Enantioselective Biotransformation of Hexabromocyclododecane by in Vitro Rat and Trout Hepatic Sub-Cellular Fractions

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ABSTRACT: α-, β-, and γ-Hexabromocyclododecanes (HBCDs) were subjected to in vitro biotransformation experiments with rat and trout liver S9 fractions for different incubation times (10, 30, and 60 min) at 2 concentration levels (1 and 10 μM). The metabolic degradation of target HBCDs followed first order kinetics. Whereas β-HBCD undergoes rapid biotransformation (t0.5 = 6.4 and 38.1 min in rat and trout, respectively), α-HBCD appears the most resistant to metabolic degradation (t0.5 = 17.1 and 134.9 min). The biotransformation rate in trout was slower than in rat. Investigation of HBCD degradation profiles revealed the presence of at least 3 pentabromocyclododecene (PBDC) and 2 tetrabromocyclododecadiene (TBCD) isomers indicating reductive debromination as a metabolic pathway for HBCDs. Both mono- and di-hydroxyl metabolites were identified for parent HBCDs, while only mono hydroxyl metabolites were detected for PBDCs and TBCDs. Interestingly, δ-HBCD was detected only in trout S9 fraction assays indicating metabolic interconversion of test HBCD diastereomers during biotransformation in trout. Finally, enantioselective analysis showed significant enrichment of the (−)-α-HBCD enantiomer (EF = 0.321 and 0.419 after 60 min incubation in rat and trout, respectively). The greater enrichment of (−)-α-HBCD in rat than in trout underlines the species-specific differences in HBCD metabolism and the need for caution when extending similar results from animal studies to humans.

INTRODUCTION

Hexabromocyclododecane (HBCD) is a brominated flame retardant widely used as an additive to expanded and extruded polystyrene foams for thermal insulation of buildings, backcoating of fabrics, and to a lesser extent in high impact polystyrene. The commercial formulations consist mainly of α-, β-, and γ-diastereomers with γ- predominant (>70% of ΣHBCDs), along with minor contributions of the δ- and ε-mesoforms (<0.5% of ΣHBCDs each). HBCD has low water solubility (49, 15, and 2 μg L−1 for α-, β-, and γ-HBCD respectively), a fairly low vapor pressure (6.27 × 10−3 Pa), and is persistent. It can bioaccumulate and undergo long-range transport. The levels of HBCDs in different biotic and abiotic matrices have been comprehensively reviewed. Of particular interest is the observed shift from predominance of the γ-HBCD in abiotic samples to predominance of the α-isomer in most biotic and human samples.

Available data from different studies on biota have revealed an enrichment of the (−)-α-HBCD in human milk and plasma, sole liver and muscles, eggs of peregrine falcon and common tern, and the blubber of white sided dolphins. On the other hand, higher accumulation of (+)-α-HBCD was observed in the livers of Bib, whiting, and guillemots. Such studies suggest that enantioselective processes may be involved in the uptake, metabolism, and/or excretion of α-HBCD in fish, predatory birds, and mammals.

Currently, very little is known about the metabolism of HBCDs in humans or other biota. Zegers et al. reported on the preferential biotransformation of β- and γ-HBCDs by liver microsomes of harbor seals and observed a shift in the diastereomeric pattern toward α-HBCD. These findings indicated the potential enrichment of α-HBCD in harbor seals in addition to the formation of monohydroxylated metabolites of the parent β- and γ-HBCDs. In a brief communication, Huhtala et al. reported the formation of 2 monohydroxylated metabolites of γ-HBCD following incubation with trout liver microsomes for 120 min.
Our research group reported previously on the presence of the HBCD degradation products pentabromocyclododecenes (PBCDs) and tetrabromocyclododecadienes (TBCDs) in indoor dust. As a result, our subsequent detection of these compounds in human milk could not be attributed conclusively to indoor transformation processes.

