DISPOSITION OF [^{14}C]-DBDE IN PREGNANT RATS

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Introduction

Polybrominated diphenyl ethers (PBDE) are used as flame retardants for industrial equipment such as TV sets, office equipment and textiles. They have emerged as a new class of environmental contaminants. PBDE congeners (BDE-47, 99, 100, 153, 154 and 209) have been shown to account for a large proportion of the PBDE found in the environment, including domestic environment[1, 2]. Decabromodiphenyl ether is now the only BDE commercial mix which use remains authorised within the European Union. Its worldwide demand was estimated over 50 000 tons/year, as reported in 2002[3]. Decabromodiphenyl ether (DBDE) readily debrominates into lower molecular weight BDEs, under U.V. exposure[4]. DBDE also debrominates in fish[5], in mammals[6] and possibly in human[7]. DBDE has been found in human breast milk[8] and tissues: plasma[9], adipose tissue[10], but also foetal liver[11]. Little is known about its metabolic fate in animals models. Though hydroxylated and methoxylated metabolites have been identified in rat[6], the corresponding metabolic profiles of DBDE residues in tissues are unavailable. Hence, the distribution and the proportion of metabolites in tissues remain unknown. We have started a study of the metabolism of DBDE in pregnant Wistar rats to address these different questions, based on the development of suitable extraction and analytical methods, with the aim to achieve the radio-chromatographic separation and structural identification of DBDE metabolites.

Materials and Methods

Radio-labelled DBDE was synthesised from ring-[^{14}C]-diphenyl ether (Moraveck Biochemicals, CA, USA; radio-purity: >99%, specific activity: 862.6 MBq.mmol^{-1}). 7.4 MBq [^{14}C]-diphenyl ether dissolved in methanol were evaporated under gentle nitrogen stream. Aluminium was added as the catalyser, then Br_2 was added in excess every 10 minutes, at 50°C. After the ninth Br_2 addition, the vial was hermetically closed and was kept 2 hr at 70°C. The reaction was quenched with water, and DBDE was extracted with toluene. DBDE was purified by SPE on C18 Macherey Nagel glass cartridges. Its radiochemical purity was checked by radio-HPLC (purity > 99.8%).

Three conventional pregnant Wistar rats (288 ±14 g) were individually housed in stainless steel metabolic cages. Animals were daily force-fed with [^{14}C]-DBDE (vehicle: peanut oil) by oral route from gestational days 16 to 19. Daily doses were adjusted to 1.95 mg/kg b.w./day (1.75 MBq/kg b.w./d). Urine and faeces were collected daily over 4 days. Animals were killed by exsanguination on day 20 of gestation, 24h after the last DBDE dosage. Blood was collected in heparinised tubes, plasma were obtained after centrifugation (3 000 g/10 min). Liver, digestive tract, kidneys, adrenals, spleen, lung, heart, brain, abdominal adipose tissu, muscle (*semitendinosus*), ovaries, uterus, placentas, amniotic fluid and foetuses (whole litter) were removed and stored at -20°C until analysis. Digestive tracts were divided into three parts: stomach, duodenum to caecum (no-included), and caecum to rectum. Digestive tracts were washed of their liquid contents with 20 mL of NaCl 9‰, which were stored separately from the tissues themselves.

Quantities of [^{14}C] in urine, plasma and amniotic fluid were determined by direct counting on a Packard liquid scintillation counter. Radioactivity in rat carcasses, homogenised faeces, fresh tissues and extraction pellets was determined by complete combustion using a Packard 307 Oxidizer, followed by $^{14}CO_2$ quantification using a liquid scintillation counter.

Following solvent extraction, samples were analysed by HPLC on a Spectra system P1000 (Thermo Finnigan) coupled, with on-line radioactivity detection (610TR Flo-one/β A500 radioactivity detector; Perkin Elmer), using a Nucleodur C18 250 x 4.6 mm (5µm) column. HPLC conditions were adapted from Debrauwer et al.[12] Metabolites were quantified by ^{14}C-monitoring and were isolated by HPLC separation and C18 glass cartridges SPE. The identification of DBDE and its metabolites was achieved by Atmospheric Pressure Photo-Ionisation

Ion Trap-MS (APPI-ITMS) or LC-APPI-ITMS. MS analyses were carried out on a quadrupole ion trap mass spectrometer (Finnigan LCQ DecaXP, Thermo Electron, Les Ulis, France) fitted with the Finnigan APCI / APPI dual ionisation source. This source generates 10 eV photons by means of a VUV Krypton discharge lamp.

