Hydroxylated PBDE metabolites in rat blood after exposure to a mixture of PBDE congeners

Tina Malmberg[1], Maria Athanasiadou[1], Göran Marsh[1], Ingvar Brandt[2], Åke Bergman[1]

[1]Department of Environmental Chemistry. Stockholm
[2]Department of Environmental Toxicology, Uppsala Universitet, Sweden

Introduction

Halogenated aromatic compounds are in general both hydrophobic and slowly reacting chemicals, often referred to as persistent organic pollutants (POPs). These chemicals are however by no means inert. They are instead undergoing metabolism in wildlife and humans leading to the formation of halogenated phenolic compounds (HPCs) and when certain structural criteria are fulfilled methyl sulfone metabolites may be formed. Interestingly, a large number of HPCs are retained in blood [1-3]and up to almost 50 of those have been structurally identified in human plasma [4] . The majority of all HPCs identified until now are OH-PCBs [4,5]. The OH-PCBs retained in plasma or serum are substituted with the hydroxyl group in preferentially the *para* position, or as in a limited number of cases the *meta* position, holding chlorine atoms on both sides of the hydroxyl group [4,5]. The OH-PCBs are retained due to strong competitive thyroxine binding to transthyretin (TTR) [6].

Until now, no studies have been performed to determine if also PBDE metabolites, OH-PBDEs, are retained in the blood of e.g. rats. The aim of of this study was to determine the potential OH-PBDE profile in blood after dosing equimolar amounts of seven PBDE congeners to rats.

Materials and Methods

Chemicals: The compounds under study; BDE-47[7], BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209 were synthesised as described elsewhere [8-10] as well as BDE-138 and BDE-66 used as internal standards (I.S.). The methoxylated PBDEs used as reference compounds for the phenolic fraction: 4'-methoxy-2,2',4-triBDE (4'-MeO-BDE-17), 3-methoxy-2,2',4,4'-tetraBDE (3-MeO-BDE-47), 6-methoxy-2,2',4,4'-tetraBDE (6-MeO-BDE-47), 2'-methoxy-2,3',4,4'-tetraBDE (2'-MeO-BDE-66), 4'-methoxy-2,2',4,5'-tetraBDE (4'-MeO-BDE-49), 2'-methoxy-2,3',4,5'-tetraBDE (2'-MeO-BDE-68), 4-methoxy-2,2',3,4'-tetraBDE (4-MeO-BDE-42), 6-methoxy-2,2',4,4',5-pentaBDE (6-MeO-BDE-99), 4-methoxy-2,2',3,4',5-pentaBDE (4-MeO-BDE-90) and 6-methoxy-2,2',3,4,4',5-hexaBDE (6-MeO-BDE-137) were prepared according to Marsh *et al* [11]. Soya phospholipon 100 was obtained from Nattermann Phospholipid GmbH (Cologne, Germany)

and Lutrol F127 was purchased from BASF Wyandotte Corp. (Wyandotte, MI, USA). Diazomethane was prepared as described by Fieser and Fieser [12]. All other chemicals and solvents were of pesticide or analytical grade and obtained commercially.

