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In vivo distribution and biotransformation of Tris (1,3-dichloro-2-propyl) phosphate in mice☆

Ting Zhu [a, b, d], Xiao-Bo Zheng [c], Xiao Yan [b], Bin Tang [b], Jing Zheng [b, *], Xiao-Jun Luo [a, e], Chun-You Zhu [a, d], Yun-Jiang Yu [b], Bi-Xian Mai [a, e]

[a] State Key Laboratory of Organic Geochemistry, Guangdong Key Laboratory of Environmental Resources Utilization and Protection, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou, 510640, People's Republic of China
[b] State Environmental Protection Key Laboratory of Environmental Pollution Health Risk Assessment, South China Institute of Environmental Sciences, Ministry of Ecology and Environment, Guangzhou, 510655, People's Republic of China
[c] College of Resources and Environment, South China Agricultural University, Guangzhou, 510642, People's Republic of China
[d] University of Chinese Academy of Sciences, Beijing, 100049, People's Republic of China
[e] Guangdong-Hong Kong-Macao Joint Laboratory for Environmental Pollution and Control, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou, 510640, People's Republic of China

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ABSTRACT

Phosphorus flame retardants (PFRs) have been widely detected in environmental media and human samples. Understanding the distribution and biotransformation of PFRs is important for toxicological research of PFRs in humans. C57BL/6 mice were administered with 300 mg/kg body weight/day of tris (1,3-dichloro-2-propyl) phosphate (TDCIPP) for 35 consecutive days. The liver, kidney, muscle, feces, urine and hair samples were collected to investigate the distribution of TDCIPP and its diester, bis (1,3-dichloro-2-propyl) phosphate (BDCIPP), as well as other potential metabolites of TDCIPP. Concentrations of TDCIPP in muscle (535.7 ± 192.2 ng/g wet weight) were significantly higher than those in liver (186.9 ± 55.0 ng/g wet weight) and kidney (43.5 ± 12.0 ng/g wet weight) ($p < 0.05$), while concentrations of BDCIPP were higher in kidney (2189.2 ± 420.7 ng/g wet weight) and liver (1337.1 ± 249.6 ng/g wet weight) than other tissues. The distribution of TDCIPP and BDCIPP in mice is tissue-specific, TDCIPP tends to accumulate in muscle, while BDCIPP tends to enrich in kidney and liver. BDCIPP was prone to be eliminated by urine, which may result in the high levels of BDCIPP in urine. Urine and feces had significantly higher concentrations of BDCIPP than TDCIPP ($p < 0.05$), which demonstrated that BDCIPP is an important metabolite of TDCIPP. Hair could serve as a suitable and reliable biomarker for TDCIPP and also metabolites of TDCIPP. Multifarious metabolites of TDCIPP were identified in various matrices of mice, especially urine. Seven novel metabolites of TDCIPP were identified for the first time. TDCIPP metabolic pathways involved oxidative dealkylation, oxidative dehalogenation, reoxidation, dehalogenation with dehydrogenation. The metabolites identified in the present study could serve as candidate biomarkers for future human biomonitoring studies.

1. Introduction

Phosphorus flame retardants (PFRs) were commonly additives in commercial products to prevent materials from burning (van der Veen and de Boer, 2012), and have been used in resins, plastics, textile coatings and latexes for over 150 years (Andrae and Thesis, 2007; Green et al., 2008; Killilea et al., 2017). The usage of PFRs is increasing year by year, as they were considered as alternatives to brominated flame retardants (BFRs), which were more persistent and bioaccumulative (van der Veen and de Boer, 2012). The estimated global production of flame retardants (FRs) in 2019 is six billion pounds, of which PFRs may be as high as 16% (Yang et al., 2019). The widespread use of PFRs has increased the risk of human exposure to PFRs. PFRs have long-range mobility and are potentially harmful, being able to interfere with thyroid hormones (Liu et al., 2019; Xu et al., 2015), immune response, lipid and steroid metabolism (Canbaz et al., 2017; Farhat et al., 2014), and also lead to

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* Corresponding author.
E-mail address: zhengjing@scies.org (J. Zheng).

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neurotoxicity, hepatotoxicity (Alzualde et al., 2018), vascular toxicity (Zhong et al., 2019) and reproductive toxicity (Liu et al., 2013). Tris (1,3-dichloro-2-propyl) phosphate (TDCIPP) is a chlorinated PFR that is generally applied in polyurethane foams, electronic products, plastics and textiles (Been et al., 2017; Stapleton et al., 2014; van der Veen and de Boer, 2012). TDCIPP is easily released into the environment because of the physical incorporation between TDCIPP and foams without chemical bond (Betts, 2013; Zhao et al., 2020). Many countries in Northern America and Europe have restricted the usage of TDCIPP (OEHHA, 2017; Canada Safety, 2018). The United States Environmental Protection Agency (US EPA) has set the daily dose limit for TDCIPP at 0.02 mg/kg (US EPA, 2017). Results showed that TDCIPP exhibited normal physiological development in juvenile rats, while disturbed the endocrine secretion in adults (Kamishima et al., 2018). In addition, ataxia gait, hyperactivity and convulsion were observed in mice after treated with TDCIPP for 14 days (Kamata et al., 1989).

