2,4,6-Tribromophenol Disposition and Kinetics in Pregnant and Nursing Sprague Dawley Rats

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ABSTRACT

2,4,6-Tribromophenol (TBP, CAS no. 118-79-6) is a brominated chemical used as a precursor, flame retardant, and wood antifungal agent. TBP is detected in environmental matrices and biota, including human breast milk, placenta, and serum. To address reports of TBP accumulation in human placenta and breast milk, studies were conducted to characterize TBP disposition and toxicokinetics in timed-pregnant or nursing Sprague Dawley rats following a single oral dose to the dam. Animals were administered [¹⁴C]-TBP (10 μmol/kg, 25 μCi/kg, 4 ml/kg) by gavage on gestation day 12 and 20, or postnatal day 12 and serially euthanized between 15 min and 24 h for collection of blood and tissues from the dam and fetuses/pups. Observed plasma TBP C_{max} (3 and 7 nmol/ml) occurred at 15 min in both GD12 and GD20 dams while C_{max} (3 nmol/ml) was observed at 30 min for PND12 dams. Concentrations in tissues followed plasma concentrations, with kidneys containing the highest concentrations at 30 min. GD12 litters contained a sustained 0.2%–0.3% of the dose (5–9 nmol/litter) between 15 min and 6 h while GD20 fetuses (2%–3%) and placentas (0.3%–0.5%) had sustained levels between 30 min and 12 h. The stomach contents (approx. 1 nmol-eq/g, 6–12 h), livers (0.04–0.1 nmol-eq/g) and kidneys (0.1–0.2 nmol-eq/g) of PND12 pups increased over time, indicating sustained exposure via milk. Systemic exposure to TBP and its metabolites occurs in both the directly exposed mother and the indirectly exposed offspring and is rapid and persistent after a single dose in pregnant and nursing rats.

Key words: biotransformation; toxicokinetics; pharmacokinetics.

2,4,6-Tribromophenol (TBP, CAS no. 118-79-6; MW = 330.801 g/mol) is a chemical used primarily as a precursor for the manufacture of brominated flame retardant (BFR), but also as a stand-alone BFR and as a wood antifungal agent (Thomsen et al., 2002). In addition to its direct manufacture, TBP may arise in the environment as other common BFRs as tetrabromobisphenol A and polybrominated diphenyl ethers degrade to form TBP (Koch et al., 2016). While most BFR are anthropogenic, TBP can also be naturally occurring and can be found in wild-caught seafood (Oliveira et al., 2009). In certain populations, it has been shown that TBP from seafood contributes more to serum

concentrations than exposure via electronic waste recycling (Eguchi et al., 2012).

The primary routes of exposure to TBP are through ingestion, inhalation, and dermal contact with contaminated dust particles, and may occur either occupationally or through environmental routes. Occupational exposure to TBP has been described in electronics workers, where exposure may arise from dust and the handling of circuit boards, and sawmill workers using solutions of TBP in antimicrobial dip baths for raw lumber. Circuit board producers and electronics disassemblers were found to have levels of 14.2–244.9 pmol TBP/g blood, lipid

normalized (Eguchi et al., 2012). In sawmill workers, urine contained between 5.7 and 37.2 μmol TBP/g creatinine (Gutierrez et al., 2005). In nonoccupational exposures, TBP in serum is positively correlated with PBDEs (Butt et al., 2016). Breakdown of these PBDEs may be a source of both external exposure through degradation products in the environment, or internally as they are metabolized in the liver (Athanasiadou et al., 2008; Krieger et al., 2017; Leonetti et al., 2016).

Maternal-fetal or maternal-infant exposure is another route of concern, given the potential for TBP to act as an endocrine disruptor. TBP was a commonly measured analyte in studies of PBDEs in maternal and fetal blood samples, indicative of one source of internal exposure for both mother and fetus, with fetal blood containing roughly 6-fold higher concentration of TBP than that of the mother (Qiu et al., 2009). In a study of human placentas collected after full-term births in Durham, North Carolina, TBP was present in all samples, with an average concentration of 47 pmol/g lipid (Leonetti et al., 2016). None of the mothers had known occupational exposure to TBP. Recent studies of postpartum mothers found TBP in breast milk samples and maternal/cord serum samples, demonstrating significant exposure to mothers and fetuses and the risk of exposure of newborns via breastfeeding (Fujii et al., 2018; Qiu et al., 2009).