Brandsma et al. identified the likely metabolites monohydroxy-HBCDs in tern eggs and seal blubber. In the same study, Wistar rats, exposed to the technical HBCD mixture for 28 days, displayed diverse monohydroxylated derivatives of HBCD, PBCD, and TBCD in addition to dihydroxylated derivatives of HBCD and PBCD. Esslinger et al. further elucidated the metabolic pathways and formation of hydroxylated metabolites for all HBCD enantiomers during phase I metabolism using in vitro rat liver microsomal enzyme assays. Recently, adult female mice exposed to dietary α-HBCD produced 4 hydroxylated metabolites of the parent compound, while those exposed to γ-HBCD produced multiple isomers of monohydroxy-PBCD and dihydroxy-PBCD in feces, in addition to a single monohydroxy-PBCD observed in liver and adipose tissue extracts. These results provided experimental evidence of species-specific differences in the stereoselective metabolism of different HBCD optical isomers.

Moreover, in 2009 our research group reported on the detection of δ-HBCD in fish samples from English lakes. Its absence from temporally and spatially consistent water and sediment samples suggested its formation via bioisomerization as a consequence of piscine HBCD metabolism.

In both fish and mammals, the liver is the major organ for xenobiotic biotransformation. Hence, the primary emphasis of several metabolic studies has been on the use of various in vitro liver models. Subcellular fractions incorporating 9000 g supernatants of liver homogenate (S9), are currently a common in vitro model used to study potential metabolic pathways of various persistent chemicals in the environment. The application of S9 liver fractions in metabolic studies is favored owing to the ease of their preparation, low cost, high stability, adaptability for high-throughput screening, and commercial availability.

Furthermore, S9 fractions contain both the microsomal and cytosolic fractions of liver cells, allowing them to provide information on both phase I and phase II metabolic pathways when phase II cofactors are supplemented to the reaction medium.

Against this background, the aims of the current study are to (a) study the metabolic profiles of HBCDs following in vitro exposure to rat and trout subcellular fractions (S9); (b) study and compare the behavior of individual HBCD isomers during phase I metabolism in rat and rainbow trout (Oncorhynchus mykiss) at different concentration levels; and (c) test the hypothesis that bioisomerization can account for the formation of δ-HBCD detected previously in fish from English lakes.

### MATERIALS AND METHODS

**Chemicals and Standards.** All solvents and reagents were purchased from Sigma-Aldrich (Poole, UK) of the highest available quality (e.g., analytical grade). Native α-, β-, and γ-HBCD standards were obtained from Cambridge Isotope Laboratories (Andover, MA, USA), while isotope-labeled 13C- and 15N-α-, β-, and γ-HBCDs were purchased from Wellington Laboratories (Guelph, ON, Canada). The δ-HBCD standard was kindly provided as a gift by Wellington Laboratories.

**In Vitro Incubation Experiments.** The S9 fractions were preincubated at the appropriate temperature (15 and 37 °C for trout and rat S9 fractions, respectively) with 100 mM sodium phosphate buffer (pH 7.4), sterile Milli-Q water, and the test compounds (solution containing equimolar concentrations of α-, β-, and γ-HBCDs in DMSO) in sterile tubes for 10 min. NADPH regenerating system was added to make a final volume of 1 mL before the tubes were incubated for the required time at the appropriate temperatures. Typical incubation experiments contained 10 μL of HBCD exposure solution in DMSO, 100 mM sodium phosphate buffer, 3 mM NADPH regenerating system (10 mM glucose-6-phosphate, 15 mM magnesium chloride, 0.1 mM ethylenediaminetetraacetic acid, 1.0 mM nicotinamide adenine dinucleotide phosphate, and 2 units/mL glucose-6-phosphate dehydrogenase), and 1 mg of microsomal protein for a total assay volume of 1 mL. At the end of the incubation period, the reaction was stopped by addition of an equal volume of ice cold methanol, then the tubes were centrifuged at 4000g for 10 min and the supernatant kept at −80 °C until further analysis. All incubation experiment batches included “non-enzymatic” controls featuring heat-inactivated S9 fractions and additional “negative control” blanks which contained pure carrier vehicle (10 μL of DMSO) in lieu of test substrate.

