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Sulfonation and glucuronidation of hydroxylated bromodiphenyl ethers in human liver

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HIGHLIGHTS

- Glucuronidation of OH-BDEs occurred more rapidly than sulfonation.
- SULT1B1 most readily catalyzed sulfonation of each studied OH-BDE.
- Individual variability was observed in rates of glucuronidation and sulfonation.

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ABSTRACT

Hydroxylated bromodiphenyl ethers (OH-BDEs) can arise from monooxygenation of anthropogenic BDEs or through natural biosynthetic processes in marine organisms, and several OH-BDEs have been shown to be toxic. OH-BDEs are expected to form sulfate and glucuronide conjugates that are readily excreted, however there is little information on these pathways. We examined the human hepatic glucuronidation and sulfonation of 6-OH-BDE47, 2-OH-BDE68, 4-OH-BDE68 and 2-OH-6′methoxy-BDE68. Human liver microsomes and cytosol were from de-identified female and male donors aged 31 to 75 under an exempt protocol. Recombinant human SULT1A1, 1B1, 1E1 and 2A1 enzymes were prepared from bacterial expression systems. Sulfonation and glucuronidation of each OH-BDE were studied using radiolabeled co-substrates, 3′phosphoadenosine-5′phospho-^{35}S-sulfate or uridine diphospho-β-D-^{14}C-glucuronic acid in order to quantify the sulfated or glucuronidated products. The OH-BDEs studied were more efficiently glucuronidated than sulfonated. Of the compounds studied, 2-OH-BDE68 was the most readily conjugated, and exhibited an efficiency (V_{max}/K_M) of glucuronidation of 0.274 ± 0.125 mL/min/mg protein, mean ± S.D., n = 3, while that for sulfonation was 0.179 ± 0.030 mL/min/mg protein. For both pathways, all K_m values were in the low μM range. Studies with human SULT enzymes showed that sulfonation of these four substrates was readily catalyzed by SULT1B1 and SULT1E1. Much lower activity was found with SULT1A1 and SULT2A1. Assuming that the glucuronide and sulfate conjugates are non-toxic and readily excreted, as is the case for most such conjugates, these studies suggest that OH-BDEs should not accumulate in people to the same extent as the parent BDEs.

1. Introduction

Since their mass production in the 1990s, brominated diphenyl ethers (BDEs) have been widely used as flame retardants in various consumer products. Common household items include plastics, electronics, furniture, home insulation, as well as many baby products (Siddiqi et al., 2003; Stapleton et al., 2011; Chen et al., 2012). These lipophilic, volatile compounds were sprayed on to the surfaces of different matrices and were not structurally bound thus were able to disperse widely into the air, settling on numerous different surfaces, and increasing human exposure. Unlike the carbon-chlorine bond in chlorinated biphenyls (CBs), BDEs contain a relatively weak carbon-bromine bond that can degrade more easily under environmental conditions (Blanksby and Ellison,

Abbreviations: BDE, bromodiphenyl ether; CB, chlorinated biphenyl; OH-BDE, hydroxylated bromodiphenyl ether; MeO-BDE, methoxy-bromodiphenyl ether; PAPS, 3′-phosphoadenosine-5′-phospho-sulfate; SULT, sulfotransferase; UGT, glucuronosyltransferase; UDPGA, uridine diphospho-glucuronic acid.

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2003). Despite this, BDEs are persistent contaminants in various environmental compartments including air, sediment, and soil, as well as in various marine organisms and wildlife (Christensen and Platz, 2001; Strandberg et al., 2001; Song et al., 2004; Yogui and Sericano, 2009). Furthermore, BDEs have been shown to accumulate in various mammalian biological samples including breast milk, placenta, adipose tissue and blood (Darnerud et al., 2001), and have been found to have endocrine disrupting properties including reproductive, thyroid, and neurodevelopmental effects (Dishaw et al., 2014). Effects on the thyroid system are thought to occur because of the similarity of BDEs and OH-BDEs to thyroid hormone, however mechanisms of toxicity are still poorly understood.

About a decade after their introduction, reports surfaced regarding the potential toxicity associated with brominated flame retardants and BDEs were discontinued and removed from mass production. BDE47, specifically, was widely used in polyurethane foam but was phased out in 2004. However, BDEs persist in household surfaces such as old furniture, upholstery, and insulation and eventually leach out into the air and can settle in dust particles. Stapleton et al. reported that BDEs were widely found in dust samples collected around the home and from there, BDEs can be absorbed dermally, inhaled, or ingested when coming into contact with foodstuffs (Stapleton et al., 2005). The National Report on Human Exposure to Environmental Chemicals and additional studies showed that out of 10 BDEs tested, BDE47 demonstrated the highest levels in blood serum (CDC, 2009; Sjodin et al., 2018). Levels for BDE47 in the U.S. population are roughly 3 to 10 times higher than levels in populations from different studies conducted in European countries (Sjodin et al., 2018). A study conducted in China in 2017 quantified BDEs and OH-BDEs in human serum and their findings showed that BDE47 and 6-OH-BDE47 were the congeners present at highest concentrations in the samples studied (Xu et al., 2018).

