IN VITRO METABOLISM OF DECABROMO DIPHENYL ETHER USING RAT MICROSOMES

Mas Sílvia[1,2], Raldua Demetrio[3] , de Juan Anna [2] , Tauler Romà [1] and Lacorte Sílvia[1]

[1] Department of Environmental Chemistry, IIQAB-CSIC, Jordi Girona 18-26, 08034 Barcelona, Catalonia, Spain
[2] Department of Analytical Chemistry, University of Barcelona, Diagonal 647, 08028 Barcelona, Catalonia, Spain.
[3] Laboratory of Environmental Toxicology, Universitat Politècnica de Catalunya, CN 150, Km. 14.5 (Institut Politècnic Campus Terrassa), 08220 Terrassa, Catalonia, Spain.

Introduction

Polybrominated diphenyl ethers (PBDEs) have been extensively used as flame retardants in a wide variety of products including plastics, textiles and electronics[1]. During the past years, concern has arisen due to the increasing occurrence of these products in the environment and to the proven toxic and endocrine disruptor activity of lowly brominated congeners[2]. Among PBDEs, Deca-BDE (BDE-209) is extensively being used in Europe and its environmental fate is still uncertain. Regarding to its elimination, it has been demonstrated that BDE-209 can be degraded photochemically, producing lower brominated BDEs[3]. Similary, the biotransformation of BDE-209 may lead to an increase of more active and persistent low brominated PBDEs in the environment. Little is known on the biotransformation of PBDEs in wildlife, but it was recently shown that CYP2B and, to a lesser extent, CYP1A and CYP4A3 were, involved in the metabolism of some PBDE congeners by rat liver microsomes[4]. In this study, the metabolism of BDE-209 by CYP2B, CYP1A and CYP4A3 was tested using phenobarbital, β-naphthoflavone and clofibrate-treated rat liver microsomes. These enzymatic systems are involved in the detoxification of many xenobiotics, producing hydroxylation, epoxidation or other reactions in phase I metabolism, capable thus to eliminate toxic compounds. In the present study we tested their ability to biotransform BDE-209. BDE-209 is a high molecular weight compound (959.17 Da) and its bioavailability and possible metabolization in vertebrates has been questioned. Given the neurotoxic effects that BDE-209 can cause in exposed rats[5], and their potential impact to other mammals, including men, studies on the metabolization pathways are needed for a better risk assessment. In an attempt to evaluate the debromination or hydroxilation processes during the microsomal degradation experiment, gas chromatography coupled to negative chemical ionization mass spectrometry (GC-NCI-MS) and liquid chromatography coupled to negative tandem mass spectrometry (LC-ISP(-)-MS/MS) were used.

Materials and Methods

Microsomes and Chemicals

Liver microsomes from Sprague-Dawley rats pre-treated with phenobarbital, β-naphthoflavone and clofibrate were purchased in Tebu-bio (Barcelona Science Park, Catalonia). Analytical grade decabromodiphenyl ether (98%) was purchased from Sigma-Aldrich (St. Louis, MO USA). Analytical grade methanol and dimethylsulfoxide (DMSO) and GC grade hexane were purchased from Merck (Darmstadt, Germany). The decachlorinated biphenyl (PCB 209) was purchased from Lab. Dr Ehrenstorfer (Augsburg, Germany) and was used as internal standard. The remaining chemicals used in this study were reagent grade or better and were purchased from Sigma (St. Louis, MO).

A stock solution of BDE-209 was prepared in DMSO at a concentration of 140 mg L^{-1}. In order to prepare the NADPH regenerating system (NRS), a stock solution A (20 mM NADP+/ 100 mM G6P/ 100 mM $MgCl_2$) and stock solution of G6PDH at 292 U mL^{-1} were made.

Microsomal assay and sample extraction

BDE-209 metabolism was assayed by incubation with induced hepatic microsomes, as described for other PBDEs[4], with slight modifications. Briefly, 1.5 µM of BDE-209 was preincubated with 1 mg mL^{-1} hepatic microsomes in a 0.1 M Tris-HCl buffer (pH 7.5) for 5 min in a shaking water bath at 37°C (shaking at 150 rpm). After preincubation, the reaction was initiated by the addition of NRS (50 µL solution A + 3.75 µL G6PDH + 196.25 µL 2% $NaHCO_3$) in a total incubation system volume of 1 ml. Metabolism was stopped after 30, 60 and

120 min by the addition of 2 ml ice-cold methanol. After centrifugation (1000 x *g* for 5 min), the supernatants were extracted three times with 2 ml diisopropyl ether by vortexing for 30 s, and then removal of the both aqueous and organic phases. The extracts were dried by evaporation under N_2, and organic residues were reconstituted in 250 µl of hexane prior to injection in GC-NCI-MS. Afterwards, the same extract and the aqueous residues were evaporated and reconstituted with 250 µl of methanol prior to injection in a HPLC-MS/MS. 100 ng of PCB 209 as internal standards was added as internal standards after stopping the reaction in order to correct the loss in the extraction procedure.
Control incubations were carried out by performing identical incubations with the BDE-209 and 250 µl of 2% $NaHCO_3$ added instead of NRS. In order to determine the possible degradation of BDE-209 three additional control incubations were also carried out without microsomes.