Protein concentrations of rat and trout S9 fractions were measured using the Bradford Assay (BCA kit) and bovine serum albumin (BSA) as a standard. The pooled S9 fractions were then diluted accordingly to give a final protein concentration of 1 mg/mL for both rat and trout preparations prior to their use for in vitro incubation experiments.

Incubation experiments were carried out using two different concentration levels of the test compounds (1 and 10 μM) representing average and high level exposure scenarios. Concentrations exceeding 10 μM were not possible due to poor solubility of HBCDs in the dosing vehicle. Dosing solutions were prepared from pure standards of individual HBCD diastereomers (200 μg/mL). Accurately measured aliquots of the pure standards were transferred to amber glass vials and solvent exchanged under a gentle stream of nitrogen to dimethyl sulfoxide (DMSO) at a concentration 100 times that of the desired final concentration to minimize exposure of S9 fractions to the dosing vehicle (DMSO). Only 10 μL of the dosing solution was added to each 1 mL of experimental media to yield the desired final concentration of test compounds (following a 1:100 dilution factor) with the total contribution of DMSO to the experimental medium not exceeding 1%. Furthermore, the dosing solutions were analyzed for their HBCDs content as a

<table>
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<th>Target Compounds</th>
<th>quantifier MRM (m/z)</th>
<th>qualifier MRM (m/z)</th>
<th>LOD (pg)</th>
<th>LOQ (pg)</th>
</tr>
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<td>native HBCDs</td>
<td>640.6→78.8</td>
<td>642.6→78.8</td>
<td>3</td>
<td>10</td>
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<td>13C-HBCDs</td>
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<td>655.4→78.8</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>d10-HBCDs</td>
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<td>6598→78.8</td>
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<tr>
<td>PBCDs</td>
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<td>5628→79.0</td>
<td>6</td>
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<tr>
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<td>4824→79.0</td>
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<td>dihydroxy-HBCDs</td>
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<td>6748→78.8</td>
<td>4</td>
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<td>4995→79.0</td>
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quality control measurement for the mass of HBCD diastereomers added to each incubation experiment and excellent results were obtained for both concentration levels (96 ± 8% and 102 ± 10%, respectively). Each concentration level (1 and 10 μM of α-, β-, and γ-HBCDs) was incubated for a maximum of 60 min, after which the enzyme activity in the S9 fractions was found to drop below 50% of its initial value under the test conditions as revealed by the results of ethoxyresorufin-O-deethylase (EROD) assay.23 To facilitate the study of HBCDs biotransformation pathways during phase I metabolism—the main focus of this study—and to avoid further complications of the produced chromatograms with conjugated metabolites, we opted not to supply the reaction media with phase II cofactors. As a result, no glucuronide or sulfate-conjugates were formed as indicated by the lack of significant difference in metabolite levels with and without the use of β-glucuronidase/aryl sulfatase enzyme during sample extraction (Supporting Information (SI) Figure SI-2). Further details are provided in the SI.

Sample Extraction and Cleanup. Samples were spiked with 10 μL of internal (or surrogate) standard (IS) solution (containing 1 ng/μL of each of 13C- α-, β-, and γ-HBCDs) then mixed with 3 mL of hexane/DCM mixture (1:1 v/v) in centrifuge tubes by vortexing for 30 s. The tubes were then ultrasonicated for 5 min followed by centrifugation at 4000 g for 3 min. The organic layer was collected while the aqueous layer was washed twice with 3 mL of hexane/DCM mixture (1:1 v/v). The combined organic extract and washings were gently reduced to ~2 mL under a gentle stream of nitrogen then washed with 2 mL of sulfuric acid. The extract was further purified by passing through a column containing 1 g of florisil topped with 1 g of anhydrous sodium sulfate through which the target compounds were eluted using 8 mL of hexane/DCM mixture (1:1 v/v). The purified extract was evaporated under a gentle stream of nitrogen before the target compounds were redissolved in 150 μL of methanol containing 3 ng of d15-γ-HBCD used as recovery determination (or syringe) standard for QA/QC purposes.

