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Disposition of the Emerging Brominated Flame Retardant, 2-Ethylhexyl 2,3,4,5-Tetrabromobenzoate, in Female SD Rats and Male B6C3F1 Mice: Effects of Dose, Route, and Repeated Administration

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ABSTRACT

2-Ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB; MW 549.92 g/mol; CAS 183658-27-7) is a brominated component of flame retardant mixtures used as substitutes for some PBDEs. EH-TBB is added to various consumer products, including polyurethane foams, and has been detected in humans. The present study characterized the fate of EH-TBB in rodents. [^{14}C]-labeled EH-TBB was absorbed, metabolized, and eliminated via the urine and feces following single administrations of 0.1–100 μmol/kg (~0.05–55 mg/kg) or repeated administration (0.1 μmol/kg/day × 5–10 days) by gavage to female Hsd:Sprague DawleySD (SD) rats. Cumulative excretion via feces increased (39–60%) with dose (0.1–10 μmol/kg) with corresponding decreases in urinary excretion (54 to 37%) after 72 h. Delayed excretion of [^{14}C]-radioactivity in urine and feces of a 100 μmol/kg oral dose was noted. Recovery was complete for all doses by 72 h. IV-injected rats excreted more of the 0.1 μmol/kg dose in urine and less in feces than did gavaged rats, indicating partial biliary elimination of systemically available compound. No tissue bioaccumulation was found for rats given 5 oral daily doses of EH-TBB. Parent molecule was not detected in urine whereas 2 metabolites, tetrabromobenzoic acid (TBBA), a TBBA-sulfate conjugate, and a TBBA-glycine conjugate were identified. EH-TBB and TBBA were identified in extracts from feces. Data from gavaged male B6C3F1/Tac mice indicated minimal sex- or species differences are likely for the disposition of EH-TBB. Approximately 85% of a 0.1 μmol/kg dose was absorbed from the gut. Overall absorption of EH-TBB is expected to be even greater at lower levels.

Key words: 2-Ethylhexyl 2,3,4,5-Tetrabromobenzoate; disposition; metabolism.

2-Ethylhexyl-2,3,4,5-tetrabromobenzoate (CAS# 183658-27-7, Pubchem CID 71316600) has been described using a number of different abbreviations in the contemporary literature, eg, EH-TBB, EHTeBB, EHTBB, or TBB. The designation "EH-TBB" is used in the present work in accordance with the abbreviation standards for flame retardants proposed by Bergman et al. (2012).

2-Ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) is a component in some commercial brominated flame retardant (BFR) mixtures (eg, Firemaster BZ-54 [BZ-54], Firemaster 550 [FM550], and Firemaster 600 [FM600]) introduced for use in polyurethane foams as a replacement for the commercial pentabrominated PBDE mixture (Dodson *et al.*, 2012; Stapleton *et al.*, 2012). BZ-54 is composed of a 2:5 mixture of BEH-TEBP & EH-TBB (Bearr *et al.*, 2012).The composition of FM550 has been described as containing isopropylated phenyl phosphate (30–60%), triphenyl phosphate (10–30%), and a 1:4 mixture of bis(2-ethylhexyl) tetrabromophthalate (BEH-TEBP) and EH-TBB (40–70%) (Chemtura, 2014; Stapleton *et al.*, 2008). FM600 is composed of isobutylenated phenol phosphate (50–70%) and an unknown ratio of EH-TBB and BEH-TEBP (50–70%) (Chemtura, 2016). It is likely the ratio of EH-TBB to BEH-TEBP is similar in both FM550 and FM600, as current press releases and material safety data sheets for these products characterize the brominated components as "1,3-Isobenzofurandione, 4,5,6,7-tetrabromo-, reaction

TABLE 1. Synopsis of Studies Conducted for Describing EH-TBB Disposition

	Dose (μmol/kg), route, species					
	0.1, IV, rat	0.1, oral, rat	0.1, oral, mouse	1, oral, rat	10, oral, rat	100, oral, rat
Dose number, duration		Single, 1 h Single, 4 h Single, 8 h Single, 24 h Single, 24 h, JVC Single, 24 h, BDC	Single, 24 h			
	Single, 72 h	Single, 72 h Repeat x 5, 24 h Repeat x 5, 72 h Repeat x 10, 24 h Repeat x 10, 72 h		Single, 72 h	Single, 72 h	Single, 72 h

Studies were primarily conducted in female SD rats. N = 4 animals per study unless otherwise noted. JVC: jugular vein cannulated; BDC: bile duct cannulated.

products with 2-ethyl-1-hexanol, CAS# 219632-53-8" (Chemtura, 2016; GreatLakes, 2016). Although risk assessments are under consideration by the US EPA, EH-TBB production and import volumes to the US are not available and are classified as "confidential business information" by the USEPA (USEPA, 2015); similarly, international production volumes are not publicly available. EH-TBB is not listed in the US EPA High Production Volume Information System, indicating its US production and import volumes are less than the threshold of "1 million pounds or more per year"; however, EH-TBB is subject to a USEPA significant new use rule (USEPA, 2015). EH-TBB has been detected in furniture foam, baby products (mattresses and high-chair foam), house dust, outdoor dust and sediment, and wildlife (Abdallah and Harrad, 2011; Ali *et al.*, 2011, 2012; Carignan *et al.*, 2013, Davis *et al.*, 2012; Stapleton *et al.*, 2014; Zhu *et al.*, 2014). Samples of furniture foam have shown FM550 present in quantities up to 4.2% by weight (Stapleton *et al.*, 2009). EH-TBB was described as a brominated phthalate by the EPA and as such, EH-TBB was listed as one of 8 structurally similar chemicals to undergo full assessment under the Toxic Substances Control Act (TSCA) Work Plan and Action Plan (USEPA, 2011, 2012). Despite increasing scrutiny, toxicity data for EH-TBB are scant (EFSA, 2012).

Exposure to EH-TBB is essentially ubiquitous due to its use in polyurethane foams and subsequent presence in house dust (Al-Omran and Harrad, 2016; Cao *et al.*, 2014; Carignan et al., 2013; Fang and Stapleton, 2014; Harrad *et al.*, 2010; Qi *et al.*, 2014; Stapleton et al., 2014). A majority of systemic exposure to EH-TBB is expected to result from ingestion of dust, with upper range estimates of 0.2–0.4 ng EH-TBB/g dust/kg body weight (Ali et al., 2012; Al-Omran and Harrad, 2016). Sampling found a US population had serum levels of 1.3–54 ng EH-TBB/g lipid whereas amounts in hair and nail samples from these same individuals contained 7.6–4540 and 11–1210 ng EH-TBB/g, respectively (Liu *et al.*, 2016a). Urinary metabolites of EH-TBB were found in both adults and children, with detection frequency more than double for children (70%) relative to the paired adult (27%) (Butt *et al.*, 2014), consistent with previous conclusions that children have more frequent and likely higher exposures to contaminants in dust (Harrad *et al.*, 2008).

