Biotransformation of the Flame Retardant 1,2-Dibromo-4-(1,2-dibromoethyl)cyclohexane (TBECH) in Vitro by Human Liver Microsomes
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Biotransformation of the flame retardant 1,2-Dibromo-4-(1,2-dibromethyl)cyclohexane (TBECH) in vitro by Human Liver Microsomes

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The technical mixture of 1,2-Dibromo-4-(1,2-dibromomethyl)cyclohexane (TBECH or DBE-DBCH) and the pure \( \beta \)-TBECH isomer were subjected to *in vitro* biotransformation by human liver microsomes (HLM) for the first time. After 60 mins of incubation, 5 potential metabolites of TBECH were identified in microsomal assays of both the TBECH mixture and \( \beta \)-TBECH using UPLC- Q-Exactive Orbitrap™ mass spectrometry. These include mono- and di-hydroxylated TBECH, mono- and di-hydroxylated TriBECH as well as an \( \alpha \)-oxidation metabolite bromo-(1,2-dibromocyclohexyl)-acetic acid. Our results indicate potential hepatic biotransformation of TBECH via Cytochrome P450-catalyzed hydroxylation, debromination and \( \alpha \)-oxidation. Kinetic studies revealed the formation of monohydroxy-TBECH, dihydroxy-TBECH and monohydroxy-TriBECH were best fitted to a Michaelis-Menten enzyme kinetic model. Respective estimated \( V_{\text{max}} \) values (maximum metabolic rate) for these metabolites were: (11.8 ± 4), (0.6 ± 0.1) and (10.1 ± 0.8) pmol/min/mg protein in TBECH mixture and (4992 ± 1340), (14.1 ± 4.9) and (66.1 ± 7.3) pmol/min/mg protein in \( \beta \)-TBECH. This indicates monohydroxy-TBECH as the major metabolite of TBECH by human liver. The estimated intrinsic clearance (\( C_{\text{int}} \)) of TBECH mixture was slower (\( P<0.05 \)) than that of pure \( \beta \)-TBECH. While the formation of monohydroxy-TBECH may reduce the bioaccumulation potential and provide a useful biomarker for monitoring TBECH exposure, further studies are required to fully understand the levels and toxicological implications of the identified metabolites.
Introduction

Brominated flame retardants (BFRs) are anthropogenic chemicals incorporated into materials to increase their resistance to fire. Polybrominated diphenyl ethers (PBDEs) were extensively used in consumer products such as textiles, furniture, electrical devices, plastics and many other applications. However due to their toxicity, bioaccumulation, persistence and long-range atmospheric transport, commercial mixtures of Penta-BDE and Octa-BDE have been banned by the UNEP Stockholm Convention in 2009. Deca-BDE, another PBDE commercial mixture, has also been banned in Europe and voluntarily phased out in the USA. These regulations have paved the way for novel/emerging brominated flame retardants (NBFRs/EBFRs) introduced to the market as replacements for PBDEs.

1,2-Dibrom-4-(1,2-dibromethyl)cyclohexane (TBECH or DBE-DBCH) is an additive EFR produced by Albermarle Corp., U.S.A under the trade name Saytex BCL-462. The flame retardant is used in extruded polystyrene and polyurethane foam, electrical cable coatings, adhesive in fabric and construction materials. In the U.S, TBECH production volume in 2002 was 230 tons. The technical mixture of TBECH contains equimolar concentrations of two diastereoisomers, named α and β-TBECH. Although no other isomers could be detected in the technical mixture, thermal conversion into γ- and δ-TBECH was reported during incorporation into flame-retarded products at temperature of 123°C or higher. TBECH isomers have been globally detected in environmental samples including indoor air and dust, outdoor air, herring gull eggs, blubber of Canadian Arctic whale and toddler’s faeces. Recently, Tao et al. reported TBECH as the predominant emerging flame retardant (EFR) detected in all indoor air (n=35) and dust (n=92) samples from UK houses (mean = 173 pg/m³ and 21.4 ng/g in air and dust) and offices.
(mean = 320 pg/m$^3$ and 41 ng/g in air and dust)$^{12}$. TBECH also showed the highest levels of all detected EFRs in Norwegian (mean = 209 pg/m$^3$) and Swedish (mean = 43 pg/m$^3$) indoor air samples$^{5,6}$ indicating its wide application, especially in Europe. This is of concern due to its potential toxicological effects on humans and wildlife. Several toxicological in silico, in vitro (human and chicken cell lines) and in vivo (birds, fishes and rats) studies show TBECH is a strong androgen receptor agonist and endocrine disruptor$^{13-21}$. TBECH also displayed potential to disrupt thyroid and sex hormones in American kestrels$^{20}$, modulate thyroid axis in juvenile Brown Trout$^{14}$ and alter androgen receptor regulation in human ductal breast cancer and prostate cancer cell lines$^{21}$. However, very little is known about the biotransformation and fate of TBECH in humans.