Figure 1. Selected LC-ESI-MS/MS chromatograms of HBCD metabolites in rat S9 fractions following exposure to 10 μM α-, β-, and γ-HBCDs for 60 min.
Instrumental Analysis. Sample analysis was carried out using an LC-MS/MS system composed of a dual pump Shimadzu LC-20AB Prominence liquid chromatograph equipped with a SIL-20A autosampler, and a DGU-20A3 vacuum degasser coupled to a Sciex API 2000 triple quadrupole mass spectrometer equipped with an ESI ion source operated in the negative ion mode. Details of the methodology, LC columns, and mass spectrometric conditions for both chiral and achiral separation and quantification of HBCDs can be found elsewhere, while the MRM transitions used for monitoring each of the target compounds are provided in Table 1. Enantiomer fractions (EFs) reported are the ratio of the (+) enantiomer over the sum of both enantiomers corrected using responses of the corresponding 13C-labeled diastereomer standards. Further details are provided as Supporting Information.

Quality Assurance/Quality Control. All experiments were performed in triplicate on rat and trout S9 fractions separately. Heat-inactivated S9 blanks (HI-blanks), method blanks without S9 fractions, and “negative control” blanks without the test compounds were performed at each concentration level and time point. Good recoveries (73–108%) of the 13C-labeled internal standards were obtained in both QC and real samples. The use of 13C-labeled internal standards together with the good recoveries obtained precluded the need for any corrections for analyte loss during the extraction/cleanup procedure. None of the target compounds were detected in the “negative control” blanks (n = 6; no test compound added) or solvent blanks (n = 6; no test compound or S9 fractions added). Therefore, there was no need for blank correction of the results and method limits of detection (LOD) and quantification (LOQ) were estimated based on 3:1 and 10:1 S:N ratios, respectively (Table 1).

Excellent recoveries of both target compounds and 13C-labeled internal standards were obtained in HI-blanks and method blanks indicating good accuracy of the applied methodology (SI Table SI-2). In the absence of an appropriate CRM, further assurance of method accuracy and precision was achieved via replicate analysis (n = 6) of NIST SRM2585. The results obtained compared favorably with the indicative values and other results reported elsewhere (SI Table SI-3).

The identity of the target compounds was confirmed via comparing their relative retention times (RRTs) to the respective internal standards, in addition to monitoring two MRM transitions (qualifier and quantifier) for each compound where the isotope ratio for bromine must fall within ±15% of the average for the two calibration standards run before and after each sample batch.

RESULTS AND DISCUSSION

HBCD Metabolic Profiles. Our research group has reported previously on the detection and identification of PBCDs and TBCDs in indoor dust, sediment, fish, and human milk. In this study, while neither PBCDs nor TBCDs were detected in any blanks or heat-inactivated controls, 3 PBCD isomers and 2 TBCD isomers were identified (Figure 1) in both rat and trout S9 fraction assays. Given the lack of reference standards for these compounds and the isobaric nature of the isomers compounded with a theoretically large number of possible isomers, coelution of PBCDs and TBCDs cannot be excluded. We tried several mobile phase compositions using a systemic approach to alter the percentage of organic phase throughout the gradient elution program. However, no further separation of PBCDs and TBCDs could be achieved and the optimized mobile phase gradient was selected to achieve baseline separation of the parent HBCDs and their hydroxyl products (Figure 1).