Numerous studies have detected PFRs in ordinary home and workplace environments (Zheng et al., 2017; Stapleton et al., 2014). A recent study demonstrated that TDCIPP (19%) was one of the prominent organophosphorus esters in indoor floor dust (Zhao et al., 2020). In addition, significant levels of PFRs have been detected in human samples, including serum (Li et al., 2017), urine (Cooper et al., 2011), hair (Qiao et al., 2016), nail (Liu et al., 2016), breast milk (Kim et al., 2014) and placenta (Ding et al., 2016). PFRs is rapidly metabolized in biota due to their non-persistent characteristic (Lynn et al., 1981; Nomeir et al., 1981; Sasaki et al., 1984). There were few studies concerning distributions of PFRs in creatures previously. In previous studies on distribution of TDCIPP *in vivo* (rat), a radiolabel experiment from Lynn et al. (1981) demonstrated that TDCIPP significantly decreased by 30 min and less than 19% was recovered, no TDCIPP was detected in any tissue 24 h after dosing. Results showed that TDCIPP had a short half-life and a rapid biotransformation rate, and it was eliminated primarily by excretion with a recovery of 63% in urine and feces, in the form of bis (1,3-dichloro-2-propyl) phosphate (BDCIPP) (Lynn et al., 1981). The half-life of TDCIPP in rat depends on the tissues, ranging from 1.5 to 5.4 h (Nomeir et al., 1981).

Limited *in vivo* studies have demonstrated the biotransformation pathways of PFRs. The general biotransformation pathways of PFRs include phase I O-dealkylation, oxidative dehalogenation, hydroxylation, carboxylation, and phase II conjugation (such as glutathione conjugate), producing a wide variety of metabolites (Hou et al., 2016; Van den Eede et al., 2013a). BDCIPP, a di-alkyl phosphates (DAPs) via O-dealkylation biotransformation of TDCIPP, was described as the major metabolite of TDCIPP *in vitro* in human liver microsomes (HLM), human liver S9 fractions and human urine samples (Van den Eede et al., 2013a, 2013b; Carignan et al., 2013; Cooper et al., 2011; Yan et al., 2018). Therefore, BDCIPP is considered as a biomarker of human exposure to TDCIPP. In addition, previous studies also found hydroxylated TDCIPP (OH-TDCIPP), carboxylated TDCIPP (COOH-TDCIPP) and hydroxylated BDCIPP (OH-BDCIPP) in HLM and human liver S9 fractions (Van den Eede et al., 2013a), as the potential metabolites of TDCIPP.

It is important to understand the distribution and biotransformation of PFRs and metabolites of PFRs in tissues. It will not only help to understand the biotransformation and behavior of PFRs in the body, but also help to evaluate the complex toxic effects on different tissues. However, only few researches referred to the tissue-dependent enrichment of PFRs. Hou et al. (2017) found that TDCIPP was higher in liver than kidney and muscles and Tang et al. (2019) indicated a higher level of BDCIPP in liver than kidney samples in common carp (*Cyprinus carpio*). Similarly, Wang et al. (2017b) found that the concentration of BDCIPP was higher in liver than muscle in zebrafish.

Currently, the matrices for the biomonitoring of human exposure are diversified, including invasive matrices such as blood, and non-invasive alternatives such as urine, hair and nails. Blood sampling lead to invasive process and ethical problems especially when children are involved (Kucharska et al., 2015). In addition, due to the short half-lives of PFRs, urine could not be selected as a suitable biomarker during long-term exposure (Lynn et al., 1981; Cooper et al., 2011). Hair and nails, by contrast, are easier and cheaper in the sampling process. Moreover, with the characteristics of slow growing rate, contaminants levels in hair reflect the long-term exposure of contaminants (Liu et al., 2016). Human hair has been proved to be a useful indicator of PFR exposure (Qiao et al., 2016), but it is still unclear if hair can be used to monitor metabolites of PFRs.

In the present study, we investigated the concentrations of TDCIPP and relevant metabolites in various tissues (liver, kidney, muscle, and hair) and excreta (feces and urine) of mice exposed to TDCIPP (300 mg/kg body weight (bw)/day) for 35 consecutive days. The main objective of this study was to reveal the tissue distribution of TDCIPP and metabolites, and to further assess the potential of hair and excreta as indicators of PFRs and PFR metabolites.

2. Materials and methods

2.1. Chemicals and reagents

Standard of tris (1,3-dichloro-2-propyl) phosphate (TDCIPP, ≥ 97%) was purchased from Accustandard (NH, USA), TDCIPP-d_{15} (≥95%) was purchased from Toronto Research Chemicals (Toronto, Canada), bis (1,3-dichloro-2-propyl) phosphate (BDCIPP, ≥ 95%) and BDCIPP-d_{10} (≥98%) were purchased from Wellington Laboratories (Guelph, Canada). Technical product of TDCIPP for oral exposure was purchased from J&K Scientific Ltd. (Beijing, China). SPF-grade feed and laboratory animal water dispenser for mice were purchased from Beijing Keao xieli feed Co. Ltd. (Beijing, China) and Beijing muniuliuma purification Engineering Technology Co. Ltd. (Beijing, China), respectively. Bedding materials were purchased from Beijing Keao xieli feed Co. Ltd. (Beijing, China) and Guangzhou saibainuo biotechnology co. Ltd. (Guangzhou, China). All the solvents used in this experiment were HPLC-grade that purchased from Merck Chemicals (Germany). Z-SEP and DSC-18 sorbents were purchased from Supelco (Bellefonte, PA, USA). The SPE cartridges were Oasis HLB (60 mg/3 mL, Waters Corporation, Milford, USA), Strata-X-AW (60 mg/3 mL, Phenomenex, Torrance, CA), CNWBOND Florisil (500 mg/6 mL, ANPEL, Shanghai, China), CNWBOND NH_2 (60 mg/3 mL, ANPEL, Shanghai, China), CNWBOND WAX (60 mg/3 mL, ANPEL, Shanghai, China), and CNWBOND HC-C18 (60 mg/3 mL, ANPEL, Shanghai, China).