2-Ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) is lipophilic ($\log P_{est}$: 7.73–8.75) (Bergman et al., 2012; USEPA, 2016) and may cross membranes, but little is known about the disposition of EH-TBB *in vivo* for humans or animals. *In vitro*, EH-TBB was hydrolyzed to TBBA by esterases present in porcine intestine preparations, human and rat liver cytosol and microsomal protein, and rat serum (Roberts *et al.*, 2012). *In vivo*, EH-TBB was shown to bioaccumulate in aquatic organisms when administered as a mixture (FM550 or BZ-54). Bearr et al. (2012) detected EH-TBB in tissues from fathead minnows (*Pimephales promelas*) 22 days after cessation of FM550 exposure. SD rat dams administered FM550 (1 mg/day) during pregnancy and nursing were observed to have increased serum thyroxine and decreased hepatic esterase activity (Patisaul *et al.*, 2013). Furthermore, in these studies, female pups displayed high anxiety phenotypes whereas male offspring had significantly impaired glucose tolerance. The diverse effects observed in these rats may correlate to any one or a combination of the individual components of the mixture. Therefore, it is important to conduct toxicological evaluations for the specific components, including EH-TBB. The present study was designed to characterize the effects of dose, route, and repeated administration on the fate of EH-TBB in female Sprague Dawley rats (Table 1). The extent of absorption, rates and routes of elimination, tissue distribution, and metabolism are described. Preliminary assessments of enzyme induction and bioaccumulation potential in rats were also explored. Finally, an exploratory assessment of possible species- or sex-related differences in the disposition of EH-TBB was performed using male B6C3F1/Tac mice in a prior paradigm (Buchanan *et al.*, 1997). The dose range tested herein was based on high-range human exposures to EH-TBB in household dust, as described by Patisaul et al. (2013).

MATERIALS AND METHODS

Chemicals

[^{14}C]-radiolabeled EH-TBB (carboxy-labeled; Fig. 1) was purchased from Moravek Biochemicals, Inc. (Brea, California). [^{14}C]-EH-TBB radiochemical purity of >99.4% (determined by radiochemical-HPLC) with a specific activity of 55 mCi/mmol; chemical purity was determined to be >99% as compared with an EH-TBB reference standard (Accustandard, Inc., New Haven, Connecticut). Radiochemical purity was assessed by radiometric HPLC (described below). [^{14}C]-EH-TBB was diluted in toluene to a stock concentration of 0.5 mCi/mL. 2,3,4,5-Tetrabromobenzoic acid (TBBA; >98% pure) was purchased from the Duke University Small Molecule Synthesis Facility (Durham, North Carolina). Scintillation cocktails were obtained from MP Biomedicals (Ecolume; Santa Ana, California) or Perkin-Elmer (Ultima-Flo M and PermaFluor E+; Torrance, California). Corn oil

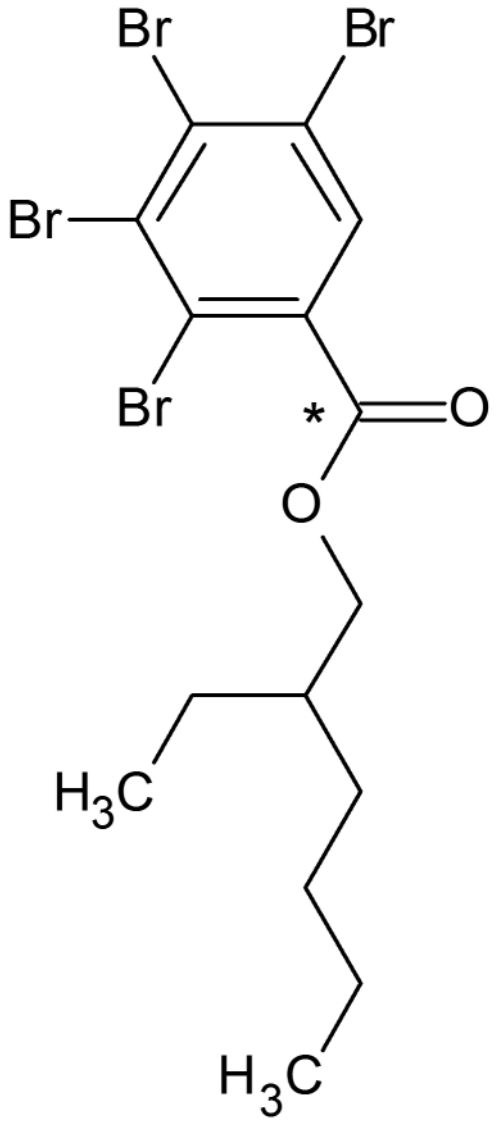

FIG. 1. Chemical structure of EH-TBB; asterisk denotes position of [^{14}C]-radiolabel.

(Mazola, ACH Food Companies, Inc., Memphis, Tennessee) was purchased from a local grocery store. Toluene, acetonitrile, ethyl acetate, ethyl alcohol, porcine carboxylesterase 3, β-glucuronidase (EC 3.2.1.31, Type B-10), ammonium acetate, D-saccharic acid 1, 4-lactone, and aryl sulfatase were purchased from Sigma-Aldrich (St. Louis, Missouri). Carbosorb E was purchased from Perkin-Elmer. All other reagents used in these studies were high performance liquid chromatography (HPLC) or analytical grade.

Animal models and dosing

Female Hsd:Sprague DawleySD rats (SD; 10 weeks, ~200 g; Harlan Laboratories, Raleigh, NC) or male B6C3F1/Tac mice (10 weeks; ~20 g; Taconic Farms, Germantown, New York) were used in this study. Animals were maintained in an AAALAC-approved animal care facility and acclimated at the facility for at least 1 week prior to being placed on study (humidity: ~49%, room temperature: ~72°F, 12 h light/dark cycle). Food (NIH #31) (Ziegler Brothers, 2010) and water (City of Durham, North Carolina) were provided for *ad libitum* consumption. All procedures were approved by the NIEHS Institutional Care and Use committee. Immediately following dosing, animals were housed individually in a ventilated glass metabolism cages for collection of excreta. [^{14}C]-radioactivity was found to adhere strongly to plastic metabolism cages at a dose level of 1 μmol/kg, necessitating the use of glass metabolism cages.

To determine the fate of systemically available EH-TBB in rats, a single intravenous (IV) bolus was injected into a lateral tail vein. For IV dosing (0.1 μmol/kg, 1 mL/kg, $N = 4$ animals), [^{14}C]-EH-TBB was added to one part Cremophore EL (Sigma-Aldrich) and toluene evaporated under nitrogen followed by the addition of 1 part ethanol and 3 parts water. Oral dosing solutions were prepared by adding appropriate volumes of [^{14}C]-EH-TBB or EH-TBB dissolved in toluene to corn oil at a dose volume of 4 mL/kg; toluene was removed by evaporation under a steady stream of nitrogen. Oral doses were administered using a #16 and #20 ball-tipped stainless steel feeding needle to rats and mice, respectively.

