METABOLISM OF α- AND γ-HEXABROMOCYCLODODECANE AND ENANTIOSELECTIVE FRACTIONS OF α-, β-, γ-ISOMERS IN MICE

Hakk H[1], Huwe JK[1], Szabo, DT[2], Diliberto, JJ[2], Birnbaum, LS[3]

[1] USDA-ARS Biosciences Research Laboratory, 1605 Albrecht Boulevard, Fargo, ND 58102-2765 USA.
[2]US-EPA. ORD, NHEERL, 109 TW Alexander Dr., Research Triangle Park, NC 27711 USA, [3]NIEHS & NCI, P.O. Box 12233, Research Triangle Park, NC 27709

Introduction

Hexabromocyclododecane (HBCD) is the third-highest production volume brominated flame retardant (BFR). It is incorporated into expanded polystyrene foam used in thermal insulation of buildings, and currently is the only suitable BFR for this application. HBCD is an additive flame retardant, which allows it to be released from consumer products and because of this is considered a ubiquitous contaminant in environmental media and biota. HBCD is an enzyme inducer[1], developmental neurotoxicant[2], and endocrine disruptor[3] in laboratory animals, and recent studies have suggested HBCD is also highly bioaccumulative[4]. Due to evidence of persistence and toxicity HBCD is currently being considered for a global ban under the framework of the Stockholm Convention on Persistent Organic Pollutants. Three major 1,2,5,6,9,10-hexabromocyclododecane diastereoisomers, denoted as alpha (α), beta (β), and gamma (γ), with the γ-diastereoisomer predominating (>70%), comprise the commercial HBCD mixture. Both β- and γ-HBCD can thermally convert to α-HBCD[5], and it is speculated that *in vivo* metabolism may also convert the γ-diastereoisomer to the α-diastereoisomer, which is the most prevalent HBCD diastereomer found in wildlife and humans[6]. The prevalence of α-HBCD as the major HBCD contaminant may be explained by differences in pharmacokinetic rates, where the γ-diastereoisomer is metabolized and/or eliminated more rapidly than the α-diastereoisomer, or by isomerization of the γ-diastereoisomer to the α-isomer. There are limited but emerging monitoring data identifying HBCD at the enantiomer level[7, 8]. This is the first study to examine the enantiomer fractions after oral exposure of two main HBCD diastereomers, α-HBCD and γ-HBCD, in the liver and feces using female mice as a mammalian model organism.

Materials and Methods

Chemicals: [^{14}C]1,2,5,6,9,10-Hexabromocyclododecane (HBCD) (2 mCi/mmol) was purchased from American Radiochemicals Corporation (ARC; St Louis, MO) as a mixture of ß- and γ-diastereoisomers. Gamma[^{14}C]1,2,5,6,9,10-hexabromocyclododecane (γ-[^{14}C]HBCD) was purified by flash chromatography on a silica gel column eluted with hexane containing increasing amounts of methylene chloride (0–50%). [^{14}C]1,2,5,6,9,10-hexabromocyclododecane alpha (α-HBCD) was prepared by thermal conversion of γ-HBCD at 170°C for up to 3 hr. It was dissolved in methylene chloride and then purified by flash chromatography on a silica gel column followed by preparative reverse phase HPLC. Both [^{14}C]stereoisomers were checked for isomeric composition by LC/MS.

Dosing solution: Stock solutions of α-HBCD and γ-HBCD were made by dissolving 19.23 mg of α-[^{14}C]HBCD or γ-[^{14}C]HBCD (3.12 µCi/mg) in toluene (400ul). A dosing solution was prepared from the stock solution that was designed to deliver approximately 0.2µCi to each mouse by adding corn oil to the vial, followed by the evaporation of toluene under vacuum (SpeedVac, Savant Instruments, Inc. Farmingdale, NY). A single dose of either α-[^{14}C]HBCD or γ-[^{14}C]HBCD (3 mg/kg) was administered by oral gavage using a curved Teflon animal feeding needle. After dosing, mice (n=4 for γ -[^{14}C]HBCD; n=6 for α-[^{14}C]HBCD) were held in metabolism cages for 4 days and urine and feces were collected daily.

