THE DISPOSITION OF BETA-HEXABROMOCYCLODODECANE (HBCD) IN MICE

Sanders JM, Knudsen GA, Birnbaum LS*

National Cancer Institute, Center for Cancer Research, Laboratory of Toxicology and Toxicokinetics, PO Box 12233, Research Triangle Park, NC 27709 USA

Introduction

1,2,5,6,9,10-Hexabromocyclododecane (HBCD) is a high production volume cycloaliphatic used as an additive flame retardant in polystyrene foam building materials and in some textile and electronic products[1]. Three pairs of diastereomeric HBCD enantiomers, designated as alpha (α)-, beta (β)-, and gamma (γ)-HBCD, predominate in technical grade products[2]. The isomeric distribution of a typical HBCD flame retardant product was reported to be approximately 12% α□HBCD, 6% β-HBCD, and 82% γ-HBCD[2]. HBCD is persistent and accumulating in abiotic and biotic environments, and therefore is of toxicological concern[3]. Studies of the isomeric mixture indicate that HBCD may be a developmental neurotoxicant and endocrine disruptor in rodents[4,5,6]. The mechanism of toxicity may involve perturbation of thyroid homeostasis. Humans may be at risk from exposure to HBCD in indoor dust and food products[7,8]. As expected, γ-HBCD is the predominant isomer detected in most abiotic samples; however, α-HBCD is generally the isomer of greatest concentration detected in humans and wildlife[3,9]. These differential profiles indicate that characterization of the *in vivo* fate of individual HBCD isomers is critical to assessing exposure risk to the mixture. To date, studies have shown that the fate of α-HBCD in female C57BL/6 mice differs from that of γ-HBCD[10,11]. Oral doses of [^{14}C]-labeled α-HBCD were less metabolized and eliminated, and retained at much higher concentrations in tissues over time than similar [^{14}C]-labeled doses of γ-HBCD[10,11]. γ-HBCD may isomerize thermally to α-HBCD, and isomerization of γ-HBCD to α-, as well as β-HBCD, was observed in mice[10,11]. In contrast, *in vivo* isomerization was not detected for α-HBCD[11]. Together, these results may explain the predominance of the α isomer in biota. Exposure to the third major isomer, β-HBCD, may involve direct contact in the environment and/or *in vivo* conversion from γ-HBCD. β-HBCD was detected in brain, liver, adipose, and feces of mice receiving oral doses of γ-HBCD[10]. Toxicokinetic data for β-HBCD are lacking and the toxicological implications of exposure are unknown. Therefore, the objective of the present study was to determine the *in vivo* fate of β-HBCD in the rodent model used in toxicokinetic studies of α- and γ-HBCD. These data are needed to support toxicological evaluations and risk assessment of HBCD isomeric mixtures.

Materials and methods

Chemicals and dosing solutions: [^{14}C]-Labeled and unlabeled β-HBCD used in the present experiments were prepared and provided by Drs. Heldur Hakk and Janice Huwe of ARS/USDA, Fargo ND. [^{14}C]-Labeled β-HBCD was obtained by separation, as described by Szabo *et al.*[10], of a mixture of [^{14}C] β- and γ-HBCD obtained from American Radiochemicals Corporation (St Louis, MO). The specific activity and radiochemical purity were 2 mCi/mmol and >99%, respectively. The identity and purity (>99%) of unlabeled β-HBCD were determined using LC/MS/MS, with comparison to an authentic standard of β-HBCD obtained from Wellington (Guelph, Ontario, Canada). Dosing solutions were formulated to administer approximately 10 μCi/kg/mouse and contained amounts of non-labeled β-HBCD for dose delivery ranging from 3 to 100 mg/kg. β-HBCD dissolved in toluene was added to a corn oil vehicle for oral administration. The toluene was removed from the dosing solution via evaporation under a stream of N_2. For iv administration, β-HBCD in toluene was added to 1 part Cremophor EL (Sigma-Aldrich, St. Louis, MO). The toluene was evaporated as described previously and 1 part ethanol and 3 parts water were added to complete the dosing solution. Dose volumes were 2 and 5 ml/kg for iv-injected and gavaged mice, respectively.