Research has demonstrated that phase I monooxygenation of BDEs gives rise to hydroxylated brominated diphenyl ethers (OH-BDEs), see Fig. 1. BDE47 undergoes cytochrome P450-dependent oxidative metabolism in human liver microsomes to several hydroxylated metabolites, 3-OH-BDE47, 5-OH-BDE47, 6-OH-BDE47, 4-OH-BDE42, 4′-OH-BDE49, and 2′-OH-BDE66 (Erratico et al., 2013; Feo et al., 2013). Other BDEs were also hydroxylated by CYP2B6 (Erratico et al., 2012; Gross et al., 2015). OH-BDEs are a topic of concern because in some cases, they are more toxic than the parent compound. For example, evaluation of the effects of several environmentally relevant OH-BDEs, including 6-OH-BDE47, on the expression of catalytic activity of aromatase using a H295R human adrenocortical carcinoma cell line showed that 6-OH-BDE47 caused a significant reduction of aromatase by 98.2% while BDE47 had no effect (Canton et al., 2005).

Not all OH-BDEs arise from monooxygenation of BDE flame retardants. OH-BDEs can also be found as natural products in marine systems (Teuten et al., 2005). Environmental samples can contain naturally occurring OH-BDEs as well as those of anthropogenic origin. Samples taken from the plasma of benthic- and pelagic-feeding fish from the highly contaminated Detroit River corridor showed multiple OH-BDE congeners including 6-OH-BDE47, 2-OH-BDE68, 4-OH-BDE49, and 4-OH-BDE42 (Valters et al., 2005). Other studies have reported 6-OH-BDE47 and 2-OH-BDE68 are present in fish (Asplund et al., 1999; Marsh et al., 2004), although most naturally occurring OH-BDEs have been typically isolated from sea sponges and tunicates (Calcul et al., 2009; Wiseman et al., 2011). Like many OH-BDEs, methoxy-BDEs (MeO-BDEs) are not produced industrially but have been found extensively in marine species. Agarwal and coworkers identified the brominated marine pyrroles/phenols (*bmp*) biosynthetic locus in marine-γ-proteobacteria genomes to be responsible for the synthesis of a wide variety of brominated aromatic compounds, including MeO-BDEs (Agarwal et al., 2014; Busch et al., 2019). The existence of naturally occurring OH-BDEs and MeO-BDEs in marine organisms has been known for many years (Carte and Faulkner, 1981; Unson et al., 1994; Agarwal et al., 2015).

Despite the presence of BDEs and related compounds in the environment, their biotransformation has not been extensively studied. The literature reports that naturally occurring OH-BDEs and MeO-BDEs contain their hydroxy and methoxy group, respectively, predominantly in the *ortho*-position of the molecule, relative to the diphenyl ether bond, for example 6-OH-BDE47 and 2-OH-BDE68. A few *para*-substituted OH-BDEs have been reported as natural products, e.g. 4-OH-BDE33, known as corallinaether (Makoto et al., 2005), while most *meta*- and *para*-substituted OH-BDEs and MeO-BDEs are human and bacterial biotransformation products. Studies to date have not determined if the structural conformation of the OH-BDE influences the toxicity or rate of metabolism. *Para*- OH-BDEs seem to be the most toxic in relation to thyroid hormone toxicity although an *ortho*-compound like 6-OH-BDE47 has been shown to also disrupt thyroid hormone homeostasis and cause neurodevelopmental defects in zebra fish (Macaulay et al., 2015; Guigueno and Fernie, 2017). Like many hydroxylated compounds, it is typically observed that OH-BDEs will undergo sulfonation and glucuronidation at their phenolic sites, which make them substrates for sulfotransferase (SULT) and glucuronosyltransferase (UGT) enzymes (Fig. 1). To the best of our knowledge, only one study has confirmed OH-BDEs as substrates

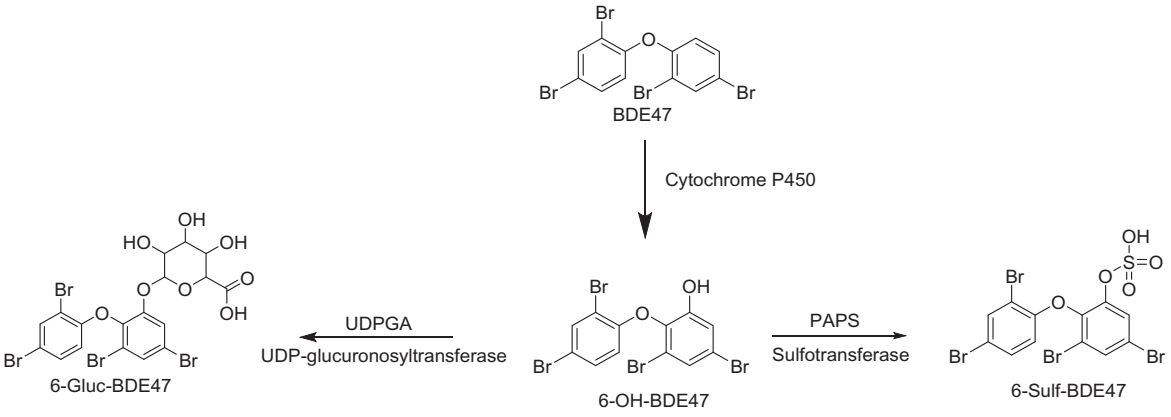

Fig. 1. Expected pathways of metabolism of BDEs and OH-BDEs. Studies have shown that CYP2B6 catalyzes the monooxygenation (Erratico et al., 2013; Feo et al., 2013). Evidence for formation of glucuronide and sulfate conjugates was presented (Erratico et al., 2015).