Based on its structure and *in vitro* assay data, TBP is an endocrine disrupting chemical. It has been shown to decrease both the alpha-estrogen receptor and androgen receptor transcriptional activity (Ezechias et al., 2012). In addition to possessing the potential to directly bind to the estrogen receptor, it can alter estrogen signaling by inhibiting estrogen sulfotransferase activity (Hamers et al., 2006). It also has the potential to interfere with or alter thyroid signaling, as has been shown to bind the thyroid hormone transport protein transthyretin (Suzuki et al., 2008).

Only a handful of studies have described the *in vivo* biological response to TBP exposure. When zebrafish (*Danio rerio*) were chronically exposed to TBP (1–10 nmol/l), offspring had negatively altered gonad morphology and reduced fertility (Deng et al., 2010); another study found TBP was transferred to eggs following administration in feed (0.1–10 μmol /g feed) to adults and the fertilized embryos showed signs of yolk-sac edema while adult offspring had significantly reduced fertilization success likely due to TBP-dependent disturbed gonad morphology. TBP was well absorbed and metabolized to form glucuronide and sulfate conjugates after oral (0.1–1000 μmol /kg) and dermal (100 nmol/cm²) administration with significant systemic bioavailability in Sprague Dawley (SD) rats and B6C3F1/Tac mice (Knudsen et al., 2019). TBP may be deconjugated by glucuronidases and sulfatases present in gut microflora where it can undergo enterohepatic circulation, increasing the overall residence time in an exposed individual. Furthermore, TBP is able to decrease the activity of protective efflux transporters at the blood brain barrier (Trexler et al., 2019), and likely in the placenta as well (Leonetti et al., 2016; Sieppi et al., 2016). In pregnant Wistar rats exposed to TBP by inhalation throughout pregnancy (0.1 and 1 mg/m³, gestation day 1–21), offspring had skeletal malformations (Lyubimov et al., 1998). It was unclear from these studies whether the reproductive and developmental toxicity observed for TBP was due to TBP exposure in the adult, developing offspring, or both.

The presence of TBP in human breast milk and serum samples and the chemical's potential for endocrine disruption prompted this study into the disposition and kinetics of TBP during pregnancy and nursing. The treatment groups measured the following exposures: disposition of TBP in pregnant dams

and embryos during mammary gland development (GD12), disposition of TBP in pregnant dams and fetuses immediately prior to parturition (GD20) and the disposition and kinetics of TBP in dams and nursing pups at the age of maximal milk consumption (postnatal day 12; PND12). In all cases, the SD rat dam was administered a single oral bolus of TBP (10 μmol /kg). The dose level was selected based on previously published kinetics studies and evidence indicating chemical disposition and kinetics can be extrapolated down at least 100-fold (Knudsen et al., 2019).

MATERIALS AND METHODS

Chemicals. [¹⁴C]-radiolabeled TBP (uniformly ring labeled; Figure 1) was purchased as a crystalline solid from Chemdepo (Camarillo, California). [¹⁴C]-TBP had a radiochemical purity of >98% (confirmed by radiochemical high-performance liquid chromatography [HPLC]) with a specific activity of 65 mCi/mmol; chemical purity was determined by HPLC to be >99% as compared to a TBP reference standard (Sigma Aldrich, St. Louis, Missouri). [¹⁴C]-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 HPLC or analytical grade.

Animal model. Timed-pregnant female SD rats (Envigo, Raleigh, North Carolina) were used in these studies. Plug-positive dams were delivered 3 days after confirmation of pregnancy and pregnancies were timed to GD12 or GD20 for *in utero* exposures or allowed to proceed through parturition. Nursing dams were housed with their litters, and pups were sexed based on anogenital distance on PND4 (Greenham and Greenham, 1977). Litters were culled and balanced to 4 males and 4 females after sexing to control for litter size and possible sex differences in litter units. Pregnant rats were housed individually and nursing dams were housed with their litters and maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal care facility at NIEHS (Research Triangle Park, North Carolina). Animals were housed in polycarbonate shoebox cages (Techniplast, West Chester, Pennsylvania) with Sani-Chip bedding (PJ Murphy Forest Products, Montville, New Jersey) throughout these studies (humidity: approx. 49%, room temperature: approx. 72°F, 12 h light/dark cycle). Food (NIH #31 [Ziegler Brothers I, 2010]) and water (City of Durham, North Carolina) were provided for *ad libitum* consumption. The NIEHS Institutional Care and Use committee approved all procedures.