Previous studies have shown some BFRs can be metabolized to more toxic lower brominated congeners$^{23-25}$. Two of the primary in vivo debrominated metabolites of decabromodiphenyl ether (BDE-209) in rainbow trout were identified as BDE-47 and BDE-99$^{23}$, which are more bioaccumulative and showed much higher toxic potential than the parent compound in goldfish and zebrafish liver cell lines$^{25,26}$. Similarly, hexabromocyclododecane (HBCD) was metabolized by rat and trout liver S9 fractions into pentabromocyclododecenes (PBCDs), which showed higher affinity for binding to the thyrotropin receptor (TSH) than the parent compound$^{24}$. Therefore, improved understanding of the biotransformation pathways, rates and products of TBECH is essential for assessment of the risk arising from human exposure to this flame retardant.

To our knowledge, only one study has investigated the potential metabolites of TBECH and moreover used in vitro rat liver microsomes (RLM)$^{22}$. Results revealed that after 60 min, 40% of
the exposure dose was metabolized by Cytochrome P450 enzymes into mono and dihydroxylated TBECH, together with some unidentified metabolites. However, this study did not provide information on the metabolic/hepatic clearance rate of TBECH. Moreover, extrapolation of results from metabolic studies in rat to human is subject to uncertainty due to inter-species variations in metabolic pathways and products. To illustrate, bioconversion from α-, β- and γ-hexabromocyclododecane (HBCD) mixture into δ-HBCD was observed in trout but not rat S9 fractions. Furthermore, 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) was metabolized significantly faster in RLM compared to HLM.

Against this background, the aims of the current study are to: (a) investigate the phase I metabolic pathways and products of TBECH following in vitro exposure to human liver microsomes (HLM); (b) compare the in vitro HLM metabolic profile of the TBECH technical mixture to that of the pure β-isomer and (c) assess the in vitro metabolic rate and intrinsic clearance of TBECH by HLM.

Materials and Methods

Chemicals and Standards

All solvents and reagents used in this study were purchased from Fisher Scientific (Loughborough, UK) and were of HPLC grade or higher. Technical TBECH was obtained as a neat powder from Accustandard, Inc. (New Haven, CT, USA). A dosing solution was prepared by dissolving technical TBECH in dimethyl sulfoxide (DMSO). High purity standards of β-TBECH, α- and β- TBECH mixture (equimolar concentrations), PBDE-77, and $^{13}$C$_{12}$-BDE100 were purchased from Wellington Laboratories (Guelph, ON, Canada). RapidStart NADPH regenerating system
was purchased from XenoTech (Kansas, KS, USA) while human liver microsomes and William’s E medium were obtained from Thermo Fisher Scientific (Paisley, UK).

**In Vitro Incubation Experiments**

Pre-incubations were performed at different HLM concentrations and different times. After optimization of the reaction parameters, the following general exposure protocol was applied: 0.5 mg of human liver microsomes, William’s E medium and 10 µL of TBECH dosing solution (final concentration 10 µM) were pre-incubated for 10 minutes at 37 °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-phosphate dehydrogenase) was added to make a final volume of 1 mL. The samples were then incubated at 37 °C, 5 % CO₂ and 98 % relative humidity for 60 min. At the end of the incubation, 1 mL of ice-cold methanol was added to stop the reaction prior to sample extraction. In all incubation experiments, experiment blanks including a non-enzymatic blank in which no NADPH regenerating system was added, a heat-inactivated blank featuring liver microsomes heated above 80 °C for 10 min and a solvent blank which contained only William’s E medium were performed and analyzed alongside the sample batch.