Animals. Female C57BL/6 mice (~20 grams) were obtained from Charles River Breeding Laboratories (Raleigh,

NC). 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 for 1 week to Nalgene metabolism cages (Nalgene, Rochester, NY). Mice were then housed individually for the remainder of the study. All mice were 60 days old at time of the treatment.

Sample analysis: Animals were euthanized by CO_2 asphyxiation followed by exsanguination via cardiac puncture. The tissues that were collected and weighed were adipose (abdominal), adrenal glands, bladder, blood, brain, kidneys, liver, lung, muscle (abdominal), skin, spleen, thymus. Radioactivity in the tissues was determined by combustion to $^{14}CO_2$ (Packard 307 Tissue Oxidizer, Downers Grove, IL) of triplicate samples when available (~0.1 g/tissue) followed by liquid scintillation counting (LSC; Beckman, Beckman Instruments, Fullerton, CA). Feces were air dried, pulverized in a mortar/pestle, weighed, and analyzed for radioactivity by combustion and LSC. Radioactivity in daily urine was measured in 100 µL aliquots in triplicate by direct addition into liquid scintillant and analysis by LSC. Bile was collected by syringe from the common bile duct.

Tissue extraction and analysis: Lyophilized livers were weighed, pulverized and then homogenized in 3 volumes of 0.9% sodium chloride solution. Serum, liver, fat, bile, urine and dried 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. Two milliliters of hexane:ether (9:1) were added, vortexed, and centrifuged to allow layer separation. The upper layers were decanted. Extractions were repeated and the upper layers were pooled for further cleanup. Silica gel thin-layer chromatography using a 1:1 hexane:methylene chloride mobile phase followed by [^{14}C]-scanning (System 2000 Imaging Scanner, Bioscan, Washington, DC) was used to resolve and quantify parent α- or γ-[^{14}C]HBCD from metabolites in tissue/fecal extracts. Fractions were collected and assayed by LSC before submission for negative ion mode LC-MS analyses. Non-extractable radioactivity in liver and feces were determined by combustion analysis. The LC-MS system consisted of an Alliance 2695 Separation Model (Waters, Beverly, MA) coupled to quadrupole-time of flight mass spectrometer (Waters Q-TOF Ultima API-US). A Symmetry C18 column (2.1 mm x 100 mm, Waters) and a Nucleodex beta-PM (4 x 100 mm; Macherey Nagel, Germany) were used for separations of diastereomers and enantiomers, respectively.

Results and Discussion

Elimination of the γ-[^{14}C]HBCD dose was very rapid in that 59% of dose was excreted in the feces and 28% in urine within 4 days of dosing[9]. Cumulative elimination was slightly less for α-[^{14}C]HBCD (45% in feces and 20% in urine). No parent γ-HBCD or α-HBCD was detectable in the urine at 1 or 2 days after treatment. Both urine samples contained at least one metabolite that was more polar than the parent compound in that the major peak by silica gel TLC occurred at Rf=0.00 (Figure 1 for γ-HBCD). The unknown metabolite did not hydrolyze with β-glucuronidase nor sulfatase (data not shown). A volatile metabolite was also evident in γ-HBCD dosed urine in that ~65% of the urinary radioactivity was routinely lost during evaporation. Size exclusion chromatography on a G75 column (Figure 2) showed that the γ-HBCD urinary radioactivity was not bound to carrier proteins as has been reported in other mammalian systems for PBDEs[10]. Bile (data not shown) and serum radioactivity from γ-HBCD dosed mice was composed of only polar metabolites by TLC (Figure 1).

