Liver	Gamma 30 Vs Mixture	63	288	1264
Liver	Mixture 30 Vs Control	19	177	336
hippocampus	Alpha 30 Vs Control	12	116	385
hippocampus	Gamma 30 Vs Control	17	132	384
hippocampus	Alpha 30 Vs Gamma 30	20	396	396
hippocampus	Alpha 30 Vs Mixture 30	11	211	211
hippocampus	Gamma 30 Vs Mixture 30	15	103	305
hippocampus	Mixture 30 Vs Control	7	89	327

*p-values are not adjusted for multiple testing

Table 5.1. Results of ANOVA on gene lists using three criteria. Each row in the table lists result from a Welch-Test, where α-HBCD, γ-HBCD or CM-HBCD exposed sample at 30mg/kg are compared to corresponding treatment group for the liver or hippocampus; n=6/treatment. The three criteria used with increasing statistical stringency are: p-value $^{*}<$ 0.05 AND fold > 1.2, p-value $^{*}<$ 0.05 AND fold > 1.5, and p-value $^{*}<$ 0.01 AND fold > 1.5.

From increasing statistical stringency (low to high), three analysis consisted of a) p-value $^{*}<$ 0.05 AND fold > 1.2; b) p-value $^{*}<$ 0.05 AND fold > 1.5, and; c) p-value $^{*}<$ 0.01 AND fold > 1.5. Altered gene candidates were observed for each analysis regardless of the level of stringency. As the stringency of statistical analysis decreased, the number of genes which passed the criteria increased. Based on the subtle dosing paradigm in this study (a

single oral exposure), duration (tissues harvested 4 days later), small amount of tissue harvested, and future pathway analysis to be performed; we decided to use the data following analysis with the criteria p-value $^{*}< 0.05$ AND fold > 1.2, to identify genes that were altered after exposure to α-HBCD, γ-HBCD or CM-HBCD. Table 1 demonstrates that there was an increase in the number of altered genes observed in the liver as compared to the hippocampal tissue across all statistical testing parameters.

Hippocampal Genes. There were a number of genes that were significantly up- and down-regulated in the hippocampus of mice at PND 14 after oral exposure to a single 30mg/kg oral dose of α-HBCD, γ-HBCD, or CM-HBCD at PND 10. Analysis of the data demonstrates that the number of genes differentially expressed (using the criteria - fold change > 1.2 up- or down-regulated AND p-value of < 0.05) in developing mice exposed to 30 mg/kg of HBCD resulted in 385 regulated genes for α-HBCD, 384 for γ-HBCD and 327 for the CM-HBCD, respectively (Table 1). An overlap of 19 genes could be observed between all three treatment groups.

Principal component analysis. Principal component analysis (PCA) was applied to the entire genomic dataset (30,000 genes) and did not reveal specific clustering of treatment groups (α-HBCD, γ-HBCD or CM-HBCD) (data not shown). However, very distinctive clustering was observed when a subset of genes differentially expressed between groups was identified (using a fold change and ANOVA p-value threshold) and then PCA performed. Each

treatment condition, α-HBCD, γ-HBCD, or CM-HBCD, was compared to controls using the subset of genes which are differentially expressed between the two groups (Figure 1a,b,c). A heat map also displays the genes altered for each exposed mouse pup (n=6).

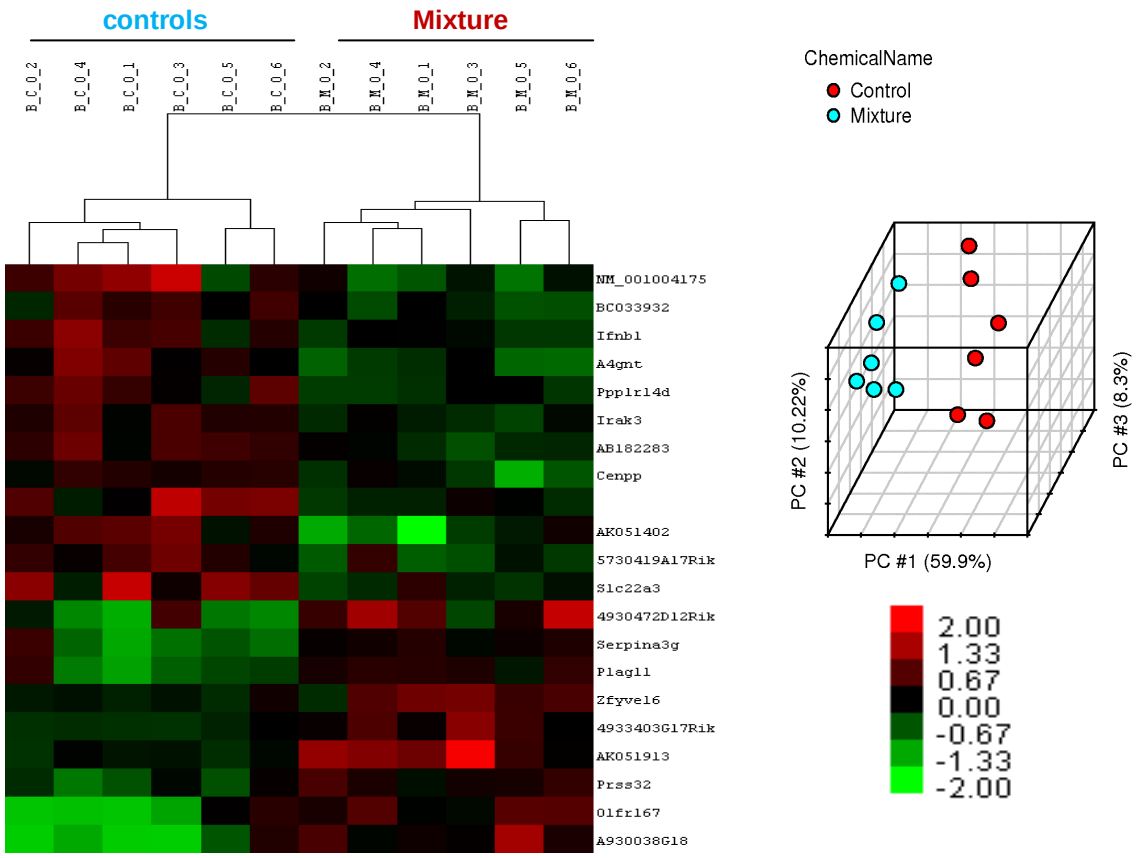

Figure 5.1a. Heat map and PCA analysis of genes of the mouse hippocampus differentially expressed between the commercial mixture of HBCD (CM-HBCD) and corn oil vehicle (controls) four days post oral exposure at PND 10. ‘Red” signifies an upregulation, “green” a downregulation while “black” a no change. (p-value < 0.01 AND fold > 1.5).

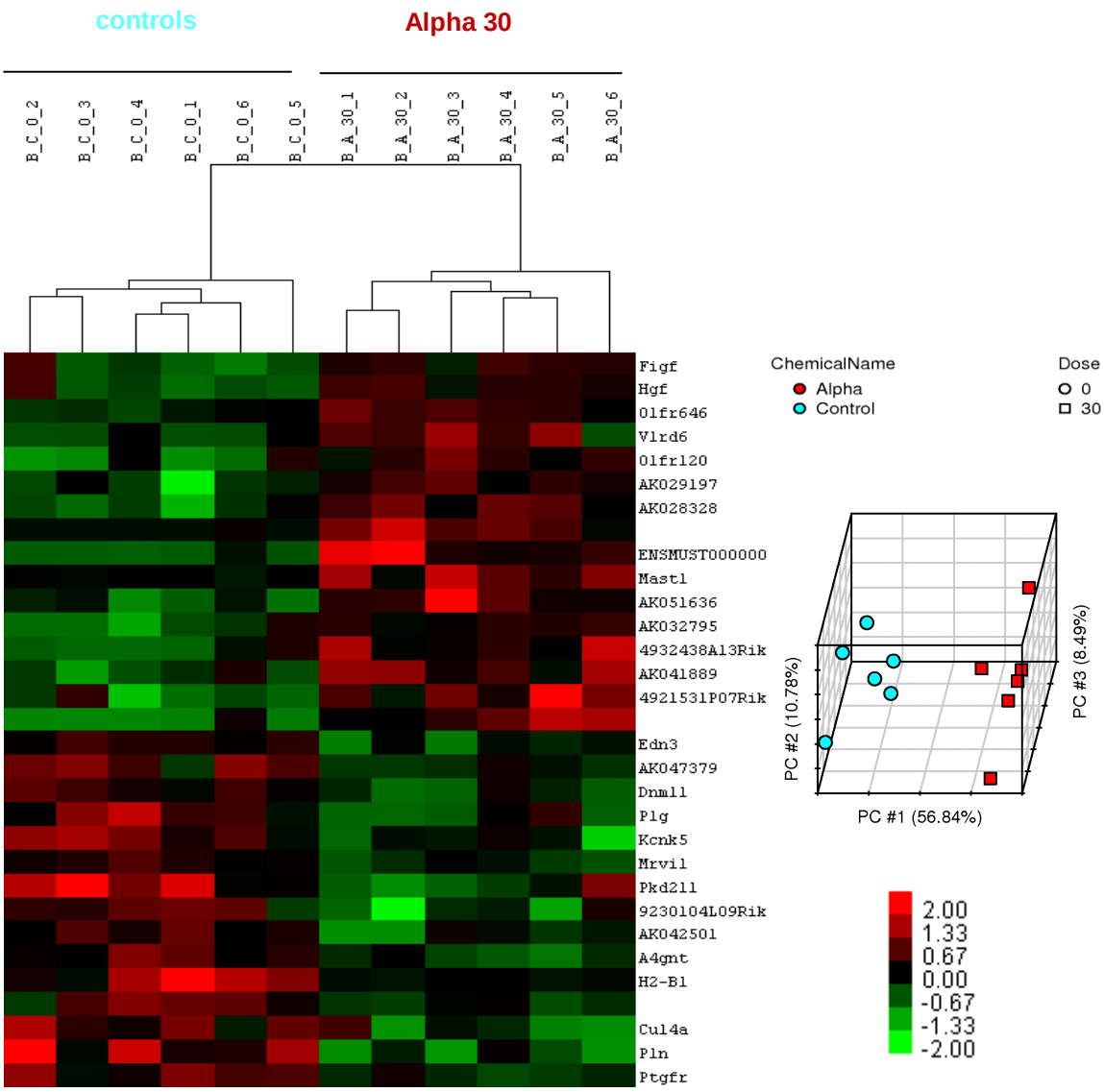

Figure 5.1b. Heat map and PCA analysis of genes of the mouse hippocampus differentially expressed between the commercial mixture of HBCD (α-HBCD) and corn oil vehicle (controls) four days post oral exposure at PND 10. 'Red" signifies an upregulation, "green" a downregulation and "black" a no change. (p-value < 0.01 AND fold > 1.5)

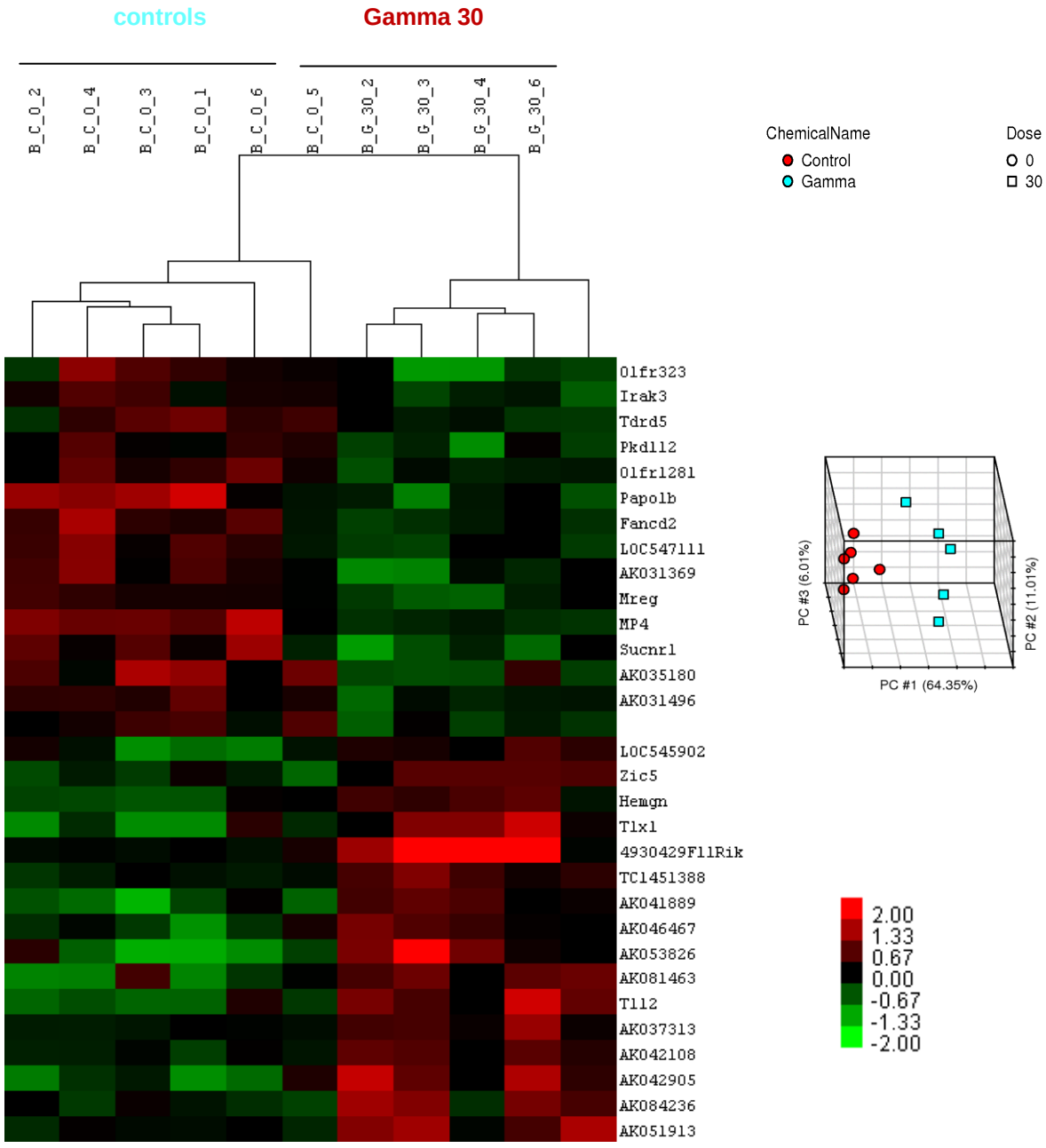
controls
Gamma 30
B_C_0_2
B_C_0_4
B_C_0_3
B_C_0_1
B_C_0_6
B_C_0_5
B_G_30_2
B_G_30_3
B_G_30_4
B_G_30_6
ChemicalName
Control
Gamma
Dose
0
30
Olfr323
Irak3
Tdrd5
Pkdl12
Olfr1281
Papolb
Fancd2
LOC547111
AK031369
Mreg
MP4
Sucnr1
AK035180
AK031496
LOC545902
Zic5
Hemgn
Tlx1
4930429F11Rik
TC1451388
AK041889
AK046467
AK053826
AK081463
Tll2
AK037313
AK042108
AK042905
AK084236
AK051913
PC #3 (6.01%)
PC #2 (11.01%)
PC #1 (64.35%)
2.00
1.33
0.67
0.00
-0.67
-1.33
-2.00

Figure 5.1c . Heat map and PCA analysis of genes of the mouse hippocampus differentially expressed between the commercial mixture of HBCD (γ -HBCD) and corn oil vehicle (controls) four days post oral exposure at PND 10. 'Red" signifies an upregulation, "green" a downregulation and "black" a no change. (p-value < 0.01 AND fold > 1.5).