2.2. Animal exposure experiment and sample collection

C57BL/6 mice (4 weeks old, $n = 20$, male) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd and raised in SPF-level laboratory (maintained at 20–22 °C, 40–60% humidity, with a 12-h light: 12-h dark cycle) in Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences. Bedding materials were mixture of wood chips and corn cobs (1:1, w/w), mice were fed with SPF-grade special feed and sterile water, the sterile water was filtered through membranes (5 μm and nano-filtration membranes) and UV sterilized. After one week of adaptive feeding, mice were randomly divided into control group ($n = 10$) and TDCIPP exposed group ($n = 10$). Subsequently, two groups were administered with intragastric gavage on 10 a. m. every day for 35 consecutive days. Control group used 0.1 mL of pure corn oil and TDCIPP exposed group used 0.1 mL of corn oil mixed with TDCIPP

(300 mg/kg bw). Body weight was recorded every week. It was reported that the LD_{50} of TDCIPP by oral intake was 2670 mg/kg for male mice (World Health Organization, 1998), and the lowest observed level (LOEL) of maternal toxicity and fetotoxicity were 200 mg/kg and 400 mg/kg, respectively (Tanaka et al., 1981). In order to identify more potential metabolites under full mass mode based on LC-Q-TOF, a relatively high dose 300 mg/kg (~1/10 LD_{50}) was chosen as the oral administered dose in present study.

By the end of 35 days of exposure, mice were transferred to individual metabolic cages, urine samples of 12 h and fresh feces samples were collected. Subsequently, mice were sacrificed, a total of six kinds of samples (liver, kidney, muscle (crureus), hair, urine and feces, $n = 20$) were collected for analysis. All the samples, except for urine, were freeze-dried after weighted, then ground into powder and stored at −20 °C until further analysis.

2.3. Extraction and clean-up of TDCIPP and metabolites

The extraction and clean-up methods of TDCIPP and metabolites in matrices except for urine were optimized based on previous studies by Liu et al. (2018) and Tang et al. (2019). Briefly, for the extraction of TDCIPP and BDCIPP in tissues, hair and feces samples, about 10–150 mg powdered sample was spiked with 10 ng internal standards (IS) mixture (TDCIPP-d_{15} and BDCIPP-d_{10}), then the mixture was extracted with 1 mL methanol for three times by ultrasonication for 30 min and centrifuged at 3500 rpm for 15 min after each extraction, the supernatant was combined and transferred to a pre-cleaned glass tube. The extract was then stored in −20 °C for 10 h to freeze the lipid after being evaporated to 500 μL with a gentle nitrogen flow. The supernatant was collected in a new glass tube. Sample was administrated with these steps above for LC-Q-TOF/MS analysis ($n = 3$ for each group), subsequently, sample was centrifuged at 10,000 rpm for 20 min and was concentrated to 100 μL under a gentle stream of nitrogen until analysis. While, further clean-up steps were performed to eliminate the remaining lipids and interferences for LC-MS/MS analysis ($n = 7$ for each group). The extract was mixed with 9.5 mL ultrapure water to form 5% methanol-aqueous solution and the mixture was cleaned on an Oasis HLB cartridge (60 mg/3 mL, Waters Corporation, Milford, USA). The SPE cartridge was pre-washed with 5 mL each of ethyl acetate, methanol and ultrapure water to condition the column. Then, loaded the sample and the cartridge was dried for 20 min under a gentle nitrogen flow. The first fraction was eluted with 5 mL ethyl acetate, the second fraction was eluted with 5 mL methanol, and these two fractions were combined. The residue was re-dissolved in 500 μL methanol after evaporation to near dryness using gentle nitrogen flow, and it was filtered through a 0.22 μm filter and finally evaporated and reconstituted in 50 μL methanol for LC-MS/MS analysis.

Urine samples were extracted and purified referred to the method reported by Zhang et al. (2020), with some modifications applied, based on the characteristics of mice urine samples. About 250 μL of urine sample was spiked with 15 ng each of internal standards (TDCIPP-d_{15} and BDCIPP-d_{10}) and diluted with 2 mL sodium acetate buffer (pH = 5), then added 10 mL enzyme (1000 units/mL glucuronidase and 33 units/mL sulfatase) and gentle vortexed for 2 min. The mixture was incubated for 12 h at 37 °C. The mixture was extracted with 5 mL methyl tert-butyl ether (MTBE) and 5 mL dichloromethane/n-hexane (4:1, v/v) with vortex oscillator at 2500 rpm for 8 min, followed by centrifugation at 4000 rpm for 8 min to separate the organic phase and aqueous phase. The above steps were repeated twice. The supernatant was organic phase that contained TDCIPP, while metabolites were contained in the aqueous phase below. The supernatant was combined in the same bottle and redissolved in n-hexane. A further clean-up procedure was performed for the aqueous phase using a Strata-X-AW cartridge before LC-Q-TOF analysis ($n = 3$ for each group). Cartridge was conditioned with 6 mL each of acetonitrile and ultrapure water, then the cartridge was loaded with aqueous phase and washed with ultrapure water. Then, the cartridge was dried for 20 min under a gentle nitrogen stream and eluted with 6 mL acetonitrile which contained 5% triethylamine (v/v) and solvent exchange to n-hexane. Combined the elution with organic phase extracted before, the mixture was evaporated and reconstituted with 500 μL methanol. Supernatant was centrifuged at 10,000 rpm for 20 min and evaporated to 100 μL after freezing at −20 °C for 10 h. The clean-up method of aqueous phase for LC-MS/MS ($n = 7$ for each group) analysis was administrated consistent with the sample preparation for LC-Q-TOF analysis above, while the organic phase required further clean-up steps as follows. First, Florisil cartridge (ANPEL, Shanghai, China, 500 mg/6 mL) was conditioned with 6 mL each of ethyl acetate and n-hexane. Then, the cartridge was eluted with 6 mL ethyl acetate after loading the organic phase. Combined the elution with the fraction eluted from Strata X-AW cartridge, extract was evaporated under a gentle nitrogen flow and reconstituted with 450 μL methanol. After that, the sample was stored at −20 °C for 10 h to precipitate impurities and filtered with a 0.22 μm filter. Finally, the extract was evaporated under a gentle nitrogen flow and reconstituted with 150 μL methanol/water (1:1, v/v) for LC-MS/MS analysis.

