2,4,6-Tribromophenol Disposition and Kinetics in Rodents: Effects of Dose, Route, Sex, and Species

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ABSTRACT

2,4,6-tribromophenol (TBP, CAS No. 118-79-6) is widely used as a brominated flame retardant and wood antifungal agent. TBP is frequently detected in environmental matrices, biota, and humans. In female SD rats, systemically available TBP (10 μ mol/kg, IV) was rapidly excreted primarily via urine, with approximately 61% of the dose recovered after 4 h, and 89%–94% in 24 h; 5% was recovered in feces; and 1%–2% in blood/tissues. TBP administered to female SD rats (0.1–1000 μ mol/kg) by gavage was well absorbed, with approximately 25% eliminated via urine after 4 h and approximately 88% after 24 h. Approximately 11% of a single oral dose was recovered in bile. Male SD rats and B6C3F1/J mice of both sexes had similar disposition profiles when administered a single oral dose of TBP (10 μ mol/kg). Following administration, fecal recoveries varied only slightly by dose, sex, or species. TBP readily passed unchanged through both human (*ex vivo* only) and rat skin with between 55% and 85% of a 100 nmol/cm² passing into or through skin. Concentrations of TBP in blood fit a two-compartment model after IV-dosing and a one-compartment model after oral dosing. Urine contained a mixture of TBP, TBP-glucuronide, and TBP-sulfate. Fecal extracts contained only parent TBP whereas bile contained only TBP-glucuronide. TBP did not appear to bioaccumulate or alter its own metabolism after repeated administration. TBP was readily absorbed at all doses and routes tested with an oral bioavailability of 23%–27%; 49% of TBP is expected to be dermally bioavailable in humans. From these data, we conclude that humans are likely to have significant systemic exposure when TBP is ingested or dermal exposure occurs.

Key words: 2,4,6-tribromophenol; disposition; pharmacokinetics; bioavailability; brominated flame retardant; persistent organic pollutant

Chemical compounds studied in this article: 2,4,6-tribromophenol (PubChem CID: 1483; CAS No. 118-79-6, FW: 330.801 g/mol, LogP: 4.13) (Hansch *et al.*, 1995).

2,4,6-Tribromophenol (TBP, CAS No. 118-79-6) is a widely used brominated flame retardant (BFR), precursor for other BFRs, and wood antifungal agent (Gutierrez *et al.*, 2005; Thomsen *et al.*, 2001, 2002). TBP is also naturally occurring in some marine fishes, such as Pacific salmon (Boyle *et al.*, 1992). Due to this wide variety of natural and anthropogenic sources, TBP is a frequently detected (60%–100% detection frequency) contaminant

in environmental matrices and biota, including human breast milk, placenta, and serum (Butt *et al.*, 2016; Dufour *et al.*, 2017; Fujii *et al.*, 2014, 2018; Gutierrez *et al.*, 2005; Koch and Sures, 2018; Leonetti *et al.*, 2016a,b; Oliveira *et al.*, 2009; Thomsen *et al.*, 2002; Whitfield *et al.*, 1999; Xiong *et al.*, 2015, 2016). High levels of TBP have been reported in indoor air and in household dust, highlighting the potential for TBP exposure via inhalation and

ingestion of dust (Sha et al., 2018; Takigami et al., 2009). In addition to potential oral or dermal exposure through dust, TBP is likely to be consumed in a diet rich in wild-caught fish (Fuller et al., 2008).

Brominated flame retardants are a prominent source of TBP. TBP has been shown to be formed as a degradation product of the polymeric flame retardant, PolyFR, a butadiene styrene brominated copolymer (Koch et al., 2016). Experimental systems designed to degrade the BFR Tetrabromobisphenol A (TBBPA) resulted in the formation of TBP (Qu et al., 2015). Furthermore, biotic degradation of TBBPA or other brominated phenolic chemicals results in the formation of TBP and other phenolic byproducts (Gu et al., 2018).

TBP is found as a naturally occurring bromophenol in salt-water sportfish (Whitfield et al., 1998) and wild-harvested seafood such as Pacific salmon, Dungeness crab, and clams (Boyle et al., 1992; Oliveira et al., 2009) and contributes to the “shrimp” flavor in processed foodstuffs (Fuller et al., 2008; Oliveira et al., 2009). In one study, coastal residents with high marine fish consumption had higher serum TBP concentrations than those seen in electronics recyclers, indicating dietary intake can be a major source of the chemical (Eguchi et al., 2012). TBP and other bromophenols are not biosynthesized in finfish and shellfish that are commercially harvested and consumed; biosynthesis of TBP occurs further down the food chain, most significantly in polychaetes (marine bristle worms) and bryozoans (filter feeding aquatic invertebrates).

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In exposed workers, circuit board producers and electronics disassemblers had blood concentrations that ranged from 14.2 to 244.9 pmol TBP/g lipid (Thomsen et al., 2001). Sawmill workers exposed to TBP were found to have urinary concentrations of 5.7–37.2 μ mol TBP/g creatinine (Gutierrez et al., 2005). In non-occupational exposures, serum levels of TBP correlated positively with those of polybrominated diphenyl ethers (PBDEs), suggesting similar sources of exposure, or that TBP may result from metabolism of PBDEs (Butt et al., 2016). Accumulation of TBP in human placenta is expected to contribute to prenatal exposures (Leonetti et al., 2016b).