Figure 1. Chemical structure for [¹⁴C]-radiolabeled TBP. *Radiolabel was uniformly distributed on the phenolic ring.

Dosing. Animals were weighed the morning of dosing and randomized by weight for assigning animals into the various time-point groupings. On GD 12, GD20, or PND12, SD dams ($N=4-5$ per time-point) were administered a single dose of [^{14}C]-labeled TBP in corn oil ($10\ \mu\text{mol/kg}$, $25\ \mu\text{Ci/kg}$, $4\ \text{ml/kg}$) by gavage and returned to their home cage. Dosing solutions were prepared by adding appropriate volumes of [^{14}C]-TBP and nonradiolabeled TBP dissolved in acetone to corn oil and acetone was removed by evaporation under a steady stream of nitrogen. Dosing solutions were prepared on the day of administration. Oral doses were administered using a 16 ball-tipped stainless steel feeding needle. Dams and pups were euthanized by CO_2 inhalation at 0.25, 0.5, 1, 2, 3, 6, 12 or 24 h post dose for sample collection; euthanasia was confirmed by exsanguination via cardiac puncture.

Sample collection. Following CO_2 asphyxiation, blood and tissues were collected from the dam and fetuses or nursing pups. Dam blood samples were collected via cardiac puncture immediately following euthanasia. Complete necropsies were performed on dams, with pooled adipose, adrenals, brain, heart, kidneys, large intestine and contents, liver, lung, mammary, muscle, pancreas, ovaries, skin, small intestine and contents, spleen, stomach and contents, thymus, thyroid, urinary bladder, and uterus collected and placed in labeled, pre-weighed vials, as described previously (Knudsen et al., 2018). Gastrointestinal tract contents were separated from their associated tissue (stomach, small and large intestine) for independent analyses. In addition, pups were necropsied and selected tissues (liver, stomach, stomach contents) were collected and placed in labeled pre-weighed vials. Placentas were collected, pooled, and placed in labeled pre-weighed vials. Fetuses from pregnant dams were placed in separate labeled pre-weighed vials for individual analyses. Plasma was isolated from heparinized blood by centrifugation (5 min at 4000 RPM [$13.1 \times g$]).

Analytical methods. Samples were analyzed in parallel for quantitative and qualitative analyses. Quantitative analyses of total [^{14}C]-radioactivity content were determined using a Beckman Coulter LS6500 Multi-Purpose Scintillation spectrometer. Placenta and fetuses, along with livers and stomach contents from dams and pups were analyzed for levels of radioactivity. Individual fetal samples were snap frozen and homogenized using a BioPulverizer Cryogenic Tissue Crusher ($N=3$ per sex, 3 liters per time-point). All other samples were individually minced, mixed, and sampled in triplicate (minimum $N=3$ individuals sampled per time-point). Triplicate aliquots (approx. 1 g) were placed in labeled pre-weighed vials and weights determined gravimetrically. The aliquots were then combusted to $^{14}\text{CO}_2$ using a Packard 307 Biological Sample Oxidizer followed by quantitation of total radioactivity using a Beckman Coulter LS6500 multipurpose scintillation counter. Aliquots of plasma were analyzed by radiochemical HPLC.

Duplicate samples were weighed and placed in glass vials for exhaustive extraction. Whole blood and tissues containing [^{14}C]-radioactivity were sampled and subjected to extractions using a previously described method (Knudsen et al., 2017). Parent 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 (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 (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 re-use. HPLC flow rates were 1 ml/min and scintillation cocktail flow rates were 2 ml/min. Amounts of TBP in samples were determined based on the area under the peak that co-eluted with the [^{14}C]-TBP standard. The lower limit of detection for the radiochemical detector was 3 times background peak height (LLOD = approx. 100 DPM).