2.4. Instrumentation and LC-MS/MS conditions

TDCIPP and BDCIPP were measured by 1260 Agilent (Santa Clara, California, USA) liquid chromatography with a Poroshell 120 EC-C18 column (4.6 mm × 50 mm, 2.7 μm (Agilent, Santa Clara, California, USA), and coupled with an AB SCIEX API4000 + MS/MS (Applied Biosystems, Foster City, CA) in selected ion monitoring mode. The mobile phases were methanol (A) and water containing 10 mM ammonium acetate (B). Isocratic elution program was used for TDCIPP and BDCIPP analysis.

The elution program for TDCIPP analysis was as follows: 0–6 min, 90% A and 10% B. The flow rate was 0.25 mL/min with an injection volume of 5 μL. TDCIPP was quantified under multiple reaction monitoring (MRM) condition, using an electrospray ionization (ESI) source with electrospray positive ion mode (+). The parameters were as follows: gas temperature, 250 °C; gas flow, 10 L/min; nebulizer, 40 psi; capillary voltage, 3000 V; dwell time, 200 ms. The details of m/z of compounds and other instrument parameters were provided in Supplementary Information (SI), Table S1. The elution program for BDCIPP analysis was as follows: 0–4 min, 70% A and 30% B. The flow rate was 0.5 mL/min with an injection volume of 5 μL. BDCIPP was quantified under multiple reaction monitoring (MRM) conditions, using an electrospray ionization (ESI) source with an electrospray negative ion mode (−). The optimized parameters were as follows: gas temperature, 250 °C; gas flow, 10 L/min; nebulizer, 40 psi; capillary voltage, 3000 V; dwell time, 200 ms. The details of m/z and other instrument parameters were provided in Tables S1 and SI.

2.5. Instrumentation and LC-Q-TOF/MS conditions

Separation of TDCIPP and metabolites was achieved using an ACQUTY UPLC I-Class system (Waters Corp., Milford, MA, USA) with an ACQUITY UPLC@ HSS T3 column (2.1 × 100 mm, 1.8 μm) (Waters Corp., Milford, MA, USA). Detailed descriptions of LC-Q-TOF/MS procedures were given in the SI.

2.6. Quality control (QC)

Quality control included spiking of internal standards (IS),

regular analysis of procedural blanks, low- and high-level spiked blanks, low- and high-level spiked matrices, and replicate samples. Mice liver from control group and solvent blank were spiked with low-level (1 ng) and high-level (15 ng) of both TDCIPP and BDCIPP, pooled urine and pure water blanks were spiked with low-level (15 ng) and high-level (150 ng) of both TDCIPP and BDCIPP. Details of recoveries and matrix effects were described in SI. IS recoveries and method quantification limits (MQLs) were calculated. The MQLs were set as the mean value of target compounds detected in procedure blanks plus three times of standard deviations.

The linearity of the LC-MS/MS method was tested with standard mixtures at eleven levels of concentration for TDCIPP ranged from 0.5 to 1000 ng/mL, and nine levels for BDCIPP ranged from 2 to 1000 ng/mL. The correlation coefficients of standard curve ranged from 0.99920 to 0.99995. Spiked matrixes and blanks were used to verify the feasibility of the developed method. The recoveries of TDCIPP and BDCIPP were expressed as mean values ± relative standard deviation (mean ± RSD%). The samples were repeatedly extracted ($n = 3$).

The recoveries of all compounds were relative recoveries after adjusted with internal standards. TDCIPP recovery was 58.0 ± 3.1% in low-level spiked liver and 84.6 ± 0.3% in high-level spiked liver, respectively. Due to the high concentrations of TDCIPP in mice samples, and the recovery of high-level spiked liver reached 84.6 ± 0.3%. Therefore, we consider the current method to be acceptable for quantification of TDCIPP in mice samples. BDCIPP recoveries were 144.0 ± 3.5% in low-level spiked liver and 113.2 ± 2.6% in high-level spiked liver. In urine samples, TDCIPP recoveries were 107.6 ± 0.9% and 101.2 ± 0.6% in low and high spiked level, respectively; BDCIPP recoveries were 92.3 ± 0.02% and 78.4 ± 0.4% in low and high spiked level, respectively. The recoveries were provided in Tables S6 and SI. Matrix effects were analysis according to Gosetti et al. (2010), matrix effects (ME%) lower or higher than 100% indicated the suppression or enhancement of analyte signals by co-eluted contents, respectively. Details of matrix effects were shown in Tables S7 and SI. Ionization suppression was observed in liver samples, mean ME% of liver samples was 42.3% for TDCIPP and 50% for BDCIPP, and for internal standards, ME% showed similar ionization suppression. We also found suppression of matrix effects for BDCIPP in urine samples, with mean ME% of 64.6%. While a slight enhance of ionization was observed for TDCIPP in urine samples, with mean ME% of 102.1%.

