Toxicokinetics of the Flame Retardant Hexabromocyclododecane Gamma: Effect of Dose, Timing, Route, Repeated Exposure, and Metabolism

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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 (3 mg/kg) was absorbed after po exposure. Disposition was dose independent and did not significantly change after 10 days of exposure. Liver was the major depot (< 0.3% of dose) 4 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 fat and 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 po 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.

Key Words: mouse; metabolism; diastereomer; pharmacokinetics; stereoisomerization; HBCD; ADME; risk assessment; persistent organic pollutant; brominated flame retardant.

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 16,700 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 (Hale et al., 2006; Law 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 der Ven et al., 2006). Recent studies have suggested that 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 1.1 ng/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

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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 (Schechter et al., 2010), especially for humans consuming large quantities of fish, which reportedly contains relatively high HBCD levels from 9 to 1110 ng/g lipid (Janák et al., 2005; Xian et al., 2007). Besides diet, house dust is likely another important pathway of human exposure to HBCD because of high levels present indoors (Roosens et al., 2009). Nondietary 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 and Takaro, 2007).

Commercial HBCD is a mixture of different 1,2,5,6,9,10-hexabromocyclododecane diastereoisomers (Fig. 1). Previous research has focused 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). 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 (Barantoni et al., 2001). Differences in water solubility are found for α-, β-, and γ-HBCDs (48.8, 14.7, and 2.1 μg/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 physicochemical 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, whereas the α-diastereoisomer was not (Zegers et al., 2005). Results from this study further suggest the occurrence of cytochrome P450–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

[14C]-hexabromocyclododecane (HBCD) (2 mCi/mmol) was purchased from American Radiochemicals Corporation (St Louis, MO) as a mixture of β- and γ-diastereoisomer and [14C]-hexabromocyclododecane gamma (γ-[14C]HBCD) was purified at the U.S. Department of Agriculture Agricultural Research Service 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 β. The γ-[14C]HBCD was 98% radiochemically and diastereomerically pure as determined by thin-layer chromatography (TLC) using silica gel plates (250 mm; Whatman Laboratory Division, 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 C18 column (2.1 × 100 mm) (Waters, Beverly, MA) (details below). Unlabeled γ-HBCD was generously provided by Wellington Laboratories (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-[14C]HBCD (van der Ven et al., 2006; Law et al., 2008). A stock solution of γ-[14C]HBCD was made by dissolving 19.23 mg of γ-[14C]HBCD (3.12 μCi/mg) in toluene (400 μl). Aliquots were used directly from this solution for all dosing regimens. All dosing solutions were subjected to pre- and postdosing radioactivity examination to ensure proper delivery of dose. All solutions were designed to deliver approximately 0.2 μCi to each mouse. Unlabeled γ-HBCD was added to the γ-[14C]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 iv treatment, 10 ml of stock γ-[14C]HBCD was allowed to evaporate in an amber vial, and the γ-[14C]HBCD was resuspended in 95% ethanol followed by Emulphor. Deionized 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 g) were obtained from Charles River Breeding Laboratories (Raleigh, NC). Mice were chosen for these experiments because...
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). 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-$\mu$L 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 three 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 three volumes of hexane, ethyl acetate, and methanol. Samples were vortexed for 5 min, and the top organic layer was removed. Brains and adipose tissue (fat) were homogenized by hand in 2 ml of hexane:acetone (1:3.5). Two milliliters of hexane:ethanol (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 $\gamma$-$^{14}$C$-$HBCD and its metabolites in the experiments. Liquid scintillation counting was used to analyze for total $^{14}$C, whereas TLC was used to differentiate parent from metabolites of $\gamma$-$^{14}$C$-$HBCD based on polarity. Liquid scintillation counting and TLC had limits of detection (LODs) of 6.7 ng and 0.6 $\mu$g, respectively. 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. LC-MS was also used to analyze selected samples and had an LOD of 0.3 ng HBCD. Liquid scintillation counting was done using a 1900CA Scintillation Counter (Packard), and TLC analysis was done using a System 2000 Imaging Scanner (Bioscan). TLC was conducted using silicagel plates (250 mm; Whatman Laboratory Division) and a (50:50) methylene chloride:methanol mobile phase. Brain and fat 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 (40 cm height by 1 cm 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 0.5 ml/min. Fractions were individually assayed by Packard 1900CA Liquid Scintillation Counter. Fat extracts were further applied onto acid silica columns constructed in-house with 60 mesh silica gel (EMT, Gibbstown, NJ) and 40% (wt:wt) sulfuric acid (Baker, Phillipsburg, NJ). Extracts were eluted with hexane, followed by 50:50 mixture of hexane:methylene chloride. 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) equipped with a Symmetry C18 column (2.1 x 100 mm) and guard column (2.1 x 10 mm) and a quadrupole time-of-flight mass spectrometer (Waters Q-TOF Ultima API-MS; Waters). Isocratic elution conditions were used which consisted of 15% aqueous 10mM NH$_4$OAc and 85% methanol: acetoneitrile (80:20 vol/vol) in 10mM NH$_4$OAc. The flow rate of the mobile phase was 0.3 ml/min. 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 percent tissue disposition data refer to the mean of all data collected from the several dose and time phases in which (1) animals were exposed orally with a 3 mg/kg dose and (2) 4-day time points were available. Intergroup comparisons for dose and tissue levels were performed by a two-way ANOVA followed by Bonferroni posttests significant when $p < 0.05$. All data are presented as mean ± SD (Prism 5.0 is distributed by GraphPad Software, Inc., La Jolla, CA). Heane Scientific Software (Melbourne, Australia) was used for half-life calculations, to calculate the percent dose and statistical analysis.