Free TBP in plasma were used to construct a concentration-time 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. Concentration versus data were fit to a one-compartment model with first order input and output. Goodness of fit for the models and weighting schemes was assessed by comparing the sum of squared residuals.

Statistics and data visualization. The data were plotted and subjected to ANOVA statistical analyses using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, California). Values were considered significantly different at $p < .05$. A Tukey's multiple comparisons test was performed if a significant ANOVA was detected.

RESULTS

Tribromophenol was rapidly absorbed after gavage administration in all experimental groups. Maximum concentration (C_{max}) in plasma was observed at 15 min in pregnant rats at both gestation ages, while observed C_{max} occurred at 30 min for PND12 dams (GD12: $3 \pm 1\ \text{nmol/ml}$; GD20: $7 \pm 2\ \text{nmol/ml}$; PND12: $3 \pm 1\ \text{nmol/ml}$). In all dose groups, TBP concentrations fell steadily through 24 h (Figure 2).

TBP concentrations in plasma and tissues were analyzed and kinetic parameters were recorded. Systemic exposure, as indicated by area under the curve (AUC) was highest in gestation day 20 animals, but when it was normalized to nominal dose, differences were minimal (Table 1). Absorption half-lives ($t_{1/2\ \text{absorption}}$) were much shorter (around 10 min or less) than what was observed in nonpregnant SD rats (approx. 1 h), indicating time to C_{max} likely occurred well before the first sampling time in both pregnant and nursing animals. Systemic clearance of TBP was consistent across the gestation ages as was the elimination half-life ($t_{1/2\ \text{elimination}}$).

Radiochemical analyses of plasma from pregnant and nursing dams were compared to that reported for nonpregnant female SD rats (Figure 3; a full description of TBP disposition data in nonpregnant female SD rats was detailed in a previous publication [Knudsen et al., 2019]). In all cases and at all time-points evaluated, the metabolite fraction in plasma exceeded that seen in nonpregnant rats, indicating increased metabolic capacity in these populations. Both GD12 and PND12 rats appeared to have a much higher metabolite pool, representing upwards of 70% of the total radioactivity in plasma, a phenomenon that appeared to persist for the duration of the time course. We were able to approach baseline separation of the sulfate and glucuronide metabolites, and in both cases the ratio of metabolites to parent chemical stayed the same throughout the timecourse. GD20 rats had a smaller proportion of metabolites but the metabolite fraction clearly increased over time.

Maternal tissues retained concentrations of [^{14}C]-radioactivity that paralleled those measured in plasma throughout the time course at all measured exposures (Figs. 4A and 4B). Total chemical clearance (parent plus metabolites) at GD12 was

Figure 2. TBP in plasma after a single oral dose (10 $\mu\text{mol/kg}$). Data from nonpregnant/conventional animals replotted from Knudsen et al. (2019). Regression lines were generated using a 1-compartment model fit of the data ($N = 4\text{--}5$ samples/time-point). Inset: TBP plasma concentrations between 0 and 240 min.

Table 1. TBP Toxicokinetics in Plasma for Adult Female SD Rats After a Single Oral Dose (10 $\mu\text{mol/kg}$)

	Dose (nmol)	AUC (min $\cdot$ nmol/ml)	C_{max} (nmol/ml)	Clearance (ml/min)	$t_{1/2}$ absorption (min)	$t_{1/2}$ elimination (h)
	Mean $\pm$ SD	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
Nonpregnant ^a	2238 $\pm$ 185	939 $\pm$ 114	2 $\pm$ 0.2	2 $\pm$ 0.3	18 $\pm$ 5	4 $\pm$ 0.4
GD12	2626 $\pm$ 146	855 $\pm$ 281	3 $\pm$ 4	3 $\pm$ 1	2 $\pm$ 65	3 $\pm$ 0.5
GD20	3469 $\pm$ 310	1523 $\pm$ 155 ^b	3 $\pm$ 0.4	2 $\pm$ 0.2	6 $\pm$ 9	5 $\pm$ 0.6
PND12	2856 $\pm$ 255	921 $\pm$ 85	4 $\pm$ 0.3	3 $\pm$ 0.3	11 $\pm$ 3	2 $\pm$ 0.4

^aData from Knudsen et al. (2019) ($N = 4\text{--}5$ samples/time-point).

