Investigating the *in vitro* metabolism of NBFRs by trout liver microsomes using a high resolution accurate mass benchtop Q-Exactive Orbitrap mass spectrometer

Aristide P. Ganci[1], Mohamed A. Abdallah[1], Khanh-Hoang Nguyen[1], Leon I. Peters[1], Thomas Moehring[2], Stuart Harrad[1]

[1] University of Birmingham, School of Geography, Earth and Environmental Sciences, B15 2TT Birmingham, United Kingdom

[2] Thermo Fisher Scientific (GmbH) Bremen, Hanna-Kunath-Str. 11, 28199 Bremen, Germany

Introduction

Due to legislative restrictions on manufacture and use of some brominated flame retardants (BFRs), several new BFRs (NBFRs) are increasingly used and have been measured in a wide range of environmental matrices. Owing to their physico-chemical properties, they have the potential for biomagnifications in the environment and studies have indicated their presence in sewage sludge, sediments, and also aquatic biota [1]. However, little is known about the environmental fate, toxicity and metabolic behaviour of such NBFRs, especially in aquatic species. Amid strict regulations on the use of laboratory animals in toxicity studies, *in vitro* techniques can provide useful insights into the metabolism of these chemicals. Analytical methods based on ultra-performance liquid chromatography (UPLC) coupled with advanced ultra high resolution mass spectrometry facilitate accurate mass measurement and identification of NBFR metabolites. In this study, trout liver microsomes (TLM) were selected in order to simulate possible metabolic pathways of aquatic organisms exposed to NBFRs. EH-TBB and BEH-TEBP as two of the main constituents of the technical flame retardant mixture Firemaster® 550 and BZ-54, as well as BTBPE as a replacement product for OctaBDE, were chosen as the compounds of interest. The primary aim of the current study is to identify potential metabolites of the target NBFRs in trout and investigate potential species-specific metabolic trends via comparison to previous *in vitro* metabolic studies in mammals.

Materials and methods

In vitro study. Initial screening experiments were conducted to evaluate the presence of possible metabolites formed. 0.5 mg of female trout liver microsomes (TLM) were exposed to 10 µM of selected NBFRs (EH-TBB, BEH-TEBP and BTBPE - dissolved in 10 µL of either DMSO or toluene). Incubation was conducted in a William's E Medium at 15 °C for 1 hour. The reaction was initiated through the addition of XenoTech RapidStart™ NADPH regenerating system and stopped after 1 h by adding 1 mL of ice-cold 1 M HCl.

Extraction and clean-up. Post-incubation, samples were extracted using 2 mL of hexane: dichloromethane mixture (1:1 v/v) and three cycles of vortex (1 min), ultrasonication (10 min) and centrifugation steps (10 min at 4000 r.p.m.). The cell pellet was discarded and the combined extracts concentrated under a gentle stream of N_2 before reconstitution in 150 µL methanol. For quality control purposes, three negative control blanks were included

for each study, a solvent blank (no NBFR), a heat inactivated blank (heat treated TLM – 10 min at 100 °C), as well as a non-enzymatic metabolism blank (whereby no NADPH regenerating system solution was added).

HPLC-HRMS. Following concentration, extracts were separated on a Thermo Scientific™ Accucore™ RP-MS column on a Thermo Scientific UltiMate® 3000 HPLC system, using a gradient elution program with water and methanol. Samples were analyzed on a Q-Exactive™ Plus mass spectrometer in both negative atmospheric-pressure chemical ionization (APCI) and negative heated electrospray-ionization (HESI) mode at a resolution of 70,000. Raw data files were processed using Thermo Scientific Compound Discoverer™ version 2.0 and Thermo Scientific Xcalibur™ software.

Preliminary results and discussion

Initially, full scan experiments were conducted to obtain a general overview of the presence of metabolites. All ion fragmentation (AIF) was performed in parallel to obtain a Br ion trace and aid in the metabolite identification. Structural confirmation was conducted using reference standards (where available), together with accurate mass, comparison of retention times and isotopic pattern. Different quasi molecular ions were formed during the ionization process of each target NBFR.

As shown in Figure 1, the major metabolite for EH-TBB could be identified as 2,3,4,5-tetrabromobenzoic acid (TBBA). TBBA had to be measured in HESI where the characteristic $[M-H]^-$ quasi molecular ion was formed, since in APCI only weak ionization of the acidic compound was observed. Retention times were confirmed by the injection of a reference standard. The formation of TBBA has also been reported in *in vitro* studies employing human and rat tissues [2].