In experimental animals, TBP exposures resulted in neurological, reproductive, and developmentally toxic outcomes and is a suspected endocrine disrupter (Deng et al., 2010; Halden et al., 2010). Pregnant rats exposed to aerosolized TBP gave birth to offspring that exhibited skeletal malformations (Lyubimov et al., 1998). Zebrafish exposed to TBP had negatively altered gonad morphology and reduced fertility (Deng et al., 2010; Halden et al., 2010). *In vitro*, TBP reduced the transcriptional activity of both estrogen and androgen receptor at low micromolar (IC_{50} = 4–14 μ M) concentrations (Ezechias et al., 2012). TBP binds the thyroid hormone transport protein, transthyretin (prealbumin), potentially altering thyroid hormone signaling (Suzuki et al., 2008). TBP also inhibits estrogen sulfotransferase activity (Kester et al., 2000), possibly via the same mechanism as TBBPA (Gosavi et al., 2013).

However, no studies have comprehensively assessed TBP disposition or kinetics, an omission noted in the recent review of TBP by Koch and Sures (2018). Therefore, we assessed the

disposition of TBP after oral, intravenous, and dermal administration. Single oral doses of [14 C]-radiolabeled TBP were administered to female Hsd: Sprague Dawley SD (SD) rats by gavage over a 10 000-fold dose range (1000, 10, and 0.1 μ mol/kg). The bioaccumulation and enzyme induction potential of TBP were investigated using 5-day repeated oral dosing (10 μ mol/kg/d). To determine route-dependent disposition, in addition to oral dosing, TBP was administered intravenously (10 μ mol/kg) or applied to rat skin (100 nmol/cm²). TBP blood-time concentration profiles were analyzed using pharmacokinetic modeling to determine oral bioavailability. *In vivo* dermal absorption study, data were compared with data from *ex vivo* human and rat skin studies to estimate human dermal bioavailability. Finally, to investigate basic sex- and species-dependent disposition, male SD rats and both sexes of B6C3F1/J mice were administered a single dose (10 μ mol/kg) by gavage and disposition was evaluated over 24 h.

MATERIALS AND METHODS

Chemicals. [14 C]-radiolabeled TBP (ring-labeled; Figure 1) was purchased as a crystalline solid from Chemdepo (Camarillo, California). [14 C]-TBP had a radiochemical purity of >98% (confirmed by radiochemical HPLC) with a specific activity of 65 mCi/mmol; chemical purity was determined by HPLC to be >99% as compared with a TBP reference standard (Sigma-Aldrich, St. Louis, Missouri). [14 C]-TBP was dissolved in acetone to a stock concentration of 0.75 mCi/ml. Scintillation cocktails were obtained from MP Biomedicals (Ecolume; Santa Ana, California) or Perkin-Elmer (Ultima Gold, Ultima-Flo M, Hionic Fluor, and PermaFluor E+; Waltham, Massachusetts). Food-grade corn oil was purchased from Sigma-Aldrich. Isoflurane was obtained from Piramal Healthcare (Mumbai, India). All other reagents used in these studies were high performance liquid chromatography (HPLC) or analytical grade.

Animal models and dosing. Hsd: Sprague Dawley SD rats (SD; 10 weeks, approximately 200 g; Harlan Laboratories, Raleigh, North Carolina) or B6C3F1/J mice (10 weeks; approximately 20 g; Jackson Labs, Bar Harbor, Maine) were used in this study. Conventional (nonsurgically altered) animals were maintained in an AAALAC-approved animal care facility and were acclimated to the facility for 1 week prior to dosing in polycarbonate shoebox cages (Techniplast, West Chester, Pennsylvania) with Sani-Chip bedding (PJ Murphy Forest Products, Montville, New Jersey) prior to being placed on study (humidity: approximately 49%, room temperature: approximately 72°F, 12 h light/dark cycle). Feed (NIH No. 31; Ziegler Brothers, 2010) and tap water (City of Durham, North Carolina) were provided for *ad libitum* consumption. The tap water was treated by reverse-osmosis in the animal facility before use. Animals with an indwelling jugular vein or common bile duct-cannula were surgically altered by the vendor and cannula patency was checked upon notification of arrival. Surgically altered animals were acclimated for 24 h in an AAALAC approved NIEHS facility prior to dosing. All procedures were approved by the NIEHS Institutional Care and Use committee.

To determine the fate of systemically available TBP in female SD rats, a single intravenous (IV) bolus was injected into a lateral tail vein. IV dosing solutions (10 μ mol/kg, 1 ml/kg, N = 4 animals), were composed of [14 C]-TBP in acetone added to 1 part of Cremophore EL (Sigma-Aldrich) followed by evaporation of acetone under a stream of nitrogen followed by the addition of 1 part of ethanol and 3 parts of water. Animals were

Figure 1. TBP structure and primary *in vivo* metabolites, TBP-glucuronide and TBP-sulfate.

preacclimated in plastic metabolism cages (Techniplast) for 24 h prior to dosing. Immediately following dosing, animals were returned to plastic metabolism cages for collection of urine and feces. Animals were not fasted prior to dosing in any studies.

To determine the fate of orally administered TBP, a single dose of TBP by gavage (PO) at dose levels of 0.1, 10, or 1000 $\mu\text{mol/kg}$ (approximately 0.03–330 mg/kg; 4 ml/kg in corn oil; $N = 4/\text{dose group}$) was administered to groups of female SD rats. Distribution to tissues and cumulative excretion of [^{14}C]-radioactivity in excreta were evaluated at 24-h postexposure for these doses. Dosing solutions (10 or 1000 $\mu\text{mol/kg}$, 4 ml/kg) were formulated to provide approximately 100 $\mu\text{Ci/kg/rat}$ of [^{14}C]-TBP and contained nonradiolabeled TBP for delivery of doses up to 1000 $\mu\text{mol/kg}$. 0.1 $\mu\text{mol/kg}$ of dosing solutions provided 6.5 $\mu\text{Ci/kg/animal}$ of [^{14}C]-TBP (specific activity limited total radioactivity dosed per animal). Biliary excretion of TBP or its metabolites was studied using bile duct cannulated female SD rats; 4 rats were administered TBP (10 $\mu\text{mol/kg}$) 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 the rats were euthanized 24 h after dosing.