PBCDs were previously identified in whitefish, chicken egg, and male Wistar rats following dietary exposure to a HBCD technical mixture. Only one peak corresponding to PBCDs was identified in whitefish and chicken egg samples using GC/MS, while 2 PBCD peaks were separated and identified using LC-MS analysis of male Wistar rat tissues. However, the difference in number of PBCD peaks identified in this study and other metabolic studies of various species should be interpreted with caution as it is largely dependent on the analytical method used (e.g., thermal isomeric interconversion on the GC and mobile phase gradient on the LC). A recent in vivo study managed to
identify TBCD in the form of its methyl mercapturate conjugate in the urine of female mice exposed to \( \gamma \)-HBCD. Following incubation of \( \alpha \), \( \beta \), and \( \gamma \)-HBCDs with rat and trout S9 subcellular fractions, various hydroxylation products were identified using LC-ESI-MS/MS analysis which were linked to their parent compounds via full MS scan analysis (Figure 2), MS/MS analysis, and retention time windows on the basis that peaks of the more polar hydroxyl compounds will appear before the less polar parent brominated compound. Four monohydroxylated and two dihydroxylated metabolites were identified for parent HBCDs (Figure 1) in both rat and trout. Only monohydroxyl metabolites were identified for PBCDs in rat and trout S9 experiments, while a monohydroxyl TBCD metabolite was detected in rat only (Figure 1). A previous in vitro study using induced rat liver microsomes reported several mono- and dihydroxyl metabolites for different HBCD enantiomers. In theory, each HBCD enantiomer can lead to a maximum of six diastereomeric monohydroxyl HBCD structures resulting in a huge number of possible dihydroxyl HBCDs. Therefore, in the absence of reference standards for these compounds, coelution of one or more hydroxylated HBCD metabolites cannot be excluded. Brandsma et al. identified monohydroxyl metabolites of both PBCD and TBCD in male Wistar rats. Another in vivo metabolic study in female mice detected both mono- and dihydroxyl metabolites of PBCD but no hydroxyl TBCD derivatives. Collectively, these data suggest that while cytochrome P450 enzymes are involved in the stereoselective phase I oxidative metabolism of HBCDs; the detection of penta- and tetra-brominated metabolites together with their hydroxylated products indicate sequential reductive debromination (not catalyzed by cytochrome P450 enzymes) as a potential pathway of HBCD metabolism. Despite mounting evidence of the involvement of deiodinase enzymes in the metabolic debromination of PBDEs, further research is required prior to comparing these studies to HBCDs due to the aliphatic nature of HBCDs as opposed to the aromatic structure of PBDEs.

Interestingly, while the \( \delta \)-HBCD meso form was absent from the HBCD mixture used in the incubation experiments; \( \delta \)-HBCD was detected in all trout (but not rat) S9 fraction assays at concentrations of 4 \( \pm \) 2% of the test \( \alpha \), \( \beta \), and \( \gamma \)-HBCD diastereomers (Figure 3). However, our experimental setup with concomitant exposure of S9 fractions to equimolar concentrations of \( \alpha \), \( \beta \), and \( \gamma \)-HBCDs precludes accurate estimation of...
the relative contribution of each of the tested 3 diastereomers to the measured levels of δ-HBCD.

Although the suspected presence of δ-HBCD in fish and piscivorous birds has been reported previously,9,10 our research group was the first to confirm its presence in 13 fish samples from English lakes.31 Combined with its absence from water and sediment from the studied lakes, the high relative abundance of δ-HBCD in the studied fish samples (1.0–11% ΣHBCDs) compared to its reported abundance in commercial HBCD formulations (0.5% ΣHBCDs)2 led us to hypothesize that δ-
HBCD was produced as a result of biotransformation within the fish. The results of the current study support our previous hypothesis and confirm metabolic interconversion of HBCDs during biotransformation in trout. Moreover, the absence of δ-HBCD from rat S9 fraction assays highlights species-specific differences in HBCD metabolism.