The first five columns are gene expression profiles for the 6 control treated mice (vehicle; corn oil) and the next 6 columns represent the mice orally administered the HBCD solution (α-HBCD, γ-HBCD, or CM-HBCD). Heat map "boxes" displayed in the color *red* signifies an "upregulation" in gene expression, black signifies "no gene expression change", and green denotes a "downregulation" in gene expression. The degree of gene expression change translates to the color intensity, with a high level of gene expression being displayed with a more intense "red" during upregulation from controls or "green" if the gene was downregulated from controls. The heat maps and PCA analysis were performed for each of the three statistical criteria (p-value and fold change; displayed in Figure 1), however, the heat maps displayed in Figures 1a, b, c are results from the highly stringent statistical criteria; p-value < 0.01 and fold change > 1.5. This was selected for the ease of visualization as the data generated using the p-value < 0.05 and fold > 1.2 resulted in a large amount of genes which is unsuitable for display. However, all of the genes identified on the heat maps in Figures 1a, b, and c are also represented (inclusive) using the criteria of p-value < 0.05 and fold change < 1.2.

Hippocampal Gene Pathway analysis. The subset of diffentially expressed genes represented on the heat maps were used to identify common pathways involved in each gene set. Using IPA, there were a number of pathways which the genes mapped to the hippocampus of mice at PND 14. Analysis of the data showed that the number of pathways with alteration in developing mice exposed to 30 mg/kg of the HBCD resulted in 32 regulated pathways after exposure to α-HBCD, 4 pathways for γ-HBCD, and 15 for the CM-HBCD (Figure 2).

IPA Common Pathways from Gene Expression - Hippocampus

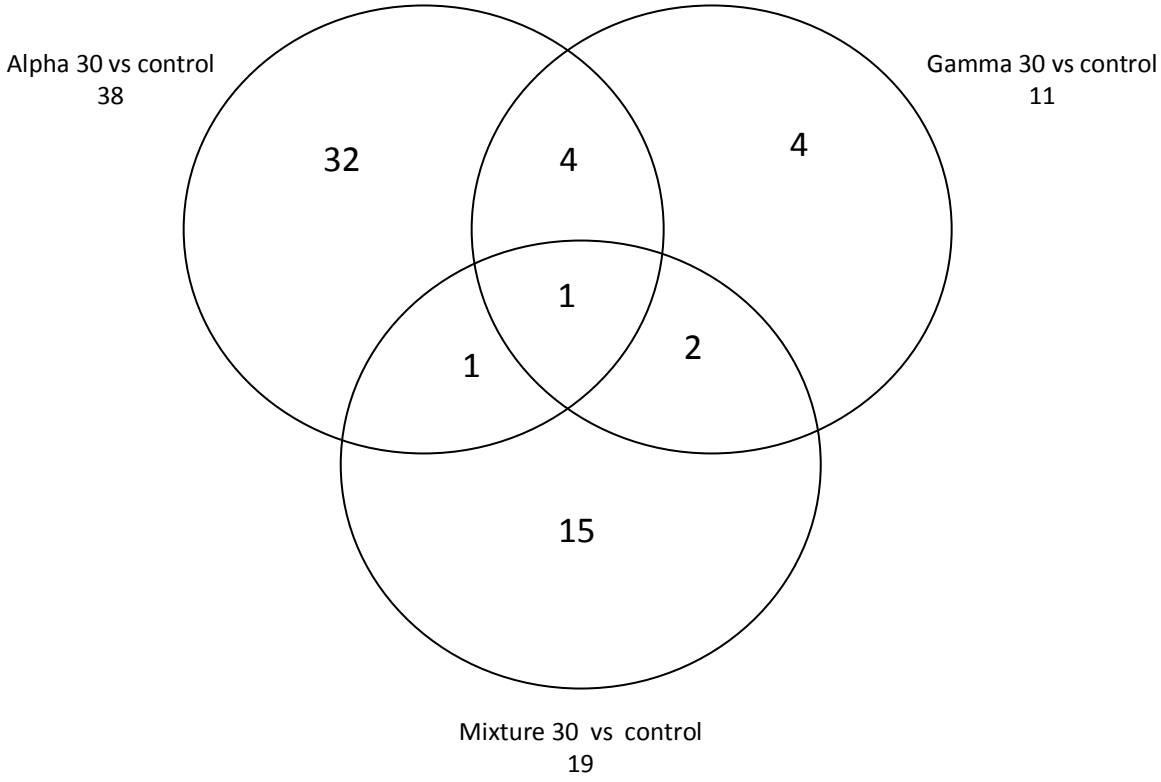

Figure 5.2. Venn diagram of hippocampal pathway analysis using gene expression changes observed in mice for all HBCD treatment groups four days after a single oral dose of 30mg/kg at PND 10. Genes were selected using an analysis of variance (Welch test) criteria of p-value = 0.05 and fold change of 1.2. IPA pathways selected had $p < 0.05$.

There were 4 specific pathways identified between the α-HBCD and γ-HBCD treated mice, 1 specific between the α-HBCD and CM-HBCD treated mice and 2 between the γ-HBCD and CM-HBCD treated mice. Interestingly there was 1 similar canonical pathway that was altered in all three treatment groups (after single oral dose of 30mg/kg; α-HBCD, γ-HBCD, or CM-HBCD).

IPA analyzed our experimental data in the context of networks, maps, and identified affected pathways. Therefore, taking a conservative approach, only the most significantly affected hippocampal pathways at this HBCD dose levels are presented as the outcome of the analysis. IPA analysis revealed that there are several pathways affected by HBCD. The top pathways based on the gene list are listed beginning with those pathways with the lowest p-values (increased confidence of relationship). After exposure to α-HBCD we observe disruption in pathways related to glutamate receptor signaling ($p = 0.002$), CREB signaling in neurons ($p = 0.005$), nitric oxide signaling ($p= 0.0069$), thyroid hormone/RXR ($p = 0.01$), synaptic long term potentiation ($p = 0.023$), retinoic acid receptor ($p = 0.038$), arachidonic acid metabolism ($p = 0.045$), and VEGF signaling ($p = 0.046$). There were considerably less neuronal specific pathways identified for γ-HBCD than for α-HBCD, only long term

potentiation ($p = 0.03$). After exposure to CM-HBCD, the genes mapped to toll like receptors signaling ($p = 0.02$) and synaptic long term potentiation ($p = 0.04$). Pathways affected by HBCD of scientific interest regarding learning and memory are shown in Tables 2-4. The genes listed in the glutametergic signaling pathway were all found in the synaptic long term potentiation pathway; these were combined and displayed together.

Pathways affected by α-HBCD, γ-HBCD, and CM-HBCD

α-HBCD

Synaptic Long -Term potentiation/
Glutamate Signaling

P= 0.002

	Gene Symbol (mouse)	Gene Name (mouse)	Gene Expression (fold)
1	**GRM6**	**Metabotro ic glutamate receptor 6**	**2.61**
2	**PRKCE**	**Protein kinase C epsilon**	**1.62**
3	**SLC1A6**	**Solute carrier family 1 (glutamate transporter)**	**1.22**
4	**CAMK4**	**Calcium/calmodulin-dependent kinase IV**	**1.33**
5	**ADCY1**	**Adenylate cyclase type 1**	**-1.24**
6	**GRIN2C**	**N-methyl D-aspartate (NMDA) receptor 2C**	**-1.27**
7	**GLUL**	**Glutamine synthetase**	**-1.37**

Nitric Oxide Signaling

P= 0.007

	Gene Symbol (mouse)	Gene Name (mouse)	Gene expression (fold)
1	**PIK3R3**	**Phosphatidylinositol 3-kinase gamma**	**1.74**
2	**FIGF**	**c-fos induced growth factor (VEGF-D)**	**1.68**
3	**PIK3CB**	**Phosphoinositide 3-kinases (PI3Ks)**	**-1.97**
4	**PLN**	**Phospholamban**	**-3.18**

Thyroid Receptor/RXR

p= 0.01

	Gene Symbol (mouse)	Gene Name (mouse)	Gene expression (fold)
1	**PIK3R3**	**Phosphatidylinositol 3-kinase gamma**	**1.74**
2	**APOA5**	**Apolipoprotein A-V**	**1.49**
3	**CAMK4**	**Calcium/calmodulin-dependent kinase IV**	**1.21**
4	**COL6A3**	**Collagen, type VI, alpha 3**	**-1.42**
5	**PIK3CB**	**Phosphoinositide 3-kinases (PI3Ks)**	**-1.97**

Retinoic Acid Receptor

p= 0.01

	Gene Symbol (mouse)	Gene Name (mouse)	Gene expression (fold)
1	**PIK3R3**	**Phosphatidylinositol 3-kinase gamma**	**1.74**
2	**PRKCE**	**Protein kinase C epsilon**	**1.62**
3	**ADCY1**	**Adenylate cyclase type 1**	**-1.24**
4	**RBP1**	**Retinol binding protein 1**	**-1.25**
5	**ALDH1A1**	**Aldehyde dehydrogenase 1 family, member A1**	**-1.33**
6	**PIK3CB**	**Phosphoinositide 3-kinases (PI3Ks)**	**-1.97**

Table 5.2. IPA pathway analysis of genes expression data from mouse hippocampus after exposure to α-HBCD.

γ-HBCD

Synaptic Long -Term Potentiatic

P= 0.03

	Gene Symbol (mouse)	Gene Name (mouse)	Gene expression (fold)
1	**CAMK4**	**Calcium/calmodulin-dependent protein kinase IV**	**1.28**
2	**PRKAR2A**	**Protein kinase type II-alpha (PKA)**	**-1.21**
3	**GRIA4**	**Glutamate Receptor 4**	**-1.28**
4	**PLCB2**	**Phospholipase C β2**	**-1.47**

Blood Brain Barrier Integrity

p=n/a

	Gene Symbol (mouse)	Gene Name (mouse)	Gene expression (fold)
1	**TJP2**	**Tight Junction Protein 2**	**-1.21**
2	**VEGFC**	**Vascular endothelial growth factor C**	**-1.33**

Programmed Cell Death

p= n/a

	Gene Symbol (mouse)	Gene Name (mouse)	Gene expression (fold)
1	**Fas**	**TNF receptor superfamily member 6**	**2.34**

Solute Carrier Transporter

p=n/a

	Gene Symbol (mouse)	Gene Name (mouse)	Gene expression (fold)
1	**SLCO1B3**	**Solute carrier organic anion transporter 1B3**	**-1.27**

Table 5.3. IPA pathway analysis of genes expression data from mouse hippocampus after exposure to γ-HBCD .

CM-HBCD

Synaptic Long-Term Potentiation

P= 0.04

	Gene Symbol (mouse)	Gene Name (mouse)	Gene expression (fold)
1	**ADCY1**	**Adenylate cyclase type 1**	**1.23**
2	**GRM2**	**Metabotropic glutamate receptor 2**	**-1.23**
3	**PRKAR2A**	**Protein kinase type II-alpha (PKA)**	**-1.24**
4	**PPP1R14D**	**Protein phosphatase 1, regulatory (inhibitor) 14D**	**-1.61**

Toll Like Receptor Signaling

P= 0.02

	Gene ymbol (mouse)	Gene Name (mouse)	Gene expression (fold)
1	**MAPK13**	**Mtogen-activated protein kinase 13**	**1.48**
2	**IRAK3**	**Interleukin-1 receptor-associated kinase 3**	**-1.51**
3	**TLR2**	**Toll like receptor 2**	**-1.29**

Solute Carrier Transporter

p= n/a

	Gene ymbol (mouse)	Gene Name (mouse)	Gene expression (fold)
1	**SLCO1B3**	**Solute carrier organic anion transporter 1B3**	**-1.37**

Table 5.4. IPA pathway analysis of genes expression data from mouse hippocampus after exposure to CM-HBCD.

Proteomics of Hippocampus

Hippocampal Proteins. There were a number of proteins that were significantly up- or down-regulated in the hippocampus of mice at PND 14 after exposure to a single oral dose of 30mg/kg of α-HBCD or γ-HBCD at PND 10. Analysis of the data shows that the number of proteins differentially expressed (fold change 1.2 up- or down-regulated) in developing mice exposed to 30 mg/kg HBCD on PND10 was 51 after exposure to α-HBCD and 159 after

exposure to γ-HBCD. An overlap of differentially expressed genes was observed with a mere 13 proteins being similar between the two treatment groups (Figure 3).

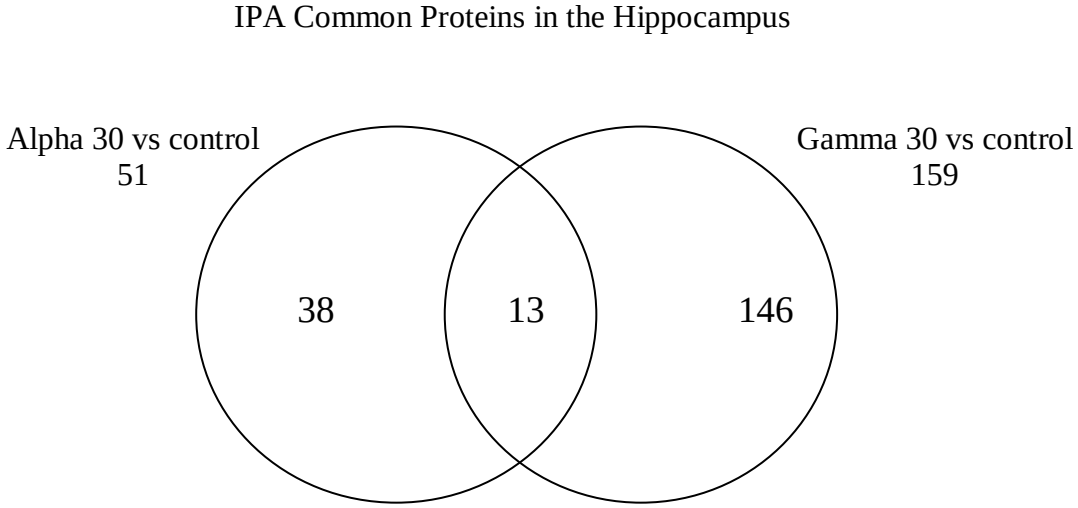

Figure 5.3. Venn diagram of hippocampal protein expression changes observed in mice for all HBCD treatment groups four days after a single oral dose of 30mg/kg at PND 10. Genes were selected using an analysis of variance (Welch test) criteria of p-value = 0.05 and fold change of 1.2.

