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

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Overview

Purpose: Identification of potential metabolites of selected NBFRs by trout liver microsome experiments to better understand their environmental fate.

Methods: *In-vitro* experiments employing trout liver microsomes exposed to NBFRs for initial screening and identification of formed metabolites, through measurement with quadrupole Orbitrap benchtop instrumentation.

Results: Insight into the formation of NBFR metabolites by exploiting the capabilities of high resolution accurate mass instrumentation.

Introduction

Due to legislative restrictions on manufacture and use of some brominated flame retardants (BFRs), several new BFRs (NBFRs) are increasingly used. However, little is known about the environmental fate of such NBFRs. *In vitro* studies can provide useful insights into the metabolism of these chemicals. Analytical methods based on liquid chromatography (LC) coupled with advances in ultra high resolution mass spectrometry facilitate accurate mass measurement and identification of NBFR metabolites.

Methods

Female trout liver microsomes (0.5 mg) 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 1mL of ice-cold 1M HCl. Post-incubation, samples were extracted using a hexane : dichloromethane mixture (1:1 v/v).) and three cycles of vortex, ultrasonication and centrifugation steps.

Following concentration and reconstitution in methanol, extracts were then separated on a Thermo Scientific Accucore™ 150x2.1mm, 2.6 µm RP-MS column on a Thermo Scientific UltiMate® 3000 HPLC system, using a gradient elution program with water and methanol as mobile phases. Samples were analyzed on a Q-Exactive™ Plus mass spectrometer in both HESI and APCI ionization mode. Raw data files were processed using Thermo Scientific Xcalibur™ software. The instrumental setup employed in this study is shown in Figure 1.

MS-parameter for screening purposes

Full Scan and All ion fragmentation (AIF)
- negative polarity
- Resolution = 70,000
- AGC target 3e6
- Maximum IT 200 ms
- Scan range 70–1050 m/z

- Optimized APCI/HESI ion source condition and gas flows

FIGURE 1. Thermo Scientific Q Exactive Plus mass spectrometer with Thermo Scientific Ultimate 3000 HPLC

Results

Screening

In this study screening experiments were initially conducted to evaluate the presence of possible metabolites formed through trout liver microsome metabolic reactions. Negative controls included a solvent blank (no NBFR), a heat inactivated blank (heat treated liver microsomes), as well as a non-enzymatic metabolism blank (whereby no NADPH solution was added). Extracts were measured both in atmospheric-pressure chemical ionization (APCI) and heated electrospray-ionization (HESI) mode.

Identification of metabolites

Initially, full scan experiments were conducted to obtain a general overview of the presence of metabolites. All ion fragmentation 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.

EH-TBB

As shown in Figure 2 a potential metabolite for EH-TBB could be identified, 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 standards

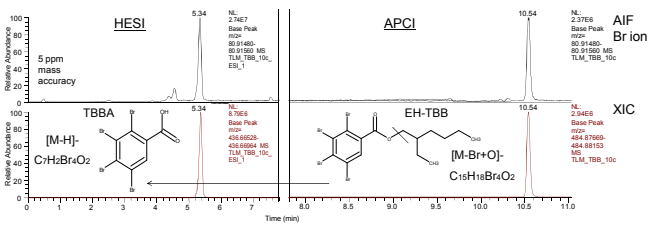


FIGURE 2. Extracted ion chromatogram (XIC) of EH-TBB (measured in APCI) and 2,3,4,5-tetrabromobenzoic acid (TBBA - measured in HESI) together with the Br trace derived from the all ion fragmentation (AIF) measurement

BEH-TEBP

For BEH-TEBP a reduction of the amount 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. Figure 3 shows the identification of the parent compound, along with the isotopic pattern .

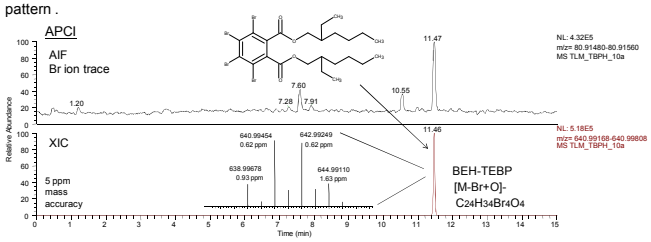


FIGURE 3. Extracted ion chromatogram of BEH-TEBP (measured in APCI) together with the Br trace derived from the all ion fragmentation (AIF) measurement

BTBPE

As shown in Figure 4, tentative metabolites were identified for BTBPE. However since no reference standards were available for the formed metabolites, retention times could not be confirmed. Further metabolites might be present (unknown marked peak) and have to be confirmed by additional data analysis.

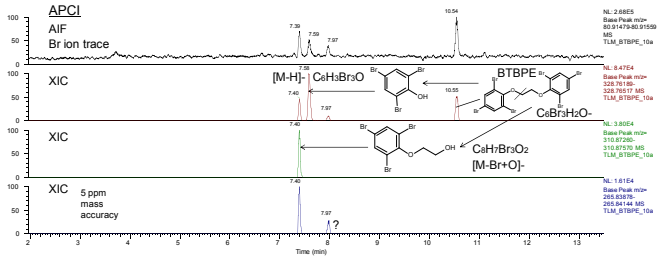


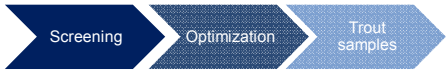
FIGURE 4. Extracted ion chromatogram of BTBPE (measured in APCI) together with the Br trace derived from the all ion fragmentation (AIF) measurement and tentative metabolites

Conclusion

The use of 70,000 FWHM resolution, sub ppm mass accuracy, as well as reference standards (when available) enabled the identification of several metabolites of the target NBFRs. Further metabolite identification will be confirmed using Thermo Fisher Compound Discoverer™ 2.0, as a software tool for the identification of unknowns. As shown in the diagram, additional experiments will include the following optimization steps:

1. Adaption of the trout liver microsome protein concentration in the range of 0.1 to 1.0 mg.
2. Variation of the NBFR concentration between 1-25 µM environmental occurrence.
3. Temperature variation in the range from 10 to 20°C 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.



Acknowledgement

The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°606857.

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