Instrumental Analysis

Gas chromatography-mass spectrometry
Gas chromatography coupled to mass spectrometry using negative chemical ionization detection (GC-NCI-MS) was performed on an Agilent 6890 gas chromatograph connected to an Agilent 5973 Network mass spectrometer in order to analyze BDE-209 debromination or other possible biotransformation products such as methoxyBDEs. An HP-5ms capillary column (15 m x 0.25 mm i.d, 0.10 µm film thickness) containing 5% phenylmethyl siloxane (Model HP DB-5Ms) was used with helium as the carrier gas at 15 psi and amonia was used as reagent gas in the NCI mode. The temperature program was from 120 ºC (held for 1 min) to 205 ºC (held for 1 min) at 6 ºC min^{-1} and then from 205 to 310 ºC (held for 15 min) at 18 ºC min^{-1}, using the splitless injection mode during 1 min. The ion source temperature and reagent gas pressure in the ion source were 250 ºC and 1.9 10^{-4} Torr respectively. Full scan data acquisition was performed by scanning from m/z 75 to 800.

Liquid chromatography-mass spectrometry
Liquid chromatography coupled to negative tandem mass spectrometry (LC-ISP(-)-MS/MS) was performed on an Agilent 1100 series HPLC system (Waldbronn, Germany) connected to an API 3000 triple quadrupole MS/MS system (PE Sciex, Concord, ON, Canada) in order to analyze decaBDE hydroxylation process. HPLC Conditions and MS/MS parameters were described previously by S. Mas et al. [6].

Results and discussion
The biotransformation of BDE-209 was tested using rat liver microsomes enriched with CYP2B, CYP1A and CYP4A3. The proposed *in vitro* assay was optimized for BDE-209 taking into consideration the low solubility of this compound in many organic solvents and the need to use and minimize the amount of solvent in the microsomal incubations to avoid denaturisation. The selected solvent was dimethysulfoxide (DMSO), widely used in this kind of bioassays and the solubility (per litter in DMSO) of BDE-209 was 140 mg. Regarding to the extraction efficiency, recoveries of BDE-209 without microsomes were of 92%±2, indicating that the procedure and the solvents used were adequate. Given the fact that both the aqueous layer and the solvent layer from LLE were analyzed, it was expected that both low brominated BDEs, methoxy, hydroxy or other metabolites of BDE-209 would be efficiently extracted.

Control without microsomes did not show any decrease in BDE-209 content, indicating that under the conditions used in the assays there was no significant abiotic degradation. Figure 1 shows the time-course of BDE-209 metabolism for the three P450 systems. After 30 min incubation, the BDE-209 content decreased 57%, 38% and 31% for the CYP2B, CYP1A1, and CYP4A3 enriched microsomes, respectively. The decrease observed in the BDE-209 content is an indirect evidence of the metabolism of this compound by the cytochrome P450. A period incubation of BDE-209 with phenobarbital (CYP2B) presented the highest metabolisation and lower DecaBDE depletion was found in the microsomal incubation with β-naphthoflavone (CYP1A) and clofibrate (CYP4A3). These results are in good agreement with previous studies that have shown that CYP2B and to a lesser extent CYP1A and CYP4A3 were involved in the metabolism of some PBDE congeners[4]. The higher activity of CYP2B is related to the fact that BDE-209 is a non-planar lipophilic molecule, common characteristics to induce the CYP2B enzyme system[7]. No further decrease of BDE-209 content was observed between 30 min and 120 min incubation time.

Although we observed decay in the BDE-209 concentration, in the GC-NCI-MS analysis only the peaks of PCB-209 (IS), BDE-209 and some decomposition products of BDE-209, due to its instability to highs temperatures, were found. This analysis revealed the total absence of any debromination product. These results are in accordance to Benedict et al.[8] who suggested that cytochrom P-450 enzymes were not the main debromination pathway for BDE-99. However, when microsomes were co-incubated with reverse thyronine, catalytic mediation via thyroid hormone deiodinases was found, generating BDE-47. In those studies, carp microsomes were used with 1 h incubation.