Results and Discussion

Metabolic Balance:

Animals were orally dosed 4 consecutive days with [^{14}C]-DBDE and were killed 24 hr after the last dosage. More than 18% of the administered dose was found to remain in the body of pregnant Wistar rat (carcass + liver + other tissues) following this 96 hr-study. In accordance with previous studies[6,13], faeces was found to be the major route of elimination of DBDE residues. Approximately 64% of the administered dose was excreted in faeces with an additional 5.3% being recovered from digestive tract contents (fig. 1). Less than 0.1% of the distributed radioactivity was excreted in urine (not shown). The highest amounts of residues were found in residual carcasses (9.2%), and in the liver (6.5%). Carcasses were further dissected to separate skin, adipose tissue, muscle and bones. Most of the radioactivity present in the carcass was associated with the skin and the adipose tissue. More than 1% of the administered [^{14}C]-DBDE dose was located in the reproductive tract (uterus, ovaries),

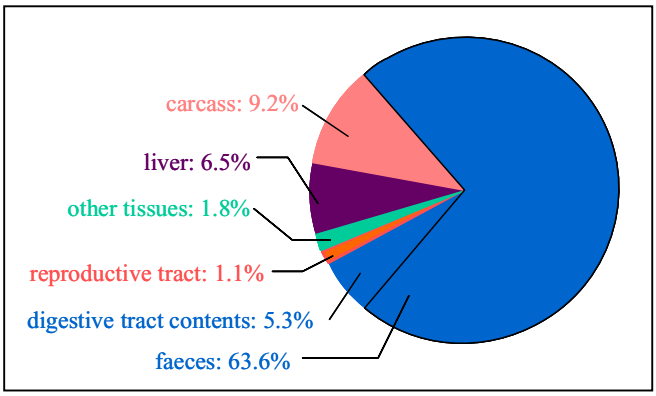

Fig.1: Radioactivity distribution in pregnant Wistar rat orally dosed with [^{14}C]-DBDE from day 16 to day 19 of gestation

