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Distribution, Metabolism, Excretion, and Lactational Transfer to Pups of Tetrabromobisphenol A and Its Metabolites in C57BL/6 Mice

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We characterized the absorption, distribution, metabolism, and excretion of tetrabromobisphenol A (TeBBPA) in C57BL/6 mice. After TeBBPA dosing, we monitored the mice and collected samples for 24 h. Most TeBBPA was excreted in the feces at 12 h. At 24 h, 71% of administered TeBBPA was found in the feces and TeBBPA-conjugates were detected at 17.8%. We also detected debrominated metabolites of TeBBPA, including tribromobisphenol A (TriBBPA), dibromobisphenol A (DiBBPA), and monobromobisphenol A (MoBBPA). TeBBPA, TriBBPA, 2,2′-DiBBPA and MoBBPA were detected in dam's blood between 30 min and 1 h. Moreover, TriBBPA and 2,2′-DiBBPA were detected in milk at 30 min. The primary metabolites of TeBBPA were formed by conjugation, whereas debromination represented a minor metabolism pathway. Moreover, our findings demonstrated that the parent TeBBPA compound and its debrominated metabolites were distributed into the maternal milk of mice and transferred to the nursing pups following TeBBPA oral administration.

Key words tetrabromobisphenol A, debrominated metabolites, lactational transfer, C57BL/6 mice

INTRODUCTION

Brominated flame retardants (BFRs) are widely used in combustible materials such as plastics, textiles, and electrical appliances.[1)] Tetrabromobisphenol A (TeBBPA) is one of the most commonly used BFRs in the world. For example, TeBBPA is a reactive BFR used for epoxy and polycarbonate resins. Additionally, TeBBPA-derivatives and oligomers are additive BFRs for ABS resins.[2)] In Japan, TeBBPA accounted for approximately 28% (10,000 tons) of the annual demand (36,000 tons) of BFRs in 2020.[3)]

Currently, TeBBPA has been registered under the European Registration, Evaluation, Authorization, and Restriction of Chemicals since June 2008.[4)] In contrast, under Japanese regulation, TeBBPA is considered a general chemical under the Chemical Substances Control Law by the Ministry of Economy, Trade, and Industry (METI).[5)] For general chemicals, the manufacturer and importing manufacturer (production or import of more than 1 ton annually) are obligated to notify the METI regarding the production volume and import amount for the previous fiscal year. Otherwise, general chemicals are considered unlimited for use. Therefore, TeBBPA is a potential contaminant to the environment during the production, usage, and waste treatment of TeBBPA-containing products.[6–8)] TeBBPA has been detected in river sediment[9)] and sludge from wastewater treatment plants,[10)] and there have been reports on the bioaccumulation of TeBBPA in aquatic organisms and marine mammals throughout the food chain.[11,12)] TeBBPA has also been found in significant amounts in human samples, such as plasma samples from Norwegian individuals working at an electronics dismantling facility[13)] and breast milk samples from Japanese mothers.[14)]

Limited information concerning the toxicological impact of TeBBPA is available. *In vitro* studies have reported that TeBBPA induces neurotoxicity,[15,16)] immunosuppressive homeostasis,[17,18)] thyroid hormone disruption,[19,20)] and estrogenic activity.[21–23)] Recently, TeBBPA has been classified in Group 2A as probably carcinogenic to humans by the International Agency for Research on Cancer.[24)] We have also reported the detection of TeBBPA and tribromobisphenol A (TriBBPA) in Japanese human milk.[14)] In that study, concentrations of TriBBPA were approximately four-fold higher than those of TeBBPA in human milk samples. Thus, debromination was identified as an important metabolic and distribution pathway in human breast milk samples; however, the underlying mechanisms responsible for the higher TriBBPA concentrations remained unclear. Notably, TeBBPA and the newly synthesized brominated bisphenol A (BPA), such as TriBBPA, 2,2′-DiBBPA, 2,6-DiBBPA, and MoBBPA, are known ligands for peroxisome proliferator-activated receptor γ (PPARγ), which is involved in human lipid and glucose metabolic processes.[25)] Little is known regarding the secondary toxic effects due to TeBBPA metabolism, particularly if TeBBPA is metabolized into TriBBPA and additional debrominated TeBBPA metabolites such as DiBBPA, MoBBPA, and BPA. Thus, it is important to clarify the metabolic pathways of TeBBPA, including its partitioning and that of its metabolites into mother's milk, and the resulting exposure to infants. In the present study, we investigated the distribution, metabolism, and excretion of TeBBPA, debrominated TeBBPA, and their conjugates in mice, particularly, using the newly synthesized brominated BPA. In addition, we characterized the lactational transfer of TeBBPA and its metabolites to nursing pups, comparing the data with the results observed in human breast milk.

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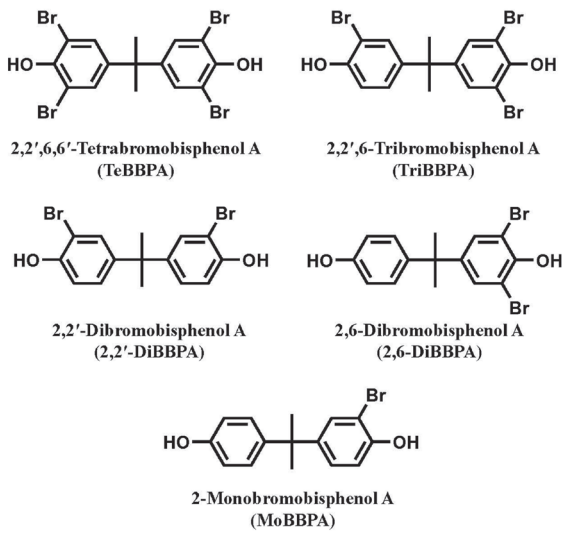

Fig. 1. Chemical Structures of the Brominated Bisphenol A Analogs Measured in This Study

TeBBPA is tetrabromobisphenol A. TriBBPA is tribromobisphenol A. 2,2′-DiBBPA is 2,2′-dibromobisphenol A. 2,6-DiBBPA is 2,6-dibromobisphenol A. MoBBPA is monobromobisphenol A.