Hippocampal Protein Pathway Analysis. Due to the limited numbers of proteins on the antibody array, IPA was not able to identify any pathways that were significantly altered in the hippocampus of mice at PND 14 exposed to a single 30mg/kg oral dose of α-HBCD or γ-HBCD at PND 10. The proteomic data was limited in numbers with 1318 proteins as compared to 30,000 genes in the whole genome array.

Comparison between transcriptomics and proteomics. The antibody array is a tool for discovery and in light of no pathway analysis, individual proteins will be used here to support and validate the transcriptomics data where applicable. There were 51 and 159 proteins found to be altered in the hippocampal brain for α-HBCD or γ-HBCD, respectively. Table 5 demonstrates that there were similarities between transcriptomics gene expression data and proteomics analysis when searching for protein counterparts to altered gene transcripts from Tables 2 and 3.

α-HBCD

	Gene Symbol (mouse)	Gene expression (fold)	Protein Expression (fold)	Protein Expression p-value
1	**PIK3R3**	**1.74**	**1.29**	**0.038**
2	**CAMK4**	**1.33**	**1.21**	**0.049**
3	**ADCY1**	**-1.24**	**-1.10**	**0.061**
4	**PRKCE**	**-1.42**	**-1.22**	**0.045**

γ-HBCD

	Gene Symbol (mouse)	Gene expression (fold)	Protein Expression (fold)	Protein Expression p-value
1	**PRKAR2A**	**-1.21**	**-1.34**	**0.023**
2	**VEGFC**	**-1.33**	**1.20**	**0.050**
3	**FAS**	**2.34**	**1.53**	**0.011**

Table 5.5. Comparison between transcriptomic and proteomics in the hippocampus of infantile mice orally exposed to 30mg/kg of either α-HBCD or γ-HBCD.

After exposure to α-HBCD, PIK3R3, CAMK4, ADCY1, and PRKCE protein levels were determined to be significantly changed and all were comparable to the transcriptomics in intensity and direction (up or down regulated). Although the protein expression data for ADCY1 ($p = 0.061$) fell below the designated significance criteria ($p=0.05$), both the gene expression and protein levels were found to be unidirectional. Similar patterns were found after exposure to γ-HBCD. PRKAR2A, VEGFC, and FAS, were all significantly changed and were comparable to the transcriptomics in intensity and direction. The only discrepancy between gene expression and protein expression was found for VEGFC (gene transcription of -1.33 and protein expression of +1.20). However, it is clear that overall these proteins further validate the transcriptomic gene expression data (previous section) and adds further confidence in this study with actual translation data.

Transcriptomics of Liver

Liver Gene Expression. There were a number of genes that were significantly up- and down-regulated in the liver of mice at PND 14 after oral exposure to a single 30mg/kg oral dose of α-HBCD, γ-HBCD, or CM-HBCD at PND 10. Analysis of the data demonstrates that the number of genes differentially expressed (using the criteria - fold change > 1.2 up- or down-regulated AND p-value of < 0.05) in developing mice exposed to 30 mg/kg of HBCD resulted in 547 regulated genes for α-HBCD, 1812 for γ-HBCD, and 336 for the CM-HBCD, respectively (Table 1). An overlap of 54 genes could be observed between all three treatment groups.

Principle component analysis. Principle component analysis (PCA) was applied on the entire genomic dataset (30,000 genes) and did not reveal specific clustering of treatment groups (α-HBCD, γ-HBCD or CM-HBCD) in the liver (data not shown). However, very distinctive clustering was observed when a subset of genes differentially expressed between groups was identified (using a fold change and ANOVA p-value threshold) and then PCA performed. Each treatment condition, α-HBCD, γ-HBCD, or CM-HBCD was compared to controls using the subset of genes which differentially expressed between the two groups (Figure 4a, b, c).

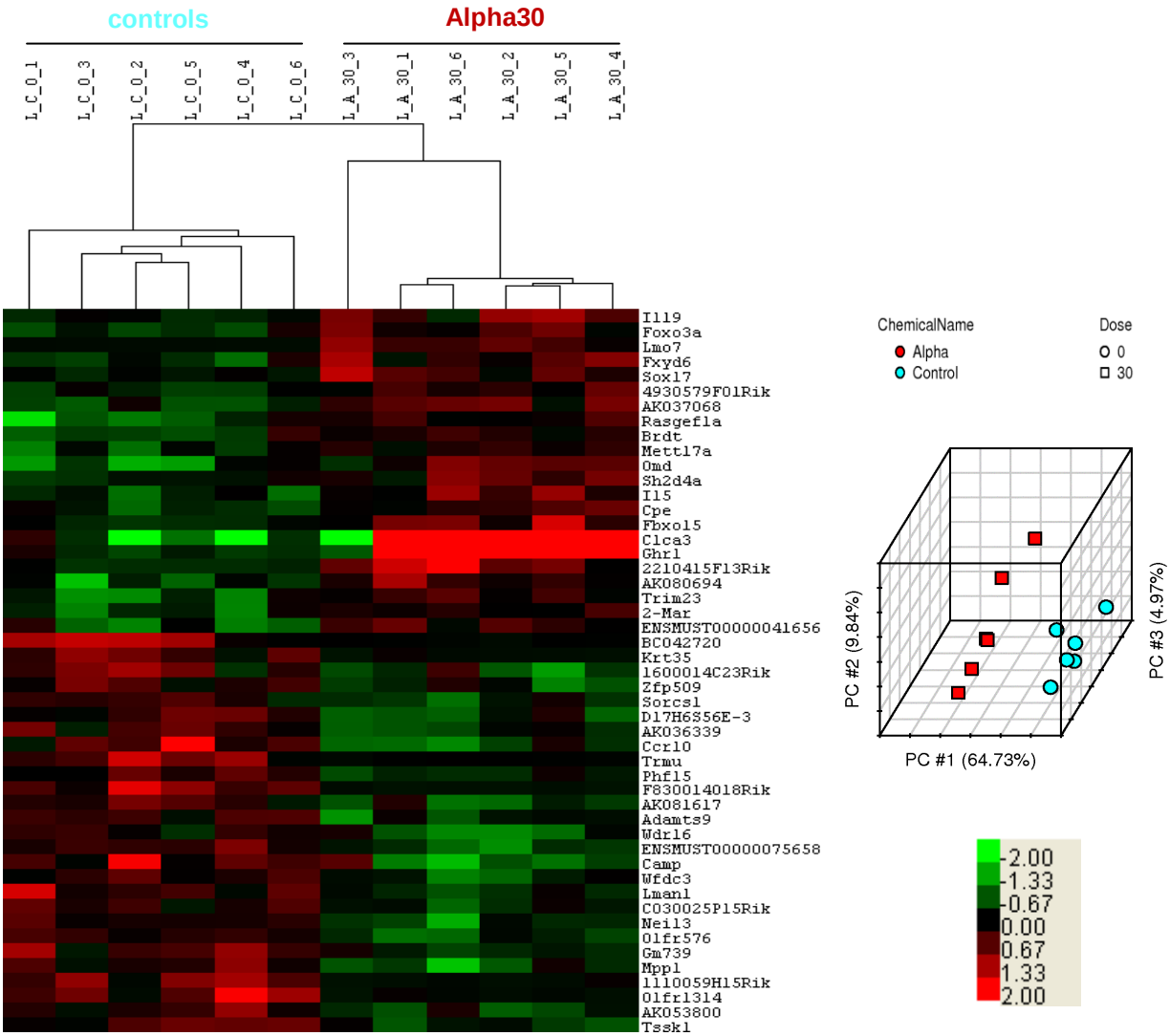

Figure 5.4a. Heat map and PCA analysis of genes of the mouse liver differentially expressed between the commercial mixture of HBCD (α-HBCD) and corn oil vehicle (controls) four days post oral exposure at PND 10. ‘Red” signifies an upregulation; “green” signifies a downregulation while “black” signifies no change. (P-value < 0.01 AND fold > 1.5)

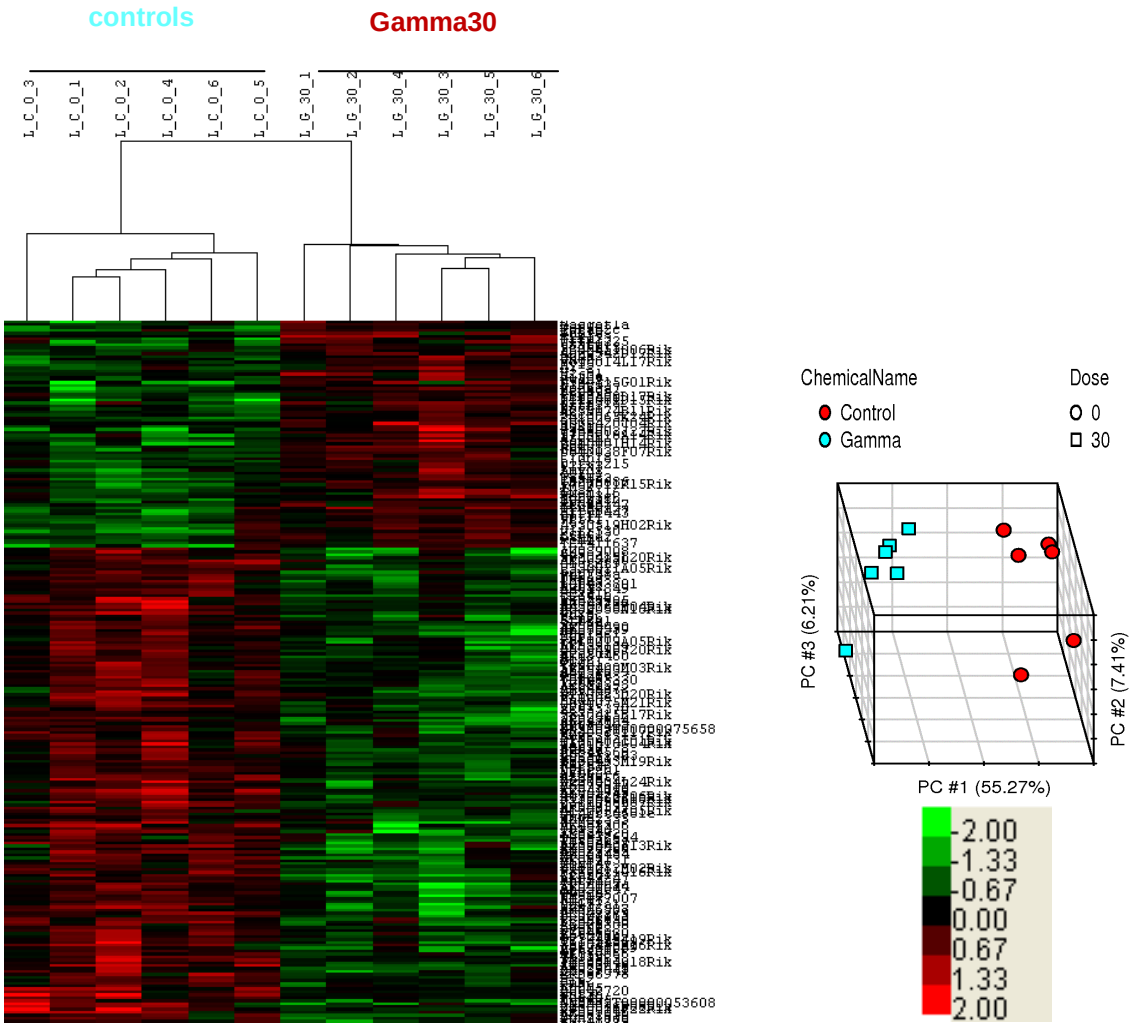

Figure 5.4b. Heat map and PCA analysis of genes of the mouse liver differentially expressed between the commercial mixture of HBCD (γ-HBCD) and corn oil vehicle (controls) four days post oral exposure at PND 10. ‘Red” signifies an upregulation; “green” signifies a downregulation while “black” signifies no change. (p-value < 0.01 AND fold > 1.5).

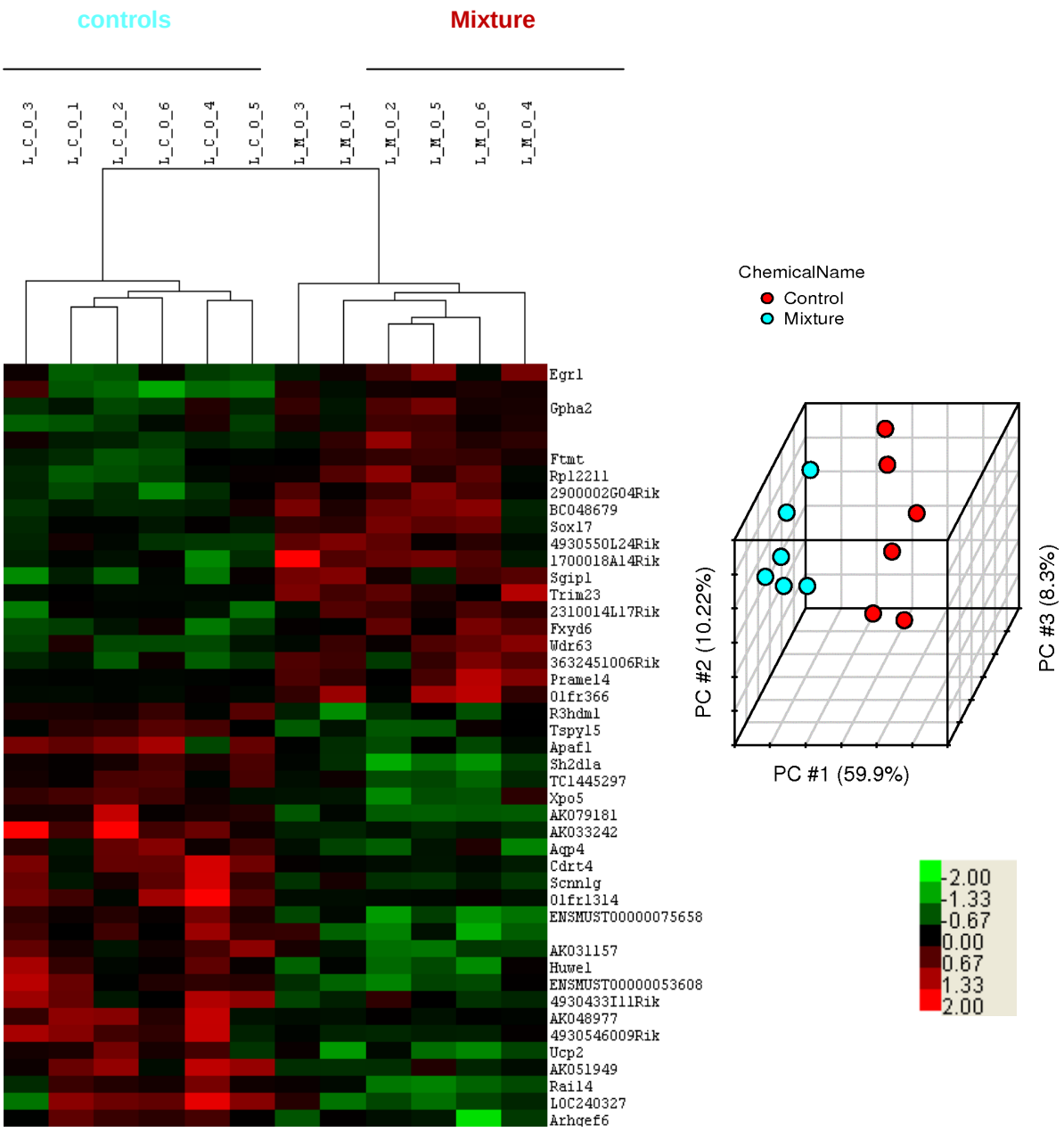

Figure 5.4c. Heat map and PCA analysis of genes of the mouse liver differentially expressed between the commercial mixture of HBCD (α-HBCD) and corn oil vehicle (controls) four days post oral exposure at PND 10. ‘Red” signifies an upregulation; “green” signifies a downregulation while “black” signifies no change. (p-value < 0.01 AND fold > 1.5)