Three procedural blanks were run simultaneously for each batch of samples to assess background contamination. Subtract the average blank level for each batch from the sample results. MQLs for TDCIPP and BDCIPP were 4.60 ng/g ww and 2.91 ng/g ww for mice tissues respectively, 8.08 ng/mL and 8.31 ng/mL for mice urine samples respectively.

2.7. Statistical analysis

Statistical analysis was performed by the SPSS 22 software (SPSS, Inc., Chicago, IL), and expressed as mean values ± standard error of the mean (SEM). The statistical differences in the weights of mice, and the levels of TDCIPP and BDCIPP among mice tissues were analyzed by one-way analysis of variance (ANOVA). The level of significance was set at $p = 0.05$.

3. Results and discussion

3.1. Optimization of extraction and clean-up conditions

Given the various matrices analyzed in the current study, it is impossible to validate the extraction methods for different matrices. Thus, we made some modifications based on the previous methods to analyze TDCIPP and metabolites in tissues (liver, kidney, muscle and hair) and feces. As for the extraction of TDCIPP and metabolites in urine samples, we only adjusted the urine sample volume, internal standard volume and the final reconstituted volume, other steps were same as reported (Zhang et al., 2020).

The optimization steps for tissues and feces contained extraction, lipid removal and solid phase extraction. In the present study, methanol and the mixture of methanol and dichloromethane (1:1, 2:1, 3:1, v/v) were used as tested extraction solvents. The polarity of BDCIPP was much higher than TDCIPP, therefore, it is difficult to extract them with the same solvent and achieve high recoveries for both. When only methanol was used, the recovery of TDCIPP and BDCIPP was 56.7 ± 9.7% and 76.1 ± 1.5%, respectively. The main reason for the low recoveries was that when the extraction solvent was optimized as a variable, the purification process was not optimized, and the effects of lipids and impurities would affect the recoveries. Considering the high repeatability of the methanol group, we considered this recovery to be acceptable. Therefore, methanol was selected as the extraction solvent. Details of the comparison of recoveries were shown in Tables S2 and SI. Reducing the lipid interferences is important in sample preparation, freezing-lipid precipitation was used to further clean-up after solvent extraction, details were shown in Section 2, SI. After freezing-lipid precipitation, there was still lipid remained in the extract and unable for the LC-MS/MS analysis, thus, we used SPE cartridges to further clean-up. The tested SPE cartridges were Oasis HLB, CNWBOND Florisil, Strata X-AW, CNWBOND NH_2, CNWBOND HC-C18 SPE and CNWBOND WAX cartridges, detailed procures can be found in Section 2, SI. After optimization, we finally chose Oasis HLB for further purification.

3.2. Body weight and tissue distribution of TDCIPP and BDCIPP

3.2.1. Body weight of mice

The body weight of mice in control group and TDCIPP exposed group during the exposure period were shown in Fig. 1, there were no significant difference in body weight between control group and TDCIPP exposed group. In addition, no abnormal behavior and no visible aberrations or abnormal behavior were observed in mice throughout the exposure period. Current results indicated that the weight gain and physiological appearance of mice were not

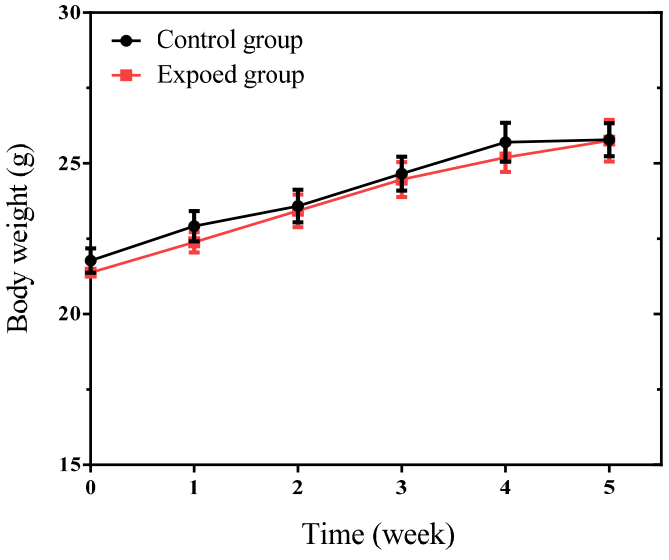


Fig. 1. Body weight of mice in control group and TDCIPP exposed group.

significantly affected by 300 mg/kg bw/day TDCIPP during 35 days.

