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APPLICATION OF ORBITRAP HRAM MASS SPECTROMETRY TO UNTARGETED IDENTIFICATION OF NBFRS AND THEIR METABOLITES

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Introduction

After the phase-out of commercial PBDEs, production volumes of many other so-called novel brominated flame retardants (NBFRs) have increased . Some representative NBFRs are bis(2-ethylhexyl) tetrabromophthalate (BEH-TEBP), 2-ethylhexyl 2,3,4,5-tetrabromobenzoate (EH-TBB), 1,2-bis(2,4,6-tibromophenoxy)ethane (BTBPE) and decabromodiphenylethane (DBDPE). A number of in vitro and in vivo studies have shown some NBFRs and/or their metabolites to be potentially toxic and estrogen disruptors [1–3]. However, until now very little is known about the fate of NBFRs and their metabolic/ degradation products in the environment, mostly limited to NBFRs such as BEH-TEBP and EH-TBB. Several in vitro studies using different models like S9 fractions, liver microsomes or liver cytosols of humans, rat, common carp, turtle and fathead minnow have been conducted [4,5]. These reveal metabolism of EH-TBB to 2,3,4,5-tetrabromobenzoic acid (TBBA) and 2,3,4,5-tetrabromomethylbenzoate (TBMB), whereas no metabolite of BEH-TEBP was identified. However by using porcine carboxylesterase, BEH-TEBP was slowly transformed to mono (2-ethylhexyl) tetrabromophthalate (TBMEHP) [4]. Understanding their metabolic pathways is crucial to effective assessment of the toxicity of NBFRs ,as well the establishment of more effective biomonitoring methods for these contaminants. In this abstract, we aim to explore the potential of a fast and reliable method to identify NBFRs and their metabolites by an untargeted approach using High Resolution Mass Accuracy (HRAM) data and post-processing by a dedicated software. The method was applied to samples from an in vitro experiment examining the metabolism of EH-TBB by mouse liver microsomes.

Materials and methods

Materials

All solvents and reagents used in this study were purchased from Fisher Scientific (Loughborough, UK) at HPLC grade. EH-TBB was obtained as a neat solution from Accustandard, Inc. (New Haven, CT, USA). RapidStart NADPH regenerating systems were purchased from XenoTech (Kansas, KS, USA), while mouse (male, CD-1) liver microsomes were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

In Vitro incubation experiments

The dosing solution of EH-TBB was prepared in DMSO with two concentration levels used: 1 and 10 μM (at final incubation concentration). Pre-incubation was carried out for 10 minutes at 37 ^{o}C in a well plate, with 0.5 mg of mouse liver microsomes, William's E medium and 10 μL of dosing solution. NADPH regenerating system (final concentration: 2.0 mM nicotinamide adenine dinucleotide phosphate, 10.0 mM glucose-6-phosphate and 2 units/mL glucose-6-phospahte dehydrogenase) was added to make a final volume of 1 mL in each well. The plate was then incubated in a bio incubator at 37 ^{o}C. After 1 hour of incubation, 1 mL ice-cold methanol was added to stop the reaction, then each well was transferred to a tube for sample extraction and cleanup.

Sample preparation

The detailed procedure is described elsewhere [6] with some modifications. Briefly, each sample tube was mixed with 3 mL of hexane/DCM mixture (1:1, v/v) then vortexed for 30 s. After that, the tubes were ultrasonicated for 5 min then centrifuged at 3500 g for 5 min and the organic layer was collected. This was repeated 3 times and the combined organic extracts were evaporated under a gentle stream of nitrogen before reconstitution in 150 μL methanol.

Instrumental analysis

Samples were analyzed by an UPLC-Orbitrap-HRMS system (Thermo Fisher Scientific, Bremen, Germany) comprising an Accela 1250 pump, an Accela Open Auto Sampler coupled to a Q Exactive Orbitrap mass spectrometer with a separate column oven (LCO 102 single, ECOM s r. o., Czech Republic). Chromatographic separation was performed on an Accucore RP-MS column (100 x 2.1 mm, 2.6 µm) with water (mobile phase A) and methanol (mobile phase B). A gradient method at 400 µL/min flow rate was applied as follows: start at 20% B; increase to 100% B over 9 min, held for 3 min; then decrease to 20% B over 0.1 min; kept constant for a total run time of 15 min. The injection volume was 10 µL and the column oven was set at 30°C.
The Q Exactive instrument used an APCI source to ionize samples in full scan negative ion mode. The parameters were set as follows: resolution 70000 , AGC target 1e6, maximum injection time 100 ms, scan range 150 to 1000 m/z, sheath gas flow rate 10, spray voltage 3.60 kV, capillary temperature 320°C and S-lens RF level 50.

