Biotransformation of flame retardant 1,2-Dibromo-4-(1,2-dibromethyl)cyclohexane (TBECH) *in vitro* by Human Liver Microsomes

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Introduction

1,2-Dibrom-4-(1,2-dibromethyl)cyclohexane (TBECH or DBE-DBCH) is an addictive brominated flame retardant produced by Albermarle Corp., U.S.A under the trade name Saytex BCL-462. The flame retardant is used in extruded polystyrene and polyurethane[1]. In the US, TBECH production volume in 2002 was 230 tons[2] . Technical mixture of TBECH contains equimolar concentrations of two diasteroisomers: α and β-TBECH[3]. The compounds have been globally detected in environmental samples including indoor air and dust[4–6], outdoor air[7] and toddler's faeces[8]. Moreover, TBECH has been reported as one of the predominant novel brominated flame retardants (NBFRs) in both Norwegian and UK indoor air[4,9]. These findings suggest its wide application, especially in Europe. There are evidences from *in vitro* and *in vivo* experiments suggested that TBECH is an androgen receptor agonist as well as an endocrine disruptor[10–12]. However, very little is known about human exposure to TBECH and its biological fate in humans. In this study, we exposed technical TBECH to human liver microsomes (HLM) at environmentally-relevant concentrations. We used UHPLC coupled to high resolution q-exactive Orbitrap mass spectrometry to investigate the metabolic pathways of TBECH *in vitro*. Enzymatic modelling was applied assess the overall metabolic rate of this compound by HLM.

Materials and Methods

Dosing Experiments

Preliminary *In vitro* experiments were performed in triplicate at 2 TBECH concentration levels: 1 and 10 µM in dimethyl sulfoxide (DMSO). Pre-incubations were performed at different HLM concentrations and different times. After optimization of the reaction parameters, the following general exposure was applied: 0.5 mg of human liver microsomes, William's E medium and 10 µL of TBECH dosing solution were pre-incubated for 10 minutes at 37 ^{o}C. 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. The samples were then incubated at 37 ^{o}C, 5 % CO_2 and 98 % relative humidity. After 1 hour of incubation, 1 mL ice-cold methanol was added to stop the reaction prior to sample extraction. For enzymatic modelling, similar experiments were carried at various concentrations of TBECH: 1, 2, 5, 10 and 15 µM.

Sample extraction

Incubated samples were spiked with ^{13}C-labelled brominated diphenyl ether 100 (^{13}C-BDE100) and extracted using a method described elsewhere[13] however without clean-up steps. The extract was

evaporated to dryness under a gentle stream of nitrogen then reconstituted in 100 µL of methanol containing ^{13}C-BDE-77 as recovery standard.

Chemical analysis

Samples were analyzed on a UPLC-Orbitrap-HRMS system (Thermo Fisher Scientific, Bremen, Germany). 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; maintained constant for a total run time of 15 min. The Orbitrap parameters were set as follows: (-) APCI full scan mode, resolution 17500, AGC target 1e6, maximum injection time 100 ms and scan range 75 to 700 m/z. Compound Discoverer 2.0 software (Thermo Fisher Scientific, Bremen, Germany) was used to detect potential metabolites and elucidate their chemical formula.

QA/QC

No potential metabolites were found in experiment blanks comprising a non-enzymatic blank without NADPH regenerating system, a heat inactivated blank where liver microsomes were heated above 80 ^{o}C and a solvent blank without TBECH. None of the target compounds were found in instrument and solvent blanks. Internal standard recoveries were within 60-110%.

Results and Discussion

Metabolite identification

Two monohydroxylated and two dihydroxylated metabolites of the parent flame retardant TBECH were identified (Fig. 1). Some dihydroxylated debrominated TBECH and unidentified metabolites with the chemical formula $C_8H_{11}Br_3O_2$ were also found (Fig. 1). Structural elucidation was confirmed via suggestive metabolite framework of the Compound Discoverer 2.0 software, accurate mass comparison (1 ppm) and isotope cluster matching. These 4 types of metabolite are similar to those reported previously for TBECH metabolism by rat liver microsomes[14]. Interestingly, we discovered another type of metabolite: monohydroxylated debrominated TBECH (peaks 6 and 7 in Fig. 1). This is the first time such *in vitro* biotransformation products of TBECH were reported. The presence of this monohydroxy-TriBECH helps explain better the formation of dihydroxy-TriBECH in both this and a previous study[10]. It is believed that a TriBECH isomer is produced prior to the formation of monohydroxy-TriBECH; however such a TriBECH isomer was not detected in our samples despite applying a high resolution GCxGC-ToF/MS platform in a separate analysis dedicated specifically to isolae this potential metabolite.