for SULT and UGT enzymes, but kinetic rates were not calculated (Erratico et al., 2015). In this present study, we examined the human hepatic phase II metabolism of three natural product OH-BDEs (6-OH-BDE47, 2-OH-BDE68, and 2-OH-6′MeO-BDE68) and a synthetic OH-BDE (4-OH-BDE68), which was synthesized to study structure-metabolism activity relationships. We hypothesized that glucuronidation would be the major metabolizing/detoxifying pathway for OH-BDEs. Furthermore, wished to compare our findings to previously published results for the glucuronidation of other prevalent hydroxylated environmental toxicants.

2. Materials and methods

2.1. Chemicals and reagents

The hydroxylated BDEs, 6-OH-BDE47, 2-OH-BDE68, 4-OH-BDE68, 2-OH,6′-MeO-BDE68 (Fig. 2) were isolated from marine *Dysideidae* sponges (Agarwal et al., 2015) or synthesized as described previously (Marsh et al., 2003). Each compound was characterized for purity by NMR and mass spectrometry, as detailed previously (Agarwal et al., 2015).

The uridine diphospho-^{14}C-glucuronic acid (UDPGA), specific activity 250 mCi/mmol and 3′-phosphoadenosine-5′-phospho-^{35}S-sulfate (PAPS), specific activity 2.99 Ci/mmole were purchased from Perkin-Elmer, Waltham, MA and diluted with unlabeled UDPGA (Sigma-Aldrich) or PAPS (Sigma) for use in assays. The unlabeled PAPS from Sigma contained PAP and therefore was purified using an anion-exchange cartridge (Sep-pak Accell plus QMA, Waters, Milford, MA) and eluting with increasing concentrations of sodium chloride. The eluted fractions were analyzed by HPLC using an anion-exchange column, Zorbax SAX, 4.6 × 250 mm, 5 μ (Agilent) with a mobile phase of 0.25 M ammonium phosphate pH 3.0, flow rate 1 mL/min and UV detection at 260 nm, selective for adenosine. Under these conditions PAP eluted at 5.0 min and PAPS at 7.5 min. Fractions shown by HPLC to contain >90% PAPS, i.e. UV_{260} peak at 7.5 min and no PAP were used in assays. All other chemicals were purchased from Sigma–Aldrich or Fisher Scientific (Orlando, FL).

2.2. Preparation of individual human liver subcellular fractions

Liver fractions used for these studies were prepared from de-identified human livers obtained from the University of Florida tissue bank or through the Cooperative Human Tissue Network, University of Alabama, under an IRB-approved exempt protocol. The liver donors were aged 45 to 71, and included 5 males and 3 females. A standard differential centrifugation method was used in order to separate cytosolic and microsomal fractions (Wang et al., 2004). Using a glass-Teflon homogenizer, a sample of liver, 1–5 g was homogenized in four volumes of ice-cold homogenizing buffer containing 1.15% KCl dissolved in 50 mM potassium phosphate, pH 7.4. The homogenate was centrifuged at 13,300 x g for 20 min and the supernatant was collected. The collected supernatant was then centrifuged again at 155,000 x g for 45 min. The cytosolic supernatant was collected and aliquots stored in cryogenic tubes at −80 °C for future use in sulfonation studies. The remaining pellet was resuspended in homogenizing buffer and centrifuged again at 155,000 x g for 40 min. The resulting pellet was resuspended in a buffer composed of 0.25 M sucrose, 0.01 M HEPES pH 7.4, 5% glycerol, 0.1 mM DTT, 0.1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. Aliquots of the resuspended microsomes were placed into cryogenic tubes and flushed with nitrogen for storage at −80 °C until used for glucuronidation studies. Prior to assay use, protein concentrations for cytosolic and microsomal fractions were determined using a bicinchoninic acid protein assay kit from Thermo Fisher Scientific with known concentrations of bovine serum albumin as standards.