**Sample extraction**

Due to the unavailability of isotopically-labelled TBECH, incubated samples were spiked with 20 ng of 13C-BDE-100 as internal standard and extracted according to a previously reported method. Briefly, samples were mixed with 3 mL of hexane:DCM mixture (1:1 v/v) by vortexing for 30 s, followed by ultrasonication for 5 min and centrifuged at 4000 g for 5 min. The organic layer was collected and the extraction procedure was repeated twice. The combined extracts
were evaporated to dryness under a gentle stream of nitrogen then reconstituted in 100 µL of methanol containing 20 ng of BDE-77 as a syringe standard for QA/QC purposes. Full details are provided in the SI section.

**Instrumental analysis**

Samples were analyzed on a UPLC-Orbitrap-HRMS system (Thermo Fisher Scientific, Bremen, Germany) composed of a Dionex Ultimate 3000 liquid chromatography equipped with a HPG-3400RS dual pump, a TCC-3000 column oven and a WPS-3000 auto sampler coupled to a QExactive Plus Orbitrap mass spectrometer. 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 programme 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 parent compound was analyzed in negative atmospheric pressure chemical ionization (APCI) mode. The Orbitrap parameters were set as follows: (-) APCI full scan mode, resolution 17500, AGC target 1e6, maximum injection time 100 ms, scan range 75 to 700 m/z, sheath gas flow rate 25 AU, aux gas flow rate 5, discharge current 30 µA, capillary temperature 250°C and S-lens RF level 50. Accurate masses of 80.91629, 512.73847 and 420.78975 were used to monitor TBECH, 13C12-BDE-100 (internal standard) and BDE-77 (syringe standard), respectively.

The more universal, softer electrospray ionisation (ESI) mode was used for screening and identification of the produced metabolites. The optimised parameters were: (-) ESI full scan mode, resolution 17500, AGC target 1e6, maximum injection time 100 ms, scan range 75 to 750 m/z, sheath gas flow rate 20 AU, discharge voltage 2.5 kV, capillary temperature 320°C.
Compound Discoverer 2.0 software (Thermo Fisher Scientific, Bremen, Germany) was used to detect potential metabolites and elucidate their chemical formulae while quantification of target compounds was performed using Quan Browser 3.0 (Thermo Fisher Scientific, Bremen, Germany).

**QA/QC**

Quality control samples where the William’s E medium was spiked with TBECH at all dosing concentration levels were analyzed, with recoveries of TBECH falling between 96 to 113 % of the theoretical dosing concentration. In incubation experiments, internal standard recoveries were within 60-110 %. Metabolite identification was achieved via 4 successive filters established within the compound discoverer 2.0 software. Specifically, these were:

1. Peak signal to noise ratio (S/N) must exceed 10:1.
2. $m/z$ value of the molecular ion peak must be within 5 ppm of its theoretical value at resolution power of 17500 FWHM (full width at half mass).
3. Br isotope pattern must match within 5 % of the theoretically predicted abundances of the predicted chemical formula.
4. $\log_2$ fold change (calculated as $\log_2$ of the peak area ratio between *in vitro* samples and experiment blanks) to be > 1.

Instrument blanks (10 µL methanol) were run before and after analysis of incubation experiment and experiment blank samples. No parent compounds or metabolites were found in instrument and solvent blanks. Additionally, no metabolites were found in the non-enzymatic and heat-inactivated blanks. Principal component analysis results from Compound Discoverer
2.0 software also showed very distinctive separation between LC/MS chromatograms of in vitro samples compared to those of experimental and instrument blanks (Figure SI-1).

Results and Discussion

Metabolite identification

Compound Discoverer 2.0 software (Thermo Fisher Scientific, Bremen, Germany) was used to interpret our data. The software workflow implemented in this study is shown in Figure S2. Briefly, the software extracted spectra from raw LC/MS files and aligned the retention times of detected peaks based on mass tolerance and maximum time shift criteria. To narrow down the hits reported by the software and confirm metabolite identity, we added the filters mentioned in the QA/QC section as pre-requisite features. The detected compounds were then grouped based on their retention times across all files and subjected to elemental composition prediction, online ChemSpider library search and offline mass list search. With this approach, a total of 5 TBECH metabolites were identified, including both hydroxylated and debrominated products (Table 1).