A heat map also displays the genes altered for each exposed mouse pup (n=6). The first five columns are gene expression profiles for the 6 control treated mice (vehicle; corn oil) and the next 6 columns represent the mice orally administered the HBCD solution (α-HBCD, γ-HBCD, or CM-HBCD) at 30 mg/kg. Heat map "boxes" displayed in the color *red* signify an "upregulation" in gene expression, black signifies "no gene expression change", and green "denotes a "downregulation" in gene expression. The degree of gene expression change translates to the color intensity, with a high level of gene expression being displayed with a more intense "red" during upregulation from controls or "green" if the gene was downregulated from controls. The heat maps and PCA analysis were performed for each of the three statistical criteria (p-value and fold change; displayed in Figure 1), however, the heat maps displayed in Figures 4 a, b, c are results from the highly stringent statistical criteria: p-value < 0.01 and fold change > 1.5, as also displayed for the hippocampus. This was selected for the ease of visualization as the data generated using the p-value < 0.05 and fold > 1.2 resulted in a large amount of hepatic genes which is unsuitable for display. However, all of the genes identified on the heat maps in Figures 4 a, b, c are also represented (inclusive) using the criteria of p-value < 0.05 and fold change < 1.2.

Liver Gene Pathway analysis. The subset of diffentially expressed genes represented on the heat maps were used to identify common pathways involved in each gene set. Using IPA, there were a number of pathways which the genes mapped to in the liver of mice at PND 14. Analysis of the data showed that the number of pathways with alteration in developing mice exposed to 30 mg/kg of the HBCD resulted in 55 regulated pathways after exposure to α-HBCD, 25 pathways for γ-HBCD, and 6 for the CM-HBCD (Figure 5).

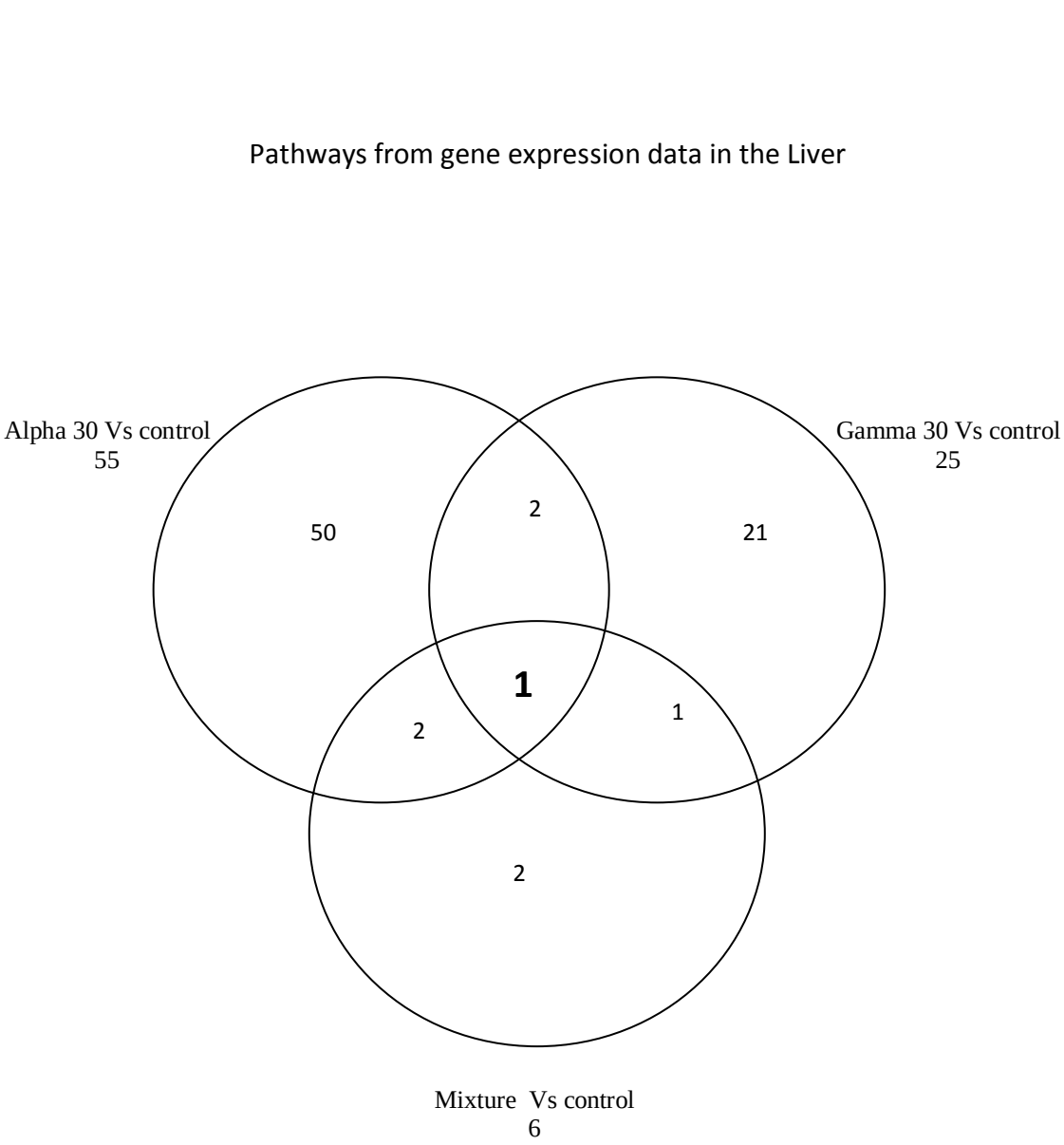

Figure 5.5. Venn diagram of liver pathway analysis using gene expression changes observed in mice for all HBCD treatment groups four days after a single oral dose of 30mg/kg at PND 10. Genes were selected using an analysis of variance (Welch test) criteria of p-value = 0.05 and fold change of 1.2. IPA pathways selected had $p < 0.05$.

There were 2 specific pathways identified between the α-HBCD and γ-HBCD treated mice, 6 specific between the α-HBCD and CM-HBCD treated mice, and 1 between the γ-HBCD and CM-HBCD treated mice. Interestingly, similar to the hippocampal data, there was 1 similar canonical pathway that was altered in all three treatment groups (single oral dose of 30mg/kg of α-HBCD, γ-HBCD, or CM-HBCD).

IPA analyzed our experimental data in the context of networks, maps, and identified affected pathways. Therefore, taking a conservative approach, only the most significantly affected liver pathways at this HBCD dose level are presented as the outcome of the analysis. IPA analysis revealed that there are several pathways affected by HBCD. The top pathways based on the gene list are listed beginning with those pathways with the lowest p-values (increased confidence of relationship). After exposure to α-HBCD, we observe disruption in pathways related to PTEN signaling ($p = 0.0019$), Type II Diabetes Mellitus Signaling ($p = 0.0038$), FAK Signaling ($p= 0.01$), PI3K/AKT Signaling ($p = 0.0135$), VEGF Signaling ($p = 0.03$), xenobiotic metabolism signaling ($p = 0.03$), NRF2-mediated oxidative stress response ($p = 0.04$), glycerolipid metabolism ($p = 0.046$) and tight junction signaling ($p = 0.047$). There were considerably less hepatic specific pathways identified for γ-HBCD than for α-HBCD: PI3K/AKT Signaling ($p = 0.002$), p53 signaling ($p = 0.008$), cysteine metabolism ($p = 0.016$), ERK/MAPK signaling ($p = 0.015$) and FAK signaling ($p = 0.03$). After exposure to CM-HBCD, the genes mapped to FAK signaling ($p = 0.04$) only.

Proteomics of Liver

Liver Proteins. There were a number of proteins that were significantly up- or down-regulated in the hippocampus of mice at PND 14 after exposure to a single oral dose of 30mg/k of α-HBCD or γ-HBCD at PND 10. Analysis of the data demonstrates that the number of proteins differentially expressed (fold change 1.2 up- or down-regulated) in developing mice exposed to 30 mg/kg HBCD on PND10 was 10 after exposure to α-HBCD and 14 after exposure to γ-HBCD. An overlap of differentially expressed proteins was observed with only 1 protein being similar between the two treatment groups (Figure 6).

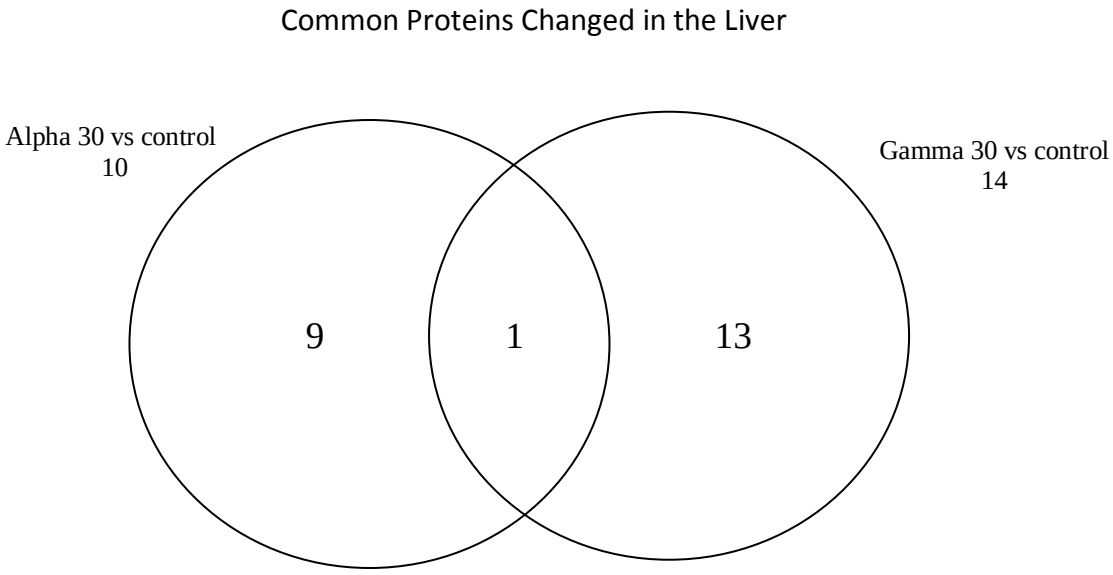

Figure 5.6. Venn diagram of liver protein expression changes observed in mice for all HBCD treatment groups four days after a single oral dose of 30mg/kg at PND 10. Genes were selected using an analysis of variance (Welch test) criteria of p-value = 0.05 and fold change of 1.2.

Liver Protein Pathway Analysis. Due to the limited numbers of protein on the antibody array, IPA was not able to identify any pathways that were significantly altered in the liver of mice at PND 14 exposed to a single 30mg/kg oral dose of α-HBCD or γ-HBCD at PND 10. The proteomic data was limited in numbers beginning with 1318 as compared to 30,000 genes in the whole genome array. Direct comparison of protein of interest will be conducted against transcriptomics results but is currently beyond the scope of this chapter. As mentioned in the introduction, this chapter will focus on the hippocampal effects.

Metabolomics of Serum

Metabolomics analysis was conducted on 60 ul serum aliquots derived from mice administered 0, 3, 10, or 30 mg/kg of α-HBCD. Analysis was conducted on serum from mice administered 0, 3, or 30 mg/kg γ-HBCD or administered 0 or 30 mg/kg of CM-HBCD. The 950 MHz ^{1}H NMR spectrum of serum (example shown in Figure 7) contained signals that could be assigned via Chenomx library matching to compounds such as amino acids, carboxylic acids, and sugars.

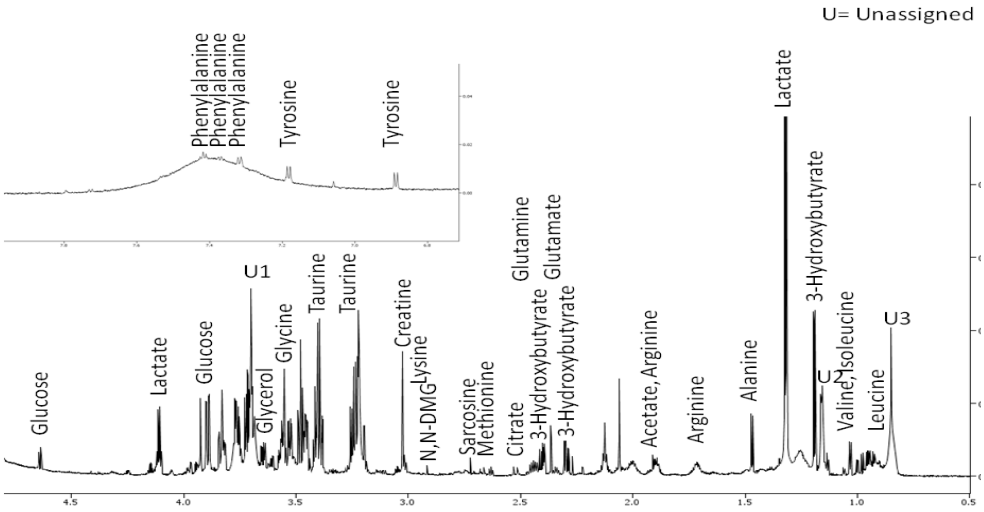

Figure 5.7. 950 MHz ^{1}H NMR spectrum of metabolites in mouse serum. Signals for the higher concentration metabolites are labeled.