RESULTS

Dose Response

Tissue distribution results as a percent of $\gamma$-$^{14}$C$-$HBCD–derived radioactivity in female C57BL/6 mice are presented in Table 1. The Table 1A represents the dose-response (where response is a function of disposition) after administration of a single and repeated po dose of $\gamma$-$^{14}$C$-$HBCD at a concentration of 3, 10, 30, or 100 mg/kg. Tissue distribution was analyzed 4 days after the administration of $\gamma$-$^{14}$C$-$HBCD. All tissues examined had low but measurable levels 4 days after dosing. We found tissue disposition to be independent of dose for the 3, 10, 30, and 100 mg/kg doses. The largest percentage of the dose in the mice was localized in the liver and ranged between 0.21 and 0.29%. This was followed by skin (0.14–0.17%) and muscle (0.09–0.10%). Low levels were detected in the blood (0.06–0.09%), brain (0.01%), and then fat (0.003–0.005%). These results also demonstrate the absence of tissue-specific sequestration.

Repeated Exposure

Tissue disposition was not altered after a 10-day repeated exposure in all tissues except for adipose and blood (Table 1A). Disposition was significantly increased between a single and
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<th>Table 1: Disposition of $\gamma$-HBCD in Mice</th>
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**Note:** Disposition of $\gamma$-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) four days following a 3 mg/kg dose through iv or po and (C) at multiple time points following a single 3 mg/kg po dose. 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 ($p < 0.05$).
repeated po exposure of 3 mg/kg $\gamma$-[14C]HBCD in the adipose tissue ($0.005 \pm 0.002\%$ vs. $0.010 \pm 0.001\%$, respectively) and in the blood ($0.081 \pm 0.005\%$ vs. $0.134 \pm 0.02\%$).

**Time Course**

From the dose-response study, the lowest dose was chosen (3 mg/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 1B). This 14-day time course study shows a biphasic profile with an initial steep decline on average from 1 h to 2 days and a less steep decline between 2 and 14 days for liver, kidney, blood, and brain (Fig. 2). Blood and liver tissue had the highest observable levels where maximum concentrations were measured at 1 h. Although most tissues had highest measured levels at 3 h, $\gamma$-[14C]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 po exposure to γ-[14C]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 h after po administration, 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.