Hydroxylated metabolites

Analysis of the obtained UPLC-Orbitrap™ MS chromatograms revealed a minimum of three monohydroxylated and three dihydroxylated metabolites of the parent TBECH following exposure of HLM to 10 µM of the technical mixture for 1 h (Figure 1). Bearing in mind the lack of reference standards for these metabolites, the isobaric nature of TBECH isomers in the technical mixture and the large number of theoretical isomers, co-elution of one or more metabolites in the same group (e.g. monohydroxylated TBECHs) could not be excluded.
Similarly, the specific position of the hydroxyl groups could not be elucidated. It is well known that Cytochrome P450-catalyzed hydroxylation usually retains the stereochemical configuration at the substrate’s reaction site. Therefore, we carried a parallel strand of experiments, where HLM were exposed to pure β-TBECH (the only purified isomer available commercially) in order to gain further information on the metabolic hydroxylation process. Comparisons of LC/MS chromatograms between β-TBECH and technical TBECH exposure experiments (Figures 1a and 1b) revealed peak M1-2 as monohydroxy-β-TBECH (β-OH-TBECH). Since the applied commercial mixture contained α- and β-TBECH isomers, it can be concluded that peaks M1-1 and M1-3 are α-OH-TBECH isomers (Figures 1a and 1b). Similarly, peak M2-4 was identified as α-(OH)$_2$-TBECH, while peaks M2-5 and M2-6 originated from the β-isomer (Figures 1c and 1d).

Our findings are generally in agreement with those reported using rat liver microsomes (RLM), where two monohydroxy- and two dihydroxy- isomers were identified following exposure to the TBECH technical mixture. While the difference in the number of isomers in each metabolite group may be attributed to inter-species variations, this hypothesis cannot be confirmed in the absence of authentic metabolite standards.

**Debrominated metabolites**

In addition to the hydroxylated metabolites of the parent TBECH, we also identified hydroxylated biotransformation products of debrominated TBECH with the formulae: C$_8$H$_{13}$Br$_3$O (M3), C$_9$H$_{15}$Br$_3$O$_2$ (M4), C$_8$H$_{13}$Br$_3$O$_2$ (M5), C$_9$H$_{16}$Br$_2$O$_4$ (M6) and C$_9$H$_{15}$Br$_3$O$_3$ (M7) (Table 1).

Metabolites M3 and M4 were assigned the chemical structures of mono- and dihydroxy-triBECH (table 1). While dihydroxy-triBECH (M4; (OH)$_2$-triBECH) was previously reported in in vitro RLM experiments, this is the first study to identify monohydroxy-triBECH (M3; (OH)-
triBECH). It is reasonable to believe that M3 can be formed by direct debromination of M1 and/or through debromination of parent TBECH followed by hydroxylation (Figure 3). This is similar to previously reported in vitro metabolic pathways for hexabromocyclododecane isomers (HBCDD) in rat and human, where both hydroxylation and debromination were observed.

Interestingly, two separate peaks were identified for M3 following HLM exposure to technical TBECH (Figure 2a), while one peak (M3-7) was observed upon exposure to pure β-TBECH. Therefore, peak M3-7 was assigned as β-OH-triBECH and peak M3-8 was attributed to α-OH-triBECH. We hypothesized that the observed M3 metabolites may be produced – at least partially - from hydroxylation of a tribrominated metabolite (i.e. a tribromoethyl cyclohexane derivative or triBECH) with a molecular formula of C$_8$H$_{13}$Br$_3$. However, such triBECH metabolites could not be detected in our samples even using the ultimate high separation and resolution power of a GC x GC-ToF/MS platform in an independent analysis dedicated specifically to identify this potential metabolite (Figure SI-3). Similar observations were reported in muscle and liver samples of juvenile brown trout exposed to β-TBECH in their diet, where no debrominated metabolites were detected. While our experimental approach could not confirm the formation of triBECH, the hypothesis cannot be refuted as triBECH might be produced then transformed quickly to its hydroxylated metabolites (M3, Figure 2a) before the reaction is stopped after 60 min.