^bStatistically different from nonpregnant/conventional female rats; $p = .0006$.

Figure 3. Representative radiochromatograms of dam plasma extracts collected at 15 min, 30 min, or 3 h. A, Nonpregnant (data from Knudsen et al., 2019), B, pregnant, GD12, C, pregnant, GD20 D: nursing, PND12. Parent molecule (TBP) and/or a mixture of metabolites (TBP-glucuronide [TBP-G] and TBP-sulfate [TBP-S]) were detected ($N = 4\text{--}5$ samples/time-point).

approximately 1 ml/min in maternal tissues while embryos had an estimated clearance of 5 ml/min (Table 2). As the major organ of excretion, maternal kidney had an overall exposure (as indicated by AUC) similar to that seen in the overall systemic circulating plasma, as well as the highest tissue concentrations (21 nmol-eq/g). Maternal tissue absorption and elimination half-lives were analogous to those calculated for plasma while embryos appeared to have a 1.5-fold longer elimination half-life.

At GD20, total chemical clearance was approximately 1 ml/min in maternal plasma and liver as well as placenta and fetuses, while kidneys had a lower clearance (0.5 ml/min) and mammary tissue had an estimated clearance of 3 ml/min (Table 3). As was seen in the GD12 pregnant animals, GD20 maternal kidney was likely to be the major organ of excretion. AUC values would indicate that at GD20, tissue exposure was even greater in kidneys than seen in the overall systemic circulating plasma, as well as the highest tissue concentrations (21 nmol-eq/g). The placenta appeared to play a protective role in reducing the peak concentrations of TBP and its metabolites in fetuses to roughly 10% of that experienced in the maternal circulation (Figure 4B). However, by 24 h, concentrations in fetus were equal to or higher than those found in maternal plasma. Placental observed C_{max} occurred at 2 h, while whole-fetus concentrations were fairly consistent through 24 h after C_{max} was reached at 2 h. Maternal tissue absorption and elimination half-lives were analogous to those

Figure 4. Concentration versus time data for total chemical from select matrices between 0 and 24 h following a single oral dose to pregnant or nursing female SD rats. A, Concentration in embryos and dam plasma, liver, and kidney collected at GD12. B, Concentration in fetuses and placentas, and dam plasma, liver, and kidney collected at GD20. C, Concentration in dam plasma, liver, kidney, and mammary gland collected at PND12. D, Concentration in pup stomach contents, liver, and kidney collected at PND12 (dam mammary added for reference). Regression lines show predicted data from a 1-compartment model fit ($N = 4-5$ samples per time-point).

Table 2. Total Chemical Toxicokinetics in Plasma and Selected Tissues After a Single Oral Dose of TBP ($10 \mu\text{mol/kg}$) at GD12 ($N = 4-5$ samples/time-point)

	AUC ($\text{min} \cdot \text{nmol/ml}$)	C_{max} (nmol/ml)	Clearance (ml/min)	$t_{1/2}$ absorption (min)	$t_{1/2}$ elimination (h)
	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
GD12 dam plasma, total	3258 ± 4922	9 ± 70	1 ± 1	2 ± 358	4 ± 0.3
	AUC ($\text{min} \cdot \text{nmol/g}$)	C_{max} (nmol/g)	Clearance (g/min)	$t_{1/2}$ absorption (min)	$t_{1/2}$ elimination (h)
	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
GD12 dam liver	1400 ± 781	4 ± 9	2 ± 1	2 ± 146	4 ± 0.2
GD12 dam kidney	3776 ± 717^a	12 ± 9	1 ± 0.1	3 ± 38	3 ± 0.3
GD12 embryo	496 ± 115	1 ± 0.2	5 ± 1	3 ± 16	6 ± 2

^aStatistically different from embryo, $p = .0364$.

