INVESTIGATION OF HBCD METABOLISM IN MARINE MAMMALS FROM CANADA USING A HEPATIC MICROSOMAL *IN VITRO* BIOASSAY APPROACH AND COMPARISON WITH FIELD SAMPLES

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Introduction

Hexabromocyclododecane (HBCD, $C_{12}H_{18}Br_6$) is an aliphatic brominated flame retardant (BFR) and the second most-heavily produced BFR globally. HBCD is the principal flame retardant in extruded and expanded polystyrene foams. Technical HBCD mixtures contain three predominant diastereomers; α-, β- and γ-HBCD. The individual diastereoisomers exist as corresponding pairs of enantiomers. In general, industrially synthesized chiral pollutants are produced as racemates. The integrity of the racemic mixture is maintained when it is subjected to achiral interactions such as hydrolysis, photolysis, leaching, volatilization and atmospheric deposition. However, alterations in enantiomeric composition might occur as a result of biological processes such as metabolism, biotransformation or other degradation processes.

Over the last 5 years we have been assessing the spatial and temporal distribution of both the HBCD diastereomers and enantiomers in Canadian biota including marine mammals in the Arctic. In addition to our studies of the environmental occurrence, distribution and fate of HBCD, we are currently assessing the ability of higher-trophic level mammals to metabolize and/or biotransform HBCD. Using an *in vitro* hepatic microsomal assay, we carried out a series of microsomal incubations with two racemic mixtures of α- and γ-HBCD diastereomers for polar bear (*Ursus maritimus*), ringed seal (*Pusa hispida*), beluga whale (*Delphinapterus leucas*) and control rat (*Rattus rattus*). The investigation of α- and γ-HBCD depletion/ metabolite formation in the laboratory *in vitro* assay was compared with field measurements of these compounds in beluga whales from the St. Lawrence Estuary that were being analyzed concurrently for the determination of temporal trends in isomer-specific HBCD.

Materials and Methods

A hepatic microsomal assay approach was used where microsomes were previously extracted from cryopreserved polar bear (n=1), beluga whale (n=2) and ringed seal (n=2) liver tissues as well as the laboratory rat as a mammalian model species (fom pooled (n = 17) adult male Wister Han rats; BD Gentest) (Table 1).[1] Enzymatic viability was indicated by ethoxyresorufin-*O*-deethylase (EROD) activities ranging from 120 pmol/mg protein/min in the rat, to 199-694 pmol/mg protein/min for the seals and whales to 2194 pmol/mg protein/min for the bear. In addition to the inclusion of buffer blanks, the overall study design for α- and γ-HBCD *in vitro* depletion/metabolite formation assays for bear, whale, seal and rat microsomes included controls (enzymatically non-activated) and samples (enzymatically activated).

We hypothesized that this differential CYP enzyme activity equates to differences in the ability and capacities to metabolically deplete structural isomers of α- and γ-HBCD. Using an *in vitro* hepatic microsomal assay, we carried out a series of microsomal incubations with racemic mixtures of α- and γ-HBCD diastereomers for each individual polar bear, ringed seal, beluga whale and control rat. Prior to substrate incubation we spiked the assays with BDE153 as the non-metabolizable internal standard. Post-assay incubation, the mixtures were spiked with 2'-OH-BDE28 as the phenolic-brominated internal standard.

Table 1. Sample collection location and date and biological data

Sample ID[a]	Species	Collection region	Collection date (YYYY/MM)	Sex	Age class
PB1	Polar bear	Iceland[a]	2008/06	F	Adult
BW1	Beluga whale	Western Hudson Bay, Canada	2003/08	M	Adult
BW2	Beluga whale	Western Hudson Bay, Canada	2003/08	F	Adult
RS1	Ringed seal	Cumberland Sound, Canada	2001/07	F	Adult
RS2	Ringed seal	Cumberland Sound, Canada	2001/07	F	Adult
	Beluga whale	St. Lawrence River, Canada	1988/2006	M	Adult

[a] Samples used in previous studies, under different sample ID: BW1 = CA10 and BW2 = CA6.[3]
[b] PB1 was stranded in Iceland as the sea ice retreated during summer. It is thus likely to be an individual from the East Greenland population.

St. Lawrence River beluga samples (1g blubber) were blended with Na_2SO_4 and Soxhlet extracted in DCM. DCM extracts were reduced and subjected to a GPC procedure affording three fractions. Chromatographic separation of HBCD diastereomers in the extracts was performed on an Agilent 1100 Series LC system using a chiral LC column containing permethylated [beta]-cyclodextrin on silica (NUCLEODEX beta-PM). Injections of 5 μL were made using an Agilent autoinjector. MS analyses were carried out using an Applied Biosystems 5500 QTrap hybrid triple quadrupole/linear ion trap mass spectrometer in negative ion ESI mode using multiple reaction monitoring (MRM). The mobile phase consisted of water, methanol and acetonitrile at a flow flow rate of 500 mL/min.

Results and Discussion

We found that regardless of species, the rate of γ-HBCD metabolism is more rapid than α-HBCD, which supports the general findings in biota (e.g. beluga whale), including the present Arctic mammal species, that the α-HBCD isomer is highly enriched, compared to the γ-HBCD isomer. Incubation fractions were analyzed for the enantiomers and metabolites (OH-HBCD metabolites and debromination products) using LC/MS/MS.

In all cases, debromination of HBCD isomers appeared to be the predominant metabolic pathway in the *in vitro* microsomal assays. As in our previous studies on free-ranging field samples from beluga whale and polar bear, only trace quantities of compounds corresponding to ions associated with OH-HBCD were observed.[4] However, HBCD debromination products were prevalent in many of the samples; and in the case of free-ranging St. Lawrence River beluga whale the quantities of the [-HBr] and [-2HBr] compounds rivaled that of the α-HBCD diastereomer (Figure 1). As expected, α-HBCD was by far the most predominant parent isomer relative to β- or γ-HBCD. In the case of the St. Lawrence River beluga, α-HBCD typically comprised >99% of the total HBCD burden.