The bioaccumulation potential of TBP was investigated by administering 5 consecutive daily doses of [^{14}C]-labeled TBP (10 $\mu\text{mol/kg/d}$; 100 $\mu\text{Ci/kg/d}$) to female SD rats and collecting excreta at 24 h intervals; accumulation of TBP-dependent radioactivity in tissues was assessed 24 h after the final dose administration. To identify potential sex- or species-related differences in the *in vivo* fate of TBP, a group of male SD rats, male B6C3F₁/J mice, and female B6C3F₁/J mice ($N = 4/\text{group}$) received a single dose of 10 $\mu\text{mol/kg}$ (4 ml/kg) by gavage and excreta were collected between 0 and 24 h. Urine and feces were collected for at least 8 h prior to dosing to determine background contamination in all studies. A summary of experiments is shown in Table 1.

Pharmacokinetic modeling. An assessment of TBP pharmacokinetics in blood was performed using female SD rats containing an indwelling (Harlan Laboratories) jugular vein cannula

(10 $\mu\text{mol/kg}$, 100 $\mu\text{Ci/kg}$, $N = 4$). Blood (approximately 100 μl /time point) was obtained through the cannula using heparinized syringes. Sampled blood was replaced with equal amounts of saline. Pharmacokinetic modeling following a single oral or IV dose (10 $\mu\text{mol/kg}$) was based on 9 data points (7.5, 15, and 30 min, then at 1, 2, 4, 8, 12, and 24 h). Final pharmacokinetic values for free TBP in blood was determined by radiochemical HPLC analyses of blood collected from individual animals at each time point described earlier. These data were 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 TBP by gavage were fit to a one-compartment model with first order input and output whereas the time-concentration data from animals administered TBP intravenously was fit to a two-compartment model. Goodness of fit for the models and weighting schemes was assessed by comparing the sum of squared residuals.

Sample collections. Following administration of the compound by gavage or IV, excreta and cage rinses were collected at intervals of 0–4, 4–8, 8–12, and 12–24 h from the metabolism cages. Samples were collected at room temperature. Reverse osmosis water (PicoPure, Hydro Service and Supplies, Inc, Durham, North Carolina) was used for cage washes. When bladder urine was recovered during necropsy, it was added to the final urine collection vial. Initial experiments (1000 $\mu\text{mol/kg}$) 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 after administration of the highest dose; therefore, the collection of expired air was discontinued for all subsequent experiments. Euthanasia was achieved by CO_2 asphyxiation and confirmed by unresponsiveness to forceps pinch applied to the hindpaw. Blood samples were collected into heparinized syringes via cardiac puncture immediately following euthanasia for determination of [^{14}C]-radioactivity in

Table 1. Summary of Sex, Species, Doses, and Routes Investigated

Female SD Rat	Male SD Rat	Female B6C3F1/J Mouse	Male B6C3F1/J Mouse
0.1 $\mu\text{mol/kg}$ PO	—	—	—
0.1 $\mu\text{mol/cm}^2$ Dermal	—	—	—
10 $\mu\text{mol/kg}$ IV	—	—	—
10 $\mu\text{mol/kg}$ PO	10 $\mu\text{mol/kg}$ PO	10 $\mu\text{mol/kg}$ PO	10 $\mu\text{mol/kg}$ PO
1000 $\mu\text{mol/kg}$ PO	—	—	—

whole blood. Plasma was isolated from the remaining 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/testes, skin (ears), small intestine, small intestine contents, spleen, stomach, stomach contents, thymus, thyroid, urinary bladder, and uterus/vas deferens and epididymis) were dissected and placed in labeled preweighed 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 -20°C until aliquoting and oxidation. After aliquoting, samples were stored at -80°C until further analyses.

Excreta and tissues containing [^{14}C]-radioactivity were sampled and subjected to extractions using a previously described method (Knudsen *et al.*, 2016). Briefly, aliquots (approximately 250 mg) of ground, dried feces were placed in labeled preweighed glass screw-top tubes and resuspended in 5 ml acetone and mixed by vortexing for 1 min. Samples were then centrifuged at $830\times g$ for 10 min using a Sorvall RC6+ centrifuge (Thermo Scientific, Inc, Waltham, Massachusetts) with a SH-3000 swing bucket rotor to sediment solids; supernatants were transferred before repeating the process twice. When recoveries were $<95\%$ of expected values, the pellet was further extracted with ethyl acetate ($3\times 5\text{ ml}$) and 95% ethanol ($3\times 5\text{ ml}$). At each step, the samples were centrifuged, and the supernatants transferred and pooled. Extracts were concentrated to approximately 100 μl using a Savant SPD1010 SpeedVac (Thermo Scientific) without heating, and aliquots were analyzed by UV/radiometric HPLC. Remaining sample pellets were air dried, weighed, and [^{14}C]-radioactivity remaining in the sample (unextracted fraction) was quantified by sample combustion followed by LSC counting. Fecal extraction efficiencies approached 100%.