Instruments: Gas chromatography-mass spectrometry (GC-MS) analyses of the samples and reference standards were performed on a Finnigan TSQ 700 (Thermoquest, Bremen, Germany). The GC was a Varian 3400 gas chromatograph equipped successively with three different types of columns. For the tetrabromo diphenylether metabolite analysis an Equity 5 column (30 m x 0.25 mm i.d. and 0.1 µm film thickness from Supelco, Bellefonte, USA) was used and follow temperature program; 80°C (1 min)-10°C/min –310°C (12 min). One DB-5HT column (15 m x 0.2 mm i.d. and 0.1 µm film thickness from J&W Scientific, Folsom, CA, USA) was used for the quantification of the seven PBDEs with the column temperature program 80°C (1 min)-15°C/min – 300°C (12 min). The polar column used was an SP™-2331 (30 m x 0.25 mm x 0.2 µm) from Supelco, Bellefonte, USA) and temperature programmed as follow; 80°C (1 min) - 20°C/min - 200°C (1min) - 3°C/min - 270° (20 min). The latter column was applied for separation of co-eluting compounds and verification of the MeO-PBDE identifications. On-column injections were performed using a septum-equipped programmable injector fitted with a high performance insert. The injector temperature was 60°C and increased with 180°C/min up to 320°C for each injection. The carrier gas was helium with head pressure of 3 psi. The transfer line temperatures was 290°C and 260°C when the polar column was used. Electron capture negative ionization (ECNI) for bromine trace analysis and full scan analysis was achieved with methane (AGA, Stockholm, Sweden) of >99,995% purity and with <5 ppm O_2 as the electron thermalization buffer gas at a pressure of 6.5 torr. The electron energy was 70 eV and the temperature of the ion source was 150°C. Masses between 70-1000 amu were scanned.

Animals: Twelve female Sprague-Dawley rats (weight range 225–245 g, B&B, Sollentuna, Sweden) were kept in cages (three per cage) under a 12 h/12 h light and dark cycle with free access to food and water.

Experimental design: Ten rats were given a single intraperitoneal (i.p.) dose of a PBDE mixture including BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209. The dose of each compound was 3 µmol/kg body weight (ranging from 1.5 mg/kg [BDE-47] to 2.9 mg/kg [BDE-209]). An emulsion of 0.16 g soya phosphlipon/Lutrol (16:34, w/w) /ml water was used as dose formulation and with a dose volume of 2 ml/kg b. wt. Five rats were sacrificed 24 hours after dose administration and remaining five rats, plus two control rats after 5 days, all by heart puncture under anaesthesia. Blood samples were collected into tubes, immediately placed on ice and centrifuged to isolate the plasma. Plasma samples were stored at –18 °C until taken out for clean up and analysis.

Extraction and clean up: A previously described method, slightly modified, down-scaled to half, was used for extraction and clean up [13]. Briefly, plasma was transferred to screw-capped tubes and the weight was adjusted with potassium chloride (1%) to 2.5 g. The internal standard (BDE-138, 103 ng) for the neutral fraction was added to the samples and two references were kept for the recovery determination. The proteins were denatured with isopropanol and hydrochloric acid (6M) and the substances were extracted from plasma with *n*-hexane:methyl *tert*-butyl ether. The combined extracts were washed with a potassium chloride solution (1%) and the solvent was reduced to dryness for lipid determination. The sample residue was dissolved in *n*-hexane and HPCs were partitioned with potassium hydroxide (0.5 M) to isolate them from the neutral PBDEs. In this step the alkaline phase was reextracted three times (instead of only once according to the

original method) since the administrated PBDEs were present at rather high concentrations in the plasma. After acidification of the aqueous phase, the phenolic compounds were extracted with 10% methyl *tert*-butyl ether in *n*-hexane. In order to methylate the hydroxy group, the samples were treated with an excess of diazomethane at room temperature (3 h) and protected from light to avoid any photochemical debromination [14]. The phenolic fraction was purified on a silica gel:sulfuric acid column (2:1 by weight, 0.5 g), with dichloromethane (10 ml) as mobile phase. The samples were analysed on GC/MS ECNI for quantification of tetrabrominated diphenyl ether metabolites for which authentic reference standards were available. For full scan analysis on GC/MS EI and ECNI of metabolites, the phenolic fraction from all samples (Day 1 and Day 5) were pooled.
The neutral fraction was diluted with *n*-hexane (4 ml) and neutral analytes were partitioned with concentrated sulfuric acid (0.5 ml) to eliminate lipids prior bromine trace analysis by GC/MS ECNI. ***Analysis:*** The administrated neutral compounds were quantified relative to the corresponding individual authentic external standards. The recovery of the internal standard BDE-138 was determined by adding an injection standard BDE-66 to the samples and the two references before analysis. Three methylated phenolic metabolites were also identified and quantified in relation to the authentic synthesized standards. Two GC/MS techniques EI and ECNI were used to cross verify behaviour similarities of hydroxylated PBDEs. Two GC-columns with completely different polarities were also used to strengthen the positive identification of those OH-PBDEs.