MATERIALS AND METHODS

Chemicals TeBBPA and $^{13}C_{12}$-TeBBPA standards were obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). BPA and $^{2}H_{16}$-BPA standards were purchased from Kanto Chemical (Tokyo, Japan). As shown in Fig. 1, monobromobisphenol A (MoBBPA), 2,2′-dibromobisphenol A (2,2′-DiBBPA), 2,6-DiBBPA, and TriBBPA were newly synthesized as reported previously.[14] Oasis HLB cartridges (500 mg, 6 cc), used for purification, were purchased from Waters (Tokyo, Japan). *β*-Glucuronidase was purchased from Wako Pure Chemicals (Osaka, Japan). Sulfatase was purchased from Nacalai Tesque (Kyoto, Japan). All other reagents and solvents were purchased from Wako Pure Chemicals or Nacalai Tesque (Kyoto, Japan).

Animal Care and Treatment Pregnant (at late stage) C57BL/6 mice were purchased from SLC Inc., Shizuoka, Japan. After delivery, the weights of the dams were 20.6–21.3 g. Mice were housed at 23 ± 1.5°C with a 12-h light/dark cycle and allowed free access to standard rodent chow and water. Within the first 48 h after birth, the litters were adjusted to have five pups by removing excess pups. The litters contained pups of both sexes. Animal experiment procedures were performed in accordance with the guidelines of Setsunan University. Animals were treated humanely and with regard for the reduction of suffering.

One dam and five pups were each assigned to one of the eight treatment groups (n = 3). TeBBPA (100 μg, dissolved in 0.1 mL of corn oil) was administrated as a single oral dose. After dosing, fecal samples from each group were collected at each defined time after administration (0, 0.5, 1, 2, 3, 6, 9, 12, 24, 48, and 72 h). 0 h was treated as a control group. The dam was anesthetized and dissected at each defined time, and blood, tissues (liver, kidneys, intestine, heart, fat, and brain), and the gastrointestinal contents (stomach content and intestinal content) were collected. Pups were anesthetized and dissected at each defined time point, and milk samples were collected from the stomachs. All samples were stored at −20°C if not used immediately. TeBBPA for intravenous administration was prepared with saline to a concentration of 0.2 μg/mL. After intravenously injecting 10 μL into the tail vein, blood, bile, and urine were collected at 0.25, 0.5, 1 and 2 h.

Cell Cultures HepG2 cells, a human hepatocarcinoma cell line, and Hepa1c1c7 cells, a mouse hepatocarcinoma cell line, were obtained from American Type Culture Collection (Rockville, MD, USA) and RIKEN BioResource Center (Ibaraki, Japan), respectively. HepG2 and Hepa1c1c7 were cultured in Dulbecco's modified Eagle's medium (DMEM) and α minimum essential media (α-MEM) containing 10% fetal bovine serum (FBS), respectively. These cells were maintained in a 5% CO_2 atmosphere at 37°C. For analysis, HepG2 (2.0×10^5 cells/well) and Hepa1c1c7 (0.8×10^5 cells/well) were seeded into a 24-well plate for 16–24 h. These cells were stimulated with 1 μM of TeBBPA and TriBBPA, respectively, at the indicated time points. Medium was collected at 0, 0.5, 1, 3, 6, 12, 24, 36 and 48 h after the addition of each chemical. The stimulated time for TriBBPA was up to 6 h.

Analytical Procedures Fecal samples were freeze-dried for at least 24 h, until constant weight was achieved. Samples were extracted for 3 h with 200 mL of 50% chloroform in methanol under reflux. After extraction, extracts were filtered with glass fiber filters. Filtrates were evaporated to 10 mL. An aliquot of the extract (0.1 mL, one hundredth of the total) was dried with nitrogen. Then, the extract was dissolved with 5 mL of water. Prior to sample cleanup, two cleanup recovery standards were spiked into samples with 5 ng each of $^{13}C_{12}$-TeBBPA and $^{2}H_{16}$-BPA in a glass flask. Sample cleanup was performed by solid-phase-extraction (SPE).

For conjugated TeBBPA, such as glucuronide and sulfate, the feces extract was treated with *β*-glucuronidase and sulfatase. An aliquot of the feces extract (0.1 mL, one hundredth of the total) was dried with nitrogen after spiking with 5 ng each of $^{13}C_{12}$-TeBBPA and $^{2}H_{16}$-BPA. Samples were dissolved with 0.25 mL of acetic acid buffer (pH 5.0), followed by prewarming at 37°C for 10 min. Subsequently, samples were added to 0.05 mL of *β*-glucuronidase (500 unit/mL) and 0.1 mL of sulfatase (50 unit/mL) for hydrolysis of the conjugate. After activating for 24 h with only *β*-glucuronidase or both *β*-glucuronidase and sulfatase, or for 1 h with only sulfatase, 0.3 mL of water was added. Hydrolysis samples were extracted three times with 0.2 mL of ethyl acetate. The organic phase was separated, washed twice with water, and dried with anhydrous Na_2SO_4. Fecal extracts were treated in the same manner.

Tissues samples (liver, kidneys, intestine, heart, fat, and brain) were dried with anhydrous Na_2SO_4 (addition of 10 times the volume according to tissue weight). The stomach and intestinal contents were freeze-dried for at least 24 h, until constant weight was achieved. The dried samples were extracted with 200 mL of 50% chloroform in methanol under reflux. After extraction, the extract was filtered by glass fiber filter, and then the filtrate was evaporated to 10 mL. An aliquot of the extract (1.0 mL, one tenth of the total) was dried with nitrogen after spiking with 5.0 ng each of $^{13}C_{12}$-TeBBPA and $^{2}H_{16}$-BPA, dissolved in 5 mL of water. In the case of conjugated TeBBPA analysis in several tissues, 1 mL of an aliquot of the extract (10 mL) was dried with nitrogen. The continuous operation was performed in the same manner as described above for the feces.

Blood samples were stored at 4°C overnight, followed by sep-

aration of blood plasma by centrifuging at 3,000 rpm after spiking with 5.0 ng each of $^{13}C_{12}$-TeBBPA and $^{2}H_{16}$-BPA. Blood plasma (0.3 mL) was added to 0.6 mL of ethanol, mixed well, and centrifuged at 3,000 rpm for 5 min to remove protein. The supernatant was applied to SPE.

Milk samples were freeze-dried for at least 24 h, until constant weight was achieved. Prior to sample cleanup, two cleanup recovery standards were spiked into each sample with 5 ng each of $^{13}C_{12}$-TeBBPA and $^{2}H_{16}$-BPA in a glass flask. Samples were applied to SPE.

Bile samples were spiked with 5.0 ng each of $^{13}C_{12}$-TeBBPA and $^{2}H_{16}$-BPA. Samples were added to 0.6 mL of ethanol, mixed well, and centrifuged at 3,000 rpm for 5 min to remove protein. The supernatant was applied to SPE.