3.2.2. Pollutants in mice tissues

The concentrations of TDCIPP and BDCIPP in different tissues and excreta of mice were shown in Fig. 2 and Table 1. The concentrations were calculated by wet weight-based units, as PFRs had a weak correlation with lipid content in tissues (Tang et al., 2019; Greaves and Letcher, 2014; Kim et al., 2011). The concentrations of TDCIPP and BDCIPP were adjusted with internal standards. Due to the high concentration of compounds in the mice urine, we diluted the extract and re-added the internal standards before the LC-MS/MS analysis, so that the concentrations of the samples were within the range of the standard curve (2–1000 ng/mL). Although seven mice were set as replicates, individual differences still existed. Therefore, in the present study, we excluded two outliers in muscle samples (5805.4 ng/g ww for TDCIPP and 124.2 ng/g ww for BDCIPP) from the same mouse in control group. In control mice samples, we also detected a certain amount of TDCIPP and BDCIPP which were significantly lower than that of the exposed group. These compounds in the control group may be derived from the feed, drinking water, cages, and litter of mice, as the diet of the mice was not specially treated.

The concentration of TDCIPP was highest in muscle (535.7 ± 192.2 ng/g ww), followed by liver (186.9 ± 55.0 ng/g ww) and kidney (43.5 ± 12.0 ng/g ww) (Fig. 2 and Table 1). TDCIPP concentrations in kidney and liver were significantly lower than that in muscle ($p < 0.01$ and $p < 0.05$ for kidney and liver, respectively), while there was no significant difference between TDCIPP concentrations in kidney and liver. The order of BDCIPP concentrations in tissues was opposite to that of TDCIPP: kidney (2189.2 ± 420.7 ng/g ww) > liver (1337.1 ± 249.6 ng/g ww) > muscle (162.5 ± 37.1 ng/g ww) (Fig. 2 and Table 1). Significant differences were observed among kidney, liver and muscle ($p < 0.05$), especially between kidney and muscle ($p < 0.001$).

The order for compositions of TDCIPP was found as muscle (68.6%) > liver (11.6%) > kidney (1.9%) (Fig. 3). Ratios of BDCIPP levels/TDCIPP levels were calculated for individual types of samples to explore the different accumulation patterns of TDCIPP and BDCIPP. BDCIPP/TDCIPP ratio in kidney (59.3 ± 8.4) was much higher than those of liver (7.90 ± 0.6) and muscle (0.65 ± 0.28) ($p < 0.05$). The results exhibited that TDCIPP had a higher enrichment in muscle while BDCIPP was preferentially accumulated in kidney. Passive diffusion is the main mode of xenobiotic chemicals *in vivo*, and the distribution of compounds in tissues is mainly related to the blood perfusion rate and membrane permeability. The material exchange between tissue and blood is rapid in well blood-perfused tissues such as liver and kidney, but relatively slow between muscle and blood (Zheng et al., 2014). After long-term exposure to TDCIPP, the liver and kidney deposit more BDCIPP due to rapid blood exchange. BDCIPP was prone to be eliminated by urine, which may result in the high levels of BDCIPP in kidney. Blood perfusion is one of the possible factors affecting the distribution of compounds in mice, while more research is needed to clarify the detailed enrichment process and mechanism.

Studies focused on bioaccumulation of PFRs in tissues of human or rodents were limited. Lynn et al. (1981) showed that BDCIPP