Animals and treatment: Female C57BL/6 mice, obtained from Charles River (Raleigh, NC), were approximately 8 weeks old and 20 g at time of dosing. Dose response was investigated in mice (n=4/treatment group) receiving single oral administration of either 3, 30, or 100 mg/kg of β-HBCD. Following gavage, the

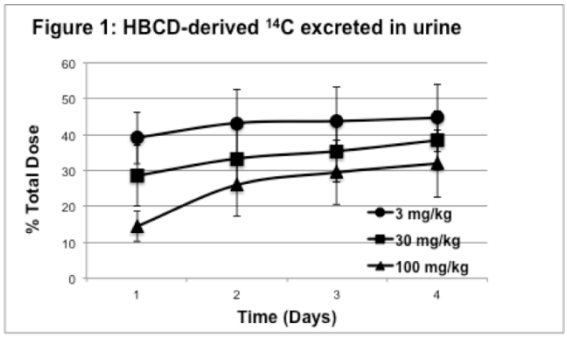
mice were housed individually in metabolism cages for daily collection of urine and feces, then sacrificed 4 days post-dosing. Distribution to, and clearance of [^{14}C] from tissues over time were investigated in treatment groups (n=4) receiving 3 mg/kg following sacrifice at timepoints of 1, 3, 8, and 24 h post-dosing. Urine and feces were collected from the 8- and 24-h mice. Three mice, sacrificed at 1 day post-dosing, received 3 mg/kg by tail-vein injection to provide comparative data for assessing the extent of absorption in gavaged mice. Food (NIH #31) and water were provided for *ad libitum* consumption. Euthanasia was by CO_2 asphyxiation. Blood was collected at time of death by cardiac puncture. Selected tissues were collected at necropsy.

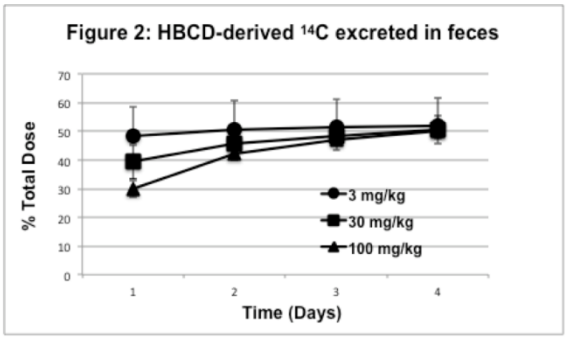
Analysis of [^{14}C] content in biological samples: The amount of β-HBCD-derived radioactivity in urine was determined by pipetting aliquots directly into vials containing Ecolume (MP Biomedicals, Solon, OH) for analysis in a liquid scintillation counter (LSC) (Beckman Coulter, Brea, CA). The [^{14}C] values from cage rinsates were added to those obtained for urine. The [^{14}C] content in tissues and feces was determined by oxidizing aliquots in a biological sample oxidizer (Perkin Elmer, Waltham, MA) prior to LSC analysis. Feces were dried, weighed and ground to a fine powder before combustion.

Analytical HPLC methods and sample preparation: The relative amounts of β-HBCD and its metabolites were determined using HPLC. The HPLC analysis was based on a method described previously[12]. The system consisted of a Waters (Milford, MA) 1525 binary pump coupled to a Waters 2487 dual absorbance detector and a IN/US β-RAM model 3 radiochemical detector (LabLogic; Brandon, FL). Samples were prepared as described below and separated using an Agilent Eclipse Plus C_{18} column (3.5 μm, 4.6 x 150 mm) with a Zorbax Reliance cartridge guard column. Mobile phases consisted of (A): 0.1% trifluoroacetic acid (TFA) in water and (B): methanol/water (80/20, v/v). A gradient of 80% A, reduced to 10% A in 2 min, held at 10% A for 33 min, then returned to initial conditions over 5 min was used. The flow rate throughout the run was 300 μl/min. For radiochemical analyses, Inflow ES scintillation cocktail (LabLogic) was used at a 3:1 ratio based on the manufacturer's recommendation. Breeze (v. 3.30, Waters) and Laura-Lite (v. 3.3.10.49, LabLogic) software packages were used for instrument control and data analyses. Prior to HPLC analysis, urine samples were filtered using non-sterile Ultrafree MC filter units (0.22 μm; Millipore; Billerica, MA) and centrifuged using an Eppendorf 5415 D centrifuge (Eppendorf; Hauppauge, NY) at 12700 RPM for 15 min. Dried fecal samples were weighed (approx. 100 μg), placed in Ultrafree MC cartridges, rehydrated with 400 μL water (containing 0.1% TFA), and centrifuged as above. This process was repeated twice more, with the filtrates transferred and pooled. Each fecal retentate was extracted with 95% ethanol (3 x 400 μl) and the filtrates transferred and pooled. Aqueous and organic extracts, maintained separately, were concentrated using a Savant SPD1010 SpeedVac (ThermoScientific; Waltham, MA) without heating, and aliquots were injected onto the HPLC. Extraction efficiencies were calculated to be approx. 70%.