Multivariate analysis was conducted using binned data normalized to the total intensity. An example analysis is shown in Figure 8 for sample collected from mice administered the vehicle control (green), or the high dose of α-HBCD (blue), γ-HBCD (orange), or CM-HBCD (red). This analysis indicates that alpha (blue) and gamma (orange) groups are more closely clustered and separated from the commercial mixture group (red).

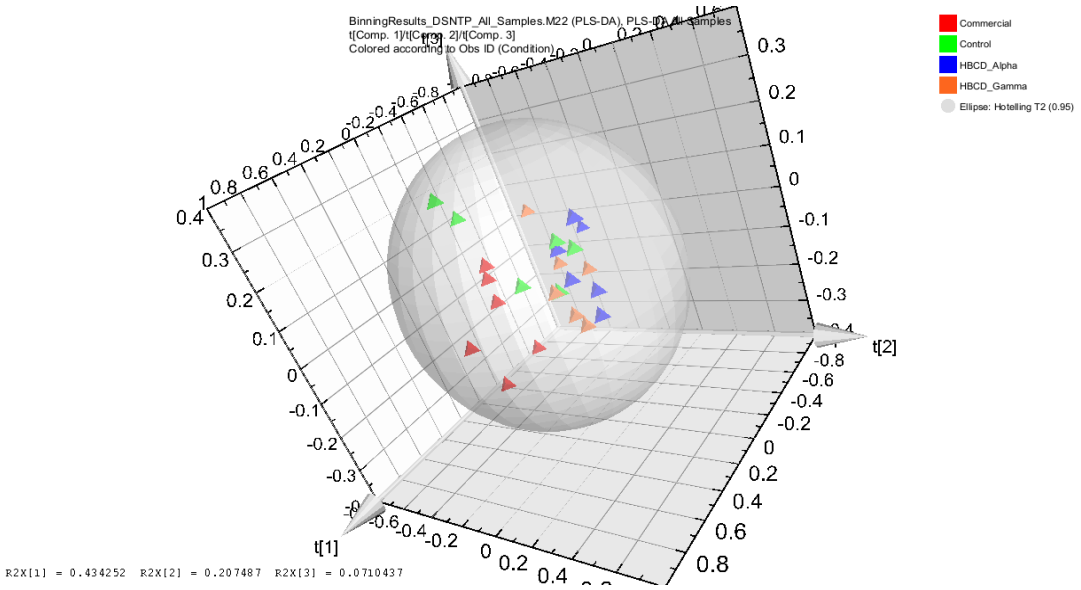

Figure 5.8. Multivariate analysis (PLS-DA score plot) of bin data obtained for serum from mice exposed to vehicle (green), α- HBCD (blue), γ-HBCD (orange), or CM-HBCD (red).

In addition to analysis by the binning approach, PCA and PLS-DA were conducted using concentration data. Library matching using NMR Suite 5.1 Professional Software (Chenomx, Edmonton, Alberta, Canada) was performed to provide a list of metabolites (and their respective concentrations) that demonstrated a match within each of the serum spectra. The concentration for each metabolite was normalized to that of formate for each serum sample. Multivariate analysis was conducted for each chemical investigated (e.g., vehicle vs low vs high dose), as well as to enable comparison among chemicals (e.g., alpha vs gamma).

The PLS-DA analysis using serum metabolite concentration data differentiated the vehicle from the low and high dose groups for mice exposed to α-HBCD (Figure 9a). PLS-DA was conducted using data obtained for the vehicle and the high dose groups (Figure 9b), and examination of loadings plots, variable importance plots, and contribution plots enabled the selection of a subset of metabolites that most contributed to the separation of the two groups (Table 6).

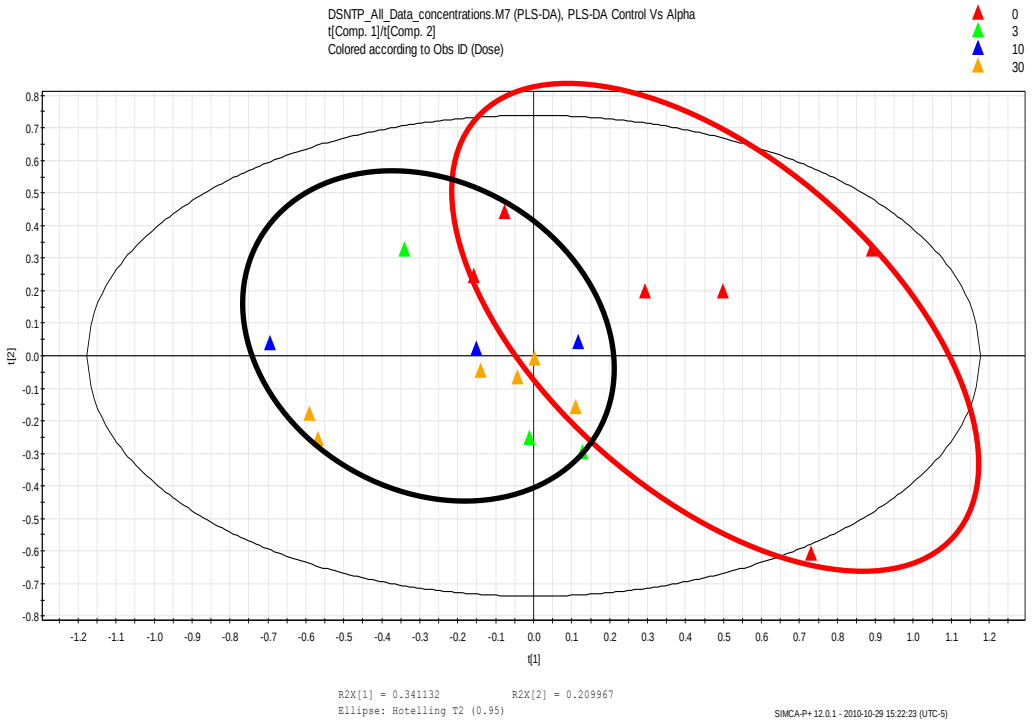

Figure 5.9a. Score plot of PLS-DA analysis of metabolite concentration data for the serum samples obtained from mice administered 0, 3, 10, and 30mg/kg doses of α-HBCD, indicating separation between the control (red) and dose groups.

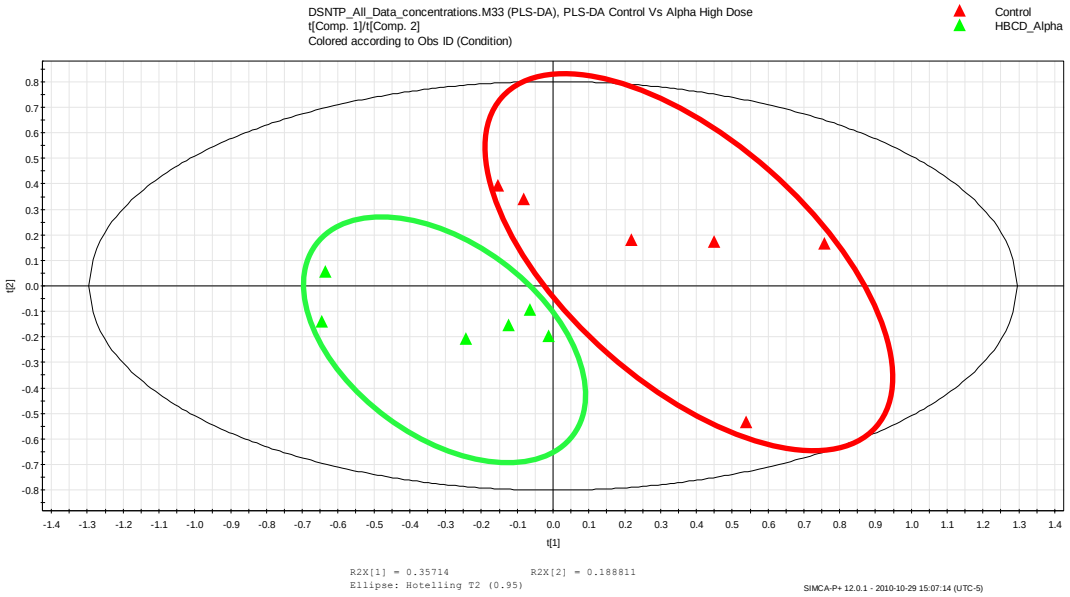

Figure 5.9b. Score plot of PLS-DA analysis of metabolite concentration data for the serum samples obtained from mice administered vehicle or 30mg/kg α-HBCD shows a clear separation of study groups (Red, Control; Green, high dose α-HBCD).

HBCD Alpha		HBCD Gamma		Commercial Mixture	
Increased	**Decreased**	**Increased**	**Decreased**	**Increased**	**Decreased**
Valine	Lactate	Glycine	Phenylalanine	Taurine	Phenylalanine
Leucine	Pyruvate	Glutamine	Glutamate	Glycerol	Methanol
3-Hydroxybutyrate	Glutamate	Asparagine	Pyruvate	Lactate	Arginine
Glutamine	Choline	3-Hydroxybutyrate	Serine	O-Phosphocholine	Alanine
Acetoacetate	Methanol	Taurine	Lactate	3-Hydroxybutyrate	N,N-Dimethylglycine
2 Hydroxybutyrate	Acetate	Citrate	Threonine	Asparagine	Glucose
	Citrate	Glycerol	Choline	Methionine	
	Alanine	Glucose	Tyrosine	Citrate	
	N,N-Dimethylglycine	2-Hydroxybutyrate	Lysine	Valine	
	Phenylalanine	Creatine	Methanol	Creatine	

Table 5.6. Serum metabolites that best differentiate the high dose and vehicle control study groups for each chemical.

Using only this subset of metabolites, the separation of the α-HBCD group from the control group in PLS-DA was improved (Figure 9c), confirming these metabolites as being significant to the separation of the study groups.

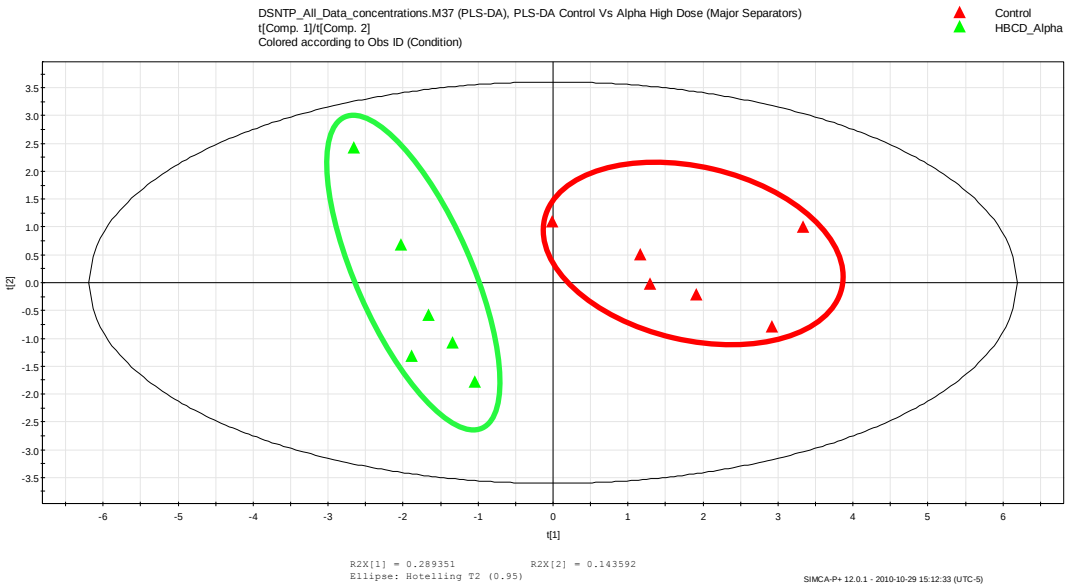

Figure 5.9c. Score plot of PLS-DA analysis using the subset of metabolites that best define the HBCD-Alpha high dose group from the control group. An improvement of the separation of the dose group from the control group (Red, Control; Green, high dose α-HBCD) was achieved using the subset of metabolites.

This process was repeated using only the γ-HBCD data (control vs each dose group) or only the CM-HBCD data (control vs each dose group) to derive a list of metabolites that best describe each group (Table 6). PLS-DA analysis of the control and all high dose group concentration data provided separation of each high dose group from the control (Figure 10).

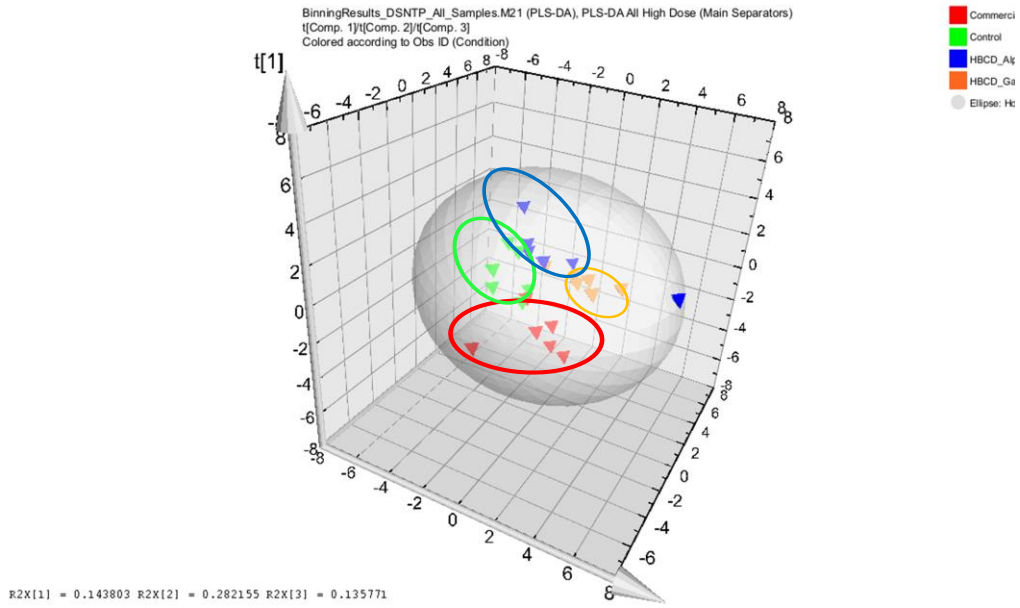

Figure 5.10. PLS-DA plot of metabolite concentration data derived for serum samples obtained from mice administered vehicle control (green), or a high dose (30mg/kg) of CM-HBCD (red), α-HBCD (blue), or γ-HBCD (orange).

The separation ability using the concentration data (Figure 10) was improved beyond that provided using bin data (Figure 8). However, in both analyses, the commercial mixture, alpha, and gamma clustered from each other and represent differences in response between each treatment. Using this analysis, it is the commercial mixture that may be more distinct from either of the two individual stereoisomers and had a trajectory pattern opposite of the alpha and gamma group's trajectory from control based on the levels of metabolites that were differentially measured in the serum (Table 6).