Additionally, we used LC-ISP(-)-MS/MS to determine the formation of any hydroxyl-BDE. The system was optimized to determine from di to tetra hydroxyl-BDEs[6] but no metabolites of this bromination level were detected. Given the recent availability of a large number of hydroxyl-BDE of different bromination levels, from penta to nona hydroxyl BDEs will be analyzed after the method is set up. Another possibility is that metabolites may be covalently bound to macromolecules, either protein or lipids, as indicated by Mörck et al. when analyzing the metabolization of BDE-209 in rats[9]. This indicates the need to determine and identify the BDE-209 phase I metabolites considering the possible interaction with cell components.

The fact that CYP2B can lead to a partial biotransformation of BDE-209 without formation of debrominated or hydroxylated compounds have important toxicological implications in mammals. On one hand, BDE-209 has been described as a developmental neurotoxicant that can produce long-term behavioral changes following a discrete period of neonatal exposure[10]. Administration of decaBDE to male rats at 3 days of age in another study was shown to disrupt normal spontaneous behavior at 2 months of age[11]. It has also been reported that lower brominated BDEs, such as BDE-47 may be recalcitrant in the environment and possess the ability to interfere with the thyroid hormonal system. Fewer studies have been performed on OH- PBDEs in mammals, although it is thought that PBDEs can compete with the binding of T4 to plasma thyroid hormone- transporter transthyretin (TTR) only when hydroxylated[4]. 6-OH-BDE 47, detected in vivo in human plasma[12] can bind to TTR with a relatively high affinity[13]. Accordingly, future studies will be enfaced to evaluate the biotransformation routes of BDE-209 by widening the spectra of possible metabolites or adducts formed.

Acknowledgements
This research has been financed by the Spanish Ministry of Education and Science (projects CTM2005-07402-C02-01/TECNO and CTQ2006_15052_C02_1/BQU). Sílvia Mas acknowledges a PhD grant UAC 2005-0071 from the Ministry of Education and Science (Associated Unit between University of Barcelona and CSIC).

References
1. World Health Organization 1994.
2. de Wit, C.A. *Chemosphere* 1991; 46: 583.
3. Mas, S., de Juan, A., Lacorte, S., Tauler, R. *Analytica Chimica Acta* 2008; 618(1):18.
4. Meerts, I.A.T.M., van Zanden, J.J., Luijks, E.A.C., van Leeeuwen-Bol, I., Marsh, G., Jakobsson, E., Bergman, A., Brouwer, A. *Toxicological Sciences* 2000; 56: 95.
5. Viberg, H., Fredriksson, A., Jakobsson, E., Orn, U., Eriksson, P., *Toxicological Sciences* 2003; 76:112
6. Mas, S., Jáuregui, O., Rubio, F., de Juan, A., Tauler, R., Lacorte, S. *Journal of Mass Spectrometry* 2007; 42:890.
7. Lewis, D.F.V., Eddershaw, P.J., Dickins, M., Tarbit, M.H., Goldfarb, P.S, *Chemico-Biological Interactions* 1998; 115:175
8. Benedict, R.T., Stapleton, H.M., Letcher, R.J., MItchelmore, C.L. *Chemosphere* 2007; 69:987
9. Mörck, A., Hakk, H., Örn, U., Wheler, E. K., *Drug Metabolism and Deposition* 2003; 31:900
10. Rice, D.C., Reeve, E.A., Herlihy, A., Thompson, W.D., Warkowski, V.P., *Neurotoxicology and Teratology* 2007; 29:511.
11. Viberg, H., Fredriksson, A., Eriksson, P., *Neurotoxicology* 2007; 28:136.
12. Hovander, L., Malmberg, T., Athanasiadou, M., Athanassiadis, I., Rahm, S., Bergman, Å., *Archives of Environmental Contamination and Toxicology* 2002; 42:105.
13. Legner, J., Cenijn, P. H., Malmberg, T., Bergman, Å., Brower, A., Organohalogen. Compounds 2002; 56:53.

