NEW INSIGHTS IN THE IN VITRO METABOLISM OF HBCD BY RAT LIVER MICROSOMES

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Introduction

Hexabromocyclododecanes (HBCDs) are the third most frequently used brominated flame retardants (BFRs) on a global scale. HBCDs are mainly applied to insulation foams and textile coatings to meet fire safety regulations (1). HBCDs are known to be persistent, bioaccumulable and toxic environmental pollutants (2) and have been proposed for listing under the Stockholm Convention as a candidate POP (3). Heeb et al. (4) predicted that there were a total of 16 different HBCD-stereoisomers, of which eight were isolated from the technical product. α-, β- and γ-Hexabromocyclododecanes (HBCDs) are the most prevalent diastereoisomers of the HBCD technical mixture, which is added to a variety of consumer products.

As a consequence of the widespread use of the HBCD mixture, HBCDs are now frequently detected in many biotic and abiotic matrices (5-6). Several temporal studies suggest that concentrations of HBCDs are continuing to increase in the environment. In particular, the varying contribution of α-, β- and γ-HBCD diastereoisomers in different matrices is of environmental and toxicological concern and might be due to metabolic processes and abiotic degradation pathways (7-9). Preliminary results of Phase I metabolism using phenobarbital induced rat liver microsomes were presented previously at Dioxin 2010 and the corresponding article is currently in preparation (10).

The objective of this follow-up study was to further investigate the metabolic transformation of HBCDs, expanding to the three main diastereomers α-, β- and γ-HBCD, using subcellular liver preparations obtained from phenobarbital treated rats. The metabolite formation from α-, β- and γ-HBCD due to Phase I was optimised and Phase II metabolism was studied through the addition of different cofactors. Clean-up and extraction procedures were improved for optimal recovery of the metabolites.

Materials and methods

Individual standards for α-, β-and γ ^{12}C-HBCD (neat, purity ≥98%) was purchased from Cambridge Isotope Laboratories and dissolved in acetone. All solvents used for the analysis (acetone, acetonitrile, dichloromethane (DCM), n-hexane (Hex), methanol (MeOH) and water (H_2O)) were of SupraSolv® grade (Merck, Darmstadt, Germany). Reduced L- Glutathione (GSH), uridine 5'-diposphoglucuronic acid trisodium salt (UDP-GA) and nicotinamide adenine dinucleotide phosphate (NADPH) were bought from Sigma-Aldrich (St. Louis, MO, USA). Hepatic microsomes and S9 fraction obtained from phenobarbital treated Sprague-Dawley rat were purchased from Celsis In Vitro Technologies (Schnelldorf, Germany). The sub-cellular fractions were shipped on dry ice and stored at -80°C until incubation. The incubation procedure was based on previously published work, with minor modifications (11).

The separation of α-, β- and γ-HBCD was achieved using a dual pump Agilent 1200 Series liquid chromatograph instrument equipped with autosampler and vacuum degasser. A Phenomenex Luna 3u-C18 analytical column (150 mm x 2.0 mm i.d., 100A particle size) was used. The mobile phase was composed of solvent A (H_2O:MeOH (1:1)) containing 2mM ammonium acetate and solvent B (MeOH).

Separation of HBCD enantiomers was obtained using the same LC instrument described above, using a chiral permethylated β-cyclodextrin LC column (200 mm × 4 mm i.d., 5 μm particle size, Nucleodex beta-PM; Macherey-Nagel, Düren, Germany). The mobile phase consisted of solvent A (MeOH:H_2O (1:1)) containing 2 mM ammonium acetate) and solvent B (MeOH:acetonitrile (1:1).

Results and discussion:

Concerning Phase I experiments: three different types of metabolites were produced, mediated by the CYP P45 enzyme system, of α- and γ-HBCDs: OH-HBCDs (major metabolites), OH-pentabromocyclododecenes (OH-PBCDEs; intermediate metabolites) and PBCDEs (minor metabolites). Co-formation of K^+-adducts and in source formation of OH-PBCDes from the major metabolites (OH-HBCDs) was detected (Figure 1). Previous experiments showed an increasing area count of all three metabolites with increasing incubation time. This result was confirmed semi-quantitatively using OH-BDE 47 as internal standard. Subsequently, all incubation parameters, including the incubation time, were optimized for a maximal metabolite yield. In accordance with previous in vitro experiments (12), Phase I metabolite formation of α-HBCD seemed to occur at a faster rate compared to that of γ-HBCD. The presence and number of HO- metabolites of α- and γ-HBCDs formed by rat liver microsomes underlines the active catalytic involvement of CYP enzymes in the preferential oxidative metabolism of γ- rather than α-HBCDs, helping to explain the higher levels of α- rather than γ-HBCDs in many mammalian species.

The presence of OH-HBCDs, as major *in vitro* metabolites, was verified in field samples (eels) which were caught in Flanders and which contained considerable HBCD levels. The *in vivo* formation of debrominated and OH debrominated metabolites could not be determined semi-quantitatively due to the lack of suitable standards, but the presence of debrominated HBCDs was verified in dust samples, originating from Flemish homes.