2.3. Glucuronidation of OH-BDEs in human liver microsomes

The kinetics of glucuronidation of OH-BDEs by human liver microsomes were determined via a previously published ion-pair extraction method (Wang et al., 2004). We used a radiochemical assay with ^{14}C-UDPGA. The solutions of OH-BDEs were prepared in methanol, added to test tubes, and dried under nitrogen gas to evaporate the methanol. A series of concentrations between 2 μM and 75 μM was tested and incubated with 3 different samples of human liver microsomes. Prior to incubation, liver microsomes were treated with a Brij-58 solution, 0.25 mg Brij/mg microsomal protein, to disrupt the microsomal membrane and access the enzyme active site. Microsomal protein, 0.01 mg, was added to each tube followed by Tris-Cl pH 7.6 (final concentration of 0.1 M in assay), $MgCl_2$ (final concentration of 5 mM in assay), and water, for a final total assay volume of 100 μL. Assay blanks contained no substrate. After vortex-mixing, tubes were centrifuged for 2 min at low speed to ensure contents were at the bottom of the tube and then pre-incubated at 37 °C for 1 min. Reaction was started by adding UDPGA, final concentration of 1 mM in assay. After a 10-min incubation with gentle shaking, the reaction was stopped using 100 μL of a mixture of 1:1 tetrabutylammonium dihydrogen phosphate and 2.5% v/v acetic acid. The glucuronide conjugate ion-pair was extracted with 2 mL of ethyl acetate and separated from the aqueous layer by centrifugation at low speed for 10 min. Two additional extractions were performed with 1 mL of ethyl acetate and all ethyl acetate fractions were pooled and dried under an air stream, followed by the addition of 4 mL of scintillation cocktail (Ecolume, MP Biomedical, Solon, OH) for quantitation. Using the specific radioactivity of the ^{14}C-UDPGA, enzymatic activity was determined after correction for the incubated microsomes blanks.

2.4. Sulfonation of OH-BDEs in human liver cytosol

The kinetics of OH-BDEs sulfonation by human liver cytosol fractions were determined via a previously published method (Wang et al., 2004). We used a radiochemical assay with ^{35}S-PAPS. The OH-BDEs were diluted in methanol, added to test tubes, and dried under nitrogen gas. A concentration series of 2 μM–50 μM was tested and incubated with 3 different samples of human liver cytosol. Tubes included 0.2% bovine serum albumin, 0.01 mg of

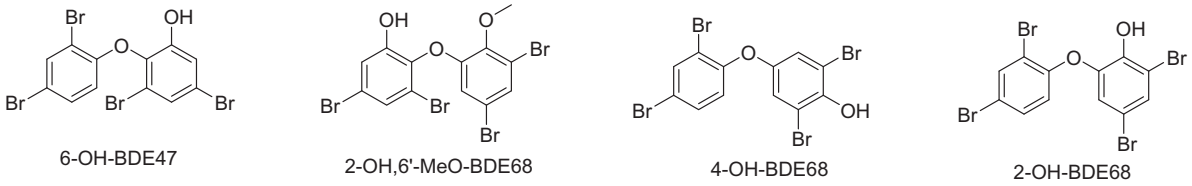

Fig. 2. Structures of OH-BDEs tested.

protein, 50 mM Tris-Cl pH 7.0, 5 mM $MgCl_2$, and water, for a final total assay volume of 100 μL. After vortex-mixing, tubes were centrifuged for 2 min at low speed to bring down all contents and then pre-incubated at 37 °C for 1 min. Reaction was started by adding 20 μM ^{35}S-PAPS. After a 10-min incubation with gentle shaking, the reaction was stopped using 100 μL of a mixture of 1:1 tetrabutylammonium dihydrogen phosphate and 2.5% v/v acetic acid. The sulfate conjugate ion-pair was extracted with 2 mL of ethyl acetate and separated from the aqueous layer by centrifugation at low speed for 10 min. Two additional extractions were performed with 1 mL of ethyl acetate and all ethyl acetate fractions were collected and dried under an air stream, followed by the addition of 4 mL of scintillant for quantitation. Using the specific radioactivity of the ^{35}S-PAPS, enzymatic activity was determined by subtracting the enzymatic activity produced by the cytosolic blanks.

2.5. Sulfonation of OH-BDEs with purified human sulfotransferases

Sulfonation kinetics of OH-BDEs by purified human sulfotransferase enzymes (SULT1B1, SULT1E1, SULT1A1 and SULT2A1) were determined initially by the ion-pair extraction method as described above, but with a range of concentrations of OH-BDEs, 0.25–100 μM. The sulfotransferases were a kind gift of C.N. Falany and were expressed in *E.coli* then purified as described previously (Wang et al., 2005). Preliminary experiments determined the optimal protein concentrations to use for each specific sulfotransferase enzyme (0.035 μg for SULT1B1, 0.05 μg for SULT1E1, 0.01 μg for SULT1A1, and 0.3 μg for SULT2A1).

Results with SULT1A1, but not SULT1B1, SULT1E1 or SULT2A1 showed that with SULT1A1 the no-substrate blanks gave results that were higher than with other SULTs, and similar to incubations with substrate, indicating sulfonation of an assay component. Therefore, a thin layer chromatography (TLC) method was used with this SULT enzyme. The assay conditions and incubation were as described above, however the reaction with SULT1A1 was stopped with 0.1 mL ice-cold methanol and vortex-mixing. Tubes were then centrifuged for 10 min at low speed to sediment protein. Aliquots of the supernatant were subjected to TLC on Analtech preabsorbent 150 silica gel plates (Miles Scientific, Newark, DE). The plates were developed in *n*-butanol: acetone: acetic acid: ammonium hydroxide: water, 50:25:9:1:15 by volume, and after drying, radioactive bands were visualized with Electronic Autoradiography (Packard Instant Imager, Meriden, CT). The percentage of radioactivity in a band at R_F 0.85, found only in incubations with OH-BDE, was used to calculate reaction rates.

