Isomer specific determination of hexabromocyclododecanes (HBCDs) in small cetaceans from the South China Sea – Levels and temporal variation

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Abstract

A diastereoisomer (α, β, and γ) specific analytical method for measuring hexabromocyclododecanes (HBCDs) was developed using liquid chromatography-tandem mass spectrometry (LC–MS–MS). The method developed was applied to analyze blubber of small cetaceans to divulge the distribution and temporal variation of HBCDs in the Asian marine environment. HBCDs were detected in all the blubber samples of finless porpoises (Neophocaena phocaenoides) and Indo-Pacific humpback dolphins (Sousa chinensis) collected from the South China Sea during 1990–2001. Concentrations of HBCDs were higher in humpback dolphins (31–380 ng/g lipid) than in finless porpoises (4.7–55 ng/g lipid), which can be attributed to habitat differences. Average concentrations of α-HBCD in finless porpoises increased from 9.5 ng/g lipid in 1990 to 35 ng/g lipid in 2000/01. α-HBCD was predominant among the three isomers with some exceptions in finless porpoises collected in 1990. This is the first report on HBCDs contamination in marine mammals from Southeast Asia.

Keywords: Brominated flame retardants; Hexabromocyclododecanes; Liquid chromatography-tandem mass spectrometry; Finless porpoise; Indo-Pacific humpback dolphin; Temporal variation

1. Introduction

Environmental contamination by brominated flame retardants (BFRs) has become a matter of serious concern because of their persistence, bioaccumulative nature, and possible adverse effects on human and wildlife. Apart from polybrominated diphenyl ethers (PBDEs), hexabromocyclododecanes (HBCDs) are also intensively used worldwide as a BFR with an annual consumption of 22,000 metric tons (BSEF, 2005). Both chemicals are additive flame retardants with different applications. HBCDs are mainly used in thermal insulation foam and upholstery furniture, whereas PBDEs are used in electronic and electrical components. According to some review papers summarizing the toxicities of BFRs (de Wit, 2002; Birnbaum and Staskal, 2004), HBCDs exhibit low acute, i.e., lethal, toxicity on experimental animals (Darnerud, 2003). At the same time, some adverse chronic effects on endocrine homeostasis, such as neurotoxic effects (Mariussen and Fonnum, 2003), induction of hepatic cytochrome P450 (CYP) (Germer et al., 2006), and disruption of the thyroid hormone system (Darnerud, 2003) were reported in some experimental studies with HBCDs. Statistical data has shown that Asian countries contributed to about 40% and 23% of the global PBDEs and HBCDs consumption in 2001, respectively

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using skipjack tuna (data on HBCDs has been reported by Ueno et al. (2006) in the Asia-Pacific region, however, the only available monitoring data is still growing; this may lead to subsequent environmental pollution whereas the consumption of HBCDs is decreasing due to effective regulation and voluntary restriction. Some researchers in Europe and US reported that HBCDs are biomagnified through the food web (Tomy et al., 2004; Janak et al., 2005; Zegers et al., 2005). From the Asia-Pacific region, however, the only available monitoring data on HBCDs has been reported by Ueno et al. (2006) using skipjack tuna (Katsuwonus pelamis) as a bioindicator. To our knowledge, there is currently no information on the levels of HBCDs in marine mammals from the Asia-Pacific region. Therefore, a widespread and detailed monitoring survey is needed from this region.

