THE EFFECTS OF 28-DAY ORAL DOSE OF HEXABROMOCYCLODODECANE ON BONE AND LIVER RETINOIDS IN WISTAR RATS

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Introduction

Hexabromocyclododecane (HBCD) is a brominated flame retardant, which has its main application in textile industry and in polysterene foam in building construction. The technical preparation is a mixture of three enantiomers; α, β, γ. It is simply blended with polymers, and therefore more likely to leach out of products[1] than covalently bound flame retardants. Indeed, this compound has been identified as a contaminant of environmental significance[2]. It is detected in various environmental compartments, including remote areas such as arctic air and biota[3]. Low but significant levels of this persistant and bioaccumulating compound are found in humans[4,5]. Food is possibly the largest source of human HBCD exposure, although inhalation and dermal routes may contribute significantly, notably in occupational exposure[4]. Therefore, the use of this compound is a reason for environmental and health concern[6].

To extend the toxicological database for HBCD we analysed the bone size and mineralization, and apolar liver retinoid levels in rats, which were exposed to HBCD in a 28 days subacute toxicity study, enhanced for endpoints affected by persistent organic pollutants, *i.e.* in the immune and endocrine domains. Both bone tissue and the retinoid system are sensitive to persistent organohalogens, such as dioxins and PCBs[7,8,9], but the mechanisms behind these effects are still poorly known.

Animals, materials and methods

Forty Wistar rats (Cpb:WU, RIVM, the Netherlands) of both sexes were housed individually to allow recording of individual feed consumption, and to avoid bias from hierarchical stress. Light/dark regime was 12/12 h. Standard pelleted rat feed without soy (Hope Farms/Arie Blok Diervoeding, Woerden, the Netherlands) and drinking water were supplied ad libitum. A technical mixture of HBCD was obtained through Bromine Science and Environmental Forum (BSEF). Target dosing was 0, 0.3, 1, 3, 10, 30, 100 or 200 mg/kg bw/day. The dose, dissolved in corn oil, was given by gavage daily during 28 days. Exposure started at 8 weeks of age. Body weight and feed consumption were monitored weekly. At the end of the exposure period, animals were killed. Euthanasia was achieved by exsanguination from the abdominal aorta under carbon dioxide anesthesia. The liver was weighed directly after dissection and was thereafter snap frozen in liquid nitrogen and stored at -80ºC. The lower part of the carcass, including lumbal vertebrae, pelvic bones and one intact hind limb, was frozen at -20ºC. At a later stage, the carcasses were thawed and left femur and tibia were dissected, cleaned from soft tissue and stored in Ringer solution[11] at -20°C until analysis. The experimental protocol followed the OECD407 28 day subacute toxicity guideline, which was enhanced for endocrine and immunological endpoints[12,13], with the exception of dose group arrangement. In contrast to the published protocol, the animals were distributed among more dose groups with fewer animals in each group for improved assessment of dose-response relationships. Each dosing group had 5 males and 5 males. Experiments were approved by the institutional Committee on Animal Experimentation, according to Dutch legislation.

C*ompound analysis:* Internal dosing was verified by analysis of liver concentrations of HBCD. A sub-sample of rat liver was taken to determine the concentrations of α-, β-, and γ-HBCD.

Bone analyses: The length of each bone was measured using an electronic sliding caliper to the nearest 0.01 mm (IP65, Sylvac SA, Crissier, Switzerland). The bones were scanned using the peripheral quantitative computed tomography (pQCT) system (Stratec XCT Research SA+) with software version 5.50 (Norland Stratec Medizintechnik, GmbH, Birkenfeld, Germany) as described elsewhere[14]. Diaphyseal pQCT scans of femur and tibia were performed at sites distanced 50% of total bone length from the end of the bone to determine cortical bone mineral density, bone mineral content and area. Metaphyseal pQCT scans, at sites distanced 15 % (male

and female) of total bone length from the distal end of femur and at 12 % (male) or 15 % (female) from the proximal end of tibia, were performed to measure total and trabecular bone mineral density, bone mineral content and area.

Liver retinoids: Apolar retinoids were extracted from liver homogenates (20% w/v in water) using diisopropyl ether and separated on a Nucleosil C_{18} 5-µm HPLC column using an ethanol:water gradient elution as described by Nilsson et al.[15] Retinol and retinyl esters were detected with a JASCO 821-FP fluorescence detector, and quantified using internal and external standards.