2.6. Data analysis

Using Prism 6 software (GraphPad Software, San Diego, CA, USA), apparent kinetic parameters were determined by fitting data to the Michaelis-Menten equation:

$$v = \frac{Vmax*[S]}{Km + [S]}$$

The efficiency of an enzyme was calculated by dividing V_{max} by K_m.

3. Results

3.1. Sulfonation of OH-BDEs in human liver cytosol

The OH-BDEs tested were sulfonated in human liver cytosol and followed Michaelis Menten kinetics in the concentration range studied. Concentrations above 50 μM were not tested to eliminate the possibility of compound insolubility. A cytosolic protein concentration of 0.1 mg/mL and an incubation time of 15 min were found to be within a linear range of sulfonated metabolite formation, data not shown. Fig. 3 shows data for sulfonation of 6-OH-BDE47 in human liver cytosol.

Apparent kinetics parameters for cytosolic sulfonation of all studied OH-BDEs are shown in Table 1. Sulfonation rates showed that the highest apparent maximum velocity (V_{max}) observed was for compound 6-OH-BDE47, followed by 2-OH-BDE68, 4-OH-BDE68, and 2-OH, 6′-MeO-BDE68. The low apparent K_M observed for 2-OH-BDE68 resulted in an apparent efficiency value that was approximately 4 times as high as that observed for the sulfonation efficiency of the three other compounds studied. Sulfonation of 2-OH, 6′-MeO-BDE68 and 4-OH-BDE68 gave similar apparent K_M values, thus similar efficiencies. Finally, although, sulfonation of 6-OH-BDE47 gave the highest apparent K_M value among all the compounds studied, the high apparent V_{max} meant that a comparable efficiency was achieved compared to that of 2-OH, 6′-MeO-BDE68 and 4-OH-BDE68 (see Table 1).

3.2. Glucuronidation of OH-BDEs in human liver microsomes

The OH-BDEs tested were glucuronidated in human microsomes. In the concentration range studied, glucuronidation followed Michaelis Menten kinetics (Table 2). A microsomal protein concentration of 0.1 mg/mL and an incubation time of 10 min were found to be within a linear range of glucuronidated metabolite formation. A representative graph of activity at different substrate concentrations is shown in Fig. 4.

Apparent kinetics parameters, both K_M and V_{max}, were higher for glucuronidation than sulfonation. The highest apparent maximum velocity (V_{max}) for glucuronidation was observed with 2-OH-BDE68 followed by 4-OH-BDE68, 6-OH-BDE47, and 2-OH,6′-MeO-BDE68. However, 2-OH,6′-MeO-BDE68 had the lowest apparent K_M, followed by 2-OH-BDE68, 6-OH-BDE47, and 4-OH-BDE68. Due to relatively lower apparent K_M values, 2-OH-BDE68 and 2-OH,6′-MeO-BDE68 achieved efficiency values that were twice as high compared to the glucuronidation efficiency of compounds 6-OH-BDE-47 and 4-OH-BDE68. 6-OH-BDE-47 and 4-OH-BDE68 had similar glucuronidation efficiency values.

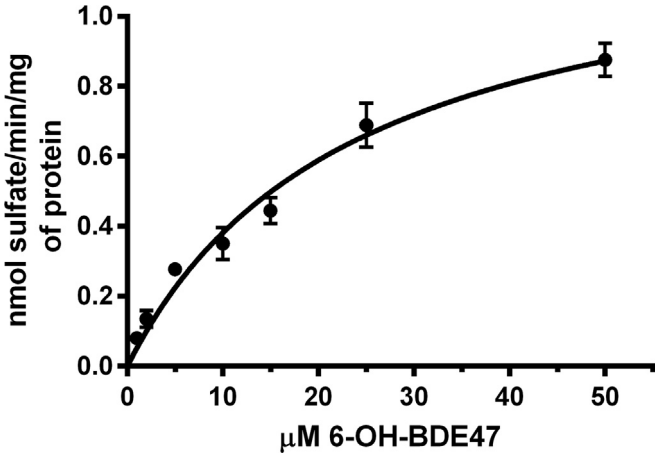

Fig. 3. Kinetics of 6-OH-BDE47 sulfonation in human liver cytosol. Results shown are duplicate determinations with a single sample. The fit of the data to the Michaelis-Menten equation is shown. Similar results were obtained with two other individual human liver cytosol samples.

Table 1
Kinetics of sulfonation of OH-BDEs by human liver cytosol fractions. Values shown are mean ± S.D., n = 3 individuals.

Substrate	Apparent K_M (μM)	Apparent V_{max} (nmol of sulfate/min/mg of protein)	Efficiency (μL/min·mg)
6-OH-BDE47	17.7 ± 4.92	1.03 ± 0.356	41.2 ± 15.5
2-OH-BDE68	1.29 ± 0.344	0.233 ± 0.079	179 ± 29.6
2-OH,6′-MeO-BDE68	3.59 ± 1.16	0.132 ± 0.035	37.3 ± 2.14
4-OH-BDE68	3.53 ± 0.843	0.169 ± 0.059	47.8 ± 9.01

Table 2
Kinetics of glucuronidation of OH-BDEs in human liver microsomes. Values shown are mean ± S.D., n = 3 individual microsomal samples.