**HBCD Metabolites in Rat vs Trout S9 Fraction Experiments.** The full metabolic profile of α-, β-, and γ-HBCDs (10 μM each) following incubation with rat S9 subcellular fraction for 60 min is provided in Figure 1. The lack of native or isotopically labeled reference standards for the isolated metabolites precluded their accurate quantification in both rat and trout experiments. However, using a semi-quantitative approach via application of an average response factor of the parent α-, β-, and γ-HBCDs, it was evident that the levels of metabolites produced by rat S9 fractions were higher (6–9 times) than those produced by trout enzymes at the studied time points. Furthermore, both monohydroxy HBCD-3 and TBCD-1 (Figure 1) were not detected in trout S9 fractions incubated with 1 μM of the test compounds. This may be attributed mainly to low levels of these metabolites below the method LOD at this low level of exposure. However, species-specific differences were evident: i.e., the detection of hydroxy TBCD in rat experiments only, while metabolic interconversion of test compounds to δ-HBCD was exclusive to trout S9 fraction experiments.

**Biotransformation Kinetics.** The levels of the studied three main HBCD diastereomers showed a rapid decrease upon incubation with both rat and trout S9 fractions (Table 2). No such degradation or formation of biotransformation products was observed in the corresponding heat-inactivated blanks where the test compound showed good stability under experimental conditions (SI Table SI-2). Despite the small number of data points in this study, this suggests that phase I metabolism of HBCDs in both rat and trout follows first order kinetics. Only one study has reported previously on the stereoselective degradation kinetics of HBCDs using an in vitro rat liver microsomal assay, where α-, β-, and γ-HBCDs underwent first order degradation.

Our results were applied to estimate separate first-order rate constants and half-lives for each HBCD diastereomer in both rat and trout (SI Figures SI-3 and SI-4). Although our results are in good agreement with those reported by Esslinger et al. for HBCD metabolism by rat liver microsomes, this is the first study of in vitro HBCD biotransformation by trout S9 fractions. The first-order rate constants and half-lives of the studied HBCDs (Table 2) show variable resistance of these diastereomers to phase I metabolic enzymes. Whereas β-HBCD undergoes rapid biotransformation, α-HBCD appears the most resistant to metabolic degradation in both rat and trout (Table 2). A recent study in female mice reported on rapid metabolism and elimination of γ- compared to α-HBCD. Furthermore, in a 90-min in vitro incubation experiment of equimolar concentrations of α-, β-, and γ-HBCDs with liver microsomes from harbor seals, Zegers et al. noticed an average decrease of 69% for δ-HBCD and 60% for γ-HBCD compared to only 17% degradation of the α-isomer. Whereas the observed difference in the metabolic rates of HBCDs may not account entirely for the observed enrichment of the α-isomer in biotic and human samples, other parameters may also contribute to such enrichment including both the higher water solubility (49, 15, 2 μg L−1 for α-, β-, and γ-HBCD, respectively) and bioaccessibility from indoor dust (92, 80, and 72% for α-, β-, and γ-HBCD, respectively) of α-HBCD. In addition, selective uptake of the α-isomer from diet has also been reported. Comparison of the results obtained from rat and trout incubation experiments (Table 2) revealed the metabolic rate in trout to be much slower than that in rat, further indicating species-specific differences in the metabolism of HBCDs.

The observed enrichment of the δ-HBCD enantiomer (24.4 min) than its α- HBCD enantiomer (24.4 min) than its β- and γ-HBCDs, respectively). This agrees with our previous findings in fish, human milk, and plasma. The observed enrichment increases with increasing incubation time (Figure 4) indicating less resistance of the (+)-α-HBCD enantiomer to phase I metabolism and a shorter half-life than the (-) enantiomer. This is generally in line with the results of Esslinger et al. who reported a longer t0.5 of the (-)-α-HBCD enantiomer (24.4 min) than its (+)-enantiomer (14.1 min) in rat microsomal enzyme assays. Although no significant change in the EFs of the β- and γ-isomers was observed, greater enrichment of the (-)-α-HBCD was observed in rat S9 assays than in trout. This further supports the species-specific differences in HBCD metabolism, and highlights the need for caution when extending similar results from animal studies to humans.

**ASSOCIATED CONTENT**

*Supporting Information*

Further details of the analytical methodology, quality assurance/ quality control parameters, and HBCD metabolic degradation. This information is available free of charge via the Internet at http://pubs.acs.org/.

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**Notes**

The authors declare no competing financial interest.

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