Four distinctive peaks of M4 (Figure 2c) were detected when HLM were exposed to either technical TBECH or pure β-TBECH. However, two additional peaks, designated as M4-13 and M4-14 were observed upon exposure to pure β-TBECH only (Figure 2d). Hence, M4-13 and M4-
14 were identified as β-(OH)$_2$-triBECH. Due to the lack of a pure authentic standard for α-TBECH, it was not possible to address the stereochemistry of peaks M4-9, M4-10, M4-11 and M4-12 (Figure 2c).

Peaks 15 and 16 of metabolite M5 were detected in both technical TBECH and β-TBECH assays at an accurate mass of 378.81864 with predicted chemical formula of C$_8$H$_{11}$Br$_3$O$_2$ (Figures 2e and 2f). As their retention times were shorter than that of most other monohydroxylated and dihydroxylated metabolites, we hypothesized they were carboxylated TriBECH metabolites (i.e. bromo-(1,2-dibromocyclohexyl) acetic acid or DBCBA) formed via α-oxidation mechanism (Figure 3). The oxidative reaction starts at C$\alpha$, transforming the terminal bromomethyl group initially to an aldehyde with subsequent oxidation to the carboxylic acid. This mechanism is similar to previous reports of metabolic α-oxidative dehalogenation of structurally-similar halogenated compounds such as halothane$^{31}$ and tris-2-chloroethyl phosphate (TCEP)$^{32}$. The aldehyde intermediate (Figure 3) however could not be identified in our samples. This is similar to the results of a previous metabolic study on TCEP using human hepatocyte cell lines, where the inability to identify the aldehyde form was attributed to potential rapid oxidation to the corresponding carboxylic acid form$^{32}$.

**Kinetics of TBECH metabolism by HLM**

Following metabolite identification, a series of assays with different technical TBECH and pure β-TBECH concentrations (1, 2, 5, 10 and 15 µM) were performed. Due to the lack of authentic standards for the metabolites, they were semi-quantified using the response factor of the parent compound. The concentrations obtained were subjected to metabolic rate modelling (including Michaelis-Menten, Hill and substrate inhibition approaches) by nonlinear regression.
analysis using SigmaPlot Enzyme Kinetics Module v1.1 (Systat Software Inc., Richmond, CA). We considered two statistical criteria: Akaike Information Criterion corrected for small sample size \( (AICc) \) and standard deviation of the residuals \( (Sy.x) \) to evaluate the goodness of fit. The best fit model was chosen as the one with lowest values for both \( AICc \) and \( Sy.x \). SigmaPlot results indicated that non-linear regressions of monohydroxy-TBECH, dihydroxy-TBECH and monohydroxy-TriBECH as well as their β isomer counterparts were best fitted to a Michaelis-Menten model (Figure 4). It should be noted that while monohydroxy-TBECH is a primary metabolite of the TBECH substrate, the lack of authentic metabolite standards precludes the confirmation of whether dihydroxy-TBECH and monohydroxy-TriBECH are primary and/or secondary metabolite. Therefore, the estimated kinetic parameters for dihydroxy-TBECH and monohydroxy-TriBECH should be considered with caution as they were derived assuming a primary metabolite status only.

The model parameters derived from non-linear regression provided useful insights into the metabolic fate of TBECH in humans (Table 2). Apparent \( V_{max} \) values (maximum metabolic rate) for the formation of monohydroxy-TBECH, dihydroxy TBECH and monohydroxy-TriBECH were 162.5, 0.64 and 10.1 pmol/min/mg protein, respectively (Table 2). This indicates monohydroxy TBECH as the major metabolite formed \textit{in vitro} by human liver microsomes. The only available information on toxicokinetics of this flame retardant suggested rapid \textit{in vivo} metabolism of β-TBECH in brown trout. Depuration of the β-isomer obeyed first order kinetics with half-lives of 22.5 ± 10.4 (low dose), 13.5 ± 5.9 (medium dose) and 13.8 ± 2.2 (high dose) days\(^{30} \). In the present study, the observed \textit{in vitro} metabolic clearance rate for β-TBECH was significantly higher \( (P < 0.05) \) than that of the TBECH mixture. Maximum metabolic formation rates of OH-β-
TBECH, (OH)$_2$-β-TBECH and (OH)-β-TriBECH were 4991.7, 14.1 and 66.1 pmol/min/mg protein, respectively (Table 2); equivalent to 31, 22 and 6.5 times of the corresponding metabolite formation rate upon exposure to the technical TBECH mixture. There are several plausible reasons for this observation including (a) slower metabolism of the α-TBECH in the technical mixture and (b) alteration of the stereoselective enzymatic metabolism process by the presence of a larger number of stereoisomers, or even other chemicals/impurities in the TBECH mixture. Nevertheless, β-TBECH was metabolized by in vitro HLM at a faster rate than the TBECH mixture.