Urine samples were extracted for 3 h with 200 mL of 50% chloroform in methanol under reflux. Prior to sample cleanup, two cleanup recovery standards were spiked into samples with 5 ng each of $^{13}C_{12}$-TeBBPA and $^{2}H_{16}$-BPA in a glass flask. After extraction, extracts were filtered with glass fiber filters. Samples were applied to SPE.

Sample cleanup was performed with SPE. First, each treated sample (feces, several tissues, blood and milk extracts, and enzyme-treated feces and several tissue extracts) was added to 5.0 mL water. Dissolved samples were sonicated for 5 min in an ultrasonic bath. Next, samples were diluted with 5.0 mL 25% 2-propanol in formic acid and 5.0 mL 50% 2-propanol in water, and sonicated with every addition for 5 min. The SPE cartridge was first washed with eluent solution (70% dichloromethane in methanol) and then preconditioned with 5.0 mL methanol, 5.0 mL dichloromethane, and 7.0 mL 70% dichloromethane in methanol. Cartridges were continuously conditioned with 5.0 mL methanol and 5.0 mL water. Treated samples were loaded onto cartridges, and the flask was rinsed with 5 mL 25% methanol in water to remove any residual sample, which was also loaded onto the cartridge. This cartridge was then washed with 4.0 mL 0.05% 2-propanol in water. After completely drying the cartridge, the adsorbed matter in the cartridge was eluted with 7.0 mL 70% dichloromethane in methanol, and the eluate was gently evaporated to dryness at 45°C under a stream of nitrogen.

The residue was added to 0.5 mL of 1 M KOH/ethanol and shaken for 30 min. Next, samples were derivatized by ethylation of the hydroxyl group using 0.5 mL diethyl sulfate for 30 min. After the addition of 4.0 mL of 1 M KOH/ethanol, samples were incubated at 70°C for 1 h. Three mL of water was added to the samples, which were then extracted twice with 2 mL of *n*-hexane. The organic phase was then dried with anhydrous Na_2SO_4. Purification was performed using a florisil column (1.0 g) and eluted with 10 mL of 4% diethyl ether in *n*-hexane. The purified extract was then concentrated to 50 µL in *n*-nonane. Extractions and purifications were conducted under light shielding conditions. The final solution was analyzed for TeBBPA related compounds by gas chromatography (GC)-high resolution mass spectrometry (HRMS).

In cell-stimulated experiments, the medium was dried with nitrogen after spiking with 5.0 ng each of $^{13}C_{12}$-TeBBPA and $^{2}H_{16}$-BPA. The dried samples were derivatized by ethylation with diethyl sulfate in the same manner as described above.

The GC-HRMS setup consisted of an Agilent 6890N gas chromatograph coupled with a JEOL JMS-700 mass spectrometer. The ion source was operated in the electron ionization (EI, 42 eV, 700 µA, 260°C) mode. The chromatographic separation was accomplished using an Agilent Technologies DB-5MS capillary column (15 m × 0.25 mm i.d., 0.10 µm film thickness). The GC oven temperature program used an initial oven temperature of 140°C with a hold for 1.5 min, followed by a temperature ramp of 10°C/min until reaching 310°C, with another hold for 3 min. The column was connected directly to the ion source of the mass spectrometer (interface temperature of 260°C). Sample introduction was performed by splitless injection (injection temperature of 260°C, 1 min splitless time) of a 1.0-µL aliquot of the sample extract. Helium was used as the carrier gas at 0.8 mL/min.

Method of QA/QC for Analytical Data TeBBPA was identified by comparing the retention times and mass spectra with those of the commercial standards, and MoBBPA, DiBBPA, and TriBBPA were identified using our synthesized standards. De-conjugated TeBBPA was obtained by treating TeBBPA-glucuronide and TeBBPA-sulfate with *β*-glucuronidase and sulfatase. This amount of TeBBPA is the total amount of conjugated and non-conjugated TeBBPA. Therefore, we defined it which the deducted TeBBPA from the total amount as conjugated TeBBPA. Selected ion monitoring (SIM) chromatograms were used to determine the peak area ratios for $[M\text{-}CH_3]^+$/$[M\text{-}C_3H_8]^+$. The acceptance criteria were set from −30% to 30% of ratios observed with the commercial and synthesized standards. Concentrations were corrected utilizing the recovery efficiency of their respective internal standards, such as $^{2}H_{16}$-BPA for MoBBPA and DiBBPA, and $^{13}C_{12}$-TeBBPA for TriBBPA and TeBBPA. Samples which had high internal standard recoveries in the range of 60–120% were used for data collection. The calibration curves were obtained with the internal standard method used by TeBBPA, TriBBPA, DiBBPA, MoBBPA, $^{13}C_{12}$-TeBBPA, and $^{2}H_{16}$-BPA. As a result, the linear range was 0.05–50 ng/mL. The limits of detections (LODs) and the limits of quantitation (LOQs) were defined as three (S/N = 3) and 10 times (S/N = 10) the noise level, respectively. The LOQs of GC-HRMS for MoBBPA, DiBBPA, TriBBPA, and TeBBPA were 0.15, 0.15, 0.15, and 0.30 pg, respectively.

RESULTS AND DISCUSSION

Distribution and Material Balance After 24 h Following Single Doses Figure 2 shows concentrations of TeBBPA and its conjugated metabolites (TeBBPA-sulfate and TeBBPA-glucuronide) in feces, liver, kidneys, intestine, heart, fat, and brain collected at 24 h after oral administration of TeBBPA. Figure 3 shows the ratio of excretion and distribution of TeBBPA and its conjugated metabolites after 24 h following single doses. TeBBPA passed through the stomach and intestine, and 71% of TeBBPA was excreted in feces as unconjugated TeBBPA after 24 h (Fig. 2 and 3). In feces, the conjugated metabolites of TeBBPA, such as TeBBPA-sulfate and TeBBPA-glucuronide, were detected at 11% and 6.8%, respectively. In total, 89 µg of TeBBPA and its conjugated metabolites were observed in feces. TeBBPA and its conjugated metabolites were excreted and detected at 96% and 98% in feces at 48 and 72 h after dosing (Table 1), respectively. Our results were similar to those reported by Kuester *et al*.,[26] who examined the TeBBPA excretion ratio in feces of male F-344 rats.