Results and Discussion

Blood was taken from five rats day 1 and another five rats at the end of the experiment, day five. The PBDE congener concentrations, given in pmol/g plasma, after one and five days are given in Table 1. The recovery of the internal standard BDE-138 was for ”Day 1” group 71 % RSD 8% and for ”Day 5” group 98 % RSD 2%. The plasma concentrations increase with number of bromine in the PBDE congeners up to BDE-183 while the BDE-209 is lower with comparable plasma concentrations as the hexaBDEs (BDE-153 and BDE-154). The reason for this lower plasma concentration may be at least twofold: either depending on a lower uptake from the intestinal cavity and/or a more rapid turnover (metabolism) of BDE 209 than of BDE-183. Both these explanations are supported by previous studies [15,16]. It has been shown that BDE-47 is quickly distributed to adipose tissues and only slowly metabolised with trace amounts of phenolic metabolites in the plasma[17].

Sixteen hydroxylated and two dihydroxylated PBDE metabolites were detected in the blood plasma after dosing of the mixture of seven PBDE congeners (Figure 1). Among these OH-PBDEs, three may be regarded as major OH-PBDE metabolites. The structural characteristics of the metabolites retained in the blood are shown by number of bromine substituents and suggested position of the hydroxyl-group, based on characteristic fragmentation pattern from GC/MS (EI) mass spectra [18] . There are several metabolites corresponding to the number of bromine substituents in the parent PBDEs. Since it is possible that the PBDEs undergoes debromination it is possible that lower brominated OH-PBDEs are formed from higher brominated congeners. It is accordingly not possible to state the origin of the OH-PBDEs shown in the chromatogram.

Table 1. Plasma mean concentrations (pmol/g plasma fresh weight) with minimum and maximum concentrations (Range) are given for the PBDE congeners day 1 and 5 after administration to rats. Concentrations are also given for three structurally identified OH-PBDE metabolites, all with four bromine atoms.

Compound	**Day 1** (*n*=4)		**Day 5** (*n*=5)	
	pmol/g fresh weight		pmol/g fresh weight	
	Mean	Range	Mean	Range
BDE-47	500	370-840	41	33-47
BDE-99	560	440-840	32	21-39
BDE-100	690	530-1090	53	39-60
BDE-153	1760	1290-2330	65	40-84
BDE-154	1380	1130-1910	36	20-53
BDE-183	2270	1890-2420	115	73-148
BDE-209	1200	360-2220	60	10-110
4-MeO-BDE-42	0.16	0.04-0.33	0.28	0.19-0.35
3-MeO-BDE-47	0.76	0.58-1.10	0.72	0.54-0.82
4'-MeO-BDE-49	0.48	0.34-0.62	0.63	0.42-0.76

Further, the OH-substituent is preferentially substituted in meta- and para-positions, as indicated in Figure 1. This is in contradiction with OH-PBDEs identified in e.g. salmon plasma where most of the OH-PBDEs are substituted with the OH-group in an *ortho*-position [18]. On the other hand it is supportive of the origin of the OH-PBDEs in each case with a natural origin of the OH-PBDEs in salmon blood and through mammalian metabolism of PBDEs in the present study. No metabolites were detected that can easily be related to BDE-209 in this study. This may be supported by the observation that BDE-209 does not form simple phenolic metabolites to any major extent [15,16].