Substrate	Apparent K_M, μM	Apparent V_{max}, nmol glucuronide/min/mg protein	Efficiency, μL/min·mg
6-OH-BDE47	35.6 ± 17.5	3.01 ± 2.01	83.3 ± 24.4
2-OH-BDE68	26.5 ± 5.82	6.87 ± 1.83	274 ± 125
2-OH,6′-OMe-BDE68	12.3 ± 7.35	2.11 ± 0.779	211 ± 99.4
4-OH-BDE68	47.8 ± 8.22	8.49 ± 0.699	116 ± 14.2

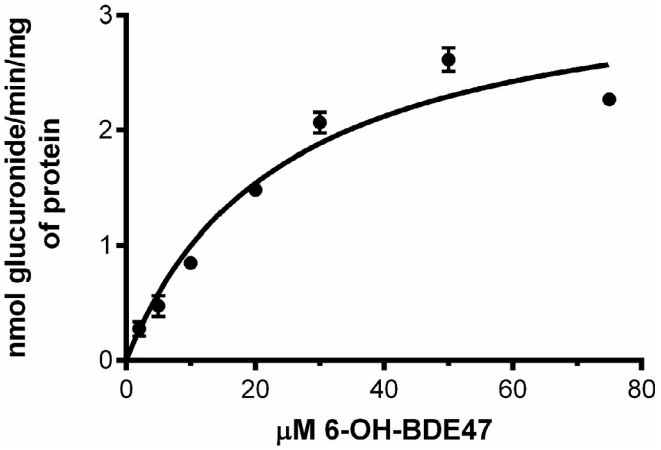

Fig. 4. Kinetics of 6-OH-BDE47 glucuronidation by human liver microsomes. Results show duplicate determinations with a single sample at each substrate concentration and the curve fit to the Michaelis-Menten equation. Similar results were found with other samples.

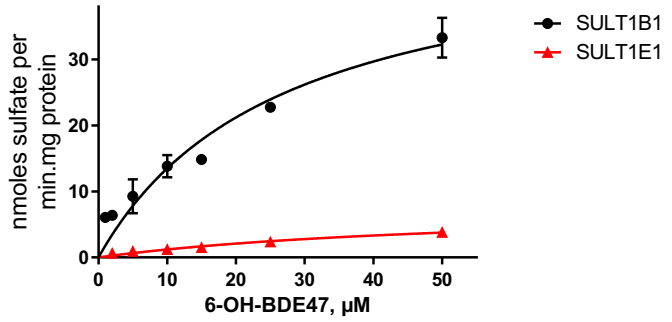

Fig. 5. Kinetics of sulfonation of 6-OH-BDE47 by expressed human SULT1B1 and SULT1E1 Each data point was studied in duplicate. The lines shown are the fit of the data to the Michaelis-Menten equation.

3.3. Sulfonation of OH-BDEs by purified human SULTs

The contributions of individual sulfotransferase enzymes were evaluated by using four SULTS present in the human liver (James and Ambadapadi, 2013). In preliminary experiments testing SULT1B1, SULT1E1, SULT1A1, and SULT2A1, very low activity was observed by SULT2A1 while higher activity was mainly produced by enzymes SULT1B1 and SULT1E1 with all compounds studied. After experimental conditions were optimized, results showed that SULT1B1 achieved the highest maximum velocities with compounds 6-OH-BDE47, 4-OH-BDE68, and 2-OH,6′-MeO-BDE68, while SULT1E1 achieved the highest maximum velocity with compound 2-OH-BDE68 (Table 3). Representative Michaelis-Menten curves obtained with 6-OH-BDE47 for sulfonation by SULT1B1 and SULT1E1 are shown in Fig. 5. Although SULT1E1 and SULT1B1 sulfonated compound 2-OH-BDE68 with similar maximum velocities, SULT1B1 achieved a higher sulfonation efficiency than SULT1E1. The ion-pair extraction method described in section 2.5 was not used to study the SULT1A1 enzyme since we observed from blanks that a component of the assay other than the substrate was also sulfonated by this enzyme, and extracted into the ethyl acetate layer along with the sulfonated OH-BDE product, giving high blanks. Using the TLC method described in section 2.5, we were able to separate and quantify the OH-BDE sulfonated product produced by SULT1A1 effectively. Rates with SULT1A1 were lower than with SULT1B1 or SULT1E1, and there was substrate inhibition at low substrate concentrations, therefore we did not complete kinetic studies. Rates of sulfonation by SULT1A1 of the four substrates at 1 μM are reported in Table 4.

4. Discussion

Once absorbed into the body, BDEs slowly undergo phase I oxidation via CYP2B6 and are converted to various hydroxylated metabolites. Other sources of OH-BDEs include seafood that

Table 3
Apparent sulfonation kinetics of OH-BDEs by purified human SULT1B1 and SULT1E1. Values shown are the mean of duplicate determinations.