For the tissue distribution of TeBBPA (Fig. 2), TeBBPA was slightly detected in the liver (0.045 µg), kidneys (0.012 µg), intestine (0.038 µg), heart (0.00093 µg), fat (0.0034 µg), and brain (0.00070 µg). Conjugated TeBBPA was also found in

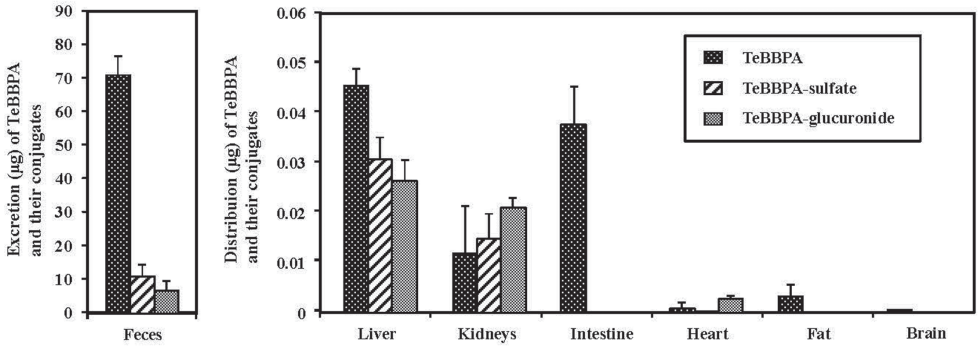

Fig. 2. Excretion and Tissue Distribution of Tetrabromobisphenol A (TeBBPA) and Its Conjugation at 24 h after Single Dosing

Feces and tissues were collected, extracted, and analyzed using gas chromatography-high resolution mass spectrometry. Data were presented as the mean ± SD from the results of three independent analyses. Limits of quantitation of monobromobisphenol A, dibromobisphenol A, TriBBPA, and TeBBPA were 0.15, 0.15, 0.15, and 0.30 pg, respectively.

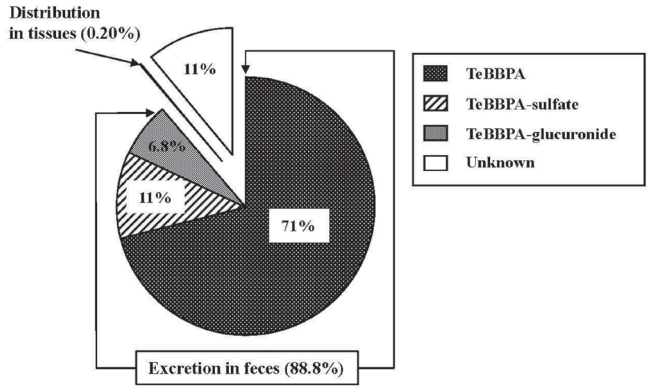

Fig. 3. Ratio of Excretion and Distribution of Tetrabromobisphenol A (TeBBPA) and Its Conjugation Metabolites After 24 h Following Single Dosing

Feces and tissues were collected, extracted, and analyzed using gas chromatography-high resolution mass spectrometry. Limits of quantitation of 2-monobromobisphenol A, dibromobisphenol A, TriBBPA, and TeBBPA were 0.15, 0.15, 0.15, and 0.30 pg, respectively.

Table 1. Time Changes of Excretion Amount (μg) of TeBBPA including Conjugated TeBBPA in Feces Following Single Dose Administration of TeBBPA[a]

Time (h)	TeBBPA including conjugated TeBBPA
0.5	< LOQ[b, c]
1	< LOQ
2	< LOQ
3	2.6
6	25
9	57
12	82
24	89
48	96
72	98

[a]Administrated TeBBPA as a single oral dose is 100 μg dissolved in 0.1 mL of corn oil.
[b]LOQ: Limit of quantitation. TeBBPA, 0.30 pg; TeBBPA-sulfate, 0.30 pg; TeBBPA-glucuronide, 0.30 pg.
[c]LOQ was defined as 10 times (S/N = 10) the noise level.

the liver and kidneys. It is likely that the conjugated metabolites of TeBBPA pass into the general circulation after liver metabolism. TeBBPA does not appear to bioaccumulate at all, although TeBBPA was slightly absorbed in the intestine and TeBBPA and its conjugated metabolites were detected at approximately 0.2% of the total amount in feces and tissues. In addition, most TeBBPA was excreted in the feces as unconjugated TeBBPA. However, 11% of administrated TeBBPA had an unknown distribution in this study (Fig. 3). We believe that TeBBPA and its conjugated metabolites were included in the stomach and intestinal contents or distributed to the skin, muscle, and blood, as reported elsewhere.[26–28]

Distribution of TeBBPA in the Stomach, Intestines, and Liver with Excretion Monitoring in Feces until 24 h after Dosing Fig. 4 shows the distribution in the stomach content, intestinal content, and liver, and excretion to the feces of mice until 24 h after TeBBPA dosing. After administration, TeBBPA began to move from the stomach immediately, and approximately 3.5% of TeBBPA moved to the intestine after 30 min (Fig 4A). With the progression of time, in accordance with the decrease of TeBBPA in the stomach contents, TeBBPA concentrations in the intestinal contents increased. The TeBBPA level in intestinal contents showed the maximum value (29%) 9 h after dosing. TeBBPA was excreted in the feces from then on with retention in the intestine for 3 h (Fig. 4B). There was a small amount of residual TeBBPA in the intestine at 12 h and most TeBBPA was excreted into the feces. TeBBPA was absorbed from the intestine after 30 min, and then it was detected in the liver (0.15%). As shown in Fig. 4C, TeBBPA was observed at low levels in the liver at 0.5–2 h. The TeBBPA level reached a maximum at 3 h after dosing, followed by a decrease of TeBBPA during the remaining 24 h. As indicated above, TeBBPA was absorbed from the intestine, and it was distributed over several tissues, primarily in the liver. From these results, we suggest that TeBBPA was metabolized through the liver and absorbed from the intestine. We also analyzed the conjugated TeBBPA in the stomach, intestinal contents, and feces (Table 2). Conjugated TeBBPA metabolites were not detected in the stomach contents, but 2.3–2.4 μg (at 1–2 h after dosing) was detected as the TeBBPA sulfate and 6.7–14 μg (at 2–6 h after dosing) as the TeBBPA glucuronide in the intestinal contents. The time at which peak levels of both conjugates were observed was 2 h after dosing. Conjugated TeBBPA was observed in feces after 6 h, and the excretion amounts were 8.0–15 μg (sulfate) and 3.7–6.8 μg (glucuronide), respectively. However, conjugated TeBBPA metabolite amounts were extremely low in liver. We think that TeBBPA is subject to entering hepatic circulation because conjugat-