Following the γ-HBCD dose, only 48% of the fecal radioactivity was extractable; however, fecal extracts showed the presence of α-, β- and γ-HBCD at 1 day, indicating that stereoisomerization of γ-[^{14}C]HBCD had occurred. Only 36% of the fecal radioactivity from α-HBCD dosed mice was extractable. In addition, fecal extracts of γ-HBCD dosed mice contained at least one monohydroxylated pentabromododecene metabolite and a dihydroxylated pentabromododecene metabolite (Table 1). The only metabolite identified in fecal extracts of α-HBCD dosed mice was a monohydroxylated-HBCD metabolite (Table 1). Others have observed multiple isomers of hydroxylated metabolites of HBCD, which could be resolved by RP-HPLC[11], but in the present study only one chromatographic peak for each metabolite was detected under the conditions used. Therefore, at present it is not possible to conclude whether one or multiple monohydroxylated isomers of HBCD were formed in mice.

A chiral LC-MS method was developed in which all six enantiomers of α-, β- and γ-HBCD could be resolved. The Enantiomeric Fraction (EF) of each enantiomeric pair was then determined, which was defined as the integrated LC/MS (M-H) peak area of the (+) enantiomer divided by the sum of the peak areas of both enantiomers[13]. For α-, β- and γ-HBCD standards (Wellington Laboratories, Inc. Guelph, ON) EFs were determined to be approximately 0.5 (0.53 to 0.55; Table 2). Liver extracts from mice orally exposed to γ-HBCD yielded an EF of 0.73 for the detected β-HBCD isomer, while parent γ-HBCD was too low to allow the determination of an EF (Table 2). Liver extracts from α-HBCD exposed mice had an EF of 0.63 for the detected α-HBCD, suggesting selective accumulation of certain HBCD enantiomers in this tissue. Similarly, stereoselectivity was suggested for the α- and γ-HBCD detected in γ-HBCD dosed fat (Table 2). A high enantioselectivity has been observed previously in livers of fish for both α- and γ-HBCD[7]. EFs for feces samples were confounding with regard to enantiomeric selectivity, although the data suggested that stereoselectivity may be occurring for γ-HBCD in γ-HBCD dosed mice (Table 2).

Collectively, these data demonstrate that γ-HBCD is well absorbed, readily eliminated, and highly metabolized in female mice. The metabolism of γ-HBCD in mice involves reductive debromination and hydroxylation, all of which have been seen in *in vivo*[11] but not *in vitro*[12] studies. In addition, the data suggest that both pharmacokinetic differences and isomerization may play a role in the α-HBCD-dominated environmental profiles of HBCD.

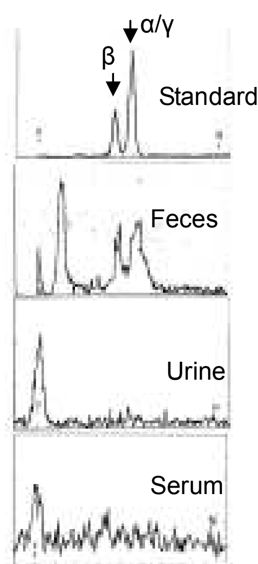

Figure 1. Silica gel TLC chromatograms of standard HBCD diastereomers, and feces, urine and serum obtained from γ-[^{14}C]HBCD dosed mice. The (polar) origin is to the left; the solvent front to the right.

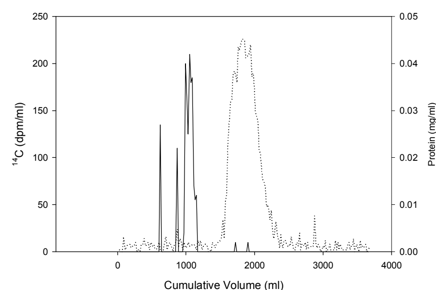

Figure 2. Gel permeation chromatograph of 0-24 h urine from γ-[^{14}C]HBCD dosed mice showing radioactivity is not associated with protein. Solid line represents protein; dashed represents [^{14}C].

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. This abstract does not reflect USEPA, NIH/NIEHS, and NCI policies.

