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In vitro metabolism of BDE-47, BDE-99, and α-, β-, γ-HBCD isomers by chicken liver microsomes

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ABSTRACT

The *in vitro* oxidative metabolism of 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99), and the individual α-, β- and γ-hexabromocyclododecane (HBCD) isomers catalyzed by cytochrome P450 (CYP) enzymes was studied using chicken liver microsomes (CLMs). Metabolites were identified using a liquid chromatography-tandem mass spectrometry method and authentic standards for the oxidative metabolites of BDE-47 and BDE-99. Six hydroxylated tetra-BDEs, namely 4-hydroxy-2,2′,3,4′-tetrabromodiphenyl ether (4-OH-BDE-42), 3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (3-OH-BDE-47), 5-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (5-OH-BDE-47), 6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE-47), 4′-hydroxy-2,2′,4,5′- tetrabromodiphenyl ether (4′-OH-BDE-49), and 2′-hydroxy-2,3′,4,4′-tetrabromodiphenyl ether (2′-OH-BDE-66), were identified and quantified after incubation of BDE-47 with CLMs. 4′-OH-BDE-49 was the major metabolite formed. Three hydroxylated penta-BDEs (5′-hydroxy-2,2′,4,4′,5-pentabromodiphenyl ether (5′-OH-BDE-99), 6′-hydroxy-2,2′,4,4′,5- pentabromodiphenyl ether (6′-OH-BDE-99), and 4′-hydroxy-2,2′,4,5,5′-pentabromodiphenyl ether, 4′-OH-BDE-101, were formed incubating BDE-99 with CLMs. Concentrations of BDE-99 metabolites were lower than those of BDE-47. More than four mono-hydroxylated HBCD (OH-HBCD), more than four di-hydroxylated HBCD (di-OH-HBCD), more than five mono-hydroxylated pentabromocyclododecenes (OH-PBCD), and more than five di-hydroxylated pentabromocyclododecenes (di-OH-PBCD) were detected when α-, β-, or γ-HBCD were individually incubated with CLMs. Response values (the ratio between the peak areas of the target compound and its internal standard) for OH-HBCD were 1–3 orders of magnitude higher than those for OH-PBCD, di-OH-HBCD, and di-OH-PBCD, suggesting that OH-HBCD might be the major metabolites of α-, β- and γ-HBCD produced by CLMs. No diastereoisomeric or enantiomeric bioisomerisation was observed incubating α-, β- or γ-HBCD with CLMs. Collectively, our data suggest that (i) BDE-47 is metabolized at a faster rate than BDE-99 by CLMs, (ii) OH-HBCD are the major hydroxylated metabolites of α-, β- and γ-HBCD produced by CLMs and (iii) the diastereoisomeric or enantiomeric bioisomerisation of α-, β- and γ-HBCD is not mediated by chicken CYP enzymes.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecane (HBCD) are brominated flame retardants (BFRs) widely used in furniture, textiles, and electrical products (Covaci et al., 2006; de Wit, 2002; Marvin et al., 2011). Numerous studies have proved PBDEs and HBCDs to be persistent, bioaccumulative, and toxic environmental pollutants (Crump et al., 2008, 2010; Fernie et al., 2009, 2011). 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) and 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) are the major PBDE congeners in the Penta-BDEs commercial formulation (de Wit, 2002). Tetra- and penta-BDEs are considered persistent organic pollutants (POPs) according to the Stockholm convention and their use was banned in Europe and U.S. since

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2004 (European Union Directive, 2003; UNEP, 2009). The technical HBCDs mixture consists mainly of three congeners: α-, β-, and γ-HBCD (Covaci et al., 2006). In 2013, HBCD has been listed under Annex A (POPRC8.3, 2013) of the Stockholm Convention on POPs (Koch et al., 2015). Despite these regulations, human exposure to these POP-BFRs is likely to continue for some time, given the ubiquity of treated products remaining in use or entering the waste stream, coupled with the environmental persistence of these BFRs. BDE-47, BDE-99, and HBCDs have been widely detected in abiotic and biotic samples including human tissues (Harrad and Abdallah, 2015; Marvin et al., 2011). The environmental occurrence and potential adverse effects of these environmental pollutants still remain a concern.