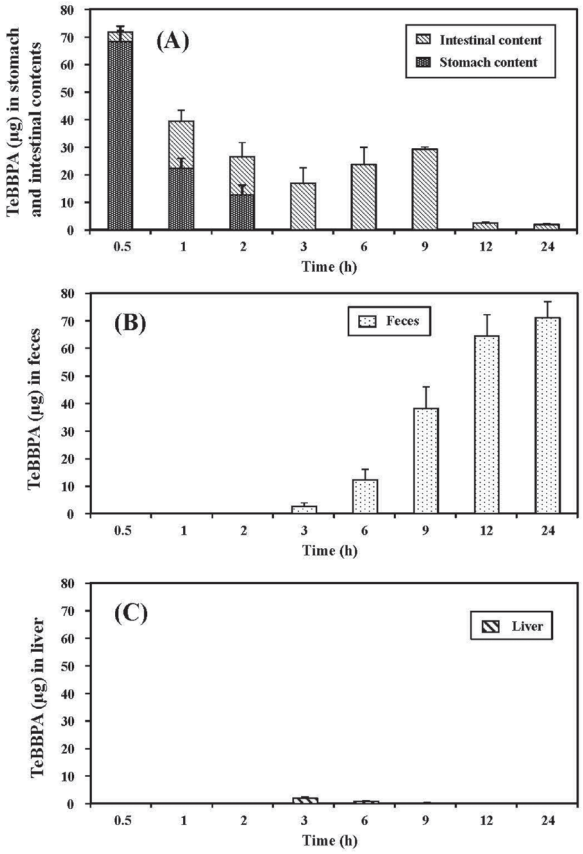

Fig. 4. Distribution Monitoring in Stomach Content, Intestinal Content, and Liver, and Excretion Monitoring in Feces until 24 h Following Single Dose of Tetrabromobisphenol A (TeBBPA)

Data are presented as the mean ± SD from the results of three independent analyses. Limits of quantitation of 2-monobromobisphenol A, dibromobisphenol A, TriBBPA, and TeBBPA were 0.15, 0.15, 0.15, and 0.30 pg, respectively. A; Intestinal content and stomach content, B; Feces, C; Liver

ed TeBBPA was found in the intestinal contents. Furthermore, intravenous administration of TeBBPA was performed to confirm excretion of TeBBPA and its metabolites into the bile and urine. As a result of intravenous administration of TeBBPA, it was confirmed that the blood concentration of TeBBPA 0.25 h after administration was 0.84 ng/mL (Table 3). After that, the blood concentrations at 0.5, 1 and 2 h were 0.99, 0.63 and 0.48 ng/mL, respectively. On the other hand, when TeBBPA in bile and urine was confirmed, it was always below the LOQ. From these results, we conclude that administered TeBBPA was absorbed in insignificant amounts from the intestine and was primarily excreted via the feces within 12 h.

Excretion Monitoring of Debrominated TeBBPA Metabolites We analyzed debrominated TeBBPA metabolites because it was clear that a part of the administered TeBBPA was absorbed from the intestine and distributed to the liver, as indicated in Fig. 4. As a preliminary study, we investigated the generation of debrominated TeBBPA using a TeBBPA standard under light shielding and normal light conditions, and we observed that TeBBPA was not debrominated into TriBBPA via photolysis under either condition (Table 4). Table 5 shows the changes with time of TeBBPA and debrominated TeBBPA metabolites (MoBBPA, DiBBPA, and TriBBPA) in feces until 24 h after dosing. TeBBPA was detected in feces at 2.6 μg from a 100-μg total dosage amount 3 h after dosing. At that time, 2,2′-DiBBPA and TriBBPA were slightly detected at 0.00026 and 0.0094 μg in feces, respectively. MoBBPA and 2,6-DiBBPA were not detected in feces. At 6–12 h, as the increase in excretion for TeBBPA in feces began, the excretion level of TriBBPA tended to increase. The TriBBPA levels in feces were 0.13, 0.38, and 0.54 μg at 6, 9, and 12 h, respectively. 2,2′-DiBBPA was similar to TriBBPA, and had a maximum level of 0.0068 μg at 12 h. Consequently, we clarified that the metabolites of TeBBPA, in addition to the sulfate and the glucuronide of TeBBPA, were also the debrominated metabolites of TeBBPA, such as TriBBPA and DiBBPA, which were detected in the feces of C57BL/6 mice. Similarly, Schauer *et al*. reported TeBBPA-glucuronide, TeBBPA-sulfate, and TriBBPA as metabolites of TeBBPA in urine and blood samples of rats.[29] Additionally, the debrominated TeBBPA was detected in TriBBPA and 2,2′-DiBBPA in our results. We believe that this phenomenon contributed to higher levels of TriBBPA than TeBBPA in human breast milk in our previous study.[14]

Transfer to Blood and Milk To understand the distribution into milk, we analyzed TeBBPA and its debrominated metabolites in the blood and milk. Fig. 5 shows a change in TeBBPA and its debrominated metabolites (TriBBPA, 2,2′-DiBBPA, and MoBBPA) in blood and milk after dosing. At first, the maximum level (0.32 μg/mL) of TeBBPA was detected in the blood of the dam 30 min following administration (Fig. 5A). Thereafter, TeBBPA levels showed a decreasing tendency as the TeBBPA concentration reduced to 33% and 31% at 2 and 3 h, respectively. The TeBBPA level gradually decreased to 3.2% (0.015% of total dosage amounts) at 24 h.

We then analyzed the debrominated TeBBPA in dam's blood. Notably, 0.5 h after dosing, MoBBPA appeared at 0.12 μg/mL (37% of observed TeBBPA at 0.5 h). In addition, 2,2′-DiBBPA (0.12 μg/mL) and TriBBPA (0.067 μg/mL) were present 1 h after dosing. While 2,2′-DiBBPA was detected in blood only at 1 h, MoBBPA and TriBBPA were detected 2 h later, but at extremely low concentrations with a decreasing tendency. These results were slightly different from our previous report for human milk, where we estimated that TeBBPA was metabolized into TriBBPA, with TriBBPA as a major metabolite transferring to human breast milk.