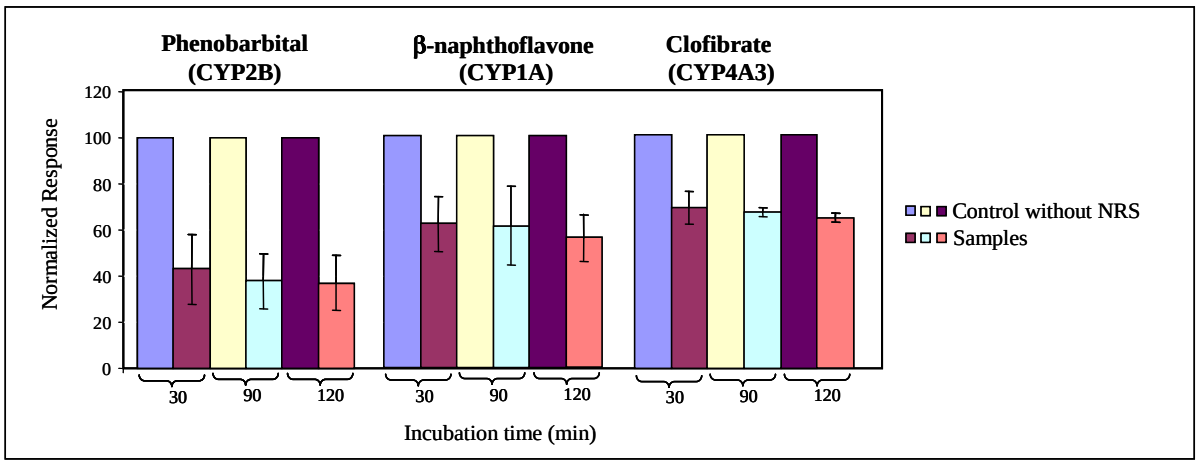

Figure 1. Time-course of BDE-209 metabolism using CYP2B, CYP1A1, and CYP4A3 enriched microsomes.

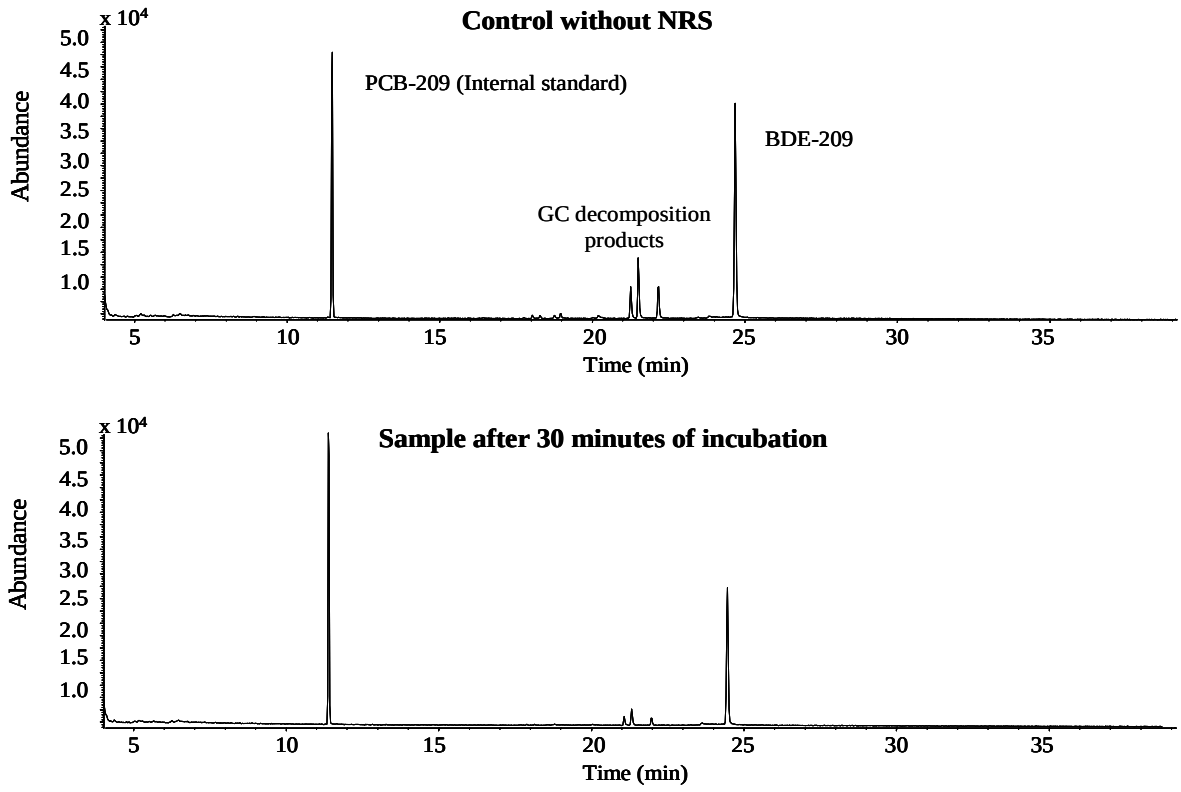

Figure 2. GC-NCI-MS analysis of BDE-209 metabolites in case of using phenobarbital Rat liver microsoma.