Hydroxylated PBDEs (OH-PBDEs) are considered to be more toxic than parent PBDEs (Wiseman et al., 2011). For example, OH-PBDEs are known to bind with a higher affinity than the native thyroid hormone thyroxine (T4) and triiodothyronine (T3) to the thyroid hormone transport protein transthyretin (TTR) (Meerts et al., 2000; Ren and Guo, 2012). OH-PBDEs also exhibited greater estrogen receptor (ER) agonistic activity than several PBDE congeners (Meerts et al., 2001). Only recently, *in vitro* oxidative metabolism of BDE-47 and BDE-99 in rat and humans was characterized (Erratico et al., 2011, 2012, 2013; Feo et al., 2013; Lupton et al., 2009; Stapleton et al., 2009). While several hydroxylated metabolites of BDE-47 and BDE-99 were formed by rat and human liver microsomes, the metabolism kinetic parameters and metabolite pattern of BDE-47 and BDE-99 was shown to be species-specific in rat and humans (Erratico et al., 2012, 2013). Less information is available on PBDEs metabolism in birds. The only available *in vitro* study investigating the oxidative metabolism of BDE-47 and BDE-99 used starling (*Sturnus vulgaris*) liver microsomes (Erratico et al., 2015a). However, chicken (*Gallus gallus domesticus*) is often the species of choice to monitor the leakage of environmental pollutants from e-waste sites into the environment and the local trophic chain (Zheng et al., 2014, 2015). Moreover, chicken liver microsomes are commercially available. Therefore, using chicken liver preparations can be an efficient strategy to provide useful information on the *in vivo* metabolism and bioaccumulation of environmental pollutants in chicken.

Metabolism of α-, β- and γ-HBCD in humans and wildlife has received even less attention than that of BDE-47 and BDE-99. In the only three studies currently available, *in vitro* metabolism of HBCDs was qualitatively investigated using liver microsomes of rat, liver S9 fraction of rat and trout and HepG2, a human liver cell line (Abdallah et al., 2014, 2015; Esslinger et al., 2011a). However, to the authors' knowledge, no information about avian metabolism of HBCDs is currently available. Given its lipophilic nature and environmental persistence, knowledge about metabolism of HBCDs in bird species is important for further understanding of their ability to bioaccumulate and to induce toxic effects in birds (Crump et al., 2010; Marteinson et al., 2011). In particular, different diastereoisomer and enantiomer patterns of HBCDs have been reported in different bird species (Covaci et al., 2006; Marvin et al., 2011). While γ-HBCD is the predominant isomer in technical mixtures, α-HBCD is the major HBCD isomer in biota samples of several bird species (Covaci et al., 2006; Marvin et al., 2011). Also, (-)-α-HBCD was preferentially accumulated over (+)-α-HBCD in eggs of herring gull (*Clupea harengus*) and peregrine falcons (*Falco peregrinus*) (Esslinger et al., 2011b; Janák et al., 2008), while the opposite was reported in eggs of white-tailed sea eagle (*Haliaeetus albicilla*) and guillemot (*Uria aalge*) (Janák et al. 2008). The enrichment of one HBCD isomer/enantiomer over the other(s) may indicate preferential metabolism of one or more HBCD isomer/enantiomer, possibly catalyzed by cytochrome P450 (CYP) enzymes. Therefore, characterization of the oxidative metabolism of HBCDs in avian species can provide valuable information on the origin, occurrence, toxicity, and isomer- or enantiomer-selective enrichment of HBCDs in birds.

In the present study, *in vitro* metabolism of BDE-47, BDE-99, α-, β-, and γ-HBCD catalyzed by CYP enzymes was investigated using chicken liver microsomes (CLMs). The specific aims of the present study were (i) to identify the oxidative metabolites of target BFRs formed by CLMs; (ii) to investigate if CYP-mediated metabolism can induce diastereoisomeric and/or enantiomeric shifts of α-, β-, and γ-HBCD profiles in chicken; and (3) to compare the oxidative metabolism of BDE-47, BDE-99, and HBCDs by CLMs with that occurring in other avian species for biomonitoring and toxicological risk assessment purposes.