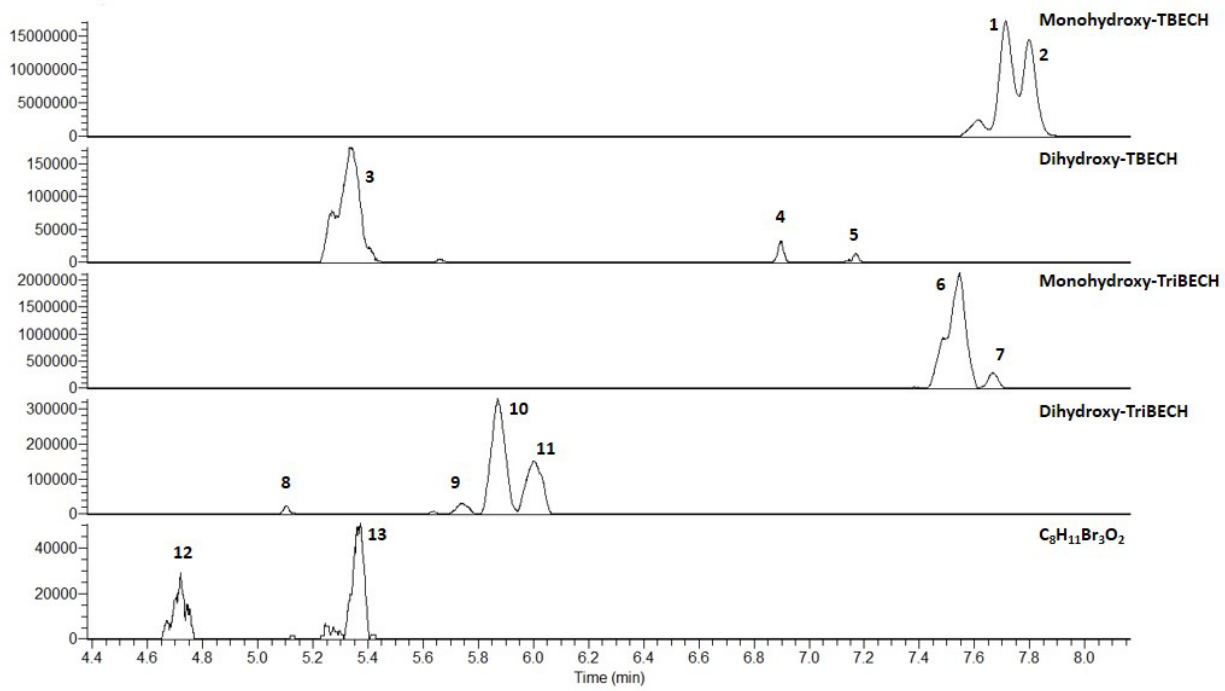

Figure 1. Selected LC - (-)ESI – Orbitrap MS chromatogram of the metabolites formed by human liver microsomes following exposure to 10 µM technical TBECH mixture for 60 minutes.

Human liver microsome *in vitro* assays with pure β-TBECH instead of TBECH technical mixture were carried out in an effort to enhance metabolite identification. Comparisons of mass chromatograms between these β-TBECH and the technical TBECH assays described above, indicate that peaks 1, 3 and 6 in Figure 1 are metabolites of α-TBECH while peaks 2, 4, 5 and 7 are derived from β-TBECH. For the unidentified metabolites, we hypothesize that they are either quinone derivatives of dihydroxy-TriBECH or dehydrobrominated derivatives of dihydroxy-TBECH. However, elucidation of the exact chemical structure of all the metabolites were not possible due to the unavailability of commercial standards.