Table 1. Putative metabolites identified in feces following a single 3 mg/kg oral dose of α-[^{14}C]HBCD or γ-[^{14}C]HBCD in mice based on negative ion LC/MS analyses.

Metabolic conversion	**LC/MS data**	**Dose received**	**Excreta**
+OH, -Br, +double bond	M-H 571 (5 Br)	γ-HBCD	Feces
+2OH, -Br, +double bond	M-H 587 (5 Br)	γ-HBCD	Feces
+OH	M-H 651 (6 Br)	α-HBCD	Feces

Table 2. Enantiomeric Fraction (EF) of each stereoisomer detected by –LC/MS in female mice following a single oral dose of γ-[^{14}C]HBCD or α-[^{14}C]HBCD (3 mg/kg). Livers were analyzed 4 days after receiving the dose, while feces samples were from days 1 and 2. Selective accumulation of one of the enantionmers over the other was suggested in some of the samples (n=1).

Sample	EF α-HBCD	EF β-HBCD	EF γ-HBCD
HBCD Standards	0.53	0.53	0.55
α-HBCD day 1 feces	*0.45*		
α-HBCD day 2 feces	*0.50*		
α-HBCD liver	*0.63*		
γ-HBCD day 1 feces	**0.50**	**0.52**	**0.69**
γ-HBCD day 2 feces	**0.77**	**0.46**	**0.62**
γ-HBCD day 3 feces	**0.80**	**0.43**	**0.94**
γ-HBCD day 4 feces	**n/a**	**n/a**	**0.81**
γ-HBCD liver		**0.73**	**n/a**
γ-HBCD fat	**0.67**		**0.84**

References

1. Hamers T, Kamstra JH, Sonneveld E, Murk AJ, Kester MH, Andersson PL, Legler J, Brouwer A. (2006) *Toxicol. Sci.* 92:157-173.
2. Eriksson P, Fisher C, Wallin M, Jakobsson E, Fredriksson A. (2006) *Environ Toxicol Pharmacol.* 21:317-322.
3. van der Ven LT, Verhoef A, van de Kuil T, Slob W, Leonards PE, Visser TJ, Hamers T, Herlin M, Håkansson H, Olausson H, Piersma AH, Vos JG. (2006) *Toxicol. Sci.* 94:281-92.
4. Law K, Palace VC, Halldorson T, Danell R, Wautier K, Evans B, Alaee M, Marvin C. (2006) *Environ. Toxicol. Chem.* 25:1757–1761.
5. Köppen R, Becker R, Jung C, Nehls I. (2008) *Chemosphere* 71:656-662.
6. Covaci A, Gerecke AC, Law RJ, Voorspoel S, Kohler M, Heeb NV, Leslie H, Allchin CR, de Boer J. (2006) *Environ. Sci. Technol.* 40:3679–3688.
7. Janaäk K, Covaci A, Voorspoels S, Becher, G.. (2005) Environ. Sci. Technol. 39, 1987-1994
8. Tomy GT, Pleskach K, Oswald T, Halldorson T, Helm PA, MacInnis, G, Marvin, C. (2008) Environ. Sci. Technol. 42, 3634–3639.
9. Szabo DT, Diliberto JJ, Hakk H, Huwe JK and Birnbaum LS. (2010) *Toxicol Sci* (accepted May 29 2010)
10. Hakk H, Larsen G, Bergman Å, Örn U. (2002) *Xenobiotica* 32:1079-1092.
11. Brandsma SH, van der Ven LTM, deBoer J, Leonards PEG. (2009) *Environ. Sci. Technol.* 43:6058-6063.
12. Zegers, BN, Mets A, van Bommel R, Minkenberg C, Hamers T, Kamstra JH, Pierce GJ, Boon JP. (2005) *Environ. Sci. Technol.* 39:2095–2100.
13. Guerraa P, Eljarrat E, Barceló D. (2008) *J. Chrom. A* 1203:81-87.