Table 3. Total Chemical Toxicokinetics in Plasma and Selected Tissues After a Single Oral Dose of TBP ($10 \mu\text{mol/kg}$) at GD20 ($N = 4-5$ samples/time-point)

	AUC ($\text{min} \cdot \text{nmol/ml}$)	C_{max} (nmol/ml)	Clearance (ml/min)	$t_{1/2}$ absorption (min)	$t_{1/2}$ elimination (h)
	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
GD20 dam plasma, total	5207 ± 338	10 ± 1	1 ± 0.04	3 ± 6	6 ± 0.4
	AUC ($\text{min} \cdot \text{nmol/g}$)	C_{max} (nmol/g)	Clearance (g/min)	$t_{1/2}$ absorption (min)	$t_{1/2}$ elimination (h)
	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
GD20 dam liver	3365 ± 199^a	6 ± 1	1 ± 0.1	2 ± 8	6 ± 0.4
GD20 dam mammary	1056 ± 103^a	1 ± 0.1	3 ± 0.3	7 ± 3	11 ± 1
GD20 dam kidney	7596 ± 464^a	17 ± 1	0.5 ± 0.03	3 ± 5	5 ± 0.3
GD20 placenta	5149 ± 820	3 ± 0.3	1 ± 0.1	18 ± 27	16 ± 3
GD20 fetus	2337 ± 386^a	2 ± 0.2	1 ± 0.2	32 ± 9	15 ± 4

^aStatistically different from dam plasma, total; $p > .0001$.

Table 4. Total Chemical Toxicokinetics in Plasma and Selected Tissues From Both Dam and Pups After a Single Oral Dose of TBP (10 μ mol/kg) at PND12 (N = 4–5 samples/time-point)

	AUC (min*nmol/ml)	C _{max} (nmol/ml)	Clearance (ml/min)	t _{1/2} absorption (min)	t _{1/2} elimination (h)
	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
PND12 dam plasma, total	3277 $\pm$ 160	10 $\pm$ 1	1 $\pm$ 0.04	<1	4 $\pm$ 0.2
	AUC (min*nmol/g)	C _{max} (nmol/g)	Clearance (g/min)	t _{1/2} absorption (min)	t _{1/2} elimination (h)
	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE	Mean $\pm$ SE
PND12 dam liver	1283 $\pm$ 95	3 $\pm$ 0.3	2 $\pm$ 0.2	<1	5 $\pm$ 0.3
PND12 dam mammary	547 $\pm$ 153	1 $\pm$ 0.1	5 $\pm$ 1	<1	7 $\pm$ 2
PND12 dam kidney	2559 $\pm$ 201	7 $\pm$ 1	1 $\pm$ 0.1	<1	4 $\pm$ 0.3
PND12 pup stomach contents	650 $\pm$ 188	0.6 $\pm$ 0.2	4 $\pm$ 1	225 $\pm$ 793	5 $\pm$ 17
PND12 pup liver	923 $\pm$ 327	1 $\pm$ 0.2	3 $\pm$ 1	304 $\pm$ 2821	6 $\pm$ 54
PND12 pup kidney	438 $\pm$ 859	0.2 $\pm$ 0.1	7 $\pm$ 13	513 $\pm$ 26 931	9 $\pm$ 502

calculated for plasma while embryos appeared to have a 1.5-fold longer elimination half-life.

Concentrations in PND12 dam plasma and tissues rose rapidly immediately after dosing (Figure 4C and Table 4). Clearance from liver and kidney was analogous to that of the systemic circulation (approx. 1 ml/min), while mammary tissue had a roughly 5-fold faster clearance rate. Stomach contents, liver, and kidney tissue from nursing pups at PND12 were assayed to assess TBP transfer from milk and internal exposure in nursing animals (Figure 4D). PND12 pup stomach contents contained 0.5 nmol-eq/g at 4 h and approx. 1.0 nmol-eq/g at 8 h. [¹⁴C]-Radioactivity in liver collected from PND12 pups decreased with time from 0.6 nmol-eq/g at 30 min post dose to less than 0.2 nmol-eq/g at 8 h post dose. Observed concentrations of total chemical in the PND20 pup kidneys and livers were low (<0.2 nmol-eq/g) but fairly level between 6 and 24 h post dose, reflected by the extended absorption and elimination half-lives.