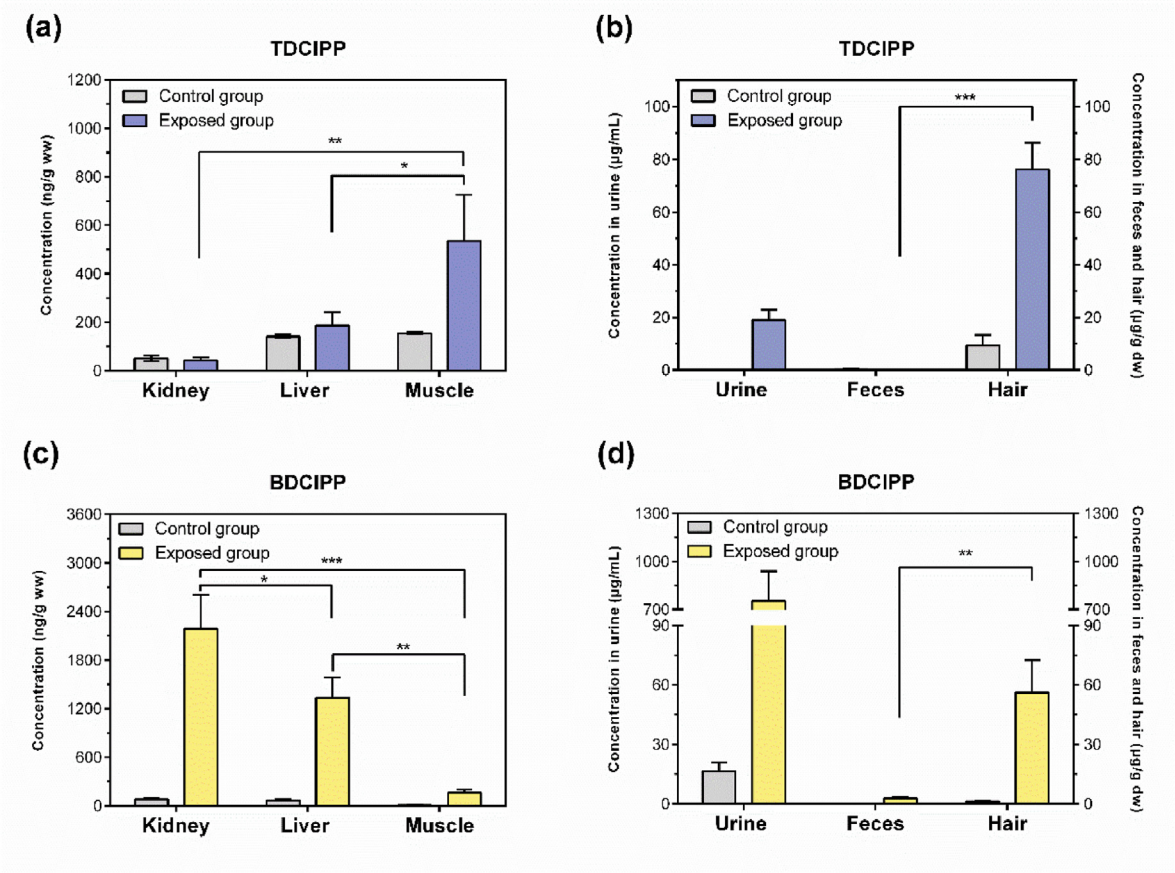


Fig. 2. Concentrations of TDCIPP and BDCIPP in different mice samples (ng/mL for urine and ng/g ww for other matrices): (a) TDCIPP in kidney, liver and muscle, (b) TDCIPP in urine, feces and hair, (c) BDCIPP in kidney, liver and muscle, (d) BDCIPP in urine, feces, and hair. "*", "**" and "***" represent significant differences between concentrations of compounds at the $p < 0.05$, $p < 0.01$ and $p < 0.001$ levels, respectively.

Table 1
The concentrations, masses and compositions of TDCIPP and BDCIPP in mice samples.

	Parameter	Kidney	Liver	Muscle	Unit	Hair	Feces	Unit	Urine	Unit
Control group										
Concentration	TDCIPP	51.6 ± 11.8 b	141.5 ± 6.7 a	155.0 ± 5.3 a	ng/g ww	9.3 ± 4.1	0.4 ± 0.2	μg/g dw	0.01 ± 0.00	μg/mL
	BDCIPP	82.2 ± 15.0 a	67.0 ± 13.5 a	27.0 ± 16.7 b	ng/g ww	1.3 ± 0.3	0.1 ± 0.1	μg/g dw	16.6 ± 4.4	μg/mL
Mass	TDCIPP	0.02 ± 0.00 b	0.19 ± 0.01 b	0.24 ± 0.02 b	μg	2.0 ± 0.8 a	–	μg	–	–
	BDCIPP	0.03 ± 0.01 b	0.09 ± 0.02 b	0.02 ± 0.01 b	μg	0.3 ± 0.1 a	–	μg	–	–
Composition	BDCIPP/TDCIPP Ratio	2.0 ± 0.5 b	0.5 ± 0.1 b	0.1 ± 0.0 b	–	0.4 ± 0.2 b	6.2 ± 4.9 b	–	2877.5 ± 1246.5 a	–
TDCIPP exposed group										
Concentration	TDCIPP	43.5 ± 12.0 b	186.9 ± 55.0 b	535.7 ± 192.2 a	ng/g ww	76.0 ± 10.2	0.24 ± 0.07	μg/g dw	19.0 ± 3.6	μg/mL
	BDCIPP	2189.2 ± 420.7 a	1337.1 ± 249.6 b	162.5 ± 37.1 c	ng/g ww	56.1 ± 16.4	2.77 ± 0.62	μg/g dw	754.4 ± 170.7	μg/mL
Mass	TDCIPP	0.02 ± 0.01 b	0.30 ± 0.09 b	0.79 ± 0.28 b	μg	17.9 ± 2.6 a	–	μg	–	–
	BDCIPP	0.98 ± 0.18 b	2.12 ± 0.41 b	0.26 ± 0.08 b	μg	13.0 ± 3.8 a	–	μg	–	–
Composition	BDCIPP/TDCIPP Ratio	59.3 ± 8.4 a	7.9 ± 0.6 cd	0.6 ± 0.3 d	–	0.8 ± 0.2 d	20.5 ± 6.9 c	–	39.5 ± 4.9 b	–

Note: Different letters within the mice tissues indicate significant differences between concentrations and masses of TDCIPP and BDCIPP at the $p < 0.05$ level.

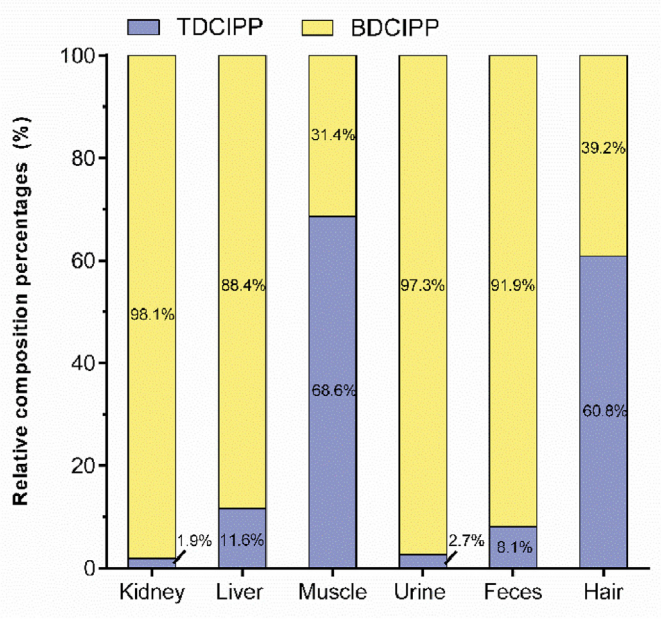


Fig. 3. Composition percentages (%) of TDCIPP and BDCIPP in different mice samples.