While theoretically 16 HBCD stereoisomers may be present (Heeb et al., 2005), the technical mixtures of HBCD consist mainly of three diastereoisomers, α-, β-, and γ-HBCD with a composition of 10–13%, 1–12%, and 75–89%, respectively (Covaci et al., 2006). The physico-chemical properties are different for these three isomers and, hence, the environmental fate and accumulation features in organisms may also be different. It is known that diastereoisomeric analysis of HBCDs cannot be achieved using gas chromatography–mass spectrometry (GC–MS) due to thermal re-arrangement of isomers above 160 °C, and poor separation within a GC column. An analytical method for measuring diastereoisomers of HBCDs using liquid chromatography-tandem mass spectrometry (LC–MS–MS) has recently been reported (Budakowski and Tomy, 2003; Cariou et al., 2005). However, Tomy et al. (2005) reported some difficulties in the method, especially on suppression of ionization in the sample extract which should be overcome to achieve accurate quantitative analysis. While applying the LC–MS–MS method to environmental and biota samples, the relative composition of the three diastereoisomers have been found to vary depending on the media used (Law et al., 2005, 2006).

The purpose of this study was to develop an analytical method for measuring individual HBCD diastereoisomers in biota samples using LC–MS–MS and to elucidate the contamination status, accumulation features and temporal variation in finless porpoises (Neophocaena phocaenoides) and Indo-Pacific humpback dolphins (Sousa chinensis) collected from the South China Sea (SCS).

2. Materials and methods

2.1. Sample collection

Seven adult male Indo-Pacific humpback dolphins (S. chinensis) and five adult male finless porpoises (N. phocaenoides) analyzed in this study were found stranded at several locations in Hong Kong between 1997 and 2001. Seven adult male finless porpoises accidentally caught in fishing nets from Dongshan, SCS in 1990 were also analyzed in this study. Biological data of the animals analyzed are given in Table 1. Blubber samples were excised from the dead animals, wrapped in aluminum foil, and stored at −20 °C in the environmental specimen bank (es-BANK) of Ehime University (Tanabe, 2006) until chemical analysis.

2.2. Chemical analysis

Analysis of HBCDs was performed following the previously reported procedure used for quantifying PBDEs (Ueno et al., 2004) with some modifications. The modified analytical procedure used in this study is shown in Fig. 1. Briefly, 2–3 g (wt. wt.) of the blubber sample was ground with anhydrous sodium sulfate and Soxhlet extracted with diethyl ether/hexane (75:25, v/v) for 7–8 h. An aliquot of the extract, after spiking with 5 ng of 13C-labeled PBDEs (13C12-BDE-3, 15, 28, 47, 99, 153, 154, 183, 197, 207, 209) and 10 ng of 13C-labeled HBCDs (13C12-α-, β-, and γ-HBCD), was loaded to a gel permeation chromatography (GPC, Bio-Beads S-X3, Bio-Rad, CA, 2 cm i.d. × 50 cm) column for lipid removal. The GPC fraction containing organohalogens was concentrated and subjected to activated silica gel column (Wakogel DX, 4 g, Wako Pure Chemicals, Tokyo) for clean-up and fractionation. PBDEs were eluted with 80 ml of dichloromethane/hexane (5:95, v/v) followed by the elution of HBCDs with 100 ml of dichloromethane/hexane mixture (25:75, v/v) from the silica gel column. Five nanograms of 13C12-BDE-139 was spiked as an internal standard to the fraction containing PBDEs and subjected to GC–MS analysis. The HBCDs fraction was evaporated and spiked with 10 ng of deuterized HBCDs (α-, β-, and γ-HBCD-d18) prior to LC–MS–MS analysis. The diastereoisomer-specific analysis of HBCDs was performed based on the reported analytical method by Tomy et al. (2004). Identification and quantification were carried out using an Alliance 2795 (Waters, Tokyo) liquid chromatograph equipped with a Quattro Micro API (Waters, Tokyo) triple quadrupole mass spectrometer. LC separation of all the three isomers (α-, β-, and γ-) of HBCDs was achieved with an Extend-C18 column (2.1 mm i.d. × 150 mm, 5 μm, Agilent, Tokyo). The mobile phase consisted of water/acetonitrile/methanol (20:30:50) at 0.2 ml/min initially for 2 min and gradually changed to acetonitrile/methanol (30:70) for 5 min and kept for 6 min. The MS–MS analysis in negative mode of electrospray ionization (ESI) was performed in multiple reaction monitoring mode (MRM). Quantification of native HBCDs was obtained using Mass Lynx 4.0 (Waters, Tokyo) software from the mean value of the response at two MRM transitions (i.e., m/z 640.6 > 81, m/z 642.6 > 81) corrected against the response of 13C12-HBCDs (i.e., m/z 652.6 > 81 MRM transition). Performance of the instrument and effect of matrices in sample extracts were
evaluated by responses of $\alpha$, $\beta$, and $\gamma$-HBCD-$d_{18}$ (i.e., $m/z$ 658.6 > 81 MRM transition) as recommended by Tomy et al. (2005). Concentrations of analytes were expressed as ng/g lipid weight unless otherwise stated.