In the present *in vitro* assay, in addition to the [-HBr] and [-2HBr] debromination compounds, [-3HBr] products were also detected in some of the incubation extracts. In the case of an assay involving hepatic microsomes from polar bear, the [-3HBr] product was readily detectable (Figure 2). This result is consistent with the preliminary results of α-HBCD that showed rapid depletion in the same 90 min. hepatic microsomal assay we previously reported for polar bears.[2]

Our results to date indicate that marine mammals are capable of metabolizing HBCD with debromination of successive bromines being the predominant mechanism. Once our data set is complete, we will have a clearer understanding of the mechanisms, as well as a more detailed analysis of potential intra- and inter-species similarities/differences. These studies facilitate a better understanding of the biological fate of isomer-specific HBCDs, and the contribution of such metabolism to HBCD bioaccumulation among these top arctic predators.

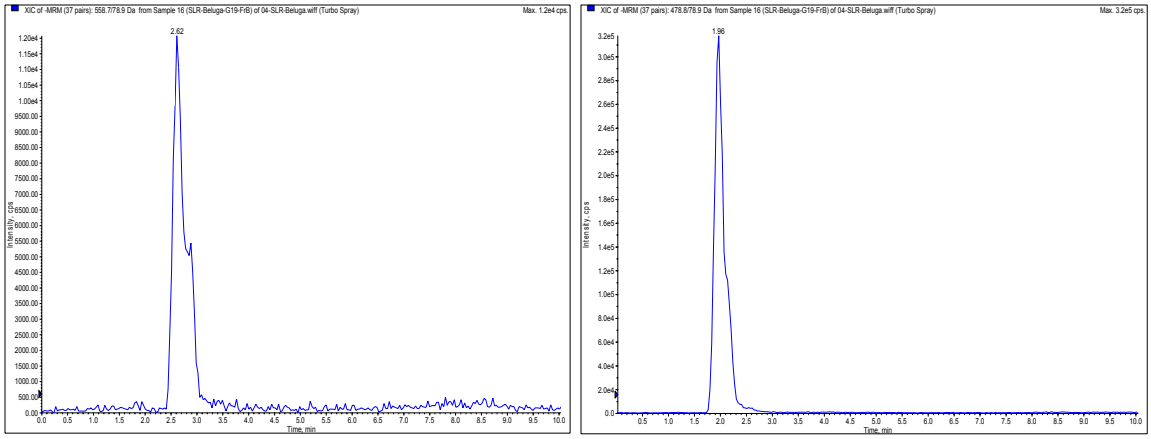

Figure 1. St. Lawrence River Beluga blubber extract showing the MRM transitions of HBCD debromination products 558.8 → 78.9 (loss of HBr; (mass chromatogram on the left) and 478.8 → 78.9 (loss of 2HBr; mass chromatogram on the right).

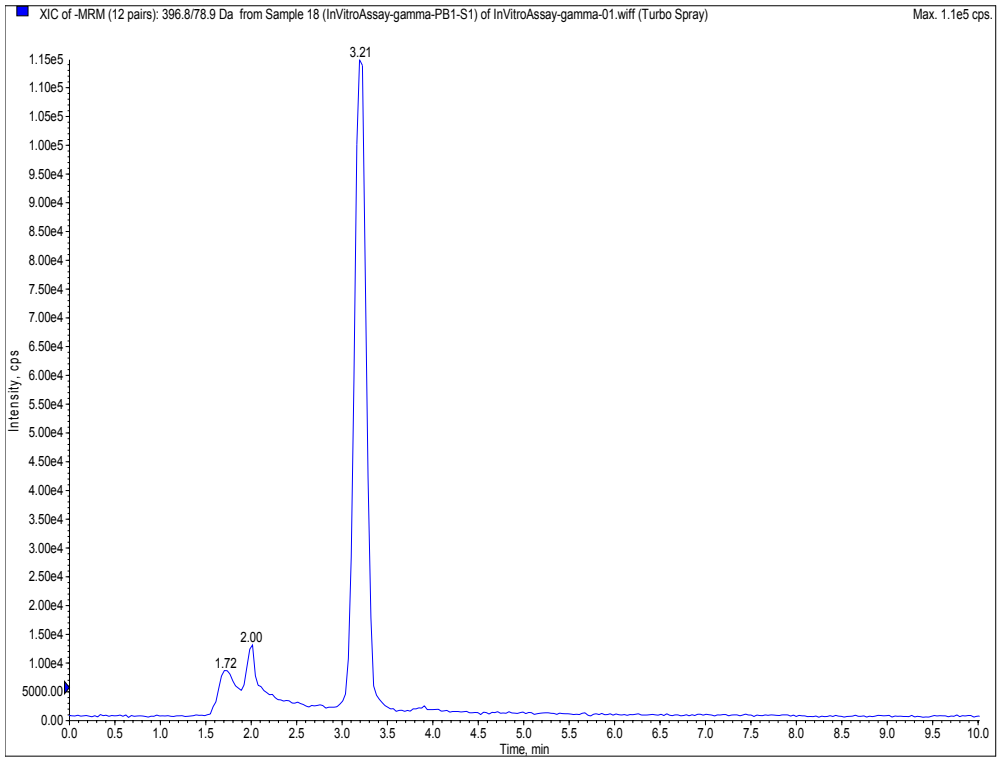

Figure 2. *In vitro* bioassay extract of polar bear showing the MRM transition of 396.8 → 78.9 (loss of 3HBr).

References

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