DISCUSSION

Exposures to endocrine active chemicals as TBP during growth and development are major causes for concern (Zoeller et al., 2012). In these studies, [¹⁴C]-radioactivity was detected in placenta and fetuses following a single maternal exposure to TBP through 24 h, indicating the placenta is not completely protective of the fetal compartment, and may act as a reservoir for a continuous exposure to the developing fetus. These data mirror cord blood studies in humans showing detectable levels of TBP and its metabolites available to the fetus. TBP has been detected in human placenta, and is frequently detected in maternal or neonatal cord blood (Kawashiro et al., 2008).

Exposure to endocrine active chemicals early in postnatal development is also a recognized hazard for development of disease later in life (Sharpe, 2001; Vaiserman, 2014). We show here that nursing dams exposed on PND12, the time of maximal milk consumption per unit pup weight, transferred TBP to the pups via nursing, as stomach contents, livers, and kidneys collected from the pups contained appreciable amounts of chemical. We therefore concluded that nursing human infants are likely to experience lactational and subsequent systemic exposure following maternal exposure to the chemical, as has been shown for other toxicants, from naturally occurring toxicants to persistent organic pollutants like perfluoroalkyl substances and pesticides (Andersson et al., 2017; Lee et al., 2018; Limon-Miro et al., 2017).

Physiological changes during pregnancy include the expansion of the central compartment, increases in cardiac output, and alterations in liver and kidney function (Ansari et al., 2016). Liver size did not change in the animals used in this study (data not shown) although some metabolic enzyme expression levels and activities have been observed to change during pregnancy (Bacq, 2000–2013; Shuster et al., 2013). Hepatic metabolism of xenobiotics is altered in pregnancy, with observed increases in sulfotransferase expression and activities and decreases in uridine diphosphate glucuronosyltransferases in mice, rats, and humans (Chen et al., 2009; Luquita et al., 2001; Wen et al., 2013; Zhang et al., 2020). Pregnancy also alters bile flow (Reyes and Kern, 1979), potentially altering systemically available TBP metabolites; it has been previously shown that approximately 10% of an oral dose of TBP is excreted via bile entirely as the glucuronide conjugate (Knudsen et al., 2019). In addition, the placenta and fetus present additional compartments with unique metabolic and depot functions for the sequestration and prolonged exposure to endocrine-active xenobiotics as TBP (Garland et al., 1996).

Well-perfused tissues like liver and kidney exhibited similar clearance values as that calculated for plasma, while apparent clearance was much higher in mammary tissue. It is well recognized that following parturition, blood volume decreases but total body water remains elevated to support the production of milk by the mammary gland (Ansari et al., 2016; Pipe et al., 1979). An increase in total body water generally increases the volume of distribution for xenobiotics along with increased clearance (Ansari et al., 2016). In addition, an expansion in the lipid portion of the mammary gland is expected to increase the likelihood of lipid-soluble chemicals (eg, TBP) partitioning into this new lipid-rich depot, enhancing lactational transfer of the chemical to the nursing infant (Fleishaker and McNamara, 1988).

CONCLUSIONS

Parent TBP was rapidly absorbed from the gut, similar to non-pregnant animals (Knudsen et al., 2019). However, TBP appeared to be conjugated more efficiently and to a greater extent to TBP-glucuronide and TBP-sulfate by both pregnant and nursing dams. Research is needed to explore at what levels we see adverse effects in offspring and what mechanisms are affected. Ongoing research is investigating the chemical nature of the [¹⁴C]-radioactivity present in the developing embryos and fetuses along with the corresponding placentas, as well as the mechanisms underlying the accumulation of the mixture of

TBP and its conjugates in the placenta. We are also investigating the interaction of TBP and efflux transporters known to be expressed in the placenta following exposures, as we have shown that TBP alters the activity of these transporters in other barrier tissues (Trexler et al., 2019).

This study demonstrated that systemic exposure to both the directly exposed mother and the indirectly exposed offspring was rapid and continuously maintained through 24 h post-dose in pregnant and nursing rats. We found that TBP is available to both the developing embryo and fetus as well as the nursing pup following maternal exposure. In addition, nursing offspring are rapidly and continuously exposed to TBP via contaminated milk, and those exposures can arise from a single maternal TBP exposure. We expect that human fetuses and nursing infants are exposed in much the same way.

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