Given the simultaneous exposure of hepatic cells to a large number of xenobiotics under real-life conditions, the in vivo metabolic rates of TBECH (and thereof, clearance rates) might be even slower than this controlled in vitro exposure experiment to a single compound. As the rates of OH-TBECH, (OH)$_2$-TBECH and OH-TriBECH formation were best described by the Michaelis Menten model, we used the corresponding equations to estimate the intrinsic in vitro hepatic clearance of TBECH and β-TBECH:

$$CL_{int} = \frac{V_{max}}{K_m} \quad (Equation \ 1)$$

$$CL_{int-liver} = CL_{int} \times 34 \text{ (mg protein/g human liver)} \quad (Equation \ 2)$$

$$CL_h = \frac{(CL_{int-liver} \times Q_h)}{(CL_{int-liver} + Q_h)} \quad (Equation \ 3)$$

Where $CL_{int}$ is the apparent intrinsic in vitro hepatic clearance, $CL_{int-liver}$ is intrinsic in vitro hepatic clearance on gram liver basis, $V_{max}$ rate and $K_m$ are the maximum metabolic rate and Michaelis Menten constant derived from the Michaelis Menten model, $CL_h$ is in vivo hepatic clearance when hepatic blood flow ($Q_h = 0.71 \text{ ml/min/g liver}$) was taken into account.

From Equation 1, the intrinsic in vitro hepatic clearance ($CL_{int}$) of the TBECH mixture due to the formation of OH-TBECH, (OH)$_2$-TBECH and OH-TriBECH were estimated as 13.8, 0.3 and 3
µL/min/mg protein, respectively. By comparison, those of β-TBECH were 302.5, 1.1 and 18.4
µL/min/mg protein, respectively. The total CL_{int-liver} from metabolic formation of all three major
metabolites was then calculated: 0.58 mL/min/g liver for the TBECH mixture and 10.94
mL/min/g liver for β-TBECH.

Despite the lack of authentic standards for TBECH metabolites, leading to the semi-quantitative
nature of these measurements, the calculated hepatic clearance rates clearly show that β-
TBECH was biotransformed at a much faster rate than the TBECH mixture. Despite reservations
on the accuracy of direct extrapolation from in vitro to in vivo clearance due to simultaneous
exposure to a large number of chemicals in vivo, we applied Equation 3 to shed some light on
the in vivo hepatic clearance of TBECH in humans. Our model calculations revealed an in vivo
hepatic clearance (CL_h) of 0.32 mL/min/g liver for the TBECH mixture, while the rapid hepatic
clearance of β-TBECH was dependent on the hepatic blood circulation (Q_h) (i.e. flow limited).

Conclusions

To our knowledge, this is the first study of TBECH metabolism by human liver microsomes. Our
in vitro experiments demonstrated that TBECH was metabolized by human liver microsomes
forming a complex mix of metabolites via cytochrome P450 enzyme-catalyzed hydroxylation
and debromination. This is the first time that a monohydroxylated debrominated metabolite of
TBECH has been detected in vitro. The other detected metabolites were OH-TBECH, (OH)_2-
TBECH, (OH)_2-TriBECH and DBCBA, with substrate concentration dependent assays showing OH-
TBECH to be the major one. The differences in TBECH metabolite profiles resulting from
incubation with HLM (this study) and RLM^{22} underscore inter-species variation in xenobiotic
metabolism. In general, more peaks of all metabolites were observed in our HLM experiments
than reported previously using RLM. Of all detected compounds, metabolic rates of OH-TBECH, 
(OH)$_2$-TBECH and OH-TriBECH were found to best fit to the Michaelis-Menten model by non-
linear regression analysis. Separate pure β-TBECH microsomal assays also demonstrated that β-
TBECH was metabolized much faster than the technical TBECH mixture. However, authentic 
standards of α-TBECH and the metabolites are needed to elucidate more precise 
pharmacokinetic parameters as well as better understanding of isomer specific metabolism.