2.3. QA/QC

Recovery throughout the method was checked by spiking known amounts of 13C-labeled HBCDs prior to Soxhlet extraction. For quality assurance and control, our laboratory participated in an intercalibration exercise organized by National Institute for Environmental Studies, Japan using the blubber of a marine mammal as the reference material. Matrix effect on signal intensity of LC–MS–MS was minimized using both 13C-labeled ($\alpha$, $\beta$, and $\gamma$-HBCD-$d_{18}$) and deuterized ($\alpha$, $\beta$, and $\gamma$-HBCD-$d_{18}$) compounds as the recovery internal standards and performance internal standards, respectively. A recent study recommends the use of both internal standards and employment of effective pretreatment steps to avoid the effect of matrices in sample extract (Tomy et al., 2005). A procedural blank was analyzed for every five samples to check for interferences and contamination.

3. Results and discussion

3.1. Validation of method

The calibration curves for the determination of the analytes were obtained by performing linear regression analysis on the ratio of native standard area to 13C-labeled surrogate area of the standard solutions. All $r^2$-values for the calibration curves of 1, 5, 10, 20, 50, 100, and 500 pg/$\mu$l for each isomer were >0.99. The instrumental detection limits (IDL) were 1.8–6.0 pg, which was calculated from standard deviations of 6 replicate analyses of 1 pg/$\mu$l standard solutions. Recoveries of four replicate analyses of spiked samples were 92 ± 14%, 99 ± 18%, and 88 ± 12% for $\alpha$, $\beta$, and $\gamma$-HBCD, respectively. As shown in Table 2, good agreement of our data were found with reported
data in an intercalibration study (Takahashi et al., 2005). Reported concentrations of total HBCDs from two institutes in the intercalibration study were 110 and 77 ng/g lipid wt. In our laboratory, the mean value of four replicate analyses was 78 ng/g lipid wt. with a relative standard deviation (RSD) of 3.8% for total HBCDs, which is within the range of reported concentrations. Since only 2 out of 10 participating institutions reported concentrations of HBCDs and no isomer-specific concentration values were available, we were unable to make a detailed comparison on the relevance of these results pending further intercalibration studies. It is also stated as the conclusion of another intercalibration exercise that several years may be needed before a sufficient number of laboratories can produce reliable results for this new contaminant (de Boer and Wells, 2006). Assuming that all our values are relevant, we believe that PBDEs and HBCDs can be quantified simultaneously from the same extract using the method developed in this study. One advantage of our method is that the time and sample amount (volume) required for the analysis are greatly reduced.

### 3.2. Contamination status

Concentrations of HBCDs as well as other previously reported organohalogenes in our samples (Ramu et al., 2005, 2006) are shown in Table 1. HBCDs were detected in all blubber samples analyzed in the present study, suggesting ubiquitous contamination by this group of flame retardants in SCS waters. To our knowledge, this is the first report on HBCDs in marine mammals from Asian waters. Among the organohalogenes analyzed so far, DDTs ranked highest followed by PCBs > CHLs > PBDEs > HCHs > HCB > HBCDs, showing that the extent of HBCDs pollution is less but widely present in this region and similar to other organohalogenes.