Dose response was investigated in female SD rats by administering a single dose of EH-TBB by gavage (PO) at dose levels of 0.1, 1, 10, or 100 μmol/kg (~0.05–55 mg/kg; 4 mL/kg; $N = 4$/dose group). Distribution to tissues and cumulative excretion of [^{14}C]-radioactivity in excreta were evaluated at 72 h post-exposure for these doses. Dosing solutions (1, 10, or 100 μmol/kg, 4 mL/kg) were formulated to provide approximately 25 μCi/kg/rat of [^{14}C]-EH-TBB and contained non-radiolabeled EH-TBB for delivery of doses up to 100 μmol/kg. About 0.1 μmol/kg of dosing solutions provided 5.5 μCi/kg/rat of [^{14}C]-EH-TBB. Male B6C3F1/Tac mice ($N = 4$) received a single dose of 0.1 μmol/kg (4 mL/kg) by gavage to identify potential sex- or species-related differences in the *in vivo* fate of EH-TBB; distribution to tissues was evaluated at 24 h post-dose for mice.

Time-course disposition of EH-TBB for a single oral dose (0.1 μmol/kg) was evaluated by analyzing radioactivity in tissues and excreta from female SD rats euthanized at 1, 4, 8, 24, or 72 h ($N = 4$ per time point). Additionally, an assessment of total radioactivity concentration over time in blood was performed in female SD rats containing an indwelling jugular vein cannula ($N = 4$). Blood (~100 μL) was obtained through the cannula using heparinized syringes. Sampled blood was replaced with equal amounts of saline. A pilot study investigating biliary elimination was conducted using bile-duct cannulated female SD rats; 2 rats were administered EH-TBB (0.1 μmol/kg) by gavage or lateral tail vein and bile was collected at hourly intervals for 8 h then continuously for the 8–12 h and 12–24 h intervals and euthanized 24 h after dosing. Both types of cannula were implanted by the vendor and the rats were fully recovered from surgery before delivery. Surgically altered animals were acclimated for 24 h in the NIEHS facility prior to dosing.

The disposition of repeated doses was assessed by 2 experimental methods. The first method investigated changes in rates or routes of elimination by administering 5 or 10 daily doses of EH-TBB (0.1 μmol/kg/day; 4 or 9 non-radiolabeled doses followed by a single [^{14}C]-radiolabeled dose, $N = 4$ animals per dose group). The [^{14}C]-radioactivity in excreta was determined over 72 h following administration of the radiolabeled dose and in tissues at this terminal time-point. The second method investigated the bioaccumulation potential of the lowest tested dose (0.1 μmol/kg/day; 5.5 μCi/kg/day) in tissues 24 h after the last of 5 consecutive daily doses of [^{14}C]-labeled EH-TBB.

Final concentrations of [^{14}C]-radioactivity were determined by assaying aliquots of dosing solution in triplicate by liquid scintillation counting using a LS6500 Scintillation Counter

(Beckman Coulter, Brea, California). Dosing solutions were subjected to HPLC-radiochemical analysis to confirm test material concentration.

Sample collections

Following administration of the compound, excreta and cage rinses were collected at 4, 8, 12, 24, 48, and 72 h. Reverse osmosis water (Picopure, Hydro Service and Supplies, Inc, Durham North Carolina) was used for cage washes. When bladder urine was recovered, it was added to the final urine collection vial. Initial experiments allowed for the collection of [^{14}C]-radioactivity excreted in expired air using a method described previously (Sanders *et al.*, 2000). However, [^{14}C]-labeled CO_2 and/or exhaled volatile organic compounds were not detected; therefore, the procedure was discontinued for all subsequent experiments. Euthanasia was achieved by CO_2 asphyxiation and confirmed by unresponsiveness to forceps pinch and exsanguination. Blood samples were collected into heparinized syringes via cardiac puncture immediately following euthanasia. Plasma was isolated from heparinized blood by centrifugation (5 min at 2300 $\times$ *g*). Tissues (adipose (pooled), adrenals, brain, heart, kidneys, large intestine, large intestine contents, liver, lung, muscle (quadriceps), pancreas, ovaries, skin (ears), small intestine, small intestine contents, spleen, stomach, stomach contents, thymus, thyroid, urinary bladder, and uterus) were dissected and placed in labeled pre-weighed vials and weights determined gravimetrically. Total mass of dispersed tissues were calculated as a fraction of body weight using previously published values (Birnbaum *et al.*, 1980). Samples were maintained at −80°C until further analyses.

Analytical methods

Quantitative analyses of total [^{14}C]-radioactivity content in plasma (50 μL), urine (10–100 μL) and cage rinses (1 mL) were determined by liquid scintillation counting (LSC). [^{14}C]-radioactivity was quantified in whole blood by combustion in a Packard 307 Biological Sample Oxidizer (Perkin-Elmer, Waltham, Massachusetts) followed by LSC counting. Feces were dried in a fume hood, weighed, and ground to a powder using a mortar and pestle. Aliquots of feces and tissues (~25 mg) were weighed and [^{14}C]-radioactivity was quantified by combustion. All samples were assayed in triplicate. Values reported for % total dose excreted in feces include [^{14}C]-radioactivity detected in large intestine content. Values reported for percent total dose excreted in urine include [^{14}C]-radioactivity from the corresponding cage wash.

Aliquots (~250 mg) of air-dried fecal samples were placed in labeled pre-weighed glass screw-top tubes and re-suspended in 5 mL toluene then centrifuged at 830 $\times$ *g* for 10 min using a Sorvall RC6+ centrifuge (Thermo Fisher Scientific, Waltham, Massachusetts) with a SH-3000 swing bucket rotor. This process was repeated twice, with the supernatants transferred and pooled. Each pellet was then further extracted with ethyl acetate (3 $\times$ 5 mL) and 1% HCl in 95% ethanol (3 $\times$ 5 mL). At each step, the samples were centrifuged and the supernatants transferred and pooled. Extracts were concentrated using a Savant SPD1010 SpeedVac (Thermo Fisher Scientific) without heating, and aliquots were analyzed by UV/radiometric HPLC.

Prior to HPLC-radiometric or MS analyses, urine samples were concentrated to near dryness using a Savant SPD1010 SpeedVac (Thermo Fisher Scientific) without heating. Concentrated samples were reconstituted in 200 μL of 0.2% formic acid in water and filtered using PVDF 0.22 μm centrifugal filters (Ultrafree MC GV, Amicon Ultra; Tullagreen, IRL). Aliquots of samples were initially profiled by HPLC with radiometric detection. To determine the nature of phase II metabolites of EH-TBB, samples were incubated at 37°C for up to 24 h with β-glucuronidase (5000 U/mL) or aryl-sulfatase (11 U/mL) as described previously (Andersen *et al.*, 1999). Urine incubated with buffer (200 μM ammonium acetate in water, pH 5.0) was incubated under identical conditions and served as controls. Carboxylesterase hydrolysis of EH-TBB was conducted using porcine carboxylesterase 3, as described previously (Roberts et al., 2012). Reactions were terminated by addition of equal volumes of acetonitrile followed by HPLC-radiometric analyses.

2-Ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) was quantified by UV/Vis absorbance and radiochemical detection following HPLC separation. The HPLC system was composed of an Agilent (Santa Clara, California) 1100 HPLC, Agilent Zorbax 150 $\times$ 4.6 mm C18 column and an in-line IN/US β-RAM model 3 Flow Scintillation Analyzer (LabLogic, Inc., Brandon FL). HPLC control and analysis software was Laura4 (LabLogic). Mobile phases consisted of (A) 0.2% formic acid in water and (B) 0.2% formic acid in acetonitrile. Sample separations were performed using a gradient from A to B; initial conditions (99% A) were maintained for 5 min then reduced to 10% over 5 min, held at 10% A for 8 min, then reduced to 1% A and held for 12 min. The column was returned to initial conditions and allowed to equilibrate for 2 min before re-use. HPLC flow rates were 1 mL/min and scintillation cocktail flow rates were 2 mL/min.