QA/QC
Triplicate experiments were performed at both dosing levels. For each experiment batch, experiment blanks consisting of a non-enzymatic blank (NE-blank) without NADPH regenerating system, a heat inactivated blank (HI-blank) where liver microsomes were heated above 70 °C and a solvent blank (S-blank) without test compounds were performed. Instrument blanks (methanol) were run before and after analysis of triplicate or experiment blank samples. None of the target compounds were found in instrument and solvent blanks.

Data analysis
Compound Discoverer 2.0 software (Thermo Fisher Scientific, Bremen, Germany) was used to interpret the data. Briefly, the software extracted spectra from input MS data files and aligned the retention times of multiple LC/MS files based on mass tolerance and maximum time shift criteria. Compound Discoverer then tried to elucidate the element compositions for each peak in every single file by predefined settings. The detected compounds were grouped based on retention time across all files and ready for further analysis nodes including background compounds filter, elemental composition prediction, online Chemspider library search, offline mass list search and isotope pattern scoring. Finally, a "Differential Analysis" node was used to provide some simple differential statistics such as PCA and ANOVA with TukeyHSD posthoc test.
Two study factors were used for data analysis: treatment and levels of treatment. For treatment, there were 2 groups: No (including HI-blank, NE-blank and individual NBFRs standard) and Yes (all other samples from in vitro experimentd with the exception of solvent blanks). Levels of treatment were 1 µM and 10 µM. Instrument blanks and S-blank were set as "blank" in Compound Discoverer for background reference.

Results
Overall, 1429 features were found in two groups: treated and untreated samples. ANOVA results showed 100 features with significant differences between two groups (adjusted-$P < 0.1$, confidence level 90%). Log2 fold changes of peak areas were then taken into account. Among 100 significant features, there were 28 features with a positive log2 fold change meaning they have higher concentrations in treated samples.

Figure 1. Overlay peaks of ion [C8H4O3Br3]- across all input files

Finally, a bromine pattern scoring filter was applied, which resulted in only 1 feature displaying a pattern that matched 4 bromines. The proposed ion composition was: $[C8H4O3Br3]^-$ which might derive from 2,3,4,5-tetrabromomethylbenzoate (C8H4O2Br4) after common (-)APCI ionization mechanism $[M-Br+O]^-$. An increase in log2 fold change between 10 and 1 µM samples was also observed: 9.71 versus 2.59. This implied $[C8H4O3Br3]^-$ was a metabolite formed during in vitro experiments. However this was different from previous study [4] where 2,3,4,5-tetrabromobenzoic acid was reported as the main metabolite. Further work is in progress to address this difference.
In conclusion, by using HRAM and Compound Discoverer software, we were able to identify a metabolite of EH-TBB by MLM through an unknown approach. This method can be applied for any other compound and potentially real samples.

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References
(1) Saunders, D. M. V.; Podaima, M.; Codling, G.; Giesy, J. P.; Wiseman, S. Aquat. Toxicol. 2015, 158, 14–21.
(2) Mankidy, R.; Ranjan, B.; Honaramooz, A.; Giesy, J. P. Toxicol. Lett. 2014, 224, 141–146.
(3) Springer, C.; Dere, E.; Hall, S. J.; McDonnell, E. V.; Roberts, S. C.; Butt, C. M.; Stapleton, H. M.; Watkins, D. J.; McClean, M. D.; Webster, T. F.; Schlezinger, J. J.; Boekelheide, K. Environ. Health Perspect. 2012, 120, 1711–1719.
(4) Roberts, S. C.; Macaulay, L. J.; Stapleton, H. M. Chem. Res. Toxicol. 2012, 25, 1435–1441.
(5) Bearr, J. S.; Mitchelmore, C. L.; Roberts, S. C.; Stapleton, H. M. Aquat. Toxicol. 2012, 124-125, 41–47.
(6) Abdallah, M. A. E.; Uchea, C.; Chipman, J. K.; Harrad, S. Environ. Sci. Technol. 2014, 48, 2732–2740.