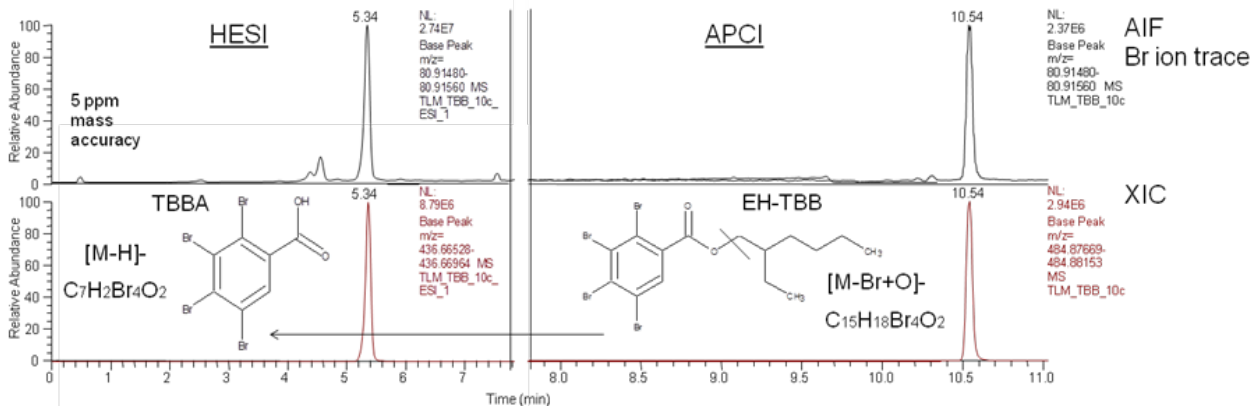

FIGURE 1. Extracted ion chromatogram (XIC) of EH-TBB ($[M-Br+O]^-$ measured in APCI) and 2,3,4,5-tetrabromobenzoic acid (TBBA - $[M-H]^-$ measured in HESI) together with the Br trace derived from the all ion fragmentation (AIF) measurement

For BEH-TEBP, a reduction of the initial dose after treatment with trout liver microsomes was observed, but no stable ions for potential metabolites were confirmed after data analysis of both APCI and HESI measured extracts.

As shown in Figure 2, the use of 70,000 FWHM resolution, sub ppm mass accuracy, as well as selectivity, elemental composition and isotopic pattern scoring enabled the identification of two metabolites for BTBPE, which were most likely formed due to a cleavage on either side of the ether linkage resulting in tribromophenol (TBP) and possibly tribromophenoxyethanol. However, since no reference standard was available for the latter metabolite, retention time could not be confirmed. Further metabolites might be present (unknown marked peak), as described in an *in vivo* study of BTBPE employing rats [3]. Apart from the two metabolites resulting from the ether bond cleavage, this group also characterized further BTBPE metabolites in rat feces: mono- and dihydroxylated, as well as debrominated on a single or both aromatic rings and combinations thereof.

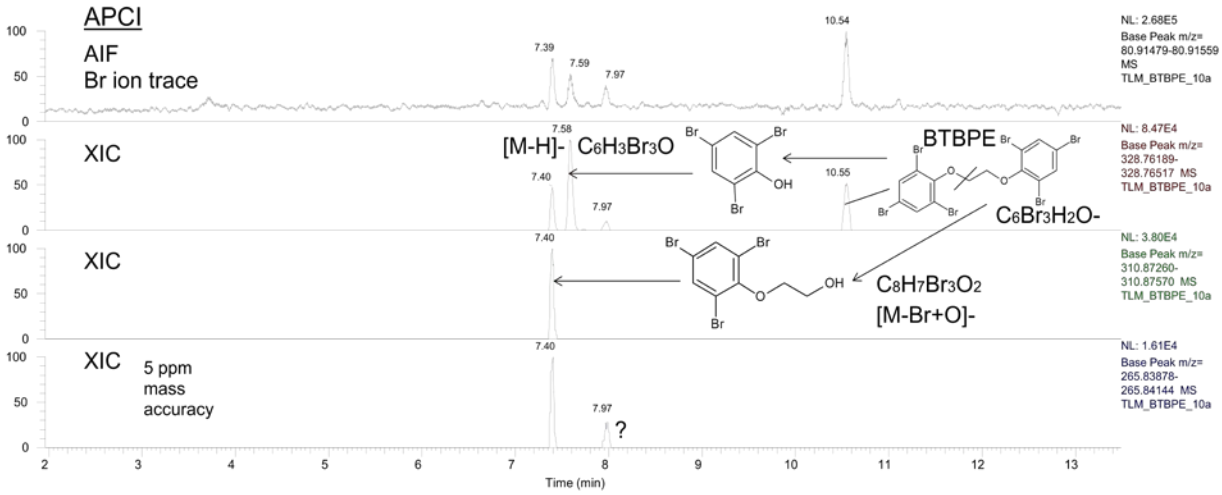

FIGURE 2. Extracted ion chromatogram of BTBPE (measured in APCI) together with the Br trace derived from the all ion fragmentation (AIF) measurement and tentative metabolites

Further metabolite identification will be confirmed using Thermo Fisher Compound Discoverer™ 2.0, as a software tool for the identification of unknowns. Additional experiments will include the understanding of the metabolism of other NBFRs such as DBE-DBCH. Optimization of the experiments can be performed by additional steps: adaption of the trout liver microsome protein concentration, variation of the NBFR concentration to reflect environmental levels and temperature variation to simulate different seasons and water temperatures. Further kinetic studies can be carried out and ultimately real trout samples analyzed to see how these *in vitro* experiments compare to real trout samples.

Acknowledgements

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References

1. Covaci, A., et al., *Novel brominated flame retardants: a review of their analysis, environmental fate and behaviour.* Environment International, 2011. **37**(2): p. 532-56.

2. Roberts, S.C., L.J. Macaulay, and H.M. Stapleton, *In vitro metabolism of the brominated flame retardants 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB) and bis(2-ethylhexyl) 2,3,4,5-tetrabromophthalate (TBPH) in human and rat tissues.* Chemical Research in Toxicology, 2012. **25**(7): p. 1435-41.
3. Hakk, H., G. Larsen, and J. Bowers, *Metabolism, tissue disposition, and excretion of 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE) in male Sprague-Dawley rats.* Chemosphere, 2004. **54**(10): p. 1367-74.