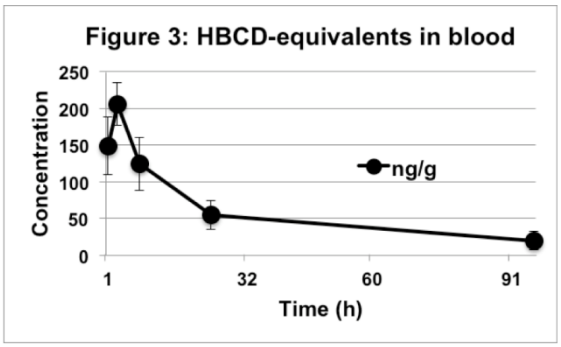
Results and discussion

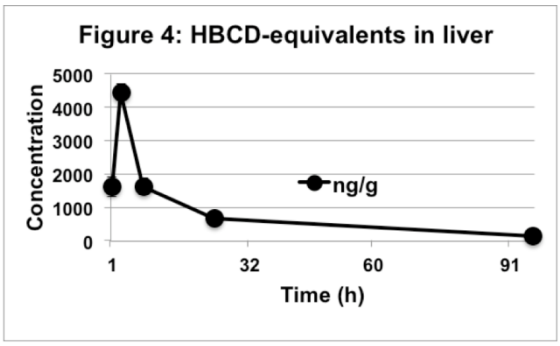
β-HBCD was rapidly absorbed from the GI tract of mice following gavage of 3 mg/kg. The extent of absorption was determined to be > 85% of the total dose, based on excreta data, feces extracts, and comparison to a similar iv dose. Approximately 90% of the total dose was excreted in urine and feces within 24 h following oral administration (Figures 1 and 2). Most of the remaining dose was excreted within 48 h, with little radioactivity remaining in tissues at the 4 day terminal timepoint. There was a dose-related trend toward delayed excretion of [^{14}C] in both urine and feces within 24 h of gavage (Figures 1 and 2). However, cumulative 4 day excretion was similar across the dosing range.





β-HBCD was absorbed into the blood (Figure 3) following oral administration of 3 mg/kg and distributed to tissues, e.g. liver, as shown in Figure 4. C_{max} for blood and liver was presumed to be between 1 and 3 h. The concentration of [^{14}C] in the liver at 3 h in these mice represented 7% of the total dose. The highest concentration of β-HBCD-derived radioactivity in the brain was observed at the 3 h timepoint (data not shown) and was similar to the concentration in blood. The concentration of [^{14}C] in assayed tissues, with the exception of adipose, was at or near (within 2-fold) background four days following gavage with either 3, 30, or 100 mg/kg (data not shown).





[^{14}C]-Labeled isomers of HBCD (α, β, γ) were separated by HPLC as shown in Panel A of Figure 5. The order of elution (α<γ<β) was found to be method and system specific. Organic extracts of feces from mice dosed with β-HBCD contained two [^{14}C]-labeled peaks co-eluting with γ- and β-HBCD standards (Figure 5, Panel B). The aqueous fraction of feces extract contained two [^{14}C]-labeled peaks (Figure 5, Panel C) with similar retention time (t_R) to that of radiolabeled peaks detected in urine samples (Figure 5, Panel D). Approximately 30% of the radioactivity contained in feces was non-extractable and was presumed to represent β-HBCD-derived metabolites. Less than 5% of the total dose was excreted as β-HBCD in feces. Neither β-HBCD, nor other HBCD isomers were detected in urine.