2. Materials and methods

2.1. Chemicals and reagents

Standards for 4′-hydroxy-2,2′,4-tribromodiphenyl ether (4′-OH-BDE-17), 2′-hydroxy-2,4,4′-tribromodiphenyl ether (2′-OH-BDE-28), 4-hydroxy-2,2′,3,4′-tetrabromodiphenyl ether (4-OH-BDE-42), 3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (3-OH-BDE-47), 5-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (5-OH-BDE-47), 6-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether (6-OH-BDE-47), 4′-hydroxy-2,2′,4,5′-tetrabromodiphenyl ether (4′-OH-BDE-49), 2′-hydroxy-2,3′,4,4′-tetrabromodiphenyl ether (2′-OH-BDE-66), 3-hydroxy-2,2′,4,4′,5-pentabromodiphenyl ether (3-OH-BDE-99), 3′-hydroxy-2,2′,4,4′,5-pentabromodiphenyl ether (3′-OH-BDE-99), 6-hydroxy-2,2′,4,4′,5-pentabromodiphenyl ether (6-OH-BDE-99), 3-hydroxy-2,2′,4,4′,6-pentabromodiphenyl ether (3-OH-BDE-100) and 2-hydroxy-2,3′,4,4′,5-pentabromodiphenyl ether (2-OH-BDE-118) were kindly donated by Dr. Goran Marsh who synthesized them as previously described (Erratico et al., 2015b; Marsh et al., 2003; Rydén et al., 2012).

Standards for BDE-47, BDE-99, 2,4-dibromophenol (2,4-DBP), 2,4,5-tribromophenol (2,4,5-TBP), 5′-hydroxy-2,2′,4,4′,5-pentabromodiphenyl ether (5′-OH-BDE-99), 6′-hydroxy-2,2′,4,4′,5-pentabromodiphenyl ether (6′-OH-BDE-99), 4′-hydroxy-2,2′,4,5,5′-pentabromodiphenyl ether (4′-OH-BDE-101), 4-hydroxy-2,3′,4,5′,6-pentachlorobiphenyl (4-OH-CB-121), and for α-, β-, and γ-HBCD were purchased from AccuStandard Inc. (DaVinci Laboratories, Rotterdam, The Netherlands). Pooled chicken liver microsomes (mixed gender, n=22) were purchased from Tebu-Bio (Boechout, Belgium) and reduced β-nicotinamide adenine dinucleotide 2′-phosphate (NADPH, purity > 95%) was purchased from Sigma-Aldrich (Diegem, Belgium). Ultrapure water (18.2 MΩ) was prepared using a Purelab flex water system by Elga (Tienen, Belgium). HPLC grade methanol, methyl-tert butyl ether and hexane were purchased from Fisher (Aalst, Belgium).

2.2. In vitro metabolism assay

Reaction mixture consisting of 100 mM phosphate buffer (pH 7.4), pooled chicken liver microsomes and individual PBDE or HBCD isomers dissolved in acetone (2% of the total reaction mixture volume) in a final volume of 0.98 mL was prepared on ice. The samples were pre-incubated for 5 min in a shaking water bath at 37 °C. The reaction was started adding NADPH (1 mM, final concentration) and quenched adding 0.1 mL of methanol containing 5% formic acid in volume and the internal standard (final concentrations of 3 μM 4-OH-CB-121 used for PBDEs metabolism assay and 100 nM 6-OH-BDE-47 for HBCD metabolism assay). Tubes were vortex mixed for 30 s and stored on ice. A 500 μL aliquot of methyl tert-butyl ether:hexane (1:1, v/v) was added to the each tube, which was vortexed for 1 min and subsequently centrifuged at 8000 revolutions per minute for 3 min. A 400 μL aliquot of the