Dermal dosing studies. Dermal TBP uptake and penetrance were investigated as previously described (Knudsen *et al.*, 2015). *Ex vivo* studies were conducted using split-thickness skin (ie, epidermis and upper portion of the dermis) from human cadaver donors (dorsal scapular skin; National Disease Research Interchange, Philadelphia, Pennsylvania, $N=4$, 3 males, 1 female, 57–69 years old) and female SD rats (Harlan Bioproducts for Science, Indianapolis, Indiana, hair clipped 24 h prior to excision, $N=4$, 11 weeks old) to match the sex, species, and strain used in the *in vivo* studies; age and sex of human donors were determined by available tissues. Briefly, *in vivo* human bioavailability was estimated as a function of *ex vivo* human exposure multiplied by a normalization factor based on the same dose applied to rat skin *in vivo* and *ex vivo*. Flux (J_{ss}) was calculated as described previously and derived from Fick's first law of diffusion (Hughes and Edwards, 2010; Niedorf *et al.*, 2008). Briefly, the maximal flux (pmol-eq per square centimeter per hour) was derived from the slopes of the penetrated mass across each barrier

plotted versus sampling period. Error was calculated as described previously (Caldwell and Vahidsafa, 2015).

For *in vivo* dermal studies, the dorsal scapular region of isoflurane-anaesthetized female SD rats was clipped 24 h prior to dosing. On the day of dosing, rats ($N=4$) were anaesthetized with isoflurane, and the dosing area was marked with a felt-tipped marker. A single dose of [^{14}C]-TBP dissolved in acetone (100 nmol/cm^2 ; 10 μl applied to approximately 1 cm^2 , 100 nmol/animal, 300 pg/animal) was applied to the dorsal scapular surface, dosing area covered using a perforated steel cap, and rats were returned to their metabolism cages. Urine, cage washes, and feces were collected at 0–6, 6–12, and 12–24 h intervals. After 24 h, animals were euthanized by CO_2 inhalation and subjected to necropsy as described earlier. Dosed skin with the attached steel mesh cap was excised and set aside for washing and tape stripping. Animals were subjected to complete necropsies, with excreta and tissues processed and analyzed as described for all other studies.

Ex vivo absorption and penetration of [^{14}C]-labeled TBP was determined using a ILC07 Automated flow-through system (PermeGear, Hellertown, Pennsylvania). A single dose of [^{14}C]-TBP (100 nmol/cm^2 ; 6.4 μl applied to 0.64 cm^2) dissolved in acetone was applied to split-thickness dorsal scapular skin and perfusion media was collected for 24 h at 6 h intervals. For *ex vivo* studies, a single dose of [^{14}C]-labeled TBP was applied to split-thickness rat or human skin with thicknesses of 356 ± 12 and $385 \pm 57\text{ }\mu\text{m}$ for rat and human skin, respectively. Human skin samples were subjected to an additional barrier test using tritiated water (1 μCi , 100 μl , 5-min exposure; PerkinElmer) prior to application of [^{14}C]-labeled TBP to determine water permeability of the samples. Penetrance of $<0.05\%$ of applied radioactivity over 1 h was indicative of an intact barrier.

After application of TBP, solvent evaporation was facilitated by gentle airflow over the dosing area and samples remained open to the air for the duration of the studies. After 24 h of perfusion media collection, the skin samples were washed in triplicate (500 μl , 1:1 Joy liquid soap: water), then 6 times with water (500 μl), and air-dried for 24 h. The flow-through system cells were rinsed in triplicate with 500 μl of ethanol. On the following day, skin samples were tape stripped 10 times to remove the stratum corneum and chemically solubilized (Solueene 350, PerkinElmer). [^{14}C]-labeled radioactivity in aliquots of perfusion media, washes, dissolved skin and tapes strips were analyzed by liquid scintillation spectrometry.

Analytical methods. Final concentrations of [^{14}C]-radioactivity in all dosing solutions were determined by assaying aliquots of dosing solution in triplicate by liquid scintillation spectrometry using a LS6500 Scintillation Counter (Beckman Coulter, Brea, California). Quantitative analyses of total [^{14}C]-radioactivity content in plasma (100 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 (100 μl) by

combustion of 50–100 μ l aliquots in a Model 307 Biological Sample Oxidizer (Perkin-Elmer) 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 (approximately 25 mg) were weighed and [14 C]-radioactivity was quantified by combustion. All samples were assayed in triplicate. Values reported for percent total dose excreted in urine include [14 C]-radioactivity from the corresponding cage wash.

Feces (dried, ground, and weighed) and dosed skin (minced and weighed) were extracted to determine the chemical nature of recovered [14 C]-radioactivity. Aliquots (approximately 250 mg) of air-dried samples were placed in labeled preweighed glass screw-top tubes and resuspended in 5 ml acetone and 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 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 and plasma samples diluted 1:1 (v/v) with 0.2% formic acid in acetonitrile and filtered using PVDF 0.22 μ m 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 TBP, undiluted urine 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). Control urine was incubated with buffer (200 μ M ammonium acetate in water, pH 5.0). Reactions were terminated by addition of equal volumes of acetonitrile followed by HPLC-radiometric analyses.

TBP was quantified by UV/Vis absorbance and radiochemical detection following HPLC separation. The HPLC system was composed of a Waters (Watertown, Massachusetts) Alliance 2695 separation module with a Waters 2487 dual wavelength detector, Phenomenex (Torrance, California) Luna 150 $\times$ 4.6 mm C18 column and an in-line Radiomatic 610TR Flow Scintillation Analyzer (PerkinElmer). HPLC control and analysis software was Laura4 (LabLogic, Brandon, Florida). Mobile phases consisted of (1) 0.2% formic acid in water and (2) 0.2% formic acid in acetonitrile. Sample separations were performed using a gradient from A to B; initial conditions (90% A) were reduced to 0% A over 5 min then held at 100% B for 5 min before returning the column to initial conditions and equilibrating the column for 2 min before reuse. HPLC flow rates were 1 ml/min and scintillation cocktail flow rates were 2 ml/min.