By analyzing the milk samples left in the stomach of the pups, we detected TriBBPA (0.0043 μg) in milk 0.5 h after dosing (Fig. 5B). Thereafter, TriBBPA showed its maximum concentration (0.023 μg) at 1 h, and it disappeared from milk after 2 h. 2,2′-DiBBPA was observed at 1 h, and the concentration (0.013 μg) was lower than that of TriBBPA. The debrominated TeBBPA detected in the milk samples was different from the composition of metabolites in the blood, with the major metabolite of TeBBPA in milk being TriBBPA. While MoBBPA was observed in the blood immediately after dosing, it was not detected in milk. Thus, we believe that the reason for the different metabolites in blood and milk is related to differences in transfer and metabolic rate. However, there are few reports to support this. Therefore, further study is needed to clarify the transfer and metabolic rate of TeBBPA and its metabolites in blood and milk. Our results reveal that absorbed TeBBPA is distributed into milk via the blood, and that the TriBBPA and 2,2′-DiBBPA metabolites are also distributed into the feces, blood, and milk.

Comparison of Metabolism by HepG2 Cells and Hepa1c1c7 Cells To compare the metabolic capacity of human and mice, we ran an experiment to metabolize TeBBPA and TriBBPA

Table 2. Time Changes of Excretion Amount (μg) of TeBBPA, TeBBPA-Sulfate, and TeBBPA-Glucuronide in Stomach Content, Intestinal Content, and Feces after TeBBPA Dosing[a]

Time (h)	Stomach content		
	TeBBPA[b, c]	TeBBPA-sulfate[b, c]	TeBBPA-glucuronide[b, c]
0.5	68 ± 5.6	< LOQ	< LOQ
1	22 ± 3.6	< LOQ	< LOQ
2	13 ± 3.5	< LOQ	< LOQ
3	< LOQ	< LOQ	< LOQ
6	< LOQ	< LOQ	< LOQ
9	< LOQ	< LOQ	< LOQ
12	< LOQ	< LOQ	< LOQ
24	< LOQ	< LOQ	< LOQ
Time (h)	Intestinal content		
	TeBBPA	TeBBPA-sulfate[b, c]	TeBBPA-glucuronide[b, c]
0.5	3.4 ± 0.64	0.94 ± 0.17	< LOQ
1	17 ± 4.2	2.4 ± 0.39	1.5 ± 1.3
2	14 ± 5.1	2.3 ± 0.67	14 ± 1.9
3	17 ± 5.7	< LOQ	6.7 ± 2.7
6	24 ± 6.2	< LOQ	8.8 ± 1.6
9	29 ± 0.86	< LOQ	< LOQ
12	2.5 ± 0.49	0.51 ± 0.28	< LOQ
24	2.0 ± 0.42	< LOQ	0.45 ± 0.13
Time (h)	Feces		
	TeBBPA[b, c]	TeBBPA-sulfate[b, c]	TeBBPA-glucuronide[b, c]
0.5	< LOQ	< LOQ	< LOQ
1	< LOQ	< LOQ	< LOQ
2	< LOQ	< LOQ	< LOQ
3	2.6 ± 1.2	< LOQ	< LOQ
6	12 ± 3.9	8.0 ± 1.6	4.3 ± 0.94
9	38 ± 7.7	15 ± 2.6	3.7 ± 1.5
12	64 ± 7.6	11 ± 2.0	6.2 ± 0.40
24	71 ± 5.7	11 ± 1.8	6.8 ± 1.4

[a]Data are the mean ± SD of three independent analyses.
[b]LOQ: Limit of quantitation. TeBBPA, 0.30 pg; TeBBPA-sulfate, 0.30 pg; TeBBPA-glucuronide, 0.30 pg.
[c]LOQ was defined as 10 times (S/N = 10) the noise level.

Table 3. Time Changes of Blood, Bile and Urine Concentration (ng/mL) of TeBBPA Following Intravenous Administration of TeBBPA[a]

Time (h)	Blood	Bile	Urine
0.25	0.84	< LOQ[b, c]	< LOQ
0.5	0.99	< LOQ	< LOQ
1	0.63	< LOQ	< LOQ
2	0.48	< LOQ	< LOQ

[a]Administrated TeBBPA as a single intravenous administration is 200 ng dissolved in saline 0.01 mL.
[b]LOQ: Limit of quantitation. TeBBPA, 0.30 pg.
[c]LOQ was defined as 10 times (S/N = 10) the noise level.

Table 4. Confirmation of Debrominated TeBBPA Using a TeBBPA Standard under Light Shielding and Normal Light Conditions[a]

Compounds	Conditions	
	Light shielding[b] (pg)	Normal light[c] (pg)
TeBBPA	96 ± 1.2	97 ± 0.58
TriBBPA	< LOQ[d, e]	< LOQ
2,2′-DiBBPA	< LOQ	< LOQ
2,6-DiBBPA	< LOQ	< LOQ
MoBBPA	< LOQ	< LOQ

[a]TeBBPA standard level is 100 pg. Data are the mean ± SD of three independent analyses.
[b]Light shielding condition was performed using brown colored glass, and the clear glass was wrapped in aluminum foil in all analytical operations.
[c]Normal light condition was performed using the clear glass instrument in analytical operation.
[d]LOQ: Limit of quantitation. TeBBPA, 0.30 pg; TriBBPA, 0.15 pg; DiBBPA, 0.15 pg; MoBBPA, 0.15 pg.
[e]LOQ was defined as 10 times (S/N = 10) the noise level.

Table 5. Time Changes of Excretion Amount (μg) of MoBBPA, 2,2′-DiBBPA, 2,6-DiBBPA, TriBBPA and TeBBPA in Feces Following Single Dose Administration of TeBBPA[a]

Time (h)	MoBBPA[b]	2,2′-DiBBPA[b]	2,6-DiBBPA[b]	TriBBPA[b]	TeBBPA[b]
0.5	< LOQ[c]	< LOQ	< LOQ	< LOQ	< LOQ
1	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
2	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
3	< LOQ	0.00026 ± 0.000092	< LOQ	0.0094 ± 0.0012	2.6 ± 1.2
6	< LOQ	0.00053 ± 0.00014	< LOQ	0.13 ± 0.025	12 ± 3.9
9	< LOQ	0.0027 ± 0.0011	< LOQ	0.38 ± 0.068	38 ± 7.7
12	< LOQ	0.0068 ± 0.0031	< LOQ	0.54 ± 0.14	64 ± 7.6
24	< LOQ	< LOQ	< LOQ	0.33 ± 0.038	71 ± 5.7

[a]Data are the mean ± SD of three independent analyses.
[b]LOQ: Limit of quantitation. MoBBPA 0.15 pg; DiBBPA, 0.15 pg; TriBBPA, 0.15 pg; TeBBPA, 0.30 pg.
[c]LOQ was defined as 10 times (S/N = 10) the noise level.