Concentrations of HBCDs in the two cetacean samples ranged from 4.7 to 380 ng/g lipid wt. When compared to reported concentrations in other marine mammals, the range was found to be higher than in California sea lions (Zalophus californianus, <0.3–12 ng/g wet wt.) from the US coast (Stapleton et al., 2006) and polar bears (Ursus maritimus, <0.03–0.85 ng/g wet wt. and <0.01–110 ng/g lipid wt.) from the arctic (Verreault et al., 2005; Muir et al., 2006), and lower than in harbor seals (Phoca vitulina, 63–2100 ng/g lipid wt.), common dolphins (Delphinus delphis, 52–3400 ng/g lipid wt.), and harbor porpoises (Phocoena phocoena, 79–9600 ng/g lipid wt.) from Europe (Morris et al., 2004; Zegers et al., 2005). This phenomenon is consistent with the fact that HBCDs are extensively used in Europe rather than in US and Asia (Watanabe and Sakai, 2003), and as a consequence, levels of environmental contamination are more serious in Europe than in other regions of the world.

Fig. 2 shows the concentrations of HBCDs in finless porpoise and humpback dolphin specimens collected during 2000/01. As shown in this figure, HBCDs accumulated more in humpback dolphins than finless porpoises (p < 0.05), as also found for PBDEs and all other organochlorines measured in the same specimens (Ramu et al., 2005). This variation can be attributed to differences in their habitat and trophic level. Humpback dolphins inhabit the estuarine waters of western Hong Kong, which are close to the Shezhen economic zone. However, finless porpoises in this region inhabit southern and eastern waters of Hong Kong, which are more ocean-influenced. A positive relationship between concentrations of HBCDs and trophic level based on δ15N was found in Lake Ontario (Tomy et al., 2004) and North Sea (Morris et al., 2004) food webs. These reports may indicate the differences in biomagnifica-

<table>
<thead>
<tr>
<th>This study (n = 4)</th>
<th>Reported concentrations (Takahashi et al., 2005)</th>
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<tbody>
<tr>
<td></td>
<td>Average</td>
</tr>
<tr>
<td>2,4,4′-TrBDE (#28)</td>
<td>26</td>
</tr>
<tr>
<td>2,2′,4,4′-TeBDE (#47)</td>
<td>200</td>
</tr>
<tr>
<td>2,2′,4,4′,5-PeBDE (#99)</td>
<td>31</td>
</tr>
<tr>
<td>2,2′,4,4′,6-PeBDE (#100)</td>
<td>32</td>
</tr>
<tr>
<td>2,2′,4,4′,5,5′-HxBDE (#153)</td>
<td>81</td>
</tr>
<tr>
<td>2,2′,4,4′,5,6′-HxBDE (#154)</td>
<td>98</td>
</tr>
<tr>
<td>2,2′,3,4,4′,5,5′-HpBDE (#183)</td>
<td>6.3</td>
</tr>
<tr>
<td>∑HBCDs</td>
<td>78</td>
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</tbody>
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* Blubber sample of finless porpoises from Japan coast.

| HBCDs            | 78      | 3  | 2  | 94      | 77  | 77       | 110     |

Table 2

Results of the intercalibration exercise for PBDEs and HBCDs (ng/g lipid wt.)

Fig. 2. Comparison of HBCD levels in Indo-Pacific humpback dolphins and finless porpoises from the South China Sea.

tion and variable levels of HBCDs found in finless porpoises and Indo-Pacific humpback dolphins.