Statistical analysis: Dose-response analysis of effects was done with the Possible Risk Obtained from Animal Studies (PROAST) software[16]. From these dose-responses, a critical effect dose (CED) was calculated at a critical effect size (CES) of 10 or 20%. CES is defined as the threshold adverse effect level, determined by expert judgment for each parameter based on knowledge of the pathophysiology of each effect, including irreversibility or adverse follow-up effects[16]. The analysis was completed with the calculation of a confidence interval with 5% and 95% confidence levels, thus enabling the definition of a Benchmark Dose at the Lower confidence level (BMD-L). The CED/BMD-L ratio was used as a measure for the statistical uncertainty in a data set, and thus for validity of the dose-response modeling.

Results

In life observations: The animals showed no signs of general toxicity during exposure to HBCD; dosing was well tolerated. All animals in all dose groups showed normal feed consumption. There were no effects on body growth during the exposure period.

Internal HBCD concentration: Analysis of HBCD α and γ in the liver showed a dose-dependent increase with a plateau at the three highest doses[11]. The concentration of HBCD was higher in female rats than in male rats over the entire dose range (on average 5.2 times). In addition, female rats in the control group had low levels of HBCD, whereas HBCD was below the limit of detection in control male rats.

Bone parameters: There was no effect of HBCD on bone length in any sex (data not shown). However, with increasing dose of HBCD there was an increase in mineral density of trabecular bone at femur (Figure 1A) and tibia (Figure 1B) metaphysis in female rats. Further changes at the femur metaphysis of female rats were increased total bone area and increased total bone mineral contents (data not shown). There were no changes in any of the bone parameters in male rats (Table 1).

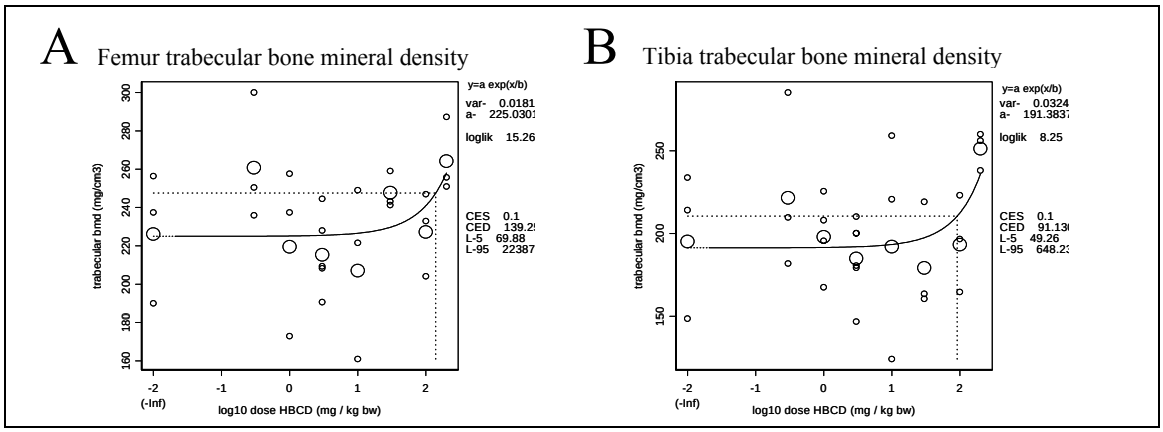

Figure 1. Dose-response curves for trabecular bone mineral density at metaphysis in (A) femur and (B) tibia in female rats after 28-day oral exposure to HBCD.

Liver weight and retinoid levels: There was a significant dose-dependent increase of the liver weight in females only, with BMD-Ls of 22.9 mg/kg bw (at 20%) (Figure 2A). Marked dose-related decreases in apolar liver retinoid concentrations were observed in female rats (data not shown). The decreases were parallel to the dose-related increase in liver-weight (Figure 2A). No effect on the apolar liver retinoid concentrations was seen in male rats (data not shown). The total content of apolar retinoids in the liver was not affected by HBCD at any dose, neither in female (Figure 2B) nor in male rats (Table 1).