DISCUSSION

We propose that CM-HBCD may alter similar molecular mechanism responsible for learning and memory as seen for BDEs and PCBs. Our objective was to investigate the effects of the CM-HBCD on gene transcription in the hippocampus. The second objective was to investigate whether the individual HBCD stereoisomers, α-HBCD and/or γ-HBCD may be responsible for driving the neurotoxicity observed after exposure to the CM-HBCD.

The exposure of CM-HBCD took place during a period of rapid brain growth, which in mice takes place during the first 1-3 weeks of life, reaching its peak near PND10 (Davison and Dobbing 1968). The multitude and complexity of processes during this rapid development makes the developing brain particularly vulnerable to the effects of xenobiotics. Mice were affected after exposure to the CM-HBCD during their performance test in the Morris water maze (Eriksson et al. 2001), commonly used as a learning task to detect effects in the hippocampus.

In the present study, we found that neonatal exposure to the CM-HBCD causes developmental effects in gene expression and protein level changes in the mouse hippocampus. Regardless of the treatment group, α-HBCD, γ-HBCD, or CM-HBCD, IPA pathway analysis on the hippocampus identified only one canonical pathway which happens to be linked directly to learning and memory in the hippocampal target tissue,

synaptic long term potentiation (LTP). Furthermore, specific changes of key postsynaptic proteins involved in glutamate receptor signaling and calcium homeostasis were revealed. The combined results suggest that synaptic changes may directly contribute to the observed defect in synaptic plasticity leading to learning and memory deficits after exposure to the CM-HBCD previously observed (Eriksson et al., 2006).

The individual HBCD stereoisomers tested in this study, α-HBCD and γ-HBCD, were both found to alter hippocampal gene expression and protein levels where pathway differences between the stereoisomers were also identified. The differences between treatment groups were overwhelmingly larger than were the similarities at all platforms (gene, protein, and pathways). This is evidence that *in vivo* stereoisomer specific effects can occur. Furthermore, the gene expression and protein data in the liver as well as the serum metabolomics data further support that there are clear effect differences between the α-HBCD and γ-HBCD stereoisomers and the mixture after exposure to infantile mice. Future efforts to map pathways differences are underway.

Our results demonstrated that administration of α-HBCD, γ-HBCD, or the CM-HBCD alters molecular mechanisms involved in LTP. Calcium-mediated processes are important for LTP (Williams and Johnston, 1989), and deletion of the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) genes in mice have resulted in impairment in LTP (Silva *et al.*, 1992). That HBCDs are able to affect molecules involved in Ca^{2+} balance in mice brain

and change CaMK protein levels may further contribute to the LTP deficit. Similar effects have been observed with PBDEs (Kodavanti and Ward, 2005; Viberg *et al.*, 2008). Second, the activation of protein kinase C (PKC) plays an important role in the expression and persistence of LTP in hippocampal neurons (Klann *et al.*, 1991; Lovinger *et al.*, 1987; Malenka *et al.*, 1989; Malinow *et al.*, 1988; Moriguchi *et al.*, 2006). The activation of postsynaptic NMDA receptors is critical for the induction of LTP in the hippocampus (Bliss and Collingridge, 1993; Burgard *et al.*, 1989; Poncer, 2003; Reymann *et al.*, 1989). From our study, HBCD, as previously seen with PBDEs, may decrease the protein levels of NMDA receptors by impairing the ratio of their subunits (Dingemans *et al.*, 2007).

It has been proposed that the cholinergic system is altered after exposure to PBDEs and HBCD (Eriksson et al., 2001; Eriksson et al., 2006) in both mouse and rat brain (Fischer *et al.*, 2008; Viberg *et al.*, 2003, 2007). This impairment may actually be an indirect mechanism involving the depression of LTP in the hippocampus (Abe *et al.*, 1994; Blitzer *et al.*, 1990; Galey *et al.*, 1994).

As previously mentioned, the data on HBCD's effect on the nervous system is limited. There is one *in vitro* study, examining the individual stereoisomer effects on synaptic transmission (Dingemans *et al.*, 2009). Effects of α- and β-HBCD were comparable to that of the technical mixture, whereas the inhibitory effect of γ-HBCD was larger. Using specific blockers of voltage-gated Ca2+ channels (VGCCs), it was shown that the inhibitory effect of

HBCD is not VGCC-specific. Additionally, the number of cells showing depolarization-evoked neurotransmitter release was markedly reduced following HBCD exposure. In this system, HBCD inhibits depolarization-evoked Ca2+ and neurotransmitter release and further adds to support the *in vivo* study presented here.

In general terms, LTP involves strengthening of synapses such that transmission between two neurons is enhanced. Following exposure to HBCDs, a number of molecular changes support inhibition of this important pathway and, as HBCDs have previously been shown to decrease rodent performance in water maze behavioral tests (Eriksson et al., 2006), may implicate these chemicals in disruption of learning and memory processes. One way in which LTP is thought to be induced at glutamatergic synapses (as can be found in the cortex and hippocampus) is through a combination of increased neurotransmitter (i.e., glutamate) release from presynaptic neurons and elevated numbers and conduction of glutamate receptors at postsynaptic sites. The top cannonical pathway identified for α-HBCD was glutamate signaling ($p = 0.002$), which was not directly observed for γ-HBCD or CM-HBCD. This finding in conjunction with synaptic long term potentiation ($p=0.01$) supports the high likelihood for this system to be altered based on previous published data. Therefore concern for effects of the individual α-HBCD stereoisomer should be considered.

The proposed molecular mechanisms for HBCD-mediated inhibition of long term potentiation (LTP) may include multiple pathways (Figure 11).

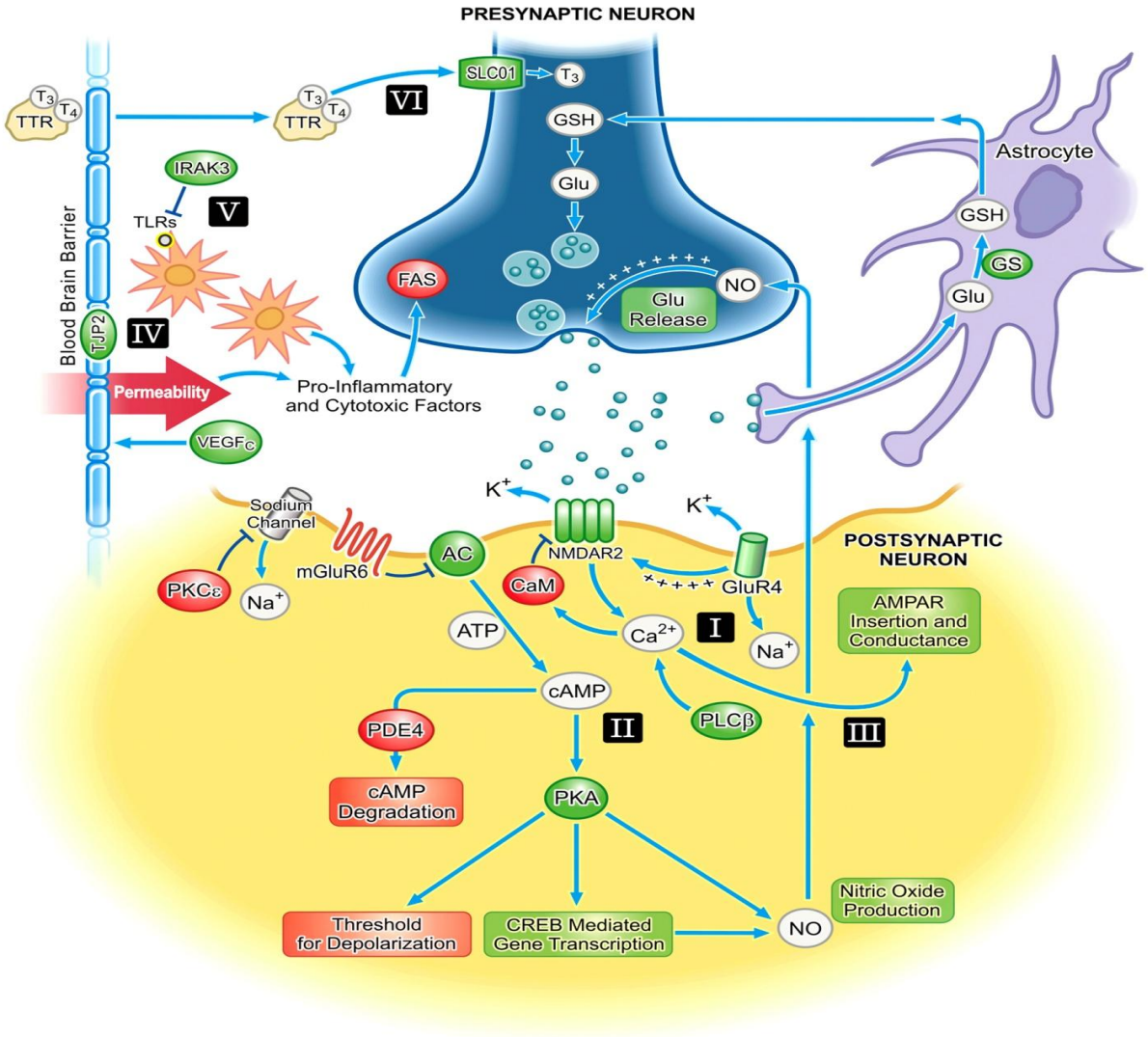

Figure 5.11. Proposed Molecular Mechanisms for HBCD-Mediated Inhibition of Long Term Potentiation (LTP) on Glutamatergic Neurons in Developing Mouse Hippocampus. Disrupted LTP is implicated in learning and memory deficits. One way in which LTP is thought to be induced at glutamatergic synapses (as can be found in the cortex and hippocampus) is through a combination of increased glutamate release from presynaptic

neurons and elevated numbers and conduction of glutamate receptors at postsynaptic sites, leading to a long-lasting increase in the magnitude and efficiency of synaptic signaling. Following exposure to HBCD, a number of molecular changes support inhibition of this process, leading to synapse weakening in the hippocampus of developing mice. Specific molecular changes and downstream signaling events of interest are identified as increased (red) or decreased (green) from controls. Lines with a blunt end represent an inhibitory process, while activation arrows with "+" symbols indicate a potentiating effect. Six major themes that may be disrupted following HBCD exposure are indicated: **(I)** Decreases in Intracellular Ca^{2+} and Na^{+}; **(II)** Decreased cyclic AMP (cAMP); **(III)** Synapse weakening via decreases in NO signaling, AMPAR insertion, receptor conductance, and probability of depolarization; **(IV)** Decreased integrity of the blood brain barrier; **(V)** Activation of pro-inflammatory microglia; and **(VI)** Reduction of T_3 to target neurons. Altogether, molecular changes by HBCD support inhibition of LTP as a significant adverse effect in the brain.

First, intracellular Ca^{2+} levels may be altered. At these synapses, glutamate first induces a weak stimulation of AMPA receptors (AMPARs), allowing an influx of Na^{+} and an efflux of K^{+} ions. With continued stimulations, activated AMPARs help displace the Mg^{2+} ion block on NMDA receptors (NMDARs). This results in a strong influx of Ca^{2+} ions upon glutamate binding (again exporting K^{+}). The net increase in Na^{+} and Ca^{2+} ions drives depolarization and neurotransmission. With HBCD exposure, transcripts for the AMPAR, GluR4, NMDAR, and NMDAR2C are decreased. In addition, phospholipase C (B2 isoform),

which, through second messenger signaling, increases intracellular Ca^{2+}, is also decreased. Together, these changes would be expected to dramatically decrease intracellular Ca^{2+} levels in the postsynaptic neuron.

Secondly, cellular cyclic AMP (cAMP) signaling is an important second messenger in neurons. cAMP has been implicated as one of the key modulators of LTP. cAMP can be synthesized by adenylate cyclase (AC) in an ATP-dependent reaction. With HBCD exposure, mRNA for AC (isoform Y1) is decreased, as is mRNA encoding mGluR6, which inhibits the activation of AC via G-protein signaling. Phosphodiesterase 4D, which breaks down cAMP, is also increased following HBCD exposure. The net result of these changes would be a depletion of cAMP in the postsynaptic neuron after HBCD exposure. Although cAMP may support LTP through multiple mechanisms, the cAMP-dependent kinase, PKA (2RA), which is also decreased by HBCD, is thought to be involved in signaling that results in CREB-mediated transcription and MAP kinase signaling that increases synthesis of nitric oxide (NO). In addition, PKA phosphorylation of postsynaptic ion channels increases the likelihood that they will open with stimulation, elevating the potential for depolarization. Thus, HBCD would be expected to make activation of these ion channels less likely (preventing depolarization) and decrease NO-mediated signaling.

Thirdly, synapse strengthening is a hallmark of LTP: decreased intracellular Ca2+ by HBCD would decrease insertion of AMPARs at the postsynaptic membrane and decrease conduction of these same receptors. NO is thought to act as a signaling molecule at these

synapses to stimulate an increased release of neurotransmitter from the presynaptic neuron and production may also be decreased following HBCD exposure. Astrocytes help to cycle glutamate (Glu) at the synapse by taking up Glu and converting it to glutathione (GSH). This GSH is then transferred to the presynaptic neuron where it is once again broken down to produce Glu for signaling. A key enzyme family involved in the production of GSH from Glu is glutathione synthase (GS, aka GLUL); HBCD decreases expression of this gene. These changes would be expected to decrease transmission efficiency at glutamatergic synapses.

Fourth, negative impact of pro-inflammatory factors can contribute to alterations in LTP. Both increases in permeability of the blood-brain barrier (BBB) and activation of microglia can result in the introduction of pro-inflammatory and cytotoxic molecules to the neuronal parenchyma. This can contribute to direct neurotoxicity and disruption of synaptic transmission. Two genes associated with permeability of the BBB are decreased by HBCD exposure; tight junction protein 2 (TJP2) and the growth factor, vascular endothelial growth factor (C, VEGFc). Over-activation of microglia can occur following stimulation of toll-like receptors (TLRs) which are inhibited by the protein IRAK3 (interleukin-1 receptor associated kinase), transcripts for which are decreased by HBCD. The pro-inflammatory and cytotoxic factors that could be released as a result of BBB breach and excessive microglial activation are capable of causing direct neurotoxicity; interestingly, HBCD exposure up regulates expression of the pro-apoptotic protein Fas.

Lastly, there is the possible contribution of direct thyroid hormone (TH) effects. It is unclear exactly how T4 and T3 regulate brain activity. Of interest is the solute carrier protein, SLCO1B3, which is known to transport T3 into cells and which is decreased by HBCD exposure in the brain. This along with genes known to be transcriptionally regulated by TH and identified by IPA analysis for α-HBCD only; indicates dysregulation of TH homeostasis in the hippocampus. Depolarization-induced release of neurotransmitters and other secretions from nerve endings is triggered by the rapid entry of Ca^{2+} through voltage-sensitive channels. Thyroid hormones are sequestered by nerve terminals and can result in changes in behavior and mood. They may therefore be involved in modulating central synaptic transmission. These results suggest a novel mechanism of action for thyroid hormones in the brain.