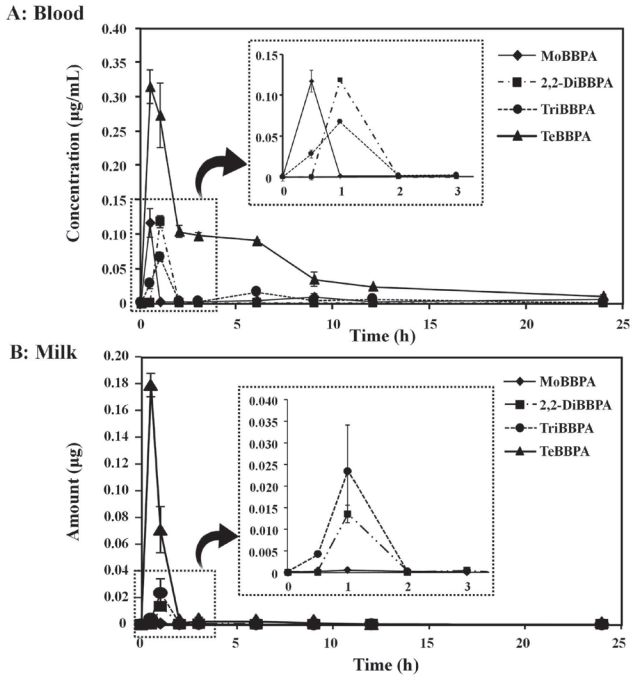

Fig. 5. Changes in 2-Monobromobisphenol A (MoBBPA), 2,2'-Dibromobisphenol A (2,2'-DiBBPA), Tribromobisphenol A (TriBBPA), and Tetrabromobisphenol A (TeBBPA) Levels in Blood and Milk after Single Dosing of TeBBPA

Data are presented as the mean ± SD from the results of three independent analyses. Limits of quantitation of MoBBPA, DiBBPA, TriBBPA, and TeBBPA were 0.15, 0.15, 0.15, and 0.30 pg, respectively. A; Blood collected from dam, B; Milk collected from pup's stomach.

by a human hepatocarcinoma cell line (HepG2) and a mouse hepatocarcinoma cell line (Hepa1c1c7). As shown in Fig. 6, it was observed that TeBBPA exposed to HepG2 was metabolized by about 30% of its initial concentration by 48 h. On the other hand, TeBBPA exposed to Hepa1c1c7 was metabolized by about 60%. When TeBBPA was exposed to HepG2 and Hepa1c1c7, the metabolite TriBBPA was detected within 0.5 to 6 h in culture medium. The metabolite TriBBPA concentration was slightly higher for Hepa1c1c7 than HepG2. Furthermore, Fig. 6 (lower) is an observation of the metabolism of TriBBPA. The metabolic rates of TriBBPA by HepG2 and Hepa1c1c7 were 54% and 7%, respectively, at 6 h. It was found that Hepa1c1c7 metabolized to DiBBPA more rapidly than HepG2. From the results, the metabolic rate from TeBBPA to TriBBPA is faster than that from TriBBPA to DiBBPA in mice. In addition, the metabolism from DiBBPA to MoBBPA is expected to be faster.

Therefore, we considered that this is the reason why MoBBPA was detected in the blood of mice earlier than DiBBPA and TriBBPA in section "Transfer to Blood and Milk". Additionally, the observation that TriBBPA was detected at high concentrations in human breast milk may be due to the difference in metabolic enzyme activity between humans and mice.[30]

Estimation of the Metabolic Pathway of TeBBPA Fig. 7 shows the GC-HRMS chromatogram of synthesized 2,2′- and 2,6-DiBBPA (upper) and detected DiBBPA in the blood sample (lower). The observed debrominated metabolite in the blood sample was identified as 2,2′-DiBBPA due to the same retention time for GC and the intensity ratio of the isotope for HRMS. In the case of TeBBPA debromination, two kinds of DiBBPA, namely 2,2′- and 2,6-DiBBPA, are regarded as debrominated compounds from TeBBPA, with TriBBPA as the intermediate. It appears that TriBBPA was metabolized from TeBBPA in this study, and then TriBBPA was debrominated into DiBBPA. During debromination from TriBBPA, bromine was eliminated from the 6-position. Subsequently, we tried to determine the metabolic pathway of TeBBPA from the results of Table 5 and Fig. 5. The metabolites of administered TeBBPA occurred as approximately 1% debrominated TeBBPA and up to 16% conjugated metabolites in feces. The detected debrominated compounds were not only TriBBPA, but also DiBBPA and MoBBPA in the blood. As shown in Fig. 8, we determined that the metabolic pathways of TeBBPA consist of two routes,

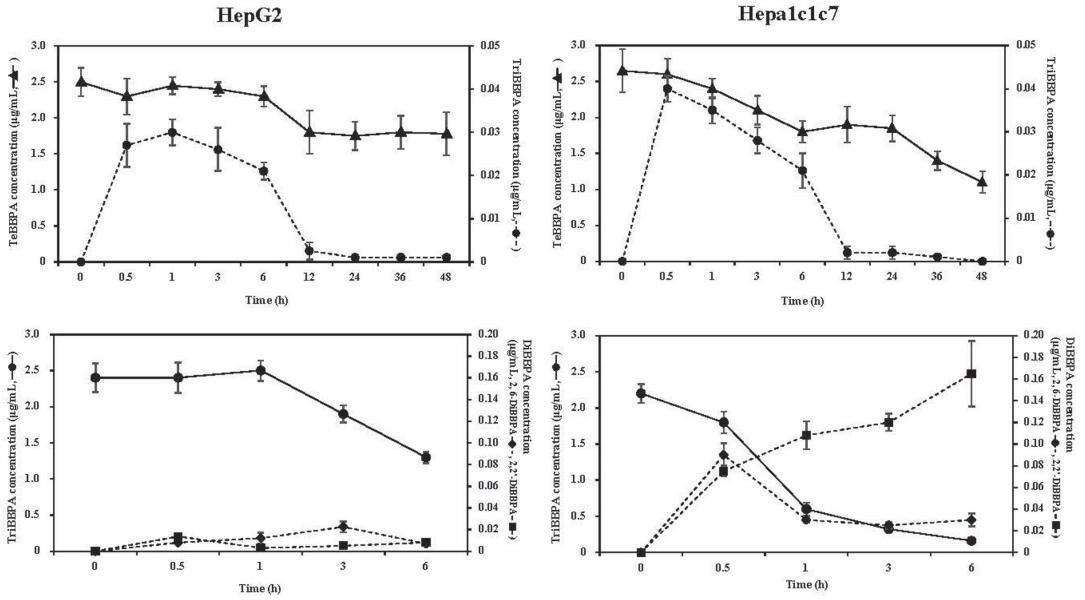

Fig. 6. Metabolic Changes in TeBBPA (Upper) and TriBBPA (Lower) by HepG2 and Hepa1c1c7 Cells