OH-BDE	Enzyme	Apparent K_M (μM)	Apparent V_{max} (nmol sulfate/min/mg protein)	Efficiency (mL/min·mg)
6-OH-BDE47	SULT1B1	26.7	49.5	1.85
	SULT1E1	63.2	8.65	0.137
4-OH-BDE68	SULT1B1	44.0	24.1	0.548
	SULT1E1	4.23	4.05	0.957
2-OH-BDE68	SULT1B1	0.172	11.0	64.0
	SULT1E1	8.87	13.9	1.57
2-OH,6′-MeO-BDE68	SULT1B1	24.9	121	4.86
	SULT1E1	1.09	9.16	8.40

Table 4
Sulfonation rates of OH-BDEs by human purified SULT1A1 at substrate concentrations of 1 μM. For most substrates duplicate assays were done, however for 2-OH,6′-MeO-BDE68 a single assay was conducted.

Compound	Rate (nmol of sulfate/min/mg protein), n = 2
6-OH-BDE47	10.50 ± 0.79
4-OH-BDE68	0.574 ± 0.041
2-OH-BDE68	1.60 ± 0.48
2-OH,6′-MeO-BDE68	0.931

contains naturally synthesized OH-BDE molecules (Agarwal et al., 2015). The objectives of the present studies were to investigate the phase II metabolism of selected OH-BDEs through *in vitro* studies, and determine kinetic rates. Our results confirmed that the OH-BDEs tested undergo efficient glucuronidation and sulfonation in human liver. As expected and in agreement with the literature for other hydroxylated compounds (Testa and Clement, 2015), glucuronidation rates were higher, with maximum velocities 3–32 times higher than OH-BDE sulfonation rates. Relative conjugation rates can be influenced by the affinity of the substrate to the metabolizing enzyme as well as the greater availability of co-substrate, uridine 5′-diphosphoglucuronic acid (UDPGA), in the liver versus 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (Rowland et al., 2013). Even at the most environmentally relevant concentration of 6-OH-BDE47 tested (1 μM), the glucuronidation rate was higher (3-fold) than its sulfonation rate, as shown in Figs. 3 and 4. Based on this data, we deduce that regardless of exposure concentration, glucuronidation appears to be the predominant hepatic detoxifying pathway for these compounds in the liver, however sulfonation is also likely to play a role.

6-OH-BDE47 and 2-OH-BDE68 are naturally occurring OH-BDEs, containing a hydroxyl group in the *ortho*-position relative to the diphenyl ether backbone. We initially hypothesized that these two compounds might demonstrate similar sulfonation and glucuronidation kinetic rates based on the position of the hydroxyl group, which is essential for catalytic activity. Interestingly, although drug metabolizing enzymes like UDP-glucuronosyltransferases (UGTs) have broad substrate selectivity which has been hypothesized to result in low affinity for substrates and exhibit K_M values in the low millimolar range or high micromolar range (Testa and Clement, 2015), K_M values for the glucuronidation of OH-BDEs were all between the 12–50 μM range. For glucuronidation, although both substrates produced similar K_M values, 2-OH-BDE68 had a V_{max} that was twice as high as that of 6-OH-BDE47, thus allowing for a higher catalytic efficiency. Structurally, 2-OH-BDE68 and 6-OH-BDE47 contain only one hydroxyl group in the same *ortho*-position and while 2-OH-BDE68 contains its two bromine atoms at the *meta*-positions of the aromatic ring relative to the diphenyl ether and 6-OH-BDE47 contains its bromine groups at the *ortho*- and *para*-positions. Thus, we reasoned that the positioning of the bromine atoms on 6-OH-BDE47 may have an inhibitory effect that produces a lower kinetic rate, perhaps as a result of unfavorable electrostatic interactions between the bromines and amino acid residues present at the catalytic active site within the UGT enzyme. Furthermore, when comparing the rates of 2-OH-BDE68 and 2-OH,6′-MeOBDE68, addition of the methoxy group at the 6′ position increased the affinity of the compound (decreased K_M) by approximately half but also decreased the maximum velocity by two-thirds, ultimately achieving a catalytic efficiency similar to that of the glucuronidation of 2-OH-BDE68. Lastly, changing the position of the hydroxyl group on 2-OH-BDE68 from the *ortho*-to the *para*-position, 4-OH-BDE68, affected the kinetic rate by increasing the K_M and slightly decreasing the V_{max}, therefore lowering the catalytic efficiency.

For the sulfonation pathway, 2-OH-BDE68 had the lowest K_M value while 6-OH-BDE47 produced the highest K_M value as well as the highest maximum velocity. Addition of a methoxy group to 2-OH-BDE68 at the 6′ position increased the apparent K_M by approximately 3-fold, reflecting decreased affinity, and decreased the maximum velocity by half, resulting in reduced catalytic efficiency of the 6′-methoxy compound to approximately one fifth of the efficiency of the 2-OH-BDE68. Similarly, switching the hydroxyl group from the *ortho*-position to the *para*-position on 4-OH-BDE68 had a similar effect by also decreasing the affinity and maximum velocity, therefore decreasing the catalytic efficiency. Within the SULT enzyme, catalytic activity is dictated by the proximity of the hydroxyl group of the substrate to a critical histidine residue within the active site, which acts as a catalytic base to deprotonate the hydroxyl group of the substrate, increasing its nucleophilic character and allowing it to attack the sulfur atom in PAPS (Tibbs et al., 2015). Positioning of the bromine atoms, hydroxyl group, and addition of the methoxy group may cause interactions that are favorable for sulfonation to occur more readily depending on the structural confirmation of each OH-BDE studied.