Qualitative analyses of extracted metabolites (described above) were carried out using ion-trap mass spectrometry with atmospheric pressure chemical ionization in negative ion mode. The mass spectrometer (MS) system was Thermo Fisher Scientific LTQXL ion trap MS coupled to a Thermo Fisher Scientific Ultimate 3000 binary UPLC with a Agilent Zorbax 150 $\times$ 4.6 mm C18 column. HPLC conditions were the same as above. MS conditions were: vaporizer temperature: 250°C; sheath gas flow rate: 20; auxiliary gas flow rate: 10; sweep gas flow rate: 2; discharge current: 5 μA; capillary temp: 275°C; capillary voltage: −15 V; tube lens voltage: −125 V.

Pharmacokinetic modeling

Pharmacokinetic modeling was based on a 9-point curve (at 7.5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h) following a single oral dose (0.1 μmol/kg). Total [^{14}C]-radioactivity collected from individual animals at each time point described above was used to construct a time–concentration data table for each animal. These data were fit to established pharmacokinetic models using the Phoenix WinNonlin (Certara USA, Inc.; St. Louis Missouri) software package. Time-concentration data from animals administered EH-TBB by gavage were fit to a one-compartment model with first order input and output. Goodness of fit was assessed by comparing the sum of squared residuals.

Statistical methods

The data were subjected to statistical analysis using paired t-tests or two-way ANOVA followed by the Tukey–Kramer test for pairwise comparisons (Graphpad Prism, Graphpad Software, Inc., La Jolla, California). Values were considered to be significantly different at $P < .05$.

RESULTS

Disposition—Single Dose

Nearly all of an administered dose of 0.1, 1, 10, or 100 μmol/kg was recovered in urine and feces of female rats within 72 h following gavage (Table 2). Although not all values were statistically significant, a trend of increased amounts of [^{14}C]-radioactivity in feces and decreased amounts in urine was observed in the range of 0.1–10 μmol/kg. Less than 1% of the total dose remained in the tissues of these rats at 72 h. In IV-dosed rats, the amount of [^{14}C]-radioactivity in urine, feces, and tissues was significantly different from amounts excreted in gavaged rats (Table 2). The elimination of EH-TBB-derived radioactivity in excreta over time in female rats is shown in Figure 2. Greater than 90% of administered [^{14}C]-radioactivity at all dose levels was eliminated in urine and feces by 48 h. However, compared with the other doses, 100 μmol/kg administered by gavage resulted in a marked decrease in elimination in both urine and feces at earlier time points.

Sampling of tissues and GI contents collected from animals dosed with a single oral administration of 0.1 μmol/kg and euthanized at 1, 4, 8, or 24 h indicated rapid absorption and distribution to tissues (Table 3). Peak concentrations of [^{14}C]-radioactivity were generally observed at the 4 h time point in major tissues, including blood (Table 3). Compartmental analysis of [^{14}C]-radioactivity in blood collected from a group of jugular vein-cannulated (JVC) rats receiving a similar dose was in close agreement with the data derived from the serial-sampled rats. The peak concentration in blood (42 ± 6 pmol-eq/mL) of the JVC rats occurred between 4.7 and 6.5 h post-dosing (Fig. 3). Based on a one-compartment model fit, the absorption half-life was approx. 4 h and the distribution/elimination half-life was approx. 5 h with a final clearance rate of 35 ± 10 mL/h.

The disposition data collected at 24 h following oral administration of EH-TBB to male B6C3F1/Tac mice (0.1 μmol/kg) was similar to that of comparably dosed female rats (Table 3). The fraction of total dose recovered in urine (49 ± 14%) and feces (47 ± 14%) were similar and comparable tissues contained the same order of magnitude of residual radioactivity. HPLC-radiometric analyses of urine and feces extracts indicated murine metabolism of EH-TBB was the same as that seen for female SD rats (Supplementary Fig. S1).

TABLE 2. Administered [^{14}C]-Radioactivity Recovery in Excreta and Tissues at 72 h Post-Dose (%)

	Dose (μmol/kg)				
	0.1—IV	0.1—oral	1—oral	10—oral	100—oral
Urine	73 ± 7[a]	54 ± 10[b]	47 ± 13	37 ± 8	44 ± 2
Feces	23 ± 6	41 ± 10	51 ± 14	60 ± 8	54 ± 2
Tissues	2.7 ± 0.3[a]	0.7 ± 0.3	0.5 ± 0.2	0.7 ± 0.3	n.d.
Blood	0.1 ± 0.02	0.06 ± 0.02	0.01 ± 0.01	0.04 ± 0.02	0.05 ± 0.01

Each value represents the mean ± SD of 4 rats per treatment group. n.d.: not determined. Recoveries in feces include gastrointestinal tract contents. Recoveries in urine include cage rinses.

[a]Significantly different from 0.1 μmol/kg oral dose, $P < .05$.

[b]Significantly different from 10 μmol/kg dose, $P < .01$.

Disposition—Repeated Dose

To investigate whether repeated exposure to EH-TBB resulted in bioaccumulation, rats were administered 5 daily oral doses of [^{14}C] EH-TBB (0.1 μmol/kg/d, 5.5 μCi/kg/d) and euthanized 24 h after the final dose. Whereas no accumulation of [^{14}C]-radioactivity in tissues was observed, repeated administration did result in an increase in the relative proportion of dose eliminated in urine and a corresponding decrease in dose recovered in feces (Table 2). To further investigate this alteration in apparent route of elimination; rats were administered 4 or 9 non-radiolabeled doses of EH-TBB by gavage (0.1 μmol/kg/d) followed by a single [^{14}C]-radiolabeled dose of EH-TBB. Cumulative amounts of [^{14}C]-radioactivity eliminated in the urine over 72 h following the radiolabeled dose in repeat-dosed rats were significantly increased ($P < .05$) over rats receiving the single dose (Fig. 4). Conversely, the excreted radioactivity significantly decreased over time in the feces of repeat-dosed rats. The excreta data were similar for rats receiving 5 or 10 doses. No effects on distribution and clearance of [^{14}C]-radioactivity in tissues were