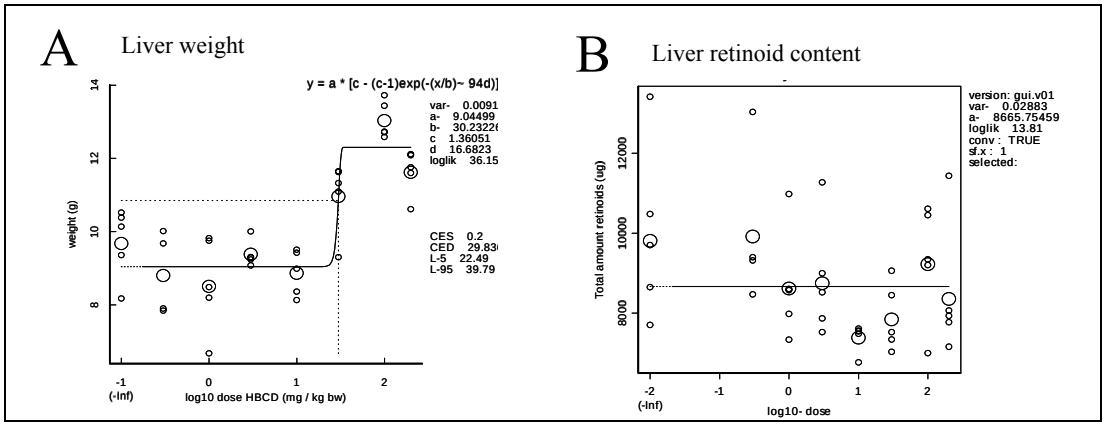

Figure 2. Dose-response curves for (A) liver weight, and (B) liver retinoid content in female rats after 28-day oral exposure to HBCD.

Table 1. Trabecular bone mineral density at femur metaphysis, liver weight and liver retinoid content in male rats after 28-day oral exposure to HBCD*.

HBCD dose (mg/kg bw)	Trabecular bone mineral density (mg/cm^2)		Liver weight (g)	Liver retinoid content (µg)
	Femur	Tibia		
0	145 ± 14[a]	143 ± 17[a]	13.9 ± 0.7	10806 ± 1352
0.3	142 ± 15	126 ± 30	17.1 ± 3.4	15534 ± 1519
1	136 ± 28[a]	131 ± 18[a]	16.2 ± 3.0	13123 ± 2826
3	174 ± 16[a]	158 ± 15[a]	15.0 ± 1.6	15371 ± 2387
10	145 ± 9[b]	136 ± 9[b]	17.7 ± 2.3	12834 ± 3101
30	151 ± 28	143 ± 19	15.7 ± 0.5	12577 ± 2530
100	184 ± 18[a]	168 ± 24[a]	16.4 ± 2.3	10227 ± 1834
200	149 ± 10[a]	135 ± 6[a]	16.4 ± 3.2	13199 ± 1816

*Figures are average ± standard deviation of n replicates per dose group. n = 5 unless otherwise stated.
[a] n = 4, [b] n = 3.

Discussion

Increased trabecular bone mineral density was observed at femur and tibia metaphysis in HBCD exposed female rats. In contrast, there was no effect of HBCD exposure on cortical bone at tibia or femur diaphysis in any sex. These data suggest that trabecular bone, which is the predominant bone type at metaphysis, is more sensitive to the perturbations by HBCD compared to cortical bone, which is the predominant bone type at diaphysis. The higher sensitivity of trabecular bone to chemical insult may be related to the higher metabolic activity and higher turnover rate as compared to cortical bone[17]. Furthermore, the 28-day study design used in the present study may only show initial effects of HBCD on bone. Possibly, longer-term exposure will reveal more pronounced bone effects. In addition, investigations of bone mechanics and more detailed geometry analyses may reveal whether also bone strength is altered by HBCD exposure. The mechanism behind these changes in bone needs further investigations.

No effect of HBCD was seen on the liver content of apolar retinoids in male and female rats. Therefore, the dose-dependent decrease in apolar liver retinoid concentrations observed in HBCD exposed female rats most likely reflects the pronounced and dose-related increase in liver weight of these animals. Hepatic retinoid reduction has been proposed to be an AhR mediated response[18]. Thus, the lack of effects on retinoids in this study suggest that HBCD does not activate the AhR, which is consistent with the observation that neither the hepatic expression of CYP1A1 nor the hepatic EROD activity are induced by HBCD[19].

The difference in effects of HBCD between the male and female rats may possibly be due to a faster elimination of HBCD in male compared to female rats, which previously has been indicated when administrating a single dose of radiolabelled HBCD[20]. Concomitantly, in this study, higher liver HBCD concentrations were observed in female rats than in male rats. The faster elimination of HBCD in male rats may also explain why HBCD was recovered from control female animals but not from control male animals. This observation further shows that these control animals, which were not experimentally exposed to HBCD, still received the compound, probably through the standard feed.
Based on a 20% increase in liver weight in females, which appeared to be the most sensitive parameter of the parameters shown in this paper, BMD-L for adverse effects by HBCD should be defined as 22.5 mg/kg bw per day. This is well below the previously proposed LOAEL of 100 mg/kg bw per day based on previous findings[21]. In conclusion, these data support that exposure to HBCD influence liver and bone after a subacute exposure regime and give rise to alterations at doses in the same range or below the previously proposed LOAEL.

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