Qualitative analyses of extracted metabolites (described earlier) were carried out using ion-trap mass spectrometry with atmospheric pressure chemical ionization in negative ion mode. The mass spectrometer (MS) system was LTQXL ion trap MS coupled to an Ultimate 3000 binary UPLC (Thermo Fisher Scientific) with an Agilent (Santa Clara, California) 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 μ A; capillary temp: 275°C; capillary voltage: –15 V; tube lens voltage: –125 V.

Parallelogram calculations for human bioavailability. *In vivo* human dermal bioavailability was calculated using a parallelogram approach as described previously (Knudsen et al., 2017; van

Figure 2. Cumulative dose recoveries in urine and feces after a single IV administration of TBP (0.1 μ mol/kg) to female SD rats (N = 4). Data represent mean $\pm$ SD.

Ravenzwaay and Leibold, 2004). Briefly, *in vivo* human bioavailability was estimated as a function of *ex vivo* human exposure multiplied by a normalization factor based on the same dose applied to rat skin *in vivo* and *ex vivo*. Flux (J_{ss}) and SD were calculated as described previously (Caldwell and Vahidsafa, 2015; Niedorf et al., 2008).

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 7, Graphpad Software, Inc, La Jolla CA). Values were significantly different at $p < .05$.

RESULTS

TBP Disposition After Single IV Dosing in Female SD Rats: Dose Recovery and Metabolite Analyses

Female SD rats were used to investigate TBP disposition after systemic (IV) administration (Figure 2). Systemically available TBP was rapidly excreted primarily via urine, with approximately 61% of the dose recovered in urine between 0 and 4 h. By 24 h, 89%–94% of the dose was recovered in urine with an additional 5% recovered in feces; the balance (1%–2%) was recovered in blood and tissues. A summary of dose recoveries after 24 h is shown in Table 2. A summary of tissue recoveries can be found in Supplementary Tables 1 and 2.

TBP Disposition After Single Oral Dosing

Dose escalation in female SD rats dose recovery and metabolite analyses at 3 dose levels.

Conventional Studies

Female SD rats were used in studies investigating dose-dependent differences in TBP disposition. In these studies, TBP appeared to be well absorbed and excreted primarily in urine at all doses tested (Figure 3). Orally administered TBP was well absorbed from the gut, with approximately 25% eliminated via urine in the first 4 h after dosing at all dose levels. Urinary excretion was essentially unchanged when elimination curves for 0.1 and 10 μ mol/kg doses were compared; 1000 μ mol/kg urinary elimination appeared to be slightly delayed between 4 and 12 h, possibly indicating overwhelmed or impaired absorption or evidence of enterohepatic circulation of biliary metabolites but was statistically indistinguishable from that of the other doses

Table 2. Dose (%) Recovered After 24 h Following a Single IV Administration of TBP to Female SD Rats

	Mean±SD
Urine	91±2
Feces	4±3
Blood	0.7±0.3
Non-GI tissues	1±0.1
GI tract and contents	1.3±0.6
Total recovery	97±3

Data represent mean±SD (N=4).

Table 3. Dose (%) Recovered after 24 h Following a Single Oral Administration of TBP to Female SD Rats

Dose	0.1 µmol/kg Mean±SD	10 µmol/kg Mean±SD	1000 µmol/kg Mean±SD
Feces	7±2	8±2	9±4
Urine	89±4	89±2	88±4
Blood	0.1±0.03	0.2±0.02	0.2±0.04
Non-GI tissues	0.3±0.5	1±0.5	1±0.4
GI tract and content	0.5±0.4	1±0.5	1±1
Total recovery	96±2	98±1	98±1

Data represent mean±SD (N=4).

Figure 3. Cumulative dose recoveries in (■) urine and (●) feces after a single oral administration of TBP to female SD rats. A, 0.1 µmol/kg PO (N=4); B, 10 µmol/kg PO (N=4); C, 1000 µmol/kg PO (N=4). Recovery in urine was significantly lower at 8-h postdose after oral administration of 1000 µmol/kg compared with 10 and 100 µmol/kg. Data represent mean±SD (N=4). Cumulative recovery was significantly lower in the highest dose group than other doses at 8 h ($p < .05$). **Figure 4.** Representative HPLC-radiochromatograms of urine and fecal extracts following oral administration of TBP (1000 µmol/kg). A, [¹⁴C]-TBP standard. B, Urine. C, Fecal extract. **Figure 5.** Mass spectra of urine collected after oral administration of TBP (1000 µmol/kg). A, Extracted ion chromatogram for peak 1 (Retention time = 4.2–4.6 min). B, Extracted ion chromatogram for peak 2 (Retention time = 6–6.5 min).

by 12 h; the dose volume was the same for all doses so we deduced this was not due to delayed gastric emptying. Fecal recoveries varied only slightly by dose, but the differences were not

significant. A summary of dose recoveries after 24 h is shown in Table 3. A summary of tissue recoveries can be found in Supplementary Tables 1 and 2.

HPLC-radiometric analyses of urine found 2 clear peaks: 1 peak that coeluted with the parent TBP standard and a second more hydrophilic peak (Figure 4). Mass spectral analyses of urine determined the major hydrophilic peak had a mass consistent with that of TBP-glucuronide (Figure 5). In addition, mass spectral analyses of urine found a third moiety that eluted

immediately before the TBP standard that had a mass consistent with TBP-sulfate. Fecal extracts contained a single peak that coeluted with parent TBP (Figure 4).