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interpretation.


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Table 1. List of TBECH metabolites produced after incubation of technical TBECH mixture with HLM for 60 min.

<table>
<thead>
<tr>
<th>Code</th>
<th>Accurate mass [M-H]^-</th>
<th>Mass deviation (ppm)</th>
<th>Chemical formula</th>
<th>Proposed chemical structure*</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>442.75136</td>
<td>1.275</td>
<td>C₈H₁₂Br₄O</td>
<td><img src="image" alt="Monohydroxy-TBECH structure" /></td>
<td>Monohydroxy-TBECH</td>
</tr>
<tr>
<td>M2</td>
<td>458.74635</td>
<td>1.395</td>
<td>C₈H₁₂Br₄O₂</td>
<td><img src="image" alt="Dihydroxy-TBECH structure" /></td>
<td>Dihydroxy-TBECH</td>
</tr>
<tr>
<td>M3</td>
<td>362.84397</td>
<td>4.521</td>
<td>C₈H₁₃Br₃O</td>
<td><img src="image" alt="Monohydroxy-TriBECH structure" /></td>
<td>Monohydroxy-TriBECH</td>
</tr>
<tr>
<td>M4</td>
<td>380.83568</td>
<td>1.267</td>
<td>C₈H₁₃Br₃O₂</td>
<td><img src="image" alt="Dihydroxy-TriBECH structure" /></td>
<td>Dihydroxy-TriBECH</td>
</tr>
<tr>
<td>M5</td>
<td>378.82054</td>
<td>2.620</td>
<td>C₈H₁₁Br₃O₂</td>
<td><img src="image" alt="DBCBA structure" /></td>
<td>DBCBA</td>
</tr>
</tbody>
</table>

* The exact position of the hydroxyl groups could not be specified via the applied standard protocol.
Table 2. Kinetic parameters derived from non-linear regression (Michaelis-Menten model) modelling of the formation of TBECH metabolites following incubation of the technical TBECH mixture and β-TBECH with HLM for 60 min.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$K_m$ (µM) ± SD</th>
<th>$V_{max}$ (pmol/min/mg protein) ± SD</th>
<th>$CL_{int}$ (µL/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Technical TBECH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OH-TBECH</td>
<td>11.78 ± 4</td>
<td>163 ± 30</td>
<td>13.8</td>
</tr>
<tr>
<td>(OH)$_2$-TBECH</td>
<td>2.2 ± 1</td>
<td>0.64 ± 0.08</td>
<td>0.3</td>
</tr>
<tr>
<td>OH-TriBECH</td>
<td>3.4 ± 0.82</td>
<td>10.1 ± 0.8</td>
<td>3</td>
</tr>
<tr>
<td><strong>β-TBECH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OH-β-TBECH</td>
<td>16.5 ± 7.1</td>
<td>4992 ± 1339</td>
<td>303</td>
</tr>
<tr>
<td>(OH)$_2$-β-TBECH</td>
<td>12.3 ± 7.5</td>
<td>14.1 ± 4.9</td>
<td>1.1</td>
</tr>
<tr>
<td>OH-β-TriBECH</td>
<td>3.6 ± 1.1</td>
<td>66.1 ± 7.3</td>
<td>18.4</td>
</tr>
</tbody>
</table>
Figure 1. Selected UPLC-ESI-Orbitrap/MS chromatograms of monohydroxy (M1) and dihydroxy (M2) metabolites formed by HLM exposure to 10 µM of technical TBECH (a and c) and β-TBECH (b and d) for 60 min.
Figure 2. Selected UPLC-Orbitrap/MS chromatograms of metabolites M3, M4 and M5 formed by HLM following exposure to 10 µM technical TBECH (a, c and e) and β-TBECH (b, d and f) for 60 min.
Figure 3. Schematic representation of α-oxidation proposed as a mechanism for biotransformation of TBEC by HLM.
Figure 4. Kinetic study of TBECH metabolite formation fit to a Michaelis-Menten model following 60 min incubation of technical TBECH mixture (A) and β-TBECH (B) with HLM at various substrate concentrations.
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