3.3. Temporal variation

To evaluate temporal change of HBCDs contamination, data obtained from finless porpoises collected in 2000/01 from Hong Kong were compared with those collected in 1990 from Dongshan (Fig. 3); the specimens collected in both years possibly belong to the same population (Zhou et al., 1995). Mean concentrations of total HBCDs, i.e., sum of the three isomers, in finless porpoises increased (twofold) from 1990 to 2000/01, although the difference was not statistically significant (Mann–Whitney U-test). \( \alpha \)-HBCD concentrations, however, increased (fourfold) significantly \( (p < 0.01) \) during this period (from 9.5 ng/g lipid in 1990 to 35 ng/g lipid in 2000/01). \( \gamma \)-HBCD was detected only in one specimen out of five collected in 2000/01 whereas it was detected in 5 of 6 samples collected in 1990. The reason for non-detection of \( \gamma \)-HBCD is unclear, but it may be due to the difference in isomeric composition of commercial mixtures and bioavailability/persistence of each isomer. The significant increase of \( \alpha \)-HBCD levels in finless porpoises from the year 1990 to 2000/01 probably suggests that HBCDs have been used extensively since the 1990s. This trend could be the result of economic development, including industrial and human activities in this region. The rate of increase of HBCD levels was less than that of PBDEs (sixfold, \( p < 0.01 \)), indicating that use of HBCDs is still not as intense in this region compared to PBDEs (Ramu et al., 2006). The SCS region is one of the fastest growing industrial areas of Asia, especially in electronic and telecommunication industries, where PBDEs are largely used. The temporal variation of HBCDs in biological samples has also been reported elsewhere. Concentrations of PBDEs and HBCDs in blubber samples from stranded male California sea lions demonstrated an increasing trend from 1993 to 2003 (Stapleton et al., 2006). A study on guillemot (Uria algea) eggs from the Baltic Sea, sampled between 1969 and 2001 showed an increasing trend in concentrations of HBCDs throughout the study period. However, HBCDs concentrations showed no significant change during the later 10 years of study, while PBDEs showed a decreasing trend after the late-1980s (Sellstrom et al., 2003). On the other hand, in a study on BFRs in peregrine falcon (Falco peregrinus) eggs from South Greenland, HBCDs decreased between 1986 and 2003, whereas PBDEs increased significantly (Vorkamp et al., 2005). Thus, temporal trends of HBCDs in the world are still not clear, as there is either an increasing or a decreasing trend, depending upon various environmental and biological factors. However, the present increasing usage of HBCDs in the world may accelerate further contamination in the ecosystem. It has already been shown by Covaci et al. (2006) that the differences in regional production and historical application of PBDEs and HBCDs leads to different temporal trends in the environment and biota.

3.4. Isomeric composition

The composition of each isomer relative to total HBCDs in blubber of cetaceans is shown in Fig. 4. The fraction of \( \alpha \)-HBCD, i.e., concentration of \( \alpha \)-HBCD divided by the sum concentration of the three isomers \( (\alpha / \sum \text{HBCDs}) \), was >0.99 in finless porpoises and humpback dolphins collected in 2000/01. This ratio in wildlife can be used as an indicator of bioaccumulation and source identification, although there are several factors affecting this ratio, such as differences in isomeric composition of source materials, their physico-chemical properties, behavior in the environment, and varying bioavailability of the three isomers. The values were reported to range from 0.49 to 0.78 in plankton to 0.76–0.88 in fish (Tomy et al., 2004), suggesting an increasing trend with trophic level. Many other studies on the isomeric composition of
HBCDs in higher trophic animals also reported the predominance of α-HBCD, as summarized by Covaci et al. (2006). This can be ascribed to isomer-specific biomagnification of α-HBCD in the upper trophic level and also low bioavailability of γ-HBCD. Our result (α-HBCD/∑HBCDs ratio >0.99) agrees well with those reports because finless porpoises and humpback dolphins are top predators in the marine environment of SCS. On the other hand, a relatively high proportion of γ-HBCD has been reported in harbor porpoises from the North Sea (Morris et al., 2004) as found in finless porpoises from 1990 used in this study. It may be concluded that γ-HBCD can also be bioaccumulated by wildlife under specific conditions. More detailed studies on the trophic transfer of each HBCD diastereoisomer should be conducted to unfold their isomer-specific behavior and fate in the food web.

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References