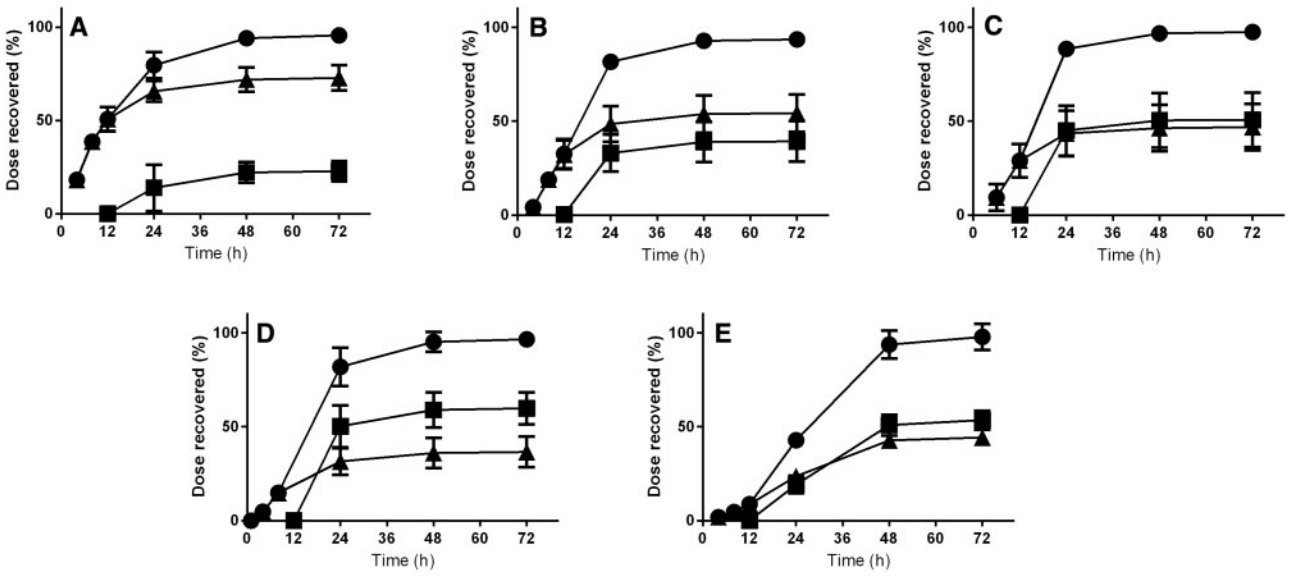

FIG. 2. Excretion of [^{14}C]-radioactivity following a single administration of EH-TBB. $N = 4$ per dose group, mean ± SD. (A) 0.1 μmol/kg, IV, (B) 0.1 μmol/kg, oral, (C) 1 μmol/kg, oral, (D) 10 μmol/kg, oral, and (E) 100 μmol/kg, oral (▲:Recovery in urine; ●:Recovery in feces; ■: Total recovery).

TABLE 3. [^{14}C]-Radioactivity Recovered in Excreta and Major Tissues after Oral Administration of EH-TBB (0.1 μmol/kg) to Female SD Rats or Male B6C3F1/Tac Mice

Tissue	Dose recovered	Single 1 h	Single 4 h	Single 8 h	Single 24 h	Single (mouse) 24 h	Repeated 24 h
Feces	%	0.2 ± 0.2[a]	16 ± 8	42 ± 11	49 ± 13[b]	49 ± 14[b]	29 ± 3[c]
Urine	%	0.3 ± 0.2	5 ± 2	19 ± 3	43 ± 13[b]	47 ± 14[b]	65 ± 4[c]
Stomach contents	%	30 ± 9	13 ± 5	3 ± 1	n.d.	n.d.	n.d.
Small intestine contents	%	46 ± 19	20 ± 6	5 ± 2	n.d.	n.d.	n.d.
Blood	%	6 ± 1	13 ± 5	10 ± 3	1 ± 0.5	0.6 ± 0.3	0.3 ± 0.1
	(pmol-eq/g)	(4 ± 1)	(9 ± 4)	(7 ± 2)	(0.001 ± 0.001)	(0.0003 ± 0.0001)	(0.4 ± 0.1)
Liver	%	2 ± 1	5 ± 2	3 ± 0.3	0.3 ± 0.1	0.1 ± 0.2	0.1 ± 0.02
	(pmol-eq/g)	(43 ± 26)	(104 ± 51)	(77 ± 3)	(8 ± 4)	(2 ± 2)	(6 ± 1)
Adipose	%	2 ± 2	2 ± 1	2 ± 2	0.6 ± 0.4	0.5 ± 0.5	0.6 ± 0.1
	(pmol-eq/g)	(15 ± 15)	(22 ± 12)	(22 ± 16)	(7 ± 4)	(3 ± 3)	(11 ± 1)
Muscle	%	2 ± 0.5	7 ± 4	4 ± 2	0.2 ± 0.2	0.1 ± 0.1	0.6 ± 0.3
	(pmol-eq/g)	(6 ± 3)	(13 ± 8)	(10 ± 4)	(0.4 ± 0.4)	(0.2 ± 0.1)	(3 ± 2)
Kidney	%	2 ± 0.6	4 ± 1	3 ± 0.5	0.4 ± 0.1	0.7 ± 0.5	0.1 ± 0.0
	(pmol-eq/g)	(226 ± 89)	(547 ± 202)	(427 ± 106)	(59 ± 19)	(41 ± 20)	(37 ± 11)
Skin	%	1 ± 0.6	6 ± 2	4 ± 0.7	0.6 ± 0.5	0.2 ± 0.2	0.4 ± 0.1
	(pmol-eq/g)	(9 ± 3)	(37 ± 19)	(31 ± 6)	(5 ± 3)	(1 ± 1)	(6 ± 1)
Lung	%	0.4 ± 0.6	0.5 ± 0.2	0.5 ± 0.2	0.05 ± 0.03	0.03 ± 0.02	0.02 ± 0.006
	(pmol-eq/g)	(57 ± 63)	(83 ± 42)	(76 ± 20)	(8 ± 5)	(3 ± 1)	(6 ± 2)
Brain	%	0.2 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.003 ± 0.01	0.003 ± 0.003	0.007 ± 0.004
	(pmol-eq/g)	(2 ± 1)	(6 ± 2)	(3 ± 2)	(0.5 ± 1)	(0.2 ± 0.2)	(2 ± 1)

[a]Each value represents the mean ± SD of 4 animals per treatment group.
[b]Significantly different from repeat dose (rat, 24 h), $P < .001$.
[c]Significantly different from single dose (rat, 24 h), $P < .0001$.
n.d.: not determined.

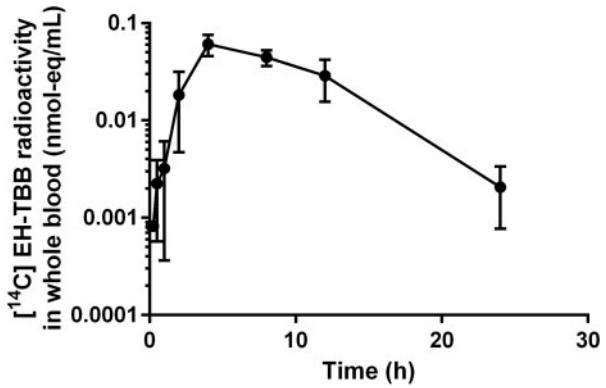

FIG. 3. Time-blood concentration curve following a single oral dose of EH-TBB to female SD rats (0.1 μmol/kg). $N = 4$, mean ± SD.

observed in these experiments (Table 4). Kidney and liver weights were not altered by repeated dosing (data not shown).