Bile Duct Cannulated Studies

To determine whether the dose fraction recovered in feces represented an unabsorbed fraction, an absorbed then hepatically cleared fraction, or a combination of the 2, female SD rats with an indwelling common bile duct cannula were orally administered a single dose of TBP (10 μ mol/kg, 100 μ Ci/kg, 4 ml/kg). After dosing, animals were placed in plastic metabolism cages for collection of excreta whereas bile was collected for quantitative and qualitative analyses. After 24 h, the rats were euthanized, blood removed by cardiac puncture, and the animals were necropsied. In this study, more than 80% of the dose was found to be absorbed and systemically available (eg, recovered in urine and tissues), consistent with previous studies using conventional animals. Bile fractions contained approximately 11% of the dose, indicative of an absorbed then hepatically cleared fraction. Feces and GI tract tissues and contents contained the remaining 4%–6% of the administered dose, indicative of an unabsorbed fraction (Figure 6). A small percentage of parent was also excreted in urine from conventional animals (Figure 7). HPLC-radiometric analyses of bile samples collected between 0- and 24-h postdosing showed only a single peak that was determined to be TBP-glucuronide.

Figure 6. Cumulative dose recovered in urine, feces, and bile after oral administration of TBP (10 μ mol/kg) to bile duct cannulated female SD rats. Data represent mean $\pm$ SD (N = 4).

Figure 7. Representative HPLC-radiochromatograms of urine and bile following administration of [14 C]-TBP (10 μ mol/kg, PO). A, 0–4 h urine. B, 0–1 h bile. Analyses of all other time points showed similar chromatography for urine and bile.

Figure 8. Cumulative dose recoveries in rats (A) and mice (B) after a single oral administration of TBP (10 μ mol/kg, 100 μ Ci/kg) to SD rats or B6C3F1/J mice. ■: female rat (N = 4); ●: male rat (N = 4); ▲: female mouse (N = 4); ▼: male mouse (N = 4). Closed symbols: urine; open symbols: feces. Male and female rat fecal recoveries were analogous and overlay almost exactly. Data represent mean $\pm$ SD.

Table 4. Dose (%) Recovered After 24 h Following a Single Oral Administration of TBP to SD Rats or B6C3F1/J Mice (10 μ mol/kg)

Dose	Female SD Rats Mean $\pm$ SD	Male SD Rats Mean $\pm$ SD	Female B6C3F1/J Mice Mean $\pm$ SD	Male B6C3F1/J Mice Mean $\pm$ SD
Feces	8 $\pm$ 2	9 $\pm$ 2	10 $\pm$ 7	14 $\pm$ 6
Urine	89 $\pm$ 2	87 $\pm$ 3	86 $\pm$ 7	82 $\pm$ 8
Blood	0.2 $\pm$ 0.02	0.1 $\pm$ 0.02	0.1 $\pm$ 0.02	0.1 $\pm$ 0.002
Non-GI tissues	1 $\pm$ 0.5	0.4 $\pm$ 0.2	0.5 $\pm$ 0.2	0.4 $\pm$ 0.1
GI tract and content	1 $\pm$ 0.5	1 $\pm$ 0.2	0.5 $\pm$ 0.2	0.2 $\pm$ 0.1
Total recovery	98 $\pm$ 1	97 $\pm$ 3	98 $\pm$ 2	96 $\pm$ 3

Data represent mean $\pm$ SD (N = 4).

Figure 9. TBP time-concentration curves following a single dose of TBP (10 μ mol/kg) to female SD rats (■: Oral; ●: IV). Lines show the predicted regression curve based on best-fit. Data represent mean $\pm$ SD (N = 4). Inset: detail of apparent oscillation in concentrations between 15 min and 4 h after oral dosing.

Figure 10. Cumulative dose recovery (%) after daily repeated oral administration of TBP (10 μ mol/kg/d) in female SD rats. Data represent mean $\pm$ SD (N = 4).

Dose Recovery in Male SD Rats, Male and Female B6C3F1/J Mice

Disposition in SD rats and B6C3F1/J mice of both sexes was compared at a single dose (10 μ mol/kg) administered by intragastric (oral) gavage (Table 4). Animals were dosed, placed in plastic metabolism cages, and excreta collected for 24 h. Male SD rats

and female B6C3F1/J mice urinary elimination overlaid that of female SD rats at all time points tested (Figure 8). Male B6C3F1/J mice displayed highly variable urinary output and corresponding overall elimination between 0 and 8 h, but by 12 h, urinary recoveries mirrored that of the other test groups. Mice eliminated more TBP in feces through 12 h but were not significantly different by 24 h. A summary of tissue recoveries can be found in Supplementary Tables 1–3.

Pharmacokinetics After Single Oral or IV Dosing

TBP systemic pharmacokinetics were determined following a single oral or IV dose of TBP in female SD rats. Rats were administered 10 μ mol/kg (100 μ Ci/kg, 4 or 1 ml/kg, respectively) as described earlier and blood sampled serially between 7.5 min and 24 h. TBP concentrations in blood were fit to one- (oral) or two-compartment (IV) models (Figure 9). After oral administration, there was an observed rapid rise to C_{max} , followed by an up-down-up oscillating concentration plateau (15 min to 4 h) that may be due to enterohepatic cycling of TBP and its metabolites. After IV administration, biphasic clearance was observed, likely due to a rapid distribution to liver as indicated by the initial 18 min half-life and an elimination half-life of 9.6 h and a final clearance of 0.5 ml/min. After oral dosing, absorption was rapid, with an absorption half-life of 18 min, a C_{max} of 2.4 nmol/ml occurring at 75-min postdose; the elimination half-life was 4.5 h with a final clearance

Table 5. Recoveries in Individual Fractions From Dermal Ex Vivo and In Vivo Studies