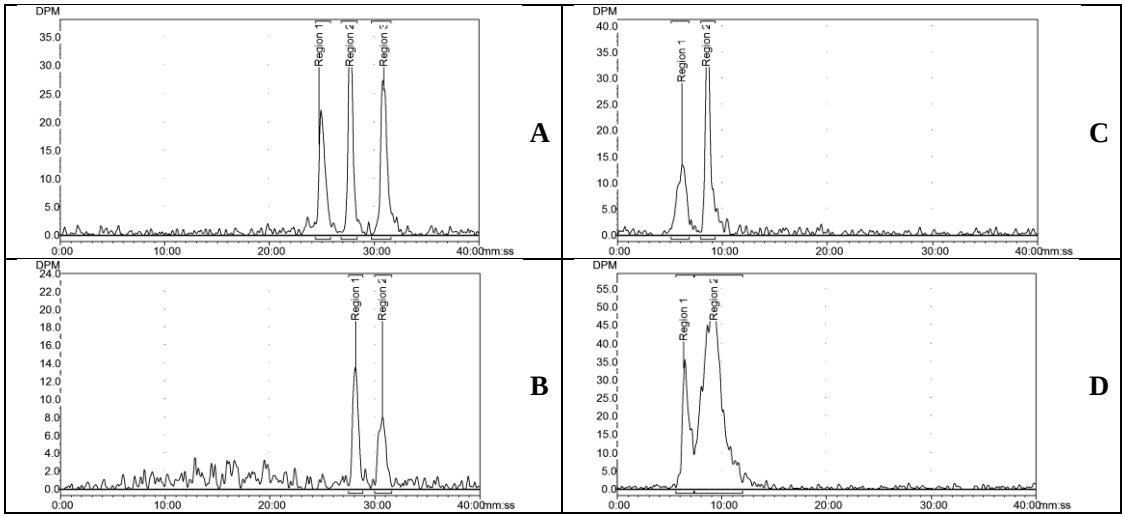


Figure 5. Representative HPLC radiochromatograms of: A) [^{14}C]-labeled HBCD isomers (α-HBCD, t_R = 24.8 min; γ-HBCD, t_R = 27.7 min; β-HBCD, t_R = 30.9 min). B) Organic phase of an extract of cumulative 24-h feces from a mouse gavaged with 3 mg/kg of β-HBCD. C) Aqueous phase of the feces extract. D) Cumulative 8-h urine from a mouse gavaged with 3 mg/kg β-HBCD.

In conclusion, the results from the present study demonstrated that β-HBCD was well absorbed, distributed to tissues, and rapidly excreted in urine and feces following oral administration to female mice. Administration of higher doses resulted in lower initial [^{14}C] excretion rates. β-HBCD was extensively metabolized, with evidence of limited *in vivo* isomerization to γ-HBCD. The disposition of β-HBCD in female mice most resembled that of γ-HBCD, not α-HBCD[10,11].

Acknowledgements
The authors thank Ms. Sherry Coulter and Mr. Abdella Sadik for excellent technical assistance. This research was supported by the Intramural Research Program of the NIH/NCI.

References:

1. Marvin CH, Tomy GT, Armitage JM, Arnot JA, McCarty L, Covaci A, Palace V. (2011) *Environ Sci Technol.* 45: 8613-23.
2. Heeb NV, Schweizer WB, Kohler M, Gerecke AC. (2005) *Chemosphere*. 61: 65-73.
3. Covaci A, Gerecke AC, Law RJ, Voorspoel S, Kokler M, Heeb NV, Leslie H, Allchin CR, de Boer J (2006) *Environ Sci Technol.* 40: 3679-88.
4. Eriksson P, Fischer C, Wallin M, Jakobsson E, Fredriksson A. (2006) *Environ Toxicol Pharmacol*. 21: 317-22.
5. van der Ven LTM, Verhoef A, van de Kuil T, Slob W, Leonards PEG, Visser TJ, Hamers T, Herlin M, Hakansson H, Olausson H, Piersma AH, Vos JG. (2006) *Toxicol Sci*. 94(2): 281-92.
6. van der Ven LTM, van de Kuil T, Leonards PEG, Slob W, Lilienthal H, Litens S, Herlin M, Hakansson H, Canton RF, van den Berg M, Visser TJ, van Loveren H, Vos JG, Piersma AH. (2009) *Toxicol Let*. 185(2): 51-62.
7. Abdallah MA, Harrad S, Covaci A. (2008) *Environ Sci Technol*. 42 : 6855-61.
8. Schecter A, Malik-Bass N, Harris TR, Malla S, Paepke O, Birnbaum L. (2011). *Organohalogen Compounds* 73: 346-7.
9. Morris S, Allchin CR, Zegers BN, Haftka JJH, Boon JP, Belpaire C, Leonards PEG, Van Leeuwen SPJ, Boer JD. (2004) *Environ Sci Technol*. 38:5497-504.
10. Szabo DT, Diliberto JJ, Hakk H, Huwe JK, Birnbaum LS. (2010) *Toxicol Sci*. 117(2) : 282-93.
11. Szabo DT, Diliberto JJ, Hakk H, Huwe JK, Birnbaum LS. (2011) *Toxicol Sci*. 121(2) : 234-44.
12. Yu Z, Peng P, Sheng G, Fu J. (2008) *J Chromatogr A.* 1190(1): 74-9.