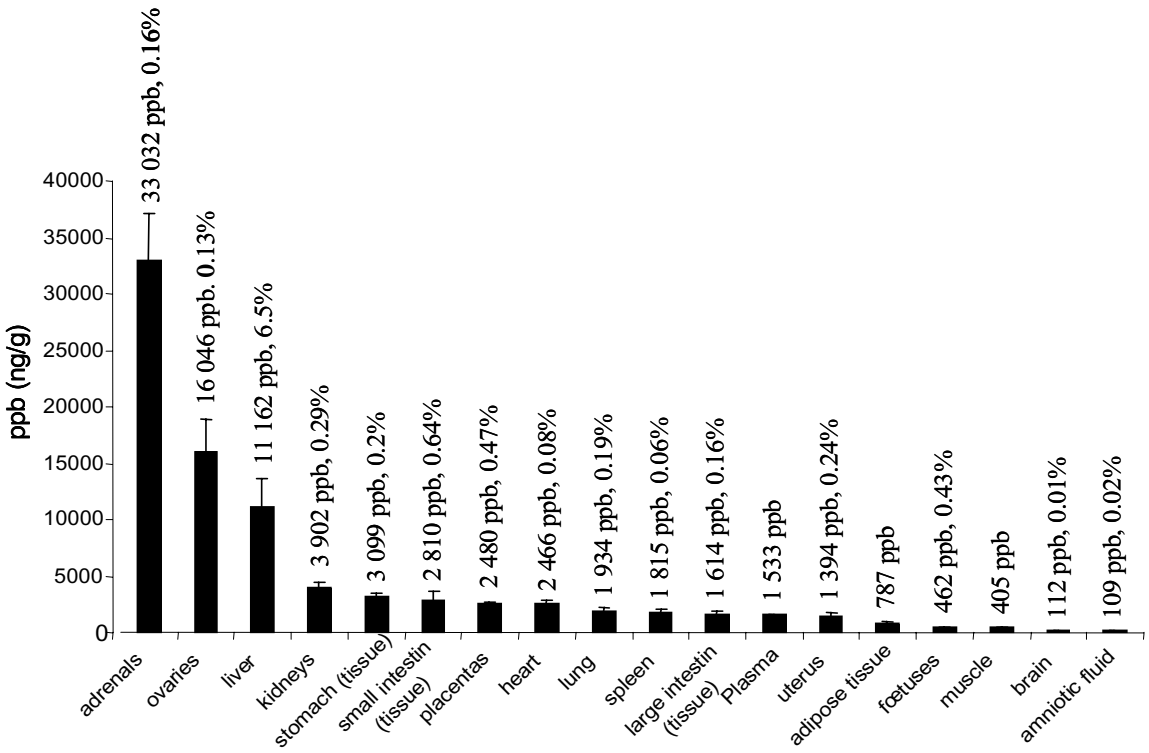

Fig. 2: Residual radioactivity levels measured in rat tissues (expressed in ng of DBDE equivalents per gram of fresh tissue, corresponding percentage of the [^{14}C]-DBDE dose administered over the 4-day study)

placentas and foetuses. Foetuses alone (whole litter) contained *ca.* 0.4% of the radioactivity distributed to mothers, demonstrating that either DBDE or its metabolites can cross the placental barrier in rat. No other tissues contained more than 1% of the distributed radioactivity. However, high concentrations of residues were found in several tissues (adrenals, ovaries, liver and kidneys) ranging from 2.5 to 34 µg/g fresh weight (fig. 2).

Radio-chromatographic profiling:
Faeces / digestive tract. The radioactivity excreted in faeces accounted for nearly two third of the total amount administered to rats. Only 81 ± 3 % of the faecal residues were found to be extractable (6 successive organic solvent extractions). The radioactivity remaining in extraction pellets was determined by combustion, confirming the presence of the remainder *ca.* 19% non-extractable residues. The covalent binding of BDEs metabolites to rat faecal macromolecules was previouly observed with BDE-47 and 99[14,15]. Radio-HPLC analyses showed that 96% of the extractable faecal radioactivity consisted of unchanged DBDE (fig. 3). Three metabolites were detected (M2, M4, M5). They were eluted before the parent compound in the reverse-phase HPLC system developed for the study. M5 was found to co-elute with standard BDE 206 and is supposed to be a nona-BDE congener. This metabolite was found to be present in the liver, but also in most of the other tissues in variable amounts (data not shown). Solvent extraction fractions of small intestine contents (less than 1% of the overall radioactivity) contained metabolites exhibiting a higher polarity than faecal metabolites and DBDE, with retention times ranging from 24 to 30 min. These could correspond to conjugated metabolites, later de-conjugated along the digestive tract into M2 or M4. This relatively hydro-soluble metabolites were not detected in tissues.