Metabolite Analyses

Three metabolite peaks were apparent in HPLC-radiometric analyses of urine, with the area of the more hydrophilic metabolite apparently increasing with dose versus the third metabolite peak, a peak that co-eluted with the TBBA standard (Fig. 5). The second metabolite peak area did not noticeably change with dose. Parent EH-TBB was not detected in urine samples at any time or dose level. Repeated dosing had no apparent effect on metabolic profile (data not shown). Three of the metabolites were identified in urine by LC-MS analysis (Fig. 6). The M1 metabolite had a mass consistent with TBBA-glycine ($m/z = 490$ Da). The M2 metabolite had a mass and fragmentation pattern consistent with TBBA-sulfate ($m/z = 516.6$ Da). The M3 peak co-eluted with the TBBA standard and had a mass consistent with that of TBBA ($m/z = 436.7$). No reference standard was available for TBBA-sulfate or TBBA-glycine. Bile collected from a female SD rat administered EH-TBB by IV had the same metabolite profile as that from extracted feces collected from non-cannulated animals (Supplementary Fig. S2). Bile collected from a female SD rat administered EH-TBB by gavage had the same metabolite profile as that from extracted feces collected from non-cannulated animals and contained no parent EH-TBB. However, feces collected from this bile-duct cannulated animal contained both parent EH-TBB and TBBA.

The principal moieties present in extracts of feces from orally dosed rats corresponded to parent EH-TBB and TBBA (Fig. 7). The ratios of parent to metabolite were affected by dose but not noticeably when single versus repeated administrations were compared. EH-TBB was not detected in extracts of feces collected from IV-dosed animals. Mass spectra for TBBA extracted from feces were analogous to that found in urine (data not shown). No TBBA-sulfate was detected in LC-MS analyses of fecal extracts.

DISCUSSION

This is the first time EH-TBB disposition has been described independent of the proprietary FR mixtures containing EH-TBB (FM550, FM600, or BZ-54). EH-TBB was absorbed, readily metabolized, and eliminated by both urinary and fecal routes in female rats following oral administration. An effect of dose was evident in the dose-dependent increases in elimination via feces in the range of 0.1–10 μmol/kg and the delay in the rate of elimination following administration of the 100 μmol/kg dose. Elimination was clearly route-dependent after administration of EH-TBB to female SD rats. Dose dependent routes of elimination showed a strong

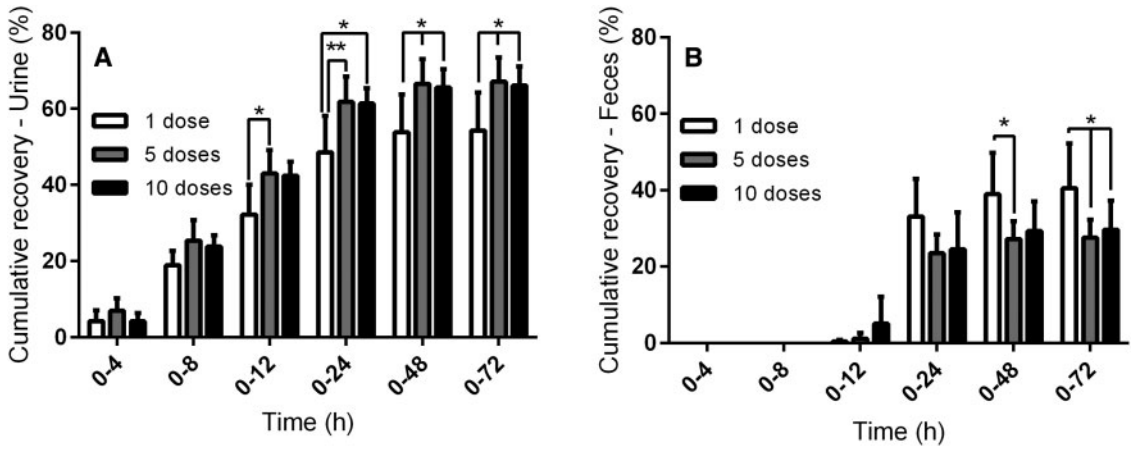

FIG. 4. Elimination of [^{14}C]-radioactivity following 1, 5, or 10 doses of EH-TBB (0.1 μmol/kg, PO). $N = 4$ per dose group, mean ± SD. (A) Recovery in urine. (B) Recovery in feces (*$P < .05$, **$P < .01$).

TABLE 4. [^{14}C]-Radioactivity Recovered in Excreta and Select Major Tissues 72 h after 1, 5, or 10 Oral Administrations of EH-TBB (0.1 μmol/kg)

Tissue	Dose recovered [a]	1 dose	5 doses	10 doses
Feces	%	41 ± 12	28 ± 5[b]	30 ± 8[b]
Urine	%	54 ± 10	67 ± 6[b]	66 ± 5[b]
Blood	%	0.06 ± 0.002	0.07 ± 0.03	0.01 ± 0.008
	(pmol-eq/g)	(0.04 ± 0.01)	(0.04 ± 0.02)	(0.004 ± 0.005)
Liver	%	0.03 ± 0.02	0.01 ± 0.02	0.005 ± 0.01
	(pmol-eq/g)	(0.7 ± 0.6)	(0.3 ± 0.4)	(0.1 ± 0.2)
Adipose	%	0.3 ± 0.2	0.5 ± 0.2	0.2 ± 0.1
	(pmol-eq/g)	(3 ± 2)	(4 ± 1)	(2 ± 1)
Muscle	%	0.4 ± 0.5	0.07 ± 0.1	0.002 ± 0.004
	(pmol-eq/g)	(1 ± 1)	(0.1 ± 0.2)	(0.004 ± 0.007)
Kidney	%	0.02 ± 0.02	0.03 ± 0.01	0.02 ± 0.002
	(pmol-eq/g)	(2 ± 2)	(4 ± 1)	(3 ± 0.4)
Skin	%	0.04 ± 0.008	0.09 ± 0.09	0.03 ± 0.04
	(pmol-eq/g)	(0.3 ± 0.05)	(0.7 ± 0.5)	(0.1 ± 0.1)
Lung	%	0.0004 ± 0.001	0.002 ± 0.003	0.001 ± 0.002
	(pmol-eq/g)	(0.07 ± 0.1)	(0.4 ± 0.5)	(0.4 ± 0.3)
Brain	%	0.002 ± 0.002	0.001 ± 0.002	0.0003 ± 0.0007
	(pmol-eq/g)	(0.2 ± 0.2)	(0.2 ± 0.2)	(0.04 ± 0.1)

[a]Each value represents the mean ± SD of 4 animals per treatment group.
[b]Significantly different from single dose, $P < .05$.

tendency toward increasing fecal recoveries as dose increased. A portion of each dose was detected in feces as parent EH-TBB indicating less than 100% absorption from the gut. This result along with comparative data from IV-injected rats indicated that the extent of absorption of the low dose was approximately 85%.