Rats have been shown to metabolize BDE-47 to five different OH-tetraBDEs present in faeces, three in liver whereas two were present in plasma[17]. BDE-99 has been reported to form two OH-pentaBDE, two OH-tetraBDE and three dihydroxylated PBDE metabolites in faeces, bile and liver[19]. Both these previous studies indicated the complexity of PBDE metabolism. If the metabolism of the six of the seven PBDEs is modelled we suggest, excluding the possibility that PBDEs are debrominated, that there may be 6 mono-OH-tetraBDEs formed from BDE-47, 11 from BDE-99, 9 from BDE-100, 5 from BDE-153, 8 from BDE-154 and 8 from BDE-183. That gives a total of 43 potential metabolites after withdrawing duplicates. In fact 16 metabolites were detected in the present study which is more than 1/3 the theoretical number. It is much more difficult to make any suggestions on metabolites formed from BDE-209, based on the current knowledge that BDE-209 is transformed to several hydroxylated/methoxylated PBDEs[15,16].

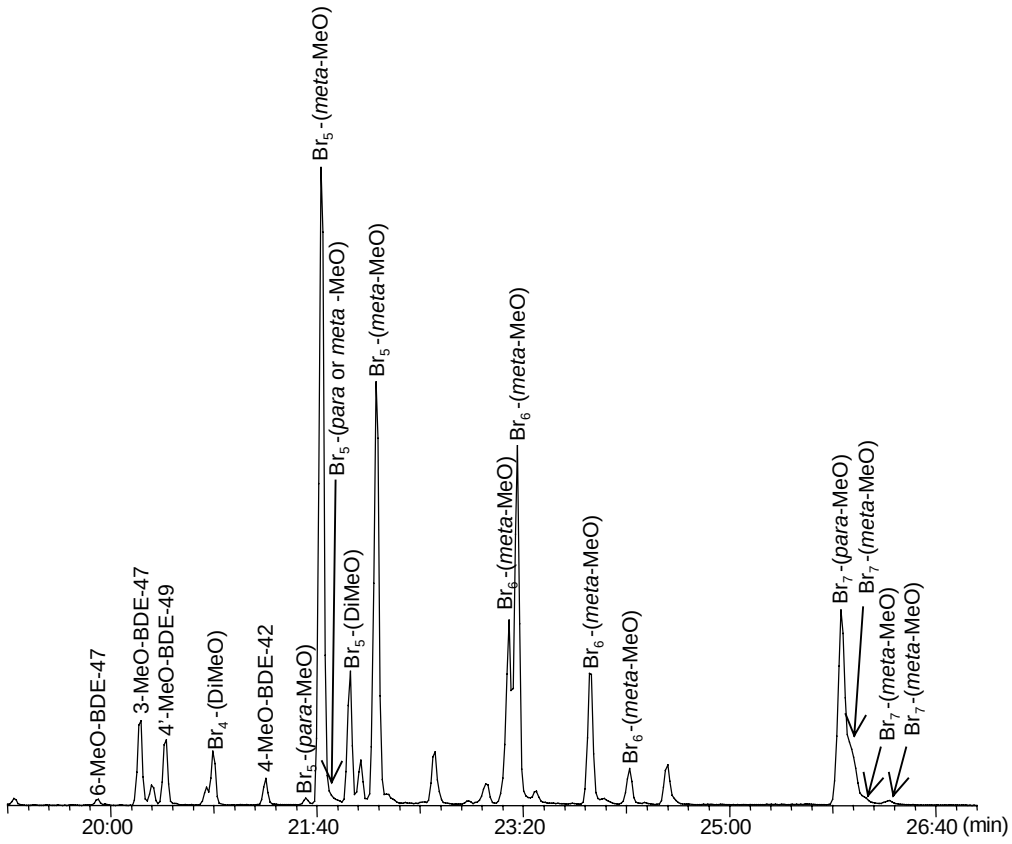

Figure 1. GC/MS (ECNI) chromatogram, m/z (79, 81), of the phenolic fraction from rat plasma is shown. The rats were administrated a mixture of equimolar amounts of seven PBDEs.