Altogether, molecular changes by HBCDs suggest inhibition of LTP as a significant adverse effect in the brain. Our findings showed that HBCD exposure at a critical window of susceptibility during development may impair synaptic plasticity. This study increases our understanding of the effects of HBCD during developmental exposure and is the first *in vivo* study demonstrating the 1) potential mechanism involved in the HBCD learning and memory deficits and 2) HBCD stereoisomers effect differences in the brain, liver and serum in any mammalian system. Stereoisomer specific contribution within the proposed pathways disrupting LTP was reasonably independent from one another but also appeared to overlap to a large extent in the postsynaptic neuron. The similarities observed between α-HBCD and γ-HBCD may be a function of stereoisomerization. The CM-HBCD, which is 70-90% γ-HBCD, appeared to have different overall response than after the administration of γ-HBCD

in the hippocampus, liver and serum and can be partly attributed to the CM-HBCD having higher levels of other stereoisomers (α- and β-HBCD) along with other minor stereoisomers not present in the γ-HBCD dosing solution. Further comparisons will include that of CM-HBCD at the same dose as γ-HBCD (30mg/kg) and with that of α-HBCD at $1/10^{th}$ of that dose (3 mg/kg) as this ratio is closer to that in the commercial mixture. To understand how and explain why HBCDs can affect synaptic plasticity, further experimental studies should focus on the underlying mechanisms proposed here. Additional studies on the stereoisomer specific effect differences observed in this study can also help in identifying specific molecular mechanisms for assessing the risk of the individual HBCD stereoisomers.

However, it is clear that we are able to distinguish differences in response between stereoisomers of the CM-HBCD in the hippocampus, liver and serum four days after a single oral exposure at PND 10 mice. The mechanisms proposed in this chapter have promise to explain the disruption of the CNS initiated during development and manifesting in adulthood.

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Chapter VI

SUMMARY AND IMPACT

SUMMARY OF THIS DISSERTATION

The idea of flame retardant materials dates back to about 450 BC, when the Egyptians used alum to reduce the flammability of wood. The Romans (about 200 BC) used a mixture of alum and vinegar to reduce combustibility (Hendersinn et al., 1990). Today there are more than hundreds of chemicals used as flame retardants. The halogenated organics have caused public health concern in the 20th century with their continued consumer use and high levels in people combined with the lack of knowledge regarding their effects and fate (Alaee et al., 2003).

The use of brominated flame retardants has increased tremendously during the last decades mainly for two reasons: 1) the need to meet strict fire safety regulations; and 2) the high demand for petroleum-based polymeric materials, which have to meet those same fire safety regulations.

Recent public health concern for the brominated flame retardant HBCD has increased because of its high environmental persistence and detection in breast milk and human serum. Limited toxicology studies suggest that CM-HBCD is a developmental, reproductive, and neurotoxicant, as well as an endocrine disrupter; however, little else is known about the overall effects of this chemical. In wildlife and humans, the high prevalence of the γ-HBCD diastereomer from the commercial mixture shifts to a dominance of the α-HBCD diastereomer. The mechanism of this is unclear. As there are currently no whole animal studies investigating the individual stereoisomers, it is difficult to determine whether this is

driven by biotransformation and/or bioaccumulation. In order to assess the human health risk of HBCD, the individual diastreomers need to be studied for the link between exposure, dose, and response to be understood.

The research presented in this dissertation specifically addresses this need for toxicokinetic data. The thrust and novelty of this research rest on the examination of two different stereoisomers of HBCD. We hypothesized that these isomers differed in pharmacokinetic and toxicological properties and moreover could interconvert, thus conclusions based on testing the commercially-available mixture might not adequately predict the outcome of real-life exposures. To test this hypothesis and provide information essential to elucidating the diastereomer shift while adding valuable information for the human health risk assessment of HBCD, specific aims included: (1) characterize basic toxicokinetics in adult female mice; (3) investigate the impact of repeated dosing on the distribution and elimination of α-HBCD and γ-HBCD in adult female mice; (4) investigate the distribution and elimination of α-HBCD and γ-HBCD in infantile mice, a developmental model; and (5) identify mechanisms leading to the reported developmental neurotoxicity after exposure to CM-HBCD and further determine whether that toxicity is driven by α-HBCD and/or γ-HBCD.

Toxicokinetics of γ-HBCD *in Female Mice*

Because previous unpublished industry toxicokinetic studies have only been performed on the CM-HBCD and are conflicting, the main diastereomer present in the commercial mixture, γ-HBCD, was studied in an effort to fully characterize absorption, distribution, and elimination parameters following a single dose with respect to dose, time, and route of exposure in adult female C57BL/6 mice. Over 85% of the administered dose was absorbed following oral administration. Liver was the major depot four-days after treatment followed by blood, fat and then brain. However, γ-HBCD was rapidly metabolized and eliminated in the urine and feces. For the first time, *in vivo* stereoisomerization was observed of the γ-diastereoisomer to the β-diastereoisomer in liver and brain tissues, and to the α- and β-diastereoisomer in feces. Polar metabolites in the blood and urine were a major factor in determining the initial whole-body half-life (1 day) after a single oral exposure. Elimination, both whole-body and from individual tissues, was biphasic. Initial half-lives were approximately 1 day, whereas terminal half-lives were up to 4 days, suggesting only limited potential for γ-diastereoisomer bioaccumulation. The toxicokinetic behavior reported here has important implications for extrapolation of toxicological studies of CM-HBCD to the assessment of HBCD risk.

Toxicokinetics of α-HBCD *in Female Mice*

Since α-HBCD constitutes the minority of the commercial mixture (<10%) but is the dominant diastereomer detected in biota (invertebrates, fish, birds, marine mammals, and

humans), this study was conducted in an effort to fully characterize absorption, distribution, and elimination parameters following a single treatment with respect to dose, time, and route of exposure in adult female C57BL/6 mice after administration of α-HBCD. Results indicate that approximately 90% of the administered dose was absorbed after an oral exposure. Disposition was 1) dictated by lipophilicity as adipose, liver, muscle and skin were major depots and 2) was dose-dependent with non-linear accumulation at higher doses. Elimination, both whole-body and from individual tissues, was biphasic. A-HBCD derived radioactivity was excreted in the feces as parent and metabolites whereas urine only contained metabolites. Presence of polar metabolites in the blood and urine were a major factor in determining the rapid initial whole-body half-life after a single oral exposure. Initial half-lives were ~1-3 days, but much longer terminal half-lives of 17 days were observed, suggesting the potential for α-HBCD bioaccumulation. Stereoisomerization previously observed after exposure to γ–HBCD was not seen after exposure of α-HBCD. The toxicokinetic behavior reported here has important implications for extrapolation of toxicological studies of CM-HBCD to assessment of risk of α-HBCD, the major stereoisomer found in wildlife and people.

Repeated Dosing on the Disposition of α-HBCD and γ-HBCD

This was the first study to investigate toxic kinetic parameters following repeated exposure to α-HBCD and γ-HBCD. The disposition and elimination of α-HBCD and γ-

HBCD was monitored in adult female C57BL/6 mice for four days following ten consecutive 3.0 mg/kg oral doses and then compared with results from our previous studies (Chapter II and III, Sabot *et al.* 2010 and 2011). The results suggest greater retention of α-HBCD in developing mice than in adults with non-linear disposition patterns following repeated exposure. Repeated exposure to α-HBCD results in higher concentrations remaining in adipose tissue which demonstrates its potential for bioaccumulation. Data also suggest that elimination of α-HBCD may be decreased following repeated exposure. This was not seen after repeated exposure to γ-HBCD where disposition was dose-independent and didn't significantly change after 10 days of exposure. These results, in combination with evidence of α-HBCD's persistence and the toxicity of the commercial mixture, underlie the need to further understand α-HBCD and γ-HBCD toxic kinetics across species under steady state conditions.

Disposition and Elimination of α-HBCD and γ-HBCD in a Developmental Mouse Model

The majority of public health concern has focused on potential hazardous effects resulting from exposure to infants and young children because of studies reporting adverse effects in rodent studies following exposure during development in combination with human exposure estimates suggesting that nursing infants and young children have the highest exposure to HBCD. This study was designed to compare differences in disposition of both α-HBCD and γ-HBCD in infantile mice reported to be susceptible to the developmental neurotoxicity of the HBCD commercial mixture.

The results show that the toxic kinetics of both α-HBCD and γ-HBCD are different in developing mice than in adult mice; while tissue distribution patterns are similar, concentrations of both α-HBCD and γ-HBCD derived radioactivity are higher in pups. Developing mice exposed to α-HBCD had an overall higher body burden than γ-HBCD at every time point measured 4 days post exposure, however total body burden differences between age groups were greater after exposure to γ-HBCD than for α-HBCD. These differences lead to higher concentrations of both HBCD diastereomers at target tissues during critical windows of development. Because CM-HBCD, and other organo-halogenated compounds (PCBs, PBDEs, etc.), have demonstrated toxicity during development, it is essential to understand the kinetic parameters in order to accurately describe the dose available to target tissues resulting from exposure, and to more accurately assess risk to human health. This study suggests that age may be a risk factor for the harmful effects of both HBCD diastereomers when developing animals may have increased susceptibility.

Molecular Mechanisms of Developmental Neurotoxicity in Mice

CM-HBCD has been shown to cause adverse effects on learning and behavior in mice (Eriksson et al., 2006). We propose that CM-HBCD may alter similar molecular mechanisms responsible for learning and memory as seen for PBDEs and PCBs. Our objective was to investigate the molecular mechanistic effects of the CM-HBCD on gene transcription in the hippocampus of developing mice. Furthermore as the toxiokinetic differences of α-HBCD

and γ-HBCD in adult (Szabo et al., 2010; Szabo et al., 2011) and developing mice (Szabo et al., 2011; submitted) were observed, we hypothesize that α-HBCD and γ-HBCD will have different biological effects *in vivo*. The second objective was to investigate whether the individual HBCD stereoisomers, α-HBCD and/or γ-HBCD, may be responsible for driving the behavioral developmental neurotoxicity observed after exposure to CM-HBCD.

We found that neonatal exposure to CM-HBCD causes effects on gene expression in the developing mouse hippocampus. The molecular changes support inhibition of long term potentiation (LTP), a process involved in learning and memory, leading to synapse weakening in the hippocampus during development. Furthermore, LTP disruption was observed for all treatment groups: α-HBCD, γ-HBCD, and CM-HBCD. Our results suggest six major cellular pathways may be disrupted following HBCD exposure: (I) decreases in intracellular Ca^{2+} and Na^{+}; (II) decreased cyclic AMP (cAMP); (III) synapse weakening via decreases in NO signaling, AMPAR insertion, receptor conductance, and probability of depolarization; (IV) decreased integrity of the blood brain barrier; (V) activation of pro-inflammatory microglia; and (VI) reduction of T_3 to target neurons. Altogether, molecular changes by HBCD, regardless of stereoisomer exposure, support inhibition of LTP as a significant adverse effect in the brain.

As stereoisomerization of γ-HBCD to β- and α-HBCD occurs in mice (Szabo et al., 2010); it is difficult to predict whether α, β, or γ is driving the neurotoxicity since genes

involved in long term potentiation were altered with each treatment (α-, γ-, or CM-HBCD). However, when we consider that α-HBCD does not stereoisomerize in mice (Szabo et al., 2011), we suggest that it is α-HBCD which may be driving the behavioral developmental neurotoxicity previously observed (Eriksson et al., 2006).

Overall Conclusions

In adult female mice, α-HBCD and γ-HBCD are both well absorbed; distribution is dictated by lipophilicity for α-HBCD but not for γ-HBCD. γ-HBCD was readily metabolized and eliminated with a terminal half- life of 4 days while α-HBCD was more biologically persistent with a terminal half-life of 17 days. Repeated exposure resulted in higher body burdens than a single exposure alone only after administration of α-HBCD and not γ-HBCD, demonstrating the potential for α-HBCD bioaccumulation. *In vivo* stereoisomerization was observed converting the orally administered γ-diastereoisomer to the β-diastereoisomer in liver and brain tissues, and to the α- and β-diastereoisomer in feces. The analytical techniques used exclude the possibility of thermal isomerism of HBCD. Stereoisomerization was not observed after exposure to α-HBCD in any tissue examined. The toxicokinetics of α-HBCD and γ-HBCD in young animals, which have been shown to be susceptible to toxicity after exposure to CM-HBCD, are different than adults. While tissue distribution is similar to adults, actual concentrations are higher in younger animals likely due to a reduced ability to metabolize and eliminate HBCD. Efforts were taken to characterize the molecular

mechanisms leading to developmental neurotoxicity in these younger mice with a higher body burden of α-HBCD and γ-HBCD. The molecular changes observed in the hippocampus support inhibition of long term potentiation (LTP) and were observed after oral exposure to, α-HBCD, γ-HBCD, and CM-HBCD.

Impact of this dissertation

Motivation for research in this dissertation was focused on the α-HBCD stereoisomer because it is the major HBCD stereoisomer found in most human and environmental biota despite its relatively minor role in commercial production and usage. The dominance of α-HBCD may be attributed to fate, transport, and exposure from the literature, and from our toxicokinetic work – it is biologically persistent and resists stereoisomerization. A wealth of data is continually becoming available demonstrating the predominance of α-HBCD in higher trophic organism, top of the food chain animals, and in human samples including blood, breast milk, and adipose tissue (previously described in the introduction section). Developing animals appear to be more susceptible than adults, both as to the window of sensitivity and to age-related toxicokinetic differences. Thus, this research provides support to the theory that daily exposure to high levels of α-HBCD from our environment are likely responsible for high body burdens observed in humans, although exposure and biotransformation of other stereoisomers may also contribute.

We observed the kinetics and effects of α-HBCD and γ-HBCD in mice. Although informative there are limitations of these studies. Female mice were used as they were observed to be more sensitive to thyroid disruption and enzyme induction; however the inclusion of male mice could help elucidate possible effect differences between sexes. Furthermore, our effects studies in the brain, liver and serum were only after exposure to α-HBCD and γ-HBCD, but not β-HBCD, which would be an interesting follow-up study since we have learned that β-HBCD, can be detected in the brain after exposure to purified γ-HBCD. In addition, these effect studies could be examined using a repeated dosing paradigm as opposed to a single exposure to aid in elucidating possible pathways. Different relevant routes of exposure can aid in understanding the behavior of HBCD stereoisomers, such as dermal and inhalation, which are currently missing from the literature.