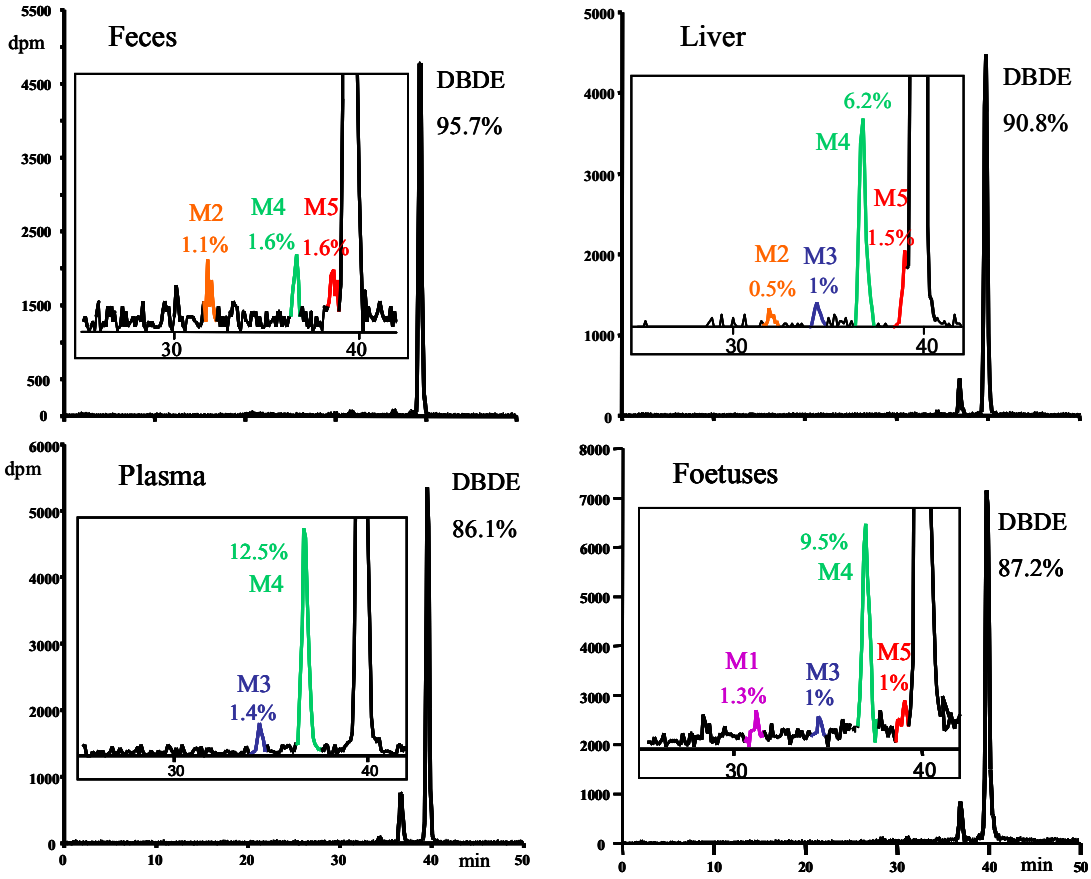

Fig. 3: Radio-HPLC chromatograms of feces, liver, serum and foetuses extracts.

Plasma / other tissues / foetuses. Solvent extraction allowed to recover more than 95% of the radioactivity present in most tissues. Radio-HPLC analyses demonstrated that more than 10%, 13% and 12% of the extractable radioactivity was associated to metabolites in liver, serum and foetuses, respectively (fig. 3).
Five metabolites (M1 to M5) were observed on the corresponding metabolic profiles. M4 was the major metabolite found in all extracts. It was much more abundant in the liver (7.8 ± 1.7 %), in plasma (12.5 ± 1.8 %) and in foetus extracts (9.5 ± 1 %) than in faeces. The high purity of the [^{14}C]-DBDE synthesised for the study (> 99.8%), allowed to quantify additional minor metabolites, among which metabolite M3, detected in almost all the analysed tissue extracts.
Fœtal metabolic profiles obtained for foetuses clearly demonstrate that DBDE and several of its metabolites can cross the placental barrier. 9.5 ± 1 % of the foetal extractable radioactivity are associated to metabolites, M4 being by far the major one (fig.3).

The direct structural identification of DBDE metabolites is currently being investigated on the basis of the analytical methods developed for the radio-chromatographic profiling of tissue extracts, by coupling liquid chromatography with APPI-ITMS. M5, purified from faeces extracts is very likely a nonabrominated diphenyl ether congener (see above). The structure of the other metabolites has not been fully elucidated yet. Preliminary results are in good accordance with Sandholm et al. study[13], in which the occurrence of BDE congeners with a high degree of bromination (octa- / nona-) was demonstrated in plasma. Hydroxylated and/or non-hydroxylated hepta- to nona-BDEs have tentatively been identified from pregnant wistar rats plasma and tissue extracts collected during this study.

Conclusions
Due to analytical difficulties (extraction, sample contamination, MS analysis), the quantification of DBDE and other high molecular weight PBDE in biological tissues is difficult to assess. Likewise, their analysis by HPLC is complicated by their weak solubility in organic solvents. Based on methods previously developed for these BFR[12] and on the synthesis of [^{14}C]-DBDE with a high purity grade, it was possible to demonstrate the formation of several DBDE metabolites in pregnant wistar rats. Previous statements about the supposed poor bioavailability of DBDE have already been contradicted by studies carried out in rat[13]. Our results confirm that a significant proportion of DBDE (*ca.* 20%) is absorbed, when administered by oral route to rats. DBDE is then biotransformed into several metabolites (probably hepta to nona-brominated molecules), which distribution depends on the target tissue. Part of these metabolites, and DBDE itself, can cross the placental barrier in rat at a late stage of gestation, implying, in this animal model, that foetuses are exposed to DBDE residues.

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