Compared with the other doses, 100 μmol/kg administered by gavage resulted in a marked decrease in elimination in both urine and feces at early time-points (4–24 h), although by 48 h dose recovery was analogous to that seen at lower doses. It was concluded that some combination of a saturation of absorption, distribution, metabolism, or elimination pathways in the gut, liver, or elsewhere contributed to this unexpected delay in excretion. There is evidence for esterase hydrolysis of EH-TBB in the intestine as well as in the liver and at high doses this processing machinery may be temporarily overwhelmed (Fang and Stapleton, 2014). This saturation effect was not apparent at lower doses; however, there was an apparent effect of dose on absorption although other disposition parameters could be involved. Alternatively, animals administered 100 μmol/kg may have simply eaten less food in the first 24 h and therefore eliminated less. Based on analyses of fecal output, the 100 μmol/kg dose group produced a smaller mass of feces than the amount produced by the 0.1 μmol/kg PO dosed animals between 12 and 24 h ($P < .01$); 1 and 10 μmol/kg animals produced approx. equal mass but less than the 0.1 μmol/kg group). Fecal mass recovered for the samples collected between 24–48 h and 48–72 h. The repeat dose data seems to indicate a possible effect on absorption although the urinary- and fecal-metabolic profiles were not different from single dose studies. Analyses of bile collected after IV or oral dosing found only metabolites were present, consistent with IV feces extract data.

Route-dependent alterations in pathways of elimination were apparent after IV administration of EH-TBB, with most, if not all, systemically available EH-TBB converted to metabolites (TBBA, TBBA-glycine, TBBA-sulfate) that were eliminated primarily in urine. TBBA-sulfate has a molecular weight of 516.6 g/mol, and adsorption to urinary proteins may contribute to its urinary, rather than fecal elimination. Free TBBA-sulfate might be expected to be cleared via bile, as the biliary molecular weight cutoff in rats is approx. 400 Da (Yang *et al.*, 2009). Similarly, glycine conjugated TBBA (molecular weight 494.7) appeared to be almost entirely eliminated in urine, indicating it too adsorbs to urinary proteins. Further evidence for protein adsorption was found during sample preparation method development, when it was observed that 80–90% of radioactivity in filtered urine samples was retained in the protein pellet. Preacidification of the samples was effective in desorbing the metabolites from the urinary protein(s). In addition, it was observed that urinary [^{14}C]-radioactivity adhered to plastic metabolism cages. Acyl sulfate conjugates are expected to be reactive and likely bind proteins but have not been widely reported in the literature (van Breemen *et al.*, 1985). Reports may be lacking due to a combination of sulfation as a high affinity but low capacity metabolism pathway (Morris and Pang, 1987) and production of a presumably reactive metabolite.

Limited retention of EH-TBB or its metabolites was observed in tissues after single administration of up to 100 μmol/kg or repeated administration of 0.1 μmol/kg to rats. These results indicate a low likelihood of bioaccumulation upon chronic exposure to the low dose. However, repeated administrations of EH-TBB resulted in increased urinary elimination indicating increased

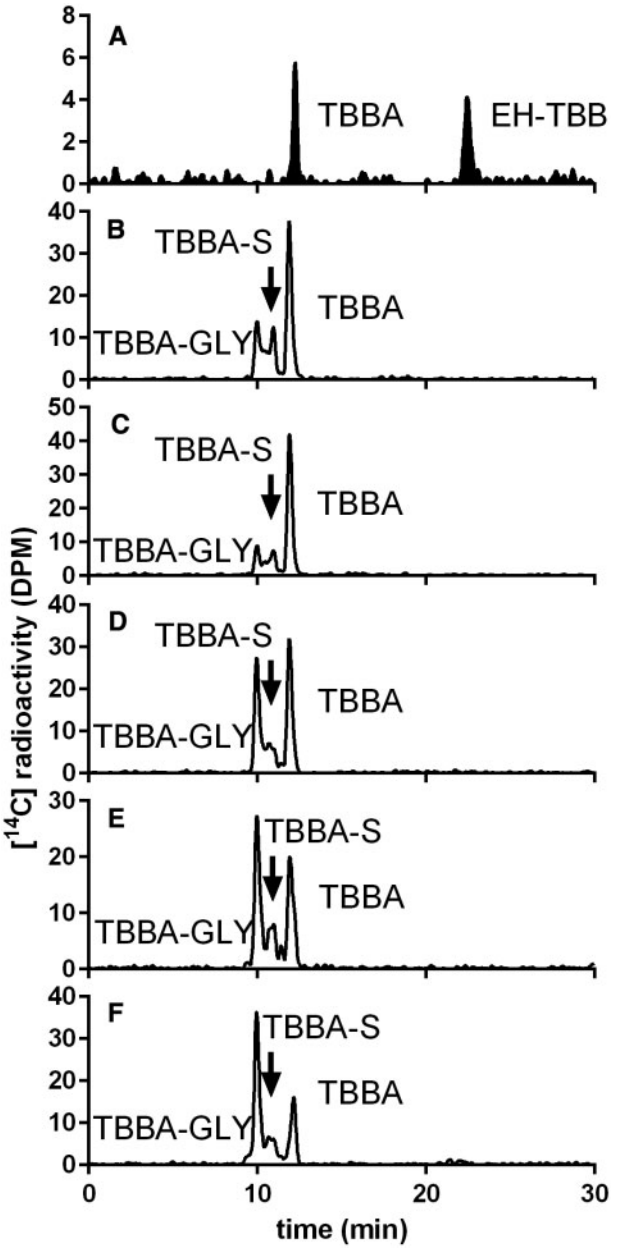

FIG. 5. Representative HPLC-radiochromatograms of [^{14}C]-radioactivity in urine collected between 12 and 24 h from female SD rats administered EH-TBB. (A) [^{14}C]-EH-TBB and TBBA standards; (B) 0.1 μmol/kg (IV); (C) 0.1 μmol/kg (oral); (D) 1 μmol/kg (oral); (E) 10 μmol/kg (oral); (F) 100 μmol/kg (oral).