**IV versus Oral: Tissue Disposition**

Environmental chemicals are rarely encountered by an iv route. However, by comparing the tissue disposition between iv and po routes, we are able to determine the percentage absorbed through the gut. To address gut absorption, a dose identical to the po 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 po and iv dose is shown in Table 1C. The percent dose and concentration (ng/g) were similar between both routes, but slight differences were observed. Although every tissue measured had higher levels after iv as compared with the po route of exposure (same values for brain), not all were statistically significant. There were slight increases in the iv as compared with the po route of exposure. Statistically significant differences are listed here as a percent of the po to iv levels: liver (83%), muscle (82%), kidney (83%), and blood (85%). The higher levels found in these tissues after iv administration implies an po absorption efficiency of 83 ± 2%. For this compound, a 4-day postexposure 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.

**Elimination**

The elimination of γ-[14C]HBCD–derived radioactivity was analyzed by daily collection of urine and feces from individual animals held from 1 to 14 days posttreatment. Total cumulative elimination after po administration in urine and feces is shown in Figure 3 for the four treatment levels. 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 14C, including that recovered from urine and feces, was 80.1 ± 4.2%. Elimination of 14C-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 (Fig. 3). Significantly less was eliminated on the second day, averaging 5% of the dose across all dose groups. By day 5, a total of 50% had been eliminated in the feces.

**IV versus Oral: Elimination**

Figure 4 compares the percent of dose in the urine and feces over four days. Excreta profiles are consistent between iv and po 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% of the dose was eliminated in the feces following po exposure and 51% following iv, an approximate 8% difference in the two routes.

The amount of 14C-derived radioactivity in the urine eliminated on the first day is relatively constant between the iv and po routes of administrations. This implies that γ-[14C]HBCD–derived radioactivity was absorbed and eliminated similarly after exposure by these two routes. The iv route of exposure had significantly higher levels of 14C-derived radioactivity in the urine than po at days 1, 3, and 4. This change reflects slightly increased po 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 po dosed animals.
Cumulatively, 31% of the dose was eliminated in the urine following exposure following iv treatment, whereas 26% was eliminated after the po route in four days. Based on fecal and urinary elimination patterns, comparison between the iv and po routes indicate that γ-[14C]HBCD may be well absorbed (85 ± 3%) orally. This agrees with the difference found in tissues deposition when comparing the iv and po 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%) that is the most likely unabsorbed material.

The TLC

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

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-[14C]HBCD–derived radioactivity was extractable from the excreted feces. This contrasts with essentially complete extraction of γ-[14C]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 HBCD diastereoisomers, α-, β-, and γ-[14C]HBCD, were spotted on TLC plates and two bands with baseline resolution formed. In this system, β-[14C]HBCD band migrated on a TLC with a shorter retention (Rf = 0.50) value than α- and γ-[14C]HBCD which comigrated as a second band with an 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 are expected. These fecal co-extractables slightly impede migration on the TLC plate.

Liver extracts were analyzed near the maximum observed concentration, 3 h. 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 Rf values to the two standard peaks (Rf = 0.50 and 0.55).
The GPC

The nature of the $\gamma$-$^{[14]}$C]HBCD–derived radioactivity in the brain and fat was initially examined by 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 migrate at the same rate. GPC chromatograms revealed one major peak (data not shown) that was further analyzed using LC-MS.