Kinetics of TBECH metabolism by HLM

Following metabolite identification, a series of assays with different technical TBECH concentrations (1, 2, 5, 10 and 15 µM) were performed. Due to the lack of authentic standards for the metabolites, they were quantified using the response factor of the parent compound. The concentrations obtained were subjected to metabolic rate modelling (including Michealis-Menten, Hill and substrate inhibition approaches). Results indicated that non-linear regressions of monohydroxy-TBECH, dihydroxy-TBECH and monohydroxy-TriBECH were best fitted to a Michealis-Menten model.

Table 1. Kinetic constant derived from non-linear regression (Michealis-Menten model) of the formation of TBECH metabolites

Metabolite	K_m (µM) ± SD	V_{max} (pmol/min/mg protein) ± SD
Mono OH TBECH	162.5 ± 29.6	11.78 ± 4
Di OH TBECH	2.2 ± 1	0.644 ± 0.08
Mono OH TriBECH	3.4 ± 0.82	10.12 ± 0.8

Apparent V_{max} values (maximum metabolic rate) for the formation of monohydroxy-TBECH, dihydroxy TBECH and monohydroxy-TriBECH were 11.78, 0.64 and 10.12 pmol/min/mg protein, respectively (Table 1). This indicated that monohydroxy TBECH is the major metabolite of TBECH formed *in vitro* by human liver microsomes among the metabolites detected here. Another metabolite, monohydroxy-TriBECH, also showed a high V_{max} value close to that of monohydroxy-TBECH but with a much lower K_m (the concentration of the substrate when the metabolic formation rate is equal to one half of V_{max}) : 3.4 µM for monohydroxy-TriBECH versus 162.5 µM for monohydroxy-TBECH. Therefore, monohydroxy-TriBECH might be a better biomarker for low level TBECH exposure.

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References

(1) Andersson, P. L.; Oberg, K.; Orn, U. *Environ. toxicol. chem.* **2006**, *25*, 1275–1282.

(2) Covaci, A.; Harrad, S.; Abdallah, M. a E.; Ali, N.; Law, R. J.; Herzke, D.; de Wit, C. a. *Environ. Int.* **2011**, *37*, 532–556.

(3) Arsenault, G.; Lough, A.; Marvin, C.; McAlees, A.; McCrindle, R.; MacInnis, G.; Pleskach, K.; Potter, D.; Riddell, N.; Sverko, E.; Tittlemier, S.; Tomy, G. *Chemosphere* **2008**, *72*, 1163–1170.

(4) Cequier, E.; Ionas, A. C.; Covaci, A.; Marcé, R. M.; Becher, G.; Thomsen, C. *Environ. Sci. Technol.* **2014**, *48*, 6827–6835.

(5) Newton, S.; Sellström, U.; de Wit, C. a. *Environ. Sci. Technol.* **2015**, 150210152433003.

(6) Hassan, Y.; Shoeib, T. *Sci. Total Environ.* **2015**, *505*, 47–55.

(7) Shoeib, M.; Ahrens, L.; Jantunen, L.; Harner, T. *Atmos. Environ.* **2014**, *99*, 140–147.

(8) Sahlström, L. M. O.; Sellström, U.; de Wit, C. A.; Lignell, S.; Darnerud, P. O. *Environ. Sci. Technol.* **2015**, *49*, 606–615.

(9) Tao, F.; Abdallah, M. A.; Harrad, S. *Environ. Sci. Technol.* **2016**, *50*, 13052–13061.

(10) Marteinson, S. C.; Letcher, R. J.; Fernie, K. J. *Environ. Toxicol. Chem.* **2015**, *34*, 2395–2402.

(11) Park, B. J.; Palace, V.; Wautier, K.; Gemmill, B.; Tomy, G. *Environ. Sci. Technol.* **2011**, *45*, 7923–7927.

(12) Curran, I. H. A.; Liston, V.; Nunnikhoven, A.; Caldwell, D.; Scuby, M. J. S.; Pantazopoulos, P.; Rawn, D. F. K.; Coady, L.; Armstrong, C.; Lefebvre, D. E.; Bondy, G. S. *Toxicology* **2017**, *377*, 1–13.

(13) Abdallah, M. A. E.; Uchea, C.; Chipman, J. K.; Harrad, S. *Environ. Sci. Technol.* **2014**, *48*, 2732–2740.

(14) Chu, S.; Gauthier, L. T.; Letcher, R. J. *Environ. Sci. Technol.* **2012**, *46*, 10263–10270.