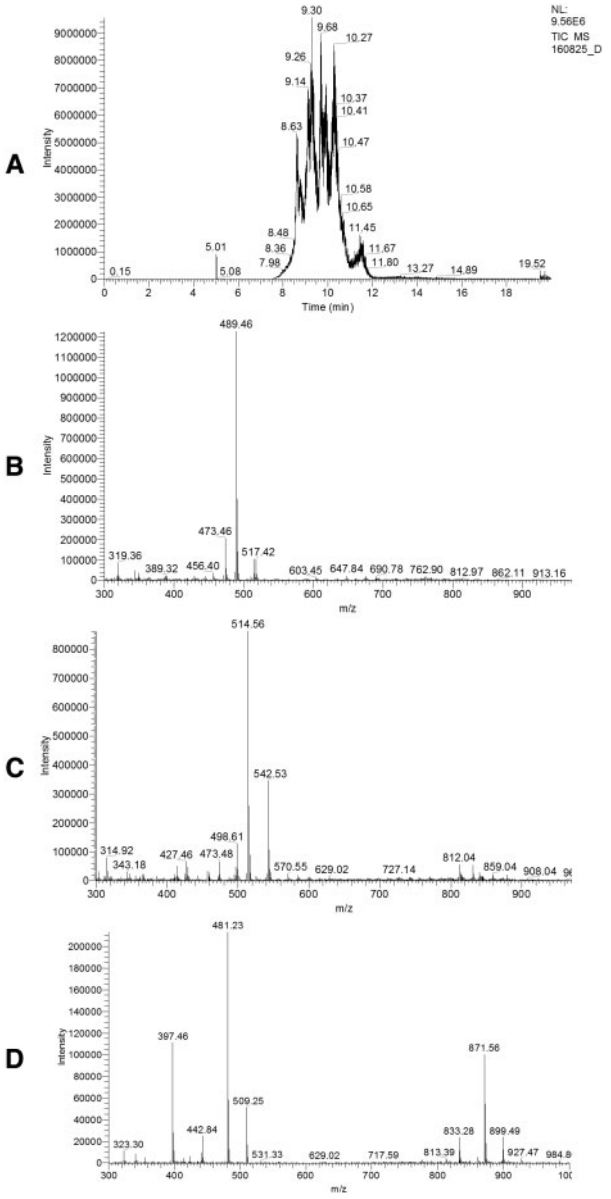

FIG. 6. Representative mass spectra of extracted urine following EH-TBB (100 μmol/kg) administration by gavage to female SD rats. (A) Total ion chromatogram for extracted urine after UPLC separation. (B) Full-scale mass spectrum for peak eluting between 9.1and 9.3 min, *m/z* of 489.46 Da is consistent with the mass of a TBBA-glycine $[M–H]^-$ ion. (C) Full-scale mass spectrum for peak eluting between 9.6 and 9.8 min, *m/z* of 514.56 Da is consistent with the TBBA-sulfate $[M–H]^-$. (D) Full-scale mass spectrum for peak eluting between 10.7 and 11.2 min, *m/z* of 481.23 Da is consistent with the TBBA $[M+H]^-$ ion with a formic acid adduct (*m/z* = 481.23 Da); a mass consistent with at TBBA dimer (*m/z* = 871.56) was formed as well (this was also seen in the TBBA standard, see Supplementary Fig. S3).

systemic exposure to EH-TBB or its metabolites. This change may be due to altered expression of esterases or transporters in the gut, liver, or kidney. Hepatic esterase protein expression may be induced following oral or intraperitoneal dosing of xenobiotics (Kaur and Ali, 1983; Khanna *et al.*, 1991; McCracken *et al.*, 1993); the increases in urinary elimination of EH-TBB metabolites were modest but fairly rapid (approx. 25% increase from a single dose, occurring in as little as 5 doses), similar to liver microsomal carboxylesterase induction by phenobarbital reported by McCracken, et al. (McCracken *et al.*, 1993). A pilot study using bile-duct cannulated rats indicated that some EH-TBB hydrolysis likely occurs in the gut, either by gut bacteria or enterocytes, potentially limiting systemic exposure to EH-TBB.

2-Ethyl hexanol (2-EH) is expected to be formed after hydrolysis of EH-TBB. Disposition of 2-EH has been well described; both parent and metabolites are excreted in urine (Deisinger *et al.*, 1994). Inhalation exposure studies found exposure to 120 ppm of 2-EH for 6 h per day for 90 days resulted in no adverse effects (Klimisch *et al.*, 1998). Rats repeatedly exposed to 2-EH were observed to have increases in liver weight and peroxisome proliferation (Keith *et al.*, 1992).

Human urine from exposed individuals has been shown to contain TBBA (Hoffman *et al.*, 2014), a metabolite formed in rats

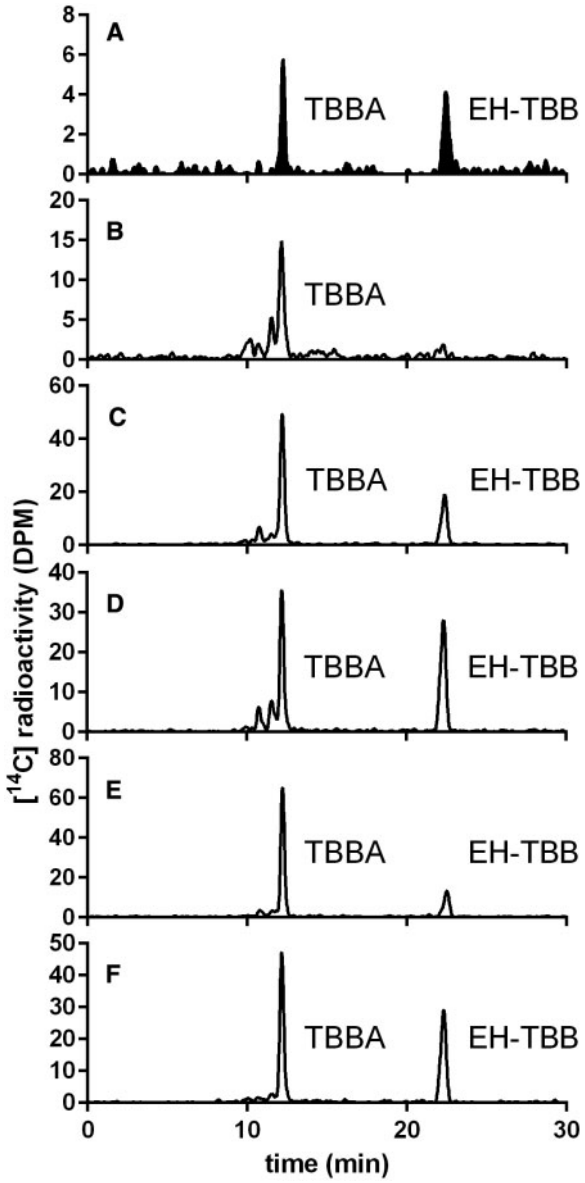

FIG. 7. Representative HPLC-radiochromatograms of [^{14}C]-radioactivity extracted from feces collected between 12 and 24 h from female SD rats administered EH-TBB. (A) [^{14}C]-EH-TBB and TBBA standards; (B) 0.1 μmol/kg (IV); (C) 0.1 μmol/kg (oral); (D) 1 μmol/kg (oral); (E) 10 μmol/kg (oral); (F) 100 μmol/kg (oral).

and mice (this study) and *in vitro* (Bearr et al., 2012; Knudsen *et al.*, in preparation). TBBA was recently shown to be a potent ligand for peroxisome proliferator activated receptor gamma (PPARγ) whereas EH-TBB was not (Fang *et al.*, 2015). Similarly, whereas EH-TBB is not likely to be a directly acting endocrine disrupting chemical, TBBA has anti-androgenic and anti-estrogenic properties (Fic *et al.*, 2014). How much these binding affinities contribute to the overall biological activity of TBBA remains to be determined.

2-Ethylhexyl-2,3,4,5-tetrabromobenzoate (EH-TBB) use and environmental prevalence are increasing, leading to potential for greater human exposure to the chemical in the future (Dodson et al., 2012; Liu *et al.*, 2016b). EH-TBB is an established BFR contaminant in homes and the environment, and may pose a risk to humans, especially to small children who are prone to ingesting dust through hand-mouth contact (Stapleton et al., 2014).

The present work has established that EH-TBB is absorbed in mammals following oral exposure. Additionally, current studies of the dermal uptake of EH-TBB indicate skin may also be an important route of exposure that contributes to systemic exposure to EH-TBB and its metabolites (Knudsen et al., in preparation). This work is expected to be relevant in determining the toxicological contribution for the major brominated component of novel BFR mixtures containing EH-TBB.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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