The LC-MS

Because of the inability of TLC to resolve $\alpha$- or $\gamma$-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 $\gamma$-HBCD was present and (2) if it had been converted to $\alpha$- or $\beta$-HBCD. This was performed by comparing the TLC bands or GPC fraction to LC-MS standards, $\alpha$-, $\beta$-, and $\gamma$-HBCD with retention times of 3.40, 4.06, and 4.89 min, respectively (Fig. 6). The TLC peaks in the liver ($R_f = 0.50$ and 0.61) and fecal extracts ($R_f = 0.44$ 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 $R_f = 0.50$ and 0.61 had retention times of 4.89 and 4.23 min, similar to $\gamma$-HBCD and $\beta$-HBCD, respectively. In the fecal extracts, TLC peak 0.44 had a retention time of 4.06 min,
which corresponds to \( \beta \)-HBCD; \( Rf = 0.54 \) resolved into two LC-MS peaks with retention times of 3.40 and 4.89 min, which corresponds to \( \alpha \)-HBCD and \( \gamma \)-HBCD, respectively. All three diastereoisomers, \( \alpha \), \( \beta \) and \( \gamma \)-HBCD, were detected in the feces 1 day postoral administration of \( \gamma \)-\([14C]HBCD \) (Fig. 6). However, in the liver, we were only able to detect the presence of \( \gamma \)-HBCD and \( \beta \)-HBCD, 3 h postoral administration of \( \gamma \)-\([14C]HBCD \). The GPC fraction of the fat on the LC-MS had molecular weight of 641 and retention times that matched all three stereoisomers, \( \gamma \)-HBCD, \( \beta \)-HBCD, and \( \alpha \)-HBCD. The brain extract had a molecular weight of 641 and a retention time that matched that of the \( \beta \)-HBCD standard only.

Summary of the parent, stereoisomer, and metabolite profiles in tissues, biological matrices, and excreta after exposure to \( \gamma \)-\([14C]HBCD \) can be 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. Stereoisomerized products were also detected in the fat (16%) and brain (100%). Low levels of the parent \( \gamma \)-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 \( \gamma \)-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 that may result in differences in pharmacokinetics, metabolism, and biological response (Covaci et al., 2006; Hunziker et al., 2004). It is therefore essential to understand the factors involved in human health risk for individual diastereoisomers of HBCD. \( \gamma \)-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 \( \alpha \)-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 \( \gamma \)-HBCD in female mice to better evaluate the toxicity and behavior of this compound. This study suggests that approximately 85% of an po dose of \( \gamma \)-HBCD was absorbed from the gut and was dose independent. This was based on the po and iv routes of administration having similar tissue and excreta concentrations of \( \gamma \)-[\(^{14}\)C]HBCD–derived radioactivity. 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 \( \gamma \)-[\(^{14}\)C]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 \( \gamma \)-HBCD exposure.

The primary route of \( \gamma \)-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 \( \gamma \)-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 \( \gamma \)-[\(^{14}\)C]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, that is, parent \( \gamma \)-HBCD and metabolites. The radioactivity patterns present in the liver and feces were similar with the majority 94–96%, being detected as metabolites, and

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<td>Bile</td>
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<td>0%</td>
<td>0%</td>
<td>100%</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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, whereas the three major diastereoisomers, that is, α-, β-, and γ-HBCD were present in the fat and feces. These preliminary results suggest that intestinal flora may alter the biliary metabolites because 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). Because the HBCD-derived radioactivity eliminated in the urine, bile and blood were in the form of polar metabolites, and storage of HBCD metabolites in the tissues was negligible, the more rapid clearance of HBCD when compared with 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 po exposure of γ-HBCD. These metabolites were found in the liver, blood, bile, urine, and feces between 3 and 24 h postexposure. Although, identification of these metabolites is beyond the scope of this report, previous reports have identified metabolites after po administration of the HBCD commercial mixture. One study identified four metabolites of HBCD of unknown structure in rats (Yu and Atallah, 1980; cited in KEMI, 2003). Another study identified four different groups of hydroxylated HBCD metabolites in rats after exposure to the commercial HBCD mixture (Brandsma et al., 2009). An in vitro study showed that 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 (Huhtala et al., 2006). Degradation/metabolite products have been observed in office dust samples, that is, 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 indicate that 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-HBCD is occurring. Furthermore, the stereoisomerization seen is rapid. Stereoisomerization is also supported by work previously reported in nonmammalian species such as fish (Law et al., 2006). In vitro experiments with rat and harbor seal microsomes have showed that biotransformation of β-HBCD and γ-HBCD was faster than that of α-HBCD (Zegers et al., 2005). The significant increase in adipose tissue deposition after 10 days of repeated po exposure may be a result of conversion to the more persistent stereoisomer alpha. 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 that 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 stereisomer profiles in biota.

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**REFERENCES**