Within the human liver, four sulfotransferase isoforms are present: SULT1A1, SULT2A1, SULT1B1 and SULT1E1. SULT1A1 is reported to be the predominant SULT in human liver, followed by SULT2A1, SULT1B1 and SULT1E1 (Riches et al., 2009). Our first hypothesis was that the SULT1 liver isoforms would predominately sulfonate OH-BDEs since their preferred substrates often contain phenolic moieties versus aliphatic alcohols (James and Ambadapadi, 2013). We began our studies by testing varying ranges of OH-BDE concentrations and initially found that the lowest rates produced were those from SULT2A1 while the highest rates were produced by SULT1B1 followed by SULT1E1, then SULT1A1. With SULT1A1, substrate inhibition was observed at concentrations greater than 2 μM, and reaction rates were lower than found with SULTs 1B1 and 1E1, thus we did not study kinetics with SULT1A1. Our results (Table 3) show that SULT1B1 and SULT1E1 readily catalyze sulfonation of OH-BDEs in the human liver. The physiological substrates of these enzymes are iodothyronines, and (for SULT1E1) estrogens. The iodothyronines have some structural similarity to OH-BDE, which may explain the greater activities observed with SULT1B1 and SULT1E1. There is variable expression of these SULTs in individuals, which will also influence rates of sulfonation of OH-BDEs.

We wished to put our results into context with published studies of glucuronidation and sulfonation of environmental phenolic chemicals. Although not an exhaustive survey of the literature, Table 5 shows calculated rates of glucuronidation and sulfonation of several environmental chemicals. The OH-BDEs studied here showed more rapid glucuronidation at 1 μM than structurally related chemicals such as triclosan, demethylated metabolites of methoxychlor and higher chlorinated polychlorobiphenylols. Rates of sulfonation were similar or lower than rates of glucuronidation at 1 μM.

Future studies that would increase our knowledge of the interactions of OH-BDEs in the body will be needed to determine if these substances influence the expression or activity of drug metabolizing enzymes such as cytochrome P450, SULTs and UGTs, and if so at what concentrations. Another important avenue of future study is determination of the origins of OH-BDE. To date, the literature suggests that OH-BDEs are mainly produced via oxidation of BDEs by phase I metabolism (Erratico et al., 2012, 2013; Feo et al., 2013). However, other possible routes of formation of these compounds include demethylation of naturally synthesized methoxy-BDEs by cytochrome P450.

Table 5
Rates of glucuronidation and sulfonation of environmental phenolic chemicals.[a].

Species, tissue	Compound	Sulfonation pmol/min/mg protein	Glucuronidation pmol/min/mg protein	Reference
Human, liver Rat, liver	Triclosan	10	7	Wang et al. (2004)
	6-OH-BDE47	55	82	This study
	2-OH-BDE68	102	250	This study
	2-OH-6′-MeO-BDE68	29	159	This study
	4-OH-BDE68	37	174	This study
	4-*t*-octylphenol		7885	Isobe et al. (2017)
	Mono-desmethyl-methoxychlor		15	Hazai et al. (2004)
	Bis-desmethyl-methoxychlor		15	Hazai et al. (2004)
	3′-OH-CB3	14	100	(Tampal et al., 2002; Wang et al., 2006)
	4′-OH-CB3	8	36	(Tampal et al., 2002; Wang et al., 2006)
	4′-OH-CB79		23	Tampal et al. (2002)
	4′-OH-CB112	11		Wang et al. (2006)
	4′-OH-CB203		2	Tampal et al. (2002)

[a] Where the information was available, rates at 1 μM substrate concentration were calculated from the kinetic constants. Rates for mono-desmethyl-methoxychlor were measured at 62.5 μM and for bis-desmethyl-methoxychlor at 31.3 μM.

5. Conclusion

We investigated the glucuronidation and sulfonation kinetics of four OH-BDEs (6-OH-BDE47, 2-OH-BDE68, 4-OH-BDE68, and 2-OH,6′-MeO-BDE68) and tested the hypothesis that glucuronidation would be the major metabolizing/detoxifying pathway in human liver. Our findings established that the predominant metabolic phase II pathway was glucuronidation, however sulfonation also occurred readily. The OH-BDEs studied were much better substrates for sulfotransferases SULT1B1 and SULT1E1 than SULT1A1 or SULT2A1. The metabolism of OH-BDEs via phase II metabolism is likely to reduce their toxicity, since efficient glucuronidation and sulfonation limits human exposure to these metabolites thus decreasing their bioaccumulation potential in various lipophilic sites and organs in the body.

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