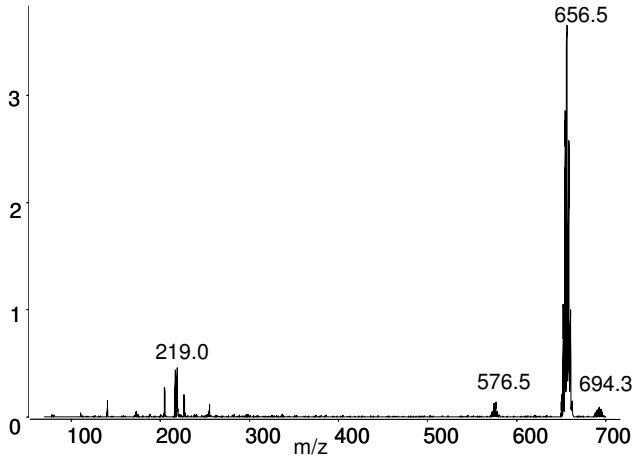

Figure 1. Mass spectrum for formation of mono OH- HBCDs (*m/z* 656.5), with co-formation of K^+-adducts (*m/z* 694.3) and in source formation of mono OH-PBCDes (*m/z* 576.5)

Phase II biotransformation reactions include, amongst others, glucuronidation and conjugation with glutathione. Most phase II reactions result in a large increase in xenobiotic hydrophilicity; hence they promote the excretion of lipophilic chemicals. The co-substrates for these reactions, such as UDP-GA and GSH, react with functional groups that either are present on the xenobiotic or are introduced/exposed during phase I biotransformation such as –OH, $-NH_2$, -SH or -COOH. Although most Phase II biotransforming enzymes are present in the cytosol, glucuronidation and glutathione conjugation, partially, occurs in microsomes (13). The phase II incubations were therefore performed with microsomal fractions and with S9 fractions (containing both microsomal and cytosolic components). An extensive range of incubation conditions were tested for the formation of Phase-II metabolites of (OH-) α-, β- and γ-HBCDs, including conjugation experiments with GSH and UDP-GA. No reports have been made concerning sulphation of α-, β- and γ-HBCDs. Metabolite gain was optimized by the addition of Alameticine. The addition of Alamethicine is known to improve the accessibility of the membrane-bound UGTs by forming pores in the microsomal membranes, and might therefore enhance the phase II metabolite formation (14). Due to the expected higher hydrophobic properties of the Phase II metabolites, the extraction procedure, applied for Phase I metabolites, had to be adapted. In a first set of experiments, 200 µl of MeOH was added additionally to the incubation mixture, after the incubation was stopped. The samples were centrifuged and 100 µl was transferred to an injection vial.

In a second set of experiments, the incubation mixture was extracted 3 times with 4 ml of ethyl acetate, after the incubation was stopped. The ethyl acetate layers were added together, evaporated to dryness, re-dissolved in 100 µl MeOH and transferred to an injection vial. In a third set of experiments, the incubation mixture was extracted 3 times with 4 ml of ethyl acetate, after the incubation was stopped. The ethyl acetate layers were combined and put over a cleaned silica cartridge. Three separate fractions were eluted, using 5.5 ml Hex, 8 ml DCM and 5 ml MEOH, respectively, evaporated to dryness, re-dissolved in 100 µl MeOH and analysed for the HBCD and metabolite content.

Whenever metabolites were detected, they were injected on the enantiomeric column. The corresponding chromatograms of the mono OH-HBCD metabolites revealed 5 separate enantiomers for mono OH-α-HBCD and 5 separate enantiomers for mono OH-γ-HBCD, which did not show any overlap (Figure 2). The corresponding neutral fractions showed a decline in ^{12}C α- and γ-HBCD as the incubation time increased. Injection on the enantiomeric column showed that the (+) enantiomer and the (-) enantiomer of γ-HBCD declined equally, indicating they are metabolized at an identical rate. The CYP450s do not seem to have a preference for either one of the γ-enantiomers. For ^{12}C α-HBCD, this trend was less clear, as the (-) enantiomer seemed to be metabolized slightly more rapidly, compared to the (+) enantiomer.

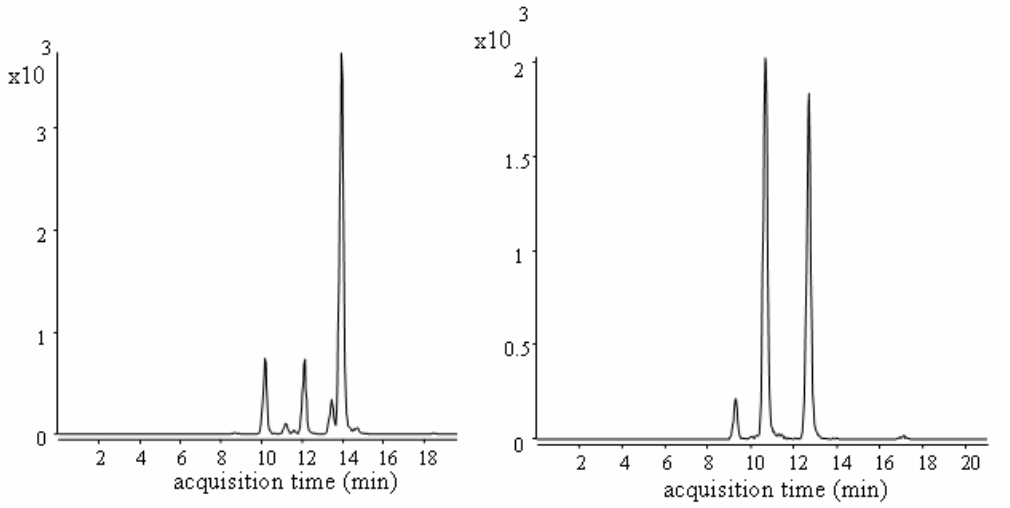

Figure 2. Enantiomeric injection of mono OH- γ-HBCD (left) and mono OH-α-HBCD (right).

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