The solid line shows the time course changes of the initial substance, and the dashed line shows the time course changes of the metabolite of the initial substance. Data are presented as the mean ± SD from the results of three independent analyses. Limits of quantitation of DiBBPA, TriBBPA, and TeBBPA were 0.15, 0.15, and 0.30 pg, respectively.

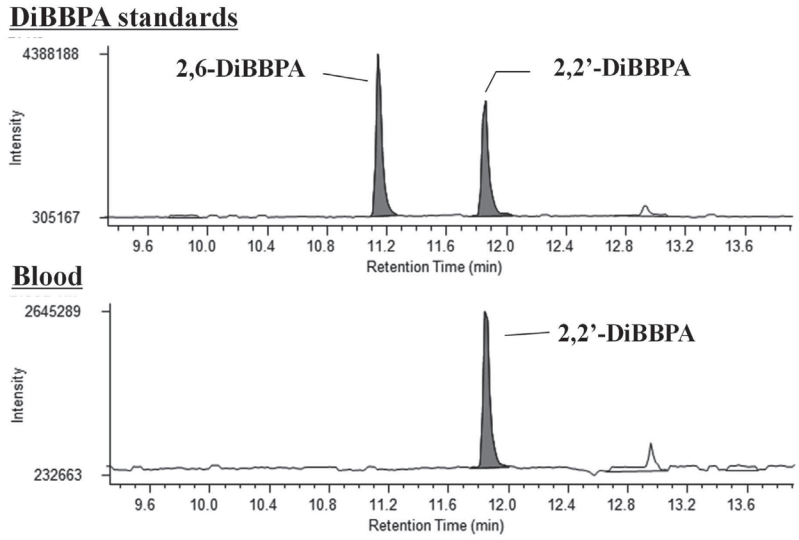

Fig. 7. Gas Chromatography-High Resolution Mass Spectrometry (GC-HRMS) Chromatogram of Synthesized 2,2'-Dibromobisphenol A (2,2'-DiBBPA) and 2,6-DiBBPA, and Detected DiBBPA in the Blood Sample

GC-HRMS analysis was performed in selected ion monitoring mode, and chromatographic separation was achieved with a DB-5MS capillary column (15 m × 0.25 mm i.d., 0.10 μm film thickness). For DiBBPA analysis, the quantitation and monitoring ions were 424.975 and 426.973, respectively.

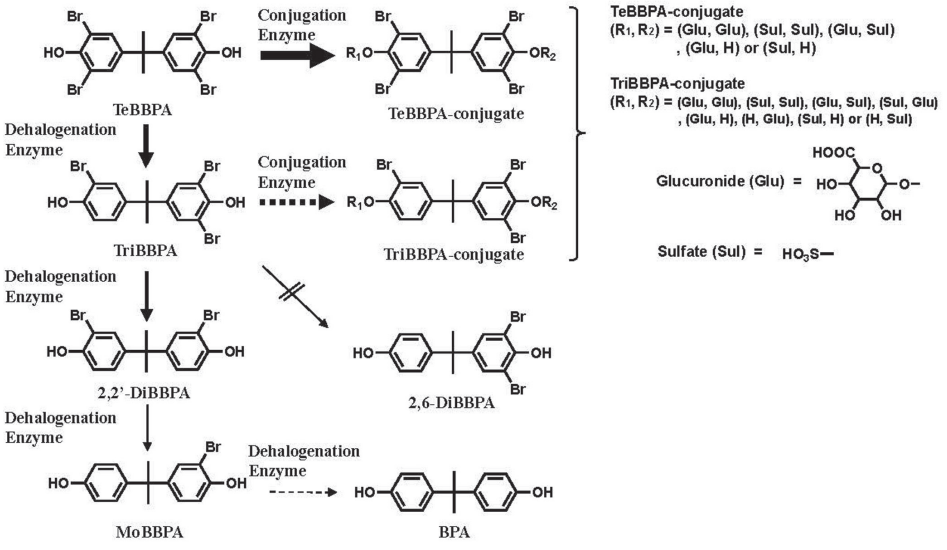

Fig. 8. Estimation of the Metabolic Pathways of Tetrabromobisphenol A (TeBBPA) in Mice

The primary metabolic pathway of TeBBPA was conjugation metabolism for sulfate and glucuronide. The suggested minor metabolic pathways were debromination such as 2-monobromobisphenol A, 2,2′-dibromobisphenol A, and tribromobisphenol A.

including conjugation and debromination, with the main metabolic pathway being conjugation. In addition, we considered that the minor metabolic pathway of TeBBPA is debromination to TriBBPA, 2,2′-DiBBPA, and MoBBPA. We speculated that the conjugation metabolites, such as TriBBPA, DiBBPA, and MoBBPA, were generated, but their metabolites were impossible to detect at trace levels.

Consequently, we confirmed that TriBBPA, as the metabolite of TeBBPA, transfers to mother's milk in humans. Gascon *et al*. reported an association between increasing polybrominated diphenyl ether (PBDE; particularly decabrominated diphenyl ether, DeBDE) concentrations in colostrum and a degraded infant mental development.[31] Moreover, they pointed out DeBDE metabolites, which are more toxic and more stable, are more likely to cross the placenta and to reach the brain than DeBDE. Our results also point out the necessity of further study into the biological effects of the parent TeBBPA and its metabolites. Particularly, Akiyama *et al*. reported that TriBBPA is an agonist for PPARγ, which is associated with glucose and lipid metabolism, and that the activity of TriBBPA is equivalent to that of TeBBPA.[25] Finally, considering that the defense mechanisms of infants are generally weaker than those of adults, the health effects of brominated bisphenol A, such as TriBBPA, DiBBPA, and MoBBPA, are a concern.

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Conflict of interest The authors declare no conflict of interest.

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