There is an interesting paradox observed between the two stereoisomers, α- and γ-HBCD investigated in this dissertation. The most water-soluble isomer is α-HBCD, which is expected to be more rapidly excreted, but undergoes less metabolism and has a longer biological half-life, whereas the least water-soluble isomer γ-HBCD, expected to accumulate most in tissues undergoes the most metabolism and has a shorter biological half-life. Although, there are slight differences in the log K_{ow} of α- β- and γ-HBCD being 5.65, 6.05 and 6.34, respectively; they are all within the range of being highly lipophilic which favors the likelihood of partitioning into lipid rich tissues, as seen for other POP such as PCBs, PBDEs and dioxins. However, the differences in biological behavior observed between the

two stereoisomers may be due to the differences in 3-D structure of the stereoisomers which appear to make γ-HBCD more readily recognized by XMEs and therefore metabolized and eliminated as opposed to α-HBCD whose structure is more resistant to metabolism and therefore partitions into fat. The increase in the number of differentially expressed hippocampal genes and pathways identified after exposure to α-HBCD (as opposed to γ-HBCD) further elicits concerns for neurotoxicity from α-HBCD which is the dominant stereoisomer found in human food (fish, beef, pork, dairy, etc).

In comparison with the database for other brominated flame retardants such as PBDEs, there is limited information on the concentration of HBCD in human tissues (Covaci et al., 2006). Several studies have addressed the concentration of HBCD in human breast milk, serum and umbilical cord serum, raising concerns about potential lactational and prenatal exposure (see Section 3.2). The isomeric composition of HBCD has been studied in commercially available preparations, environmental media, and biological samples. α-HBCD is more prevalent in biological samples, but the γ isomer predominates in abiotic environmental samples. *In vitro* studies have explained the different isomeric occurrences based upon their metabolism via the cytochrome P450 system (Zegers et al., 2005; Weiss et al., 2006).

In combination, our toxicokinetic and toxicity studies raises concerns for humans about exposure to CM-HBCD, γ-HBCD, and α-HBCD as they all have elicited, to some

degree a response in mice. However, α-HBCD appears to be more resistant to metabolism than other stereoisomers in rats and in human *in vitro* cell culture, more biologically persistent, and elicited an increased number of differentially expressed gene changes in the hippocampus which are known to be involved in learning and memory deficits. As metabolic capabilities have been observed across different species (fish, rats, humans) and similar patterns of stereoisomers are observed across biota, with α-HBCD predominating (fish, marine mamals, rats, mice, human blood, and breast milk) the likelihood of similar effects being observed in humans as found here in mice is increased and suggest a risk from this class of chemical.

In combination, our toxicokinetic studies and toxicity studies raises concerns to humans for exposure to CM-HBCD, γ-HBCD, and α-HBCD as they all have elicited, to some degree, a toxic response in mice. However, α-HBCD appears to be more resistant to metabolism than other stereoisomers in rats and human *in vitro* cell culture, more biologically persistent, and elicited an increased number of differentially expressed gene changes in the hippocampus involved in learning and memory deficits. As the metabolic capabilities are observed across different species (fish, rats, humans) and similar patterns of stereoisomers are also observed across biota with α-HBCD predominating (fish, marine mammals, rats, mice, human blood and breast milk), the likelihood of similar effects being observed in humans as found here in mice increase validity and should be considered a risk of these class of compounds.

FUTURE DIRECTIONS

Toxicokinetics and Behavior

Considering β-HBCD was detected in the brain of adult mice administered γ-HBCD, both toxicokinetic and effects studies of this stereoisomer are warranted. To help explain and support the proposed LTP mechanism, it would be important to determine whether stereoisomerism also occurs in infantile mice as was seen in adults. Furthermore, Eriksson et al (2006) performed behavioral tests after the administration of CM-HBCD in mice. To further understand the differences between the stereoisomers and whether one or all isomers are driving the developmental neurotoxicity observed with CM-HBCD, future behavioral experiments with α-HBCD, β-HBCD and γ-HBCD should be conducted.

Development of a PBPK model for HBCD

Physiologically based pharmacokinetic (PBPK) models are mathematical descriptions of the physiological and biochemical processes involved in the absorption, distribution, metabolism, and excretion (ADME) of a xenobiotic. The physiological and biochemical characteristics of a given model can be altered to reflect species-specific parameters to provide a quantitative extrapolation across species. Furthermore, PBPK models can be used to describe exposure/dose relationships in developmental scenarios such as the sensitivity of

developing rodents to HBCD stereoisomers. By understanding the processes that determine tissue dose and chemical interactions with tissues, resulting biological responses can be predicted.

Currently, no PBPK models for HBCD or their stereoisomers are available. Studies examining windows of sensitivity for HBCD-induced developmental effects have focused on both gestational and postnatal exposure. We plan to collaborate with Dr. Claude Emond from the University of Montreal) to develop a preliminary PBPK model that can describe pharmacokinetic data from acute oral exposures to HBCD stereoisomers in mice. This model will be exersized to predict disposition dose, gender, and species as well as aiding in the characterization of HBCD disposition during development (i.e. maternal and fetal body burdens during critical windows of susceptibility). Such a model can then be used in the development of a PBPK model to predict concentrations in human health risk assessments.

Validation of Transcriptomics and Proteomics

Individual quantitative RT-PCR analyses are planned to be performed for a subset of relevant genes and pathways identified as affected by HBCD in order to further confirm microarray and, indirectly, proteomic data.

Dose/Response

A systems biology approach should be taken to examine the effects of HBCD on the liver, brain, and serum in infantile mice. This study used several large data gathering tools to evaluate the transcriptomics, proteomics, and metabolomics in a developmentally susceptible mouse model. The data presented in this dissertation was limited to data from our high dose groups, 30mg/kg dose. This was the maximal concentration used for analysis chosen based on previous changes observed in mice (van den Ven, 2008) and toxicokinetic data (Szabo et al., 2010, 2011a and 2011b). However, subsequently lower doses were also tested and plans to analyze them are in our future (3mg/kg and 10mg/kg). Dose/response relationships are key data in the risk assessment process. Determination of a reference dose and the use of benchmark dosing software can be generated from such data. Arguably, the future of risk assessment is to identify mechanistic data and incorporate such data into the process of decision making purposes. One goal is to use this data in the risk assessment process for HBCD currently ongoing at the EPA/NCEA.

Long Term Potentiation

The impact of HBCD on suppression of long term potentiation, identified in Chapter 5 of this dissertation, needs additional support. Electrophysiology is a method to record the synaptic transmission between two neurons. *In vivo* extracellular recording on hippocampus synapses and could help to confirm the molecular mechanism proposed in this thesis. Our

quantitative molecular data reported here in combination with functional neurophysiologic effects can provide strong functional support for the previously reported neurobehavioral effects (Eriksson et al. 2006) and is essential for characterization of the neurotoxic hazard of this brominated flame retardant, particularly for rational risk assessment, which is required in response to the general concern about the vulnerability of the developing brain.

Metabolomics - biomarkers and systems biology

Metabolomics is the most recent systems-biology approach, to complement the genomic and proteomic efforts, to characterize an entire biological system. To aid in predicting health risks of HBCD, metabolomic analysis of serum was performed to improve our ability to draw correlations between early life exposures and developmental outcomes. Since metabolites represent end products of the function of the genome and proteome, metabolomics holds the promise of providing an integrated physiological phenotype of a system. Such metabolic profiling involves a comprehensive and accurate measurement of the types and concentrations of metabolites in a system. This current investigation was conducted to reveal the impact of HBCD exposure (α-HBCD, γ-HBCD, or CM-HBCD) on the biochemical profiles of serum from 14 day old mice, four days following dosing. Metabolomics of serum from infantile mice exposed at PND 10 to α-HBCD, γ-HBCD or CM-HBCD could be used to reveal endogenous metabolites that distinguish the treatment

groups (α-HBCD, γ-HBCD or CM-HBCD) from one another four days after a single oral exposure. Metabolites significant to the separation of dose groups were identified in biochemical pathways and significant metabolic perturbations in *energy* and *amino acid metabolism* were found.

Efforts to identify the different stereoisomer effects and pathways are underway. Attempts to link serum small molecule changes to pathway changes observed in the transcriptomics and proteomics from the liver and hippocampus are planned. However, daunting of a task, we have begun and will continue in comparing the three data sets.

1) Phenylalanine was observed to decrease in every treatment group tested. Phenylalanine is an essential amino acid that is highly concentrated in the brain and blood. It is used by the brain to make norepinephrine, a neurotransmitter involved in synaptic transduction. Depletion of norepinephrine reduces long-term potentiation in the dentate gyrus of rat hippocampus (Stanton et al., 1985). Further research to understand the relationship between phenylalanine, norepinephrine, the hippocampus, and LTP is currently ongoing.

2) 3-hydroxybutyrate is a ketone body used as an energy source by the brain when blood glucose is low. It is essential during brain development and increased levels are needed to nourish the brain during times of fasting or energy deregulation. Hepatic molecular pathways involved in energy deregulation (glycerolipid metabolism and type II diabetes) were identified, specifically after exposure to α-HBCD.

One interesting area is to investigate the mechanism by which HBCD affect hepatic glucose/energy metabolism. There is an emerging hypothesis, based on data from several chemicals in animal studies, that the obesity epidemic could be due, at least in part, to chemical exposures during vulnerable windows of development, mainly *in utero* and the first few years of life (LaMerill and Birnbaum, 2011). Indeed, in animal models there are data showing that developmental exposure to endocrine disrupting chemicals such as tributyl tin, bisphenol A, organochlorine pesticides, air pollution, lead, diethylstilbestrol, perfluorooctanoic acid, monosodium glutamate, and nicotine can lead to increased weight gain later in life. Epidemiology studies report an increased risk of obesity and/or type 2 diabetes in women who were exposed to persistent organic pollutants (including PCBs, DDE, and dioxin) during pregnancy, and there are animal data linking bisphenol A and atrazine exposures to altered glucose tolerance and insulin resistance.

Two lines of evidence suggest that BFRs, including HBCD, may promote disruptions in metabolism that could lead to obesity. The first is epidemiological: a significant association

exists between persistent organic pollutants, including BFRs, and diabetes in sport fishermen and people residing near chemical waste sites (Turyk et al., 2009, Kouznetsova et al., 2007). The second is molecular: hepatocytes from rats treated with 28 days HBCD treated rats have altered hepatic gene expression, many of which are involved in glucose metabolism (Canton et al., 2008).

In Chapter 5 of this dissertation, we observed increases in small molecules involved in ketosis (elevated levels of the ketone body 3-hydroxybutyrate after exposure to α-HBCD, γ-HBCD, and CM-HBCD). 3-hydroxybutyrate is also associated with diabetes mellitus and other defects in carbohydrate metabolism. Hepatic genes involved in glycerolipid metabolism and Type II diabetes were altered after exposure to α-HBCD as observed in our transcriptomics and proteomics data. *Whether or not BFRs alter energy metabolism and promote obesity is unknown.* Previous research has shown that BFRs suppress serum levels of thyroid hormone and can cause mild weight gain in animals. Data suggest enhanced glucose metabolism occurs compared to controls, which can promote weight gain (Zurlo et al., 1990). Fat cells from PBDE treated rats have disrupted glucose and lipid metabolism (Hoppe et al., 2007). Future studies would extend our hepatic genomic and proteomic findings as well as our serum metabolomics data to investigate the liver and its role in energy metabolism and regulating the metabolism of macronutrients. Indeed, aberrations in liver glucose and lipid metabolism are a hallmark sign of obesity (Muoio et al., 2006). We demonstrate that metabolomics is a promising approach to determine biomarkers for examining the mechanistic link between levels of HBCD exposure and disease/dysfunction.

CONCLUDING REMARKS

Hexabromocyclododecanes (HBCD) are brominated flame retardants (BFRs) that belong to a large group of organohalogen chemicals. BFRs can be highly persistent, bioaccumulative, and cause adverse effects in humans and wildlife. Although some BFRs are banned or voluntarily withdrawn from usage by the manufacturers, emerging and existing BFRs continue to be used in industrialized countries. Because of their widespread use and large quantities in consumer products and household items, indoor contamination is proposed to be a significant source of human exposure. It is widely accepted that common exposure routes are oral – both via breast milk, animal fat-containing foods, and hand to mouth activity.

Recent epidemiological studies clearly indicate that BFRs can affect human health. The human health effects include alterations in thyroid hormone homeostasis, reproductive effects, and reduced psychomotor development index and IQ performance in children at school age. . Studies have also indicated that the daily exposure to PBDEs due to inhalation, oral ingestion from food and accidentally from indoor dust, plus dermal absorption was significantly higher than that of adults. Many rodent studies have confirmed that developmental exposure to these compounds is problematic. Studies in rodents indicated that both PBDEs and HBCD are developmental neurotoxicants affecting the nervous system. Several studies have also confirmed that the PBDEs and HBCDs are endocrine disruptors,

with the potential to disrupt male and female reproductive development including altered steroidogenic activities. This has been demonstrated for HBCD in several *in vivo* studies using rodent models and *in vitro* systems which determine effects on receptor binding and on steroidogenesis. The potential modes of action may be similar between HBCD and PBDEs, as well as similar between rodents and humans. This is extremely important to consider as there have been several recent reports of adverse health consequences associated with increased PBDE exposure in humans (Meeker *et al.*, 2009; Roze *et al*., 2009; Harley *et al.*, 2010; Herbstman *et al.*, 2010).

Further research is needed to determine the long-term adverse consequences of exposures to all BFRs, especially as current laboratory studies for HBCD are limited, with no adequate human studies published to date. In the near future, it can be predicted that after the s and withdrawl of PBDEs and HBCD, levels of each will still be detected in the environment and humans. Due to their persistence and toxic effects, new alternatives are being investigated. The legacy problem emphasizes the need for proper testing before manufacturing replacement chemicals, since these compounds may also bioaccumulate and can be transplacentally and lactationally transferred. However, it appears difficult to design BFRs which are more environmental friendly but also equally effective as flame retardants and cost-effective.

Clearly, it would be better to identify fire resistant materials that are not hazardous, but replacement of DecaBDE and HBCD by other flame retardants seems not yet feasible in

some applications. For this situation, a temporary solution could be to use only the minimum effective amount of BFRs in these consumer goods, e.g. electronics, furniture upholstery, thermal insulation. New global guidelines for using the minimum concentration needed of these BFRs for fire safety in consumer goods should be implemented. Such guidelines may ultimately reduce exposure levels worldwide and reduce the remarkable differences in exposures are now observed between different geographical regions.

In their provocative review, "Facing the challenge of data transfer from animal models to humans: the case of persistent organohalogens", Suvorov and Takser (2008) make the cogent argument, for scientists whose interest is in improving public health, that "studying the mechanisms of toxicity greatly increases the relative value of animal model studies" but that "knowledge of toxicity mechanisms impacts only slightly on policy making and regulatory action". Suvorov and Takser (2008) note that after 30 years of intensive research, all of the mechanisms by which PCBs are toxic are still not known, but that this shouldn't, and didn't, preclude a ban on PCBs in the 1970's. Further, they state that when conducting environmental chemicals research, it is critical to (1) employ doses that are relevant to human exposure levels, (2) screen compounds at the most sensitive stages of development, and (3) verify exposures by internal dose measurements. These basic hallmarks were applied to the study ofthe brominated flame retardant, HBCD as reflected in this dissertation.

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