			Rat (ex vivo) Measured	Rat (in vivo) Measured	Human (ex vivo) Measured	Human (in vivo) Calculated
Unabsorbed	Dosing cell	%	0.9±0.3	15±8	2±1	
		(nmol)	(0.6±0.2)	(16±10)	(2±0.4)	
	Wash	%	15±1	18±3	37±10	44±15
Total unabsorbed		(nmol)	(10±1)	(19±3)	(23±8)	(44±16)
		%	16±1	33±6	40±11	44±14
Absorbed	Tape strips	%	9±4	10±5	2±0.4	2±1
		(nmol)	(6±3)	(10±5)	(1±0.3)	(2±1)
	Skin	%	9±6	7±5	11±2	9±9
Total absorbed		(nmol)	(6±4)	(7±5)	(7±5)	(8±8)
		%	18±5	17±10	13±2	11±7
Penetrated	Perfusate/excreta and tissues	0–6 h	31±7	19±6	9±5	5±4
		(nmol)	(20±5)	(19±5)	(5±3)	(5±3)
		6–12 h	17±3	14±2	14±5	12±5
		(nmol)	(11±2)	(14±4)	(9±3)	(12±5)
		12–18 h	10±2	—	11±2	n.d.
		(nmol)	(6±2)	—	(7±1)	(n.d.)
		18–24 h	6±1	15±6	9±1	22±10
		(nmol)	(4±1)	(16±7)	(5±0.4)	(22±11)
		%	63±3	48±4	43±12	39±12
		%	97±2	97±3	95±8	95±8
		(nmol)	(62±1)	(72±11)	(58±7)	(67±13)
		(f _{dermal} , %)	81±3	64±7	55±13	49±13

Estimates for in vivo human skin were also calculated. Dermal bioavailability was calculated as the sum of absorbed and penetrated fractions. Data represent mean±SD (N=4).

Figure 11. Cumulative penetration of [¹⁴C]-TBP through rat or human skin. A, Dose recovery in excreta after dermal application of TBP to female SD rats. B, Cumulative penetrance of TBP through in vivo and ex vivo skin samples. Estimated penetrance for in vivo human skin was calculated using the parallelogram method described earlier. Data represent mean±SD (N=4).

of 2.1 ml/min. From the areas under the curves (AUC), TBP parental bioavailability (F) was calculated to be 23%–27%. Preliminary examinations of TBP present in blood, especially at late time points, indicate TBP associates with plasma protein(s) which retains a small but measurable fraction for an appreciable amount of time.

TBP Disposition After Repeated Oral Dosing

Female SD rats were administered TBP (10 µmol/kg/d, 100 µCi/kg, 4 ml/kg) for 5 days and excreta were collected at 24 h intervals to determine bioaccumulation and metabolic enzyme alteration potential for TBP. Interday recoveries were between 79% and 94% for urine and cumulative recoveries were linear with no apparent changes in excretion patterns to indicate

bioaccumulation or enzyme alteration at the dose tested (Figure 10). Urine and feces contained the same proportions of parent and metabolites as that seen in single dose studies (Supplementary Table 4).

TBP Disposition After Single Dermal Dosing

Dermal uptake of TBP was tested ex vivo using female SD rat and human skin and in vivo using female SD rats (Figure 11). Maximal TBP flux (slopes of the penetrated mass across each barrier plotted vs sampling period) through rat skin occurred between 0- and 6-h postapplication and was 5 ± 1 nmol/cm²/h ex vivo and was 3 ± 1 nmol/cm²/h after in vivo dosing. Maximal flux for ex vivo human skin was 2 ± 1 nmol/cm²/h and was reached

Figure 12. Representative HPLC-radiochromatograms of [^{14}C]-radioactivity in studies of TBP applied to skin. A, [^{14}C]-TBP standard. B, Rat urine collected after dermal application of TBP. C, Extracts from dosed *in vivo* rat skin. D, [^{14}C]-TBP standard 2; diluted *ex vivo* dosing solution. E, *Ex vivo* rat skin perfusate. F, *Ex vivo* human skin perfusate. A single peak that coeluted with parent TBP was detected in skin extracts and perfusate whereas both parent TBP and metabolite TBP-glucuronide were detected in urine samples.

between 6 and 12 h. Computed maximal flux for *in vivo* human skin was $4 \pm 2 \text{ nmol/cm}^2/\text{h}$.

Recoveries over time showed the dose penetrating the skin increased slowly throughout the experimental 24 h for all skin matrices. Over a 24-h period, $43 \pm 12\%$ and $63 \pm 3\%$ of the dose that was administered to *ex vivo* human and rat skin penetrated the skin (Table 5). The maximum absorbed fractions for these human and rat skin samples were $13 \pm 2\%$ and $18 \pm 5\%$, respectively. *In vivo* dermal exposure to TBP found similar amounts were absorbed into the skin ($17 \pm 10\%$) and penetrated it ($48 \pm 4\%$). TBP that penetrated the skin in the *in vivo* study was largely eliminated in urine ($40 \pm 4\%$) or feces ($3 \pm 1\%$). Untreated skin (from the ears) contained $3 \pm 2\%$ of the dose ($95 \pm 57 \text{ pmol/g}$) whereas large intestine/cecum contents ($0.6 \pm 0.1\%$), spleen ($0.5 \pm 0.3\%$), and liver ($0.1 \pm 0.04\%$) were the only other tissues that contained $>0.1\%$ of the dose. In addition to undosed skin, kidney ($49 \pm 48 \text{ pmol/g}$, $0.07 \pm 0.07\%$) and liver ($12 \pm 2 \text{ pmol/g}$) retained notable concentrations of TBP after 24 h of *in vivo* dermal exposure. Based on the parallelogram calculation, a risk-averse estimate indicates approximately

$49 \pm 13\%$ of TBP may be absorbed into human skin *in vivo* with $39 \pm 12\%$ expected to reach systemic circulation after 24 h of continuous exposure. A summary of measured and calculated recoveries for individual fractions is shown in [Supplementary Tables 1, 2, and 5](#).