This study is the first report on OH-PBDE retention pattern in mammalian blood as a result of PBDE exposure. We know that several OH-PBDEs are retained in salmon blood [18] but that the origin of these OH-PBDEs seem to be dominated by naturally occurring OH-PBDEs. However, a range of OH-PBDEs have been shown in plasma from Common carp and Largemouth bass in Detroit river [20]. The PBDE contamination of our environment clearly leads to risks related to OH-PBDE exposures.

Acknowledgement

We want to particularly thank Ioannis Athanassiadis for his skilful MS work and the valuable contribution of Ankie Sundberg and Anna Sandholm to this work. The project was financially supported by the EU R&D program “Compare” (QKL4-CT2000-00261).

References

1 Bergman, Å., Klasson Wehler, E. and Kuroki, H. (1994) Environ. Health Perspect., 102, 464-469.
2 Fängström, B., Athanasiadou, M., Grandjean, P., Weihe, P. and Bergman, Å. (2002) Environ. Health Perspect., 110, 895-899.
3 Sandau, C. D., Ayotte, P., Dewailly, E., Duffe, J. and Norstrom, R. J. (2000) Environ. Health Perspect., 108, 611-616.
4 Hovander, L., Malmberg, T., Athanasiadou, M., Athanassiadis, I., Rahm, S., Bergman, Å. and Klasson Wehler, E. (2002) Arch. Environ. Contam. Toxicol., 42, 105-117.
5 Letcher, R. J., Klasson-Wehler, E. and Bergman, Å. (2000) The Handbook of Environmental Chemistry Vol. 3 Part K New Types of Persistent Halogenated Compounds. 315-359 , Springer-Verlag, Berlin Heidelberg.
6 Lans, M. C., Spiertz, C., Brouwer, A. and Koeman, J. H. (1994) European Journal of Pharmacology, 270, 129-136.
7 Ballschmiter, K., Mennel, A. and Buyten, J. (1993) Fresenius. J. Anal. Chem., 346, 396-402.
8 Marsh, G., Hu, J., Jakobsson, E., Rahm, S. and Bergman, Å. (1999) Environ. Sci. Technol., 33, 3033-3037.
9 Eriksson, J., Marsh, G., and Bergman, Å. (1-1-2001) The Second International Workshop on Brominated Flame Retardants, BFR 2001, 203-206.
10 Örn, U., Eriksson, L., Jakobsson, E. and Bergman, Å. (1996) Acta Chem. Scand., 50, 802-807.
11 Marsh, G., Stenutz, R. and Bergman, Å. (2003) Eur. J. Org. Chem.,
12 Fieser, L. F. and Fieser, M. (1967) John Wiley and Sons, New York.
13 Hovander, L., Athanasiadou, M., Asplund, L., Jensen, S. and Klasson Wehler, E. (2000) J. Anal. Toxicol., 24, 696-703.
14 Päpke, O., Bathe, L., Bergman, Å., Fürst, P., Meironyté Guvenius, D., Herrmann, T., and Norén, K. (2001) Organohalogen Compounds, 52, 197-200.
15 Mörck, A., Hakk, H., Örn, U. and Klasson-Wehler, E. (2003) Drug Metab. Dispos., 31, 900-907.
16 Sandholm, A., Emanuelsson, B.-M. and Klasson Wehler, E. (2003) Xenobiotica, 33, 1149-1158.
17 Örn, U. and Klasson Wehler, E. (1998) Xenobiotica, 28, 199-211.
18 Marsh, G., Athanasiadou, M., Bergman, Å. and Asplund, L. (2004) Environ. Sci. Technol., 38, 10-18.
19 Hakk, H., Larsen, G. and Klasson Wehler, E. (2002) Xenobiotica, 32, 369-382.
20 Letcher, R. J., D'Sa, I., Valters, K., Marsh, G., Hongxia, L., Bennett, E., and Alaee, M. (2003) Organohalogen Compounds, 61, 29-32.