TBP was not metabolized in the skin in these studies. HPLC separation and radiochemical analysis of perfusate media and extracts from skin detected only a single peak that coeluted with the parent TBP (Figure 12). Analyses of excreta collected in these studies found that dermally applied TBP was partially conjugated to TBP-glucuronide but was primarily eliminated in the urine as parent TBP. Feces contained only parent TBP (data not shown). A summary of tissue recoveries can be found in [Supplementary Table 1](#) and recoveries from individual fractions are shown in [Supplementary Table 5](#).

DISCUSSION AND CONCLUSIONS

This is the first comprehensive report of the disposition of TBP and these data support previously described high dose

disposition studies of TBP (WHO, 2005). TBP is classified as a high-volume chemical by the US EPA; exact production volumes are not publicly available but were estimated to be 2500 metric tons per year in Japan and 9500 metric tons per year worldwide in 2001 (OECD-SIDS, 2004). TBP is a current-use flame retardant and a contaminant in both environmental and indoor exposures, especially to small children who are prone to ingesting dust through hand-mouth contact (Stapleton et al., 2014). One difficulty in comprehensively assessing TBP risk is that TBP dust concentrations are infrequently reported (Sha et al., 2018; Takigami et al., 2009). This is in stark contrast to other legacy BFRs (polybrominated diphenyl ethers and hexabromocyclododecane) and currently used or novel BFRs (TBBPA, 2-ethylhexyl tetrabromobenzoate and bis(2-ethylhexyl) tetrabromophthalate) even though TBP use appears to exceed that of some of the novel BFR (Hakk and Letcher, 2003).

TBP was extensively absorbed following oral and dermal administration, the 2 most likely routes of real-world exposure. TBP is readily metabolized and eliminated primarily via renal clearance into urine but also by hepatobiliary clearance into feces. TBP had a systemic oral bioavailability of almost 30% and based on dose recoveries in bile, has a hepatic exposure of >50%. In a prospective study where male and female SD rats were administered 0.5 $\mu\text{mol/kg}$ of TBP and animals were euthanized at 4-h postdose, TBP blood concentrations were found to be highly predictable based on a linear relationship between dose and concentration. This was taken to support an assumption of dose-linear systemic exposure to TBP administration, but further studies are warranted at occupational and environmentally measured doses. There was no apparent sex- or species-dependent difference found in overall disposition. In addition, the relative distributions of peak areas did not markedly change over time, or with dose, repetition, sex, or route of administration, indicating consistent and well-conserved metabolic pathways. At least 2 metabolites were present in urine: TBP-glucuronide and TBP-sulfate. Urinary metabolite profiles differed little over a dose range from 0.1 to 1000 $\mu\text{mol/kg}$ (approximately 0.03–330 mg/kg), indicating negligible saturation of one or more metabolic pathways. We deduced that there is a role for gut microflora in modulating enterohepatic circulation through deconjugation of biliary metabolites because although fecal extracts contained only parent TBP, bile contained only TBP-glucuronide. Enterohepatic circulation was further supported by the appearance of a plateau in the blood concentrations of TBP between 10 and 120 min after oral dosing. TBP was readily absorbed from the gut when orally administered to female SD rats and is likely to be absorbed when ingested by humans. Limited tissue retention of TBP or its metabolites was observed and would indicate a low likelihood of bioaccumulation after a single dose. However, these studies also show that parent TBP is present in circulation and low, persistent levels of TBP circulate up to 24 h after a single oral or dermal exposure. Although the potential for acute toxic effects is likely low due to TBP's 30% oral bioavailability, long-term exposure may still be a concern. As has been shown in the case of TBBPA, another phenolic brominated chemical that has an even lower bioavailability (2%), long term exposure to TBBPA results in neoplasia and cancer in the NTP 2-year bioassay, and it has been judged to be a probable carcinogen to humans (IARC, 2018; NTP, 2014). Based on other studies in our laboratory that are described below, we expect TBP to possess a similar risk profile to TBBPA.

Current studies in our laboratory indicate TBP decreases efflux transporter function at the blood-brain barrier, with

significant implications for adverse drug reactions and mechanisms for endocrine disruption by TBP (Trexler et al., 2018). Future studies will include investigation of the disposition of TBP in pregnant and nursing animals as well as its impacts on thyroid stimulating hormone and thyroid hormone levels, as thyroid signaling pathways have been implicated by *in vitro* assessments (Hamers et al., 2006, 2008). The related compound TBBPA has been shown to directly interact with the ER α receptor while also altering estrogen metabolism by competing for access to tissue-specific sulfotransferases, eg, SULT1E1 (Gosavi et al., 2013), which in turn can result in alteration in estrogen signaling pathways necessary for the uterine neoplasia demonstrated in the 2-year cancer bioassay (NTP, 2014). The structural similarity of TBP to TBBPA coupled with its 10-fold greater bioavailability make it reasonable to be concerned about human exposure to TBP. More work is needed to determine the nature of these signaling pathways following repeated exposures to TBP, especially in susceptible populations and across life stages.

Ultimately, humans are likely to have an appreciable systemic exposure to ingested or dermally encountered TBP given its high volume of use, presence in seafood, and ubiquity in the environment. Although we found that TBP is unlikely to bioaccumulate, because exposure to TBP is likely to be continuous, it is probable that these chronic TBP exposures could lead to similar long-term toxicities observed for other brominated phenolic chemicals, most notably cancer.

SUPPLEMENTARY DATA

Supplementary data are available at Toxicological Sciences online.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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