concentration was the highest in liver, then the kidney and lowest in muscle, which was similar to results in the present study. On contrary, higher TDCIPP concentration was observed in kidney than liver and muscle in rat, during 5–10 days dosing (Lynn et al., 1981; Nomeir et al., 1981; Minegishi et al., 1988). Tang et al. (2019) found that TDCIPP and BDCIPP preferred to accumulate in kidney and liver, compared to other tissues in common carp (*Cyprinus carpio*). Hou et al. (2017) also found that concentrations of TDCIPP in freshwater fish ranged as: liver > kidney > muscle. Studies on tissue distribution of PFRs showed different results, which can be attributed to the species-specific accumulation of PFRs and the exposure time. In the present study, mice were exposed for 35 days rather than an acute or short-term exposure in previous studies (Lynn et al., 1981; Nomeir et al., 1981; Minegishi et al., 1988).

3.2.3. Pollutants in mice hair, feces and urine samples

Concentrations of TDCIPP and BDCIPP in urine, feces and hair were showed in Fig. 2 and Table 1. Urine and feces are the major forms of excretion in organisms that removes xenobiotic chemicals and metabolites out of body. Urinary PFRs metabolites are considered as appropriate biomarkers of human exposure (Saillenfait et al., 2018). Concentrations of TDCIPP and BDCIPP in urine were 19.0 ± 3.6 μg/mL and 754.4 ± 170.7 μg/mL, respectively. Concentrations of TDCIPP and BDCIPP in feces were 0.24 ± 0.07 μg/g dw and 2.77 ± 0.62 μg/g dw, respectively. Compositions of BDCIPP were 97.3% and 91.9% in urine and feces, respectively. The significantly higher concentrations of BDCIPP than TDCIPP ($p < 0.05$) in urine and feces were consistent with previous studies (Lynn et al., 1981; Carignan et al., 2013), suggesting that TDCIPP was easily metabolized into BDCIPP and eliminated out of the body through excreta. Compared to feces, hair contained significantly higher ($p < 0.05$) concentrations of TDCIPP (76.0 ± 10.2 μg/g dw) and BDCIPP (56.1 ± 16.4 μg/g dw) due to its long growth time. TDCIPP levels were slightly higher than BDCIPP levels in hair samples. Because of the low metabolic activities and slow growth rate of hair (Liu et al., 2016), hair seems to be a good indicator of both parent compounds and relevant metabolites of PFRs.

3.2.4. The body burden of TDCIPP and BDCIPP

In connection with exposure assessment of PFRs, it is essential to know the contents of PFRs in different body parts of mice. The weight of each tissues in mice were showed in Fig. 4a, and masses of TDCIPP and BDCIPP in mice tissues were calculated in terms of the samples available in present study (Fig. 4b and Table 1). There was no significant difference in the weight of each tissues (kidney, liver, muscle (crureus) and hair) between control group and TDCIPP exposed group ($p > 0.05$). In this study we only calculated the proportion of crureus, and according to previous reports, muscle mass accounted for 40%–45% of body weight in mammals (Cheek et al., 1971). Hair was much lighter than other tissues in mice, while hair had significantly higher loads of TDCIPP (17.9 ± 2.6 μg) and BDCIPP (13.0 ± 3.8 μg), accounting 94.2% of TDCIPP and 79.5% of BDCIPP in the body. Muscle contributed secondly to the total load of TDCIPP (0.79 ± 0.28 μg), accounting 4.2% of the total contents of TDCIPP in mice, while in terms of BDCIPP, the contribution of muscle only accounted 1.6%. The mass of TDCIPP in liver was 0.30 ± 0.09 μg, accounting 1.6% of the total content. While in terms of BDCIPP, liver contributed secondly to the total load of BDCIPP

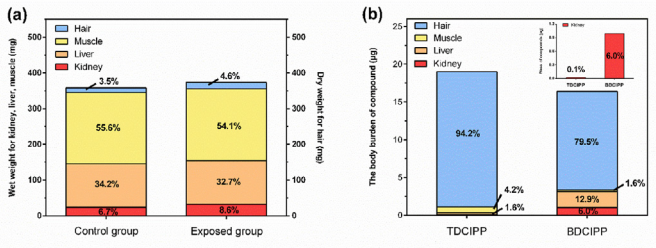


Fig. 4. Weight of mice tissues (mg) in control group and TDCIPP exposed group (a), body burden of TDCIPP and BDCIPP (μg) in mice tissues (b).

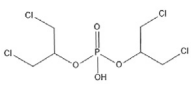
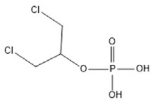
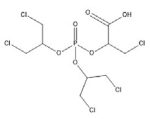
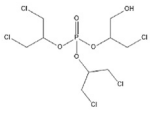
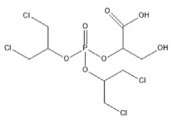
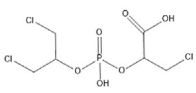
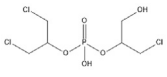
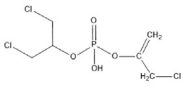
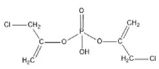
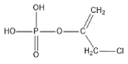
(2.12 ± 0.41 μg), accounting 12.9%. Kidney contributed negligible content of TDCIPP (0.02 ± 0.01 μg), only accounted 0.1% of the total load due to its low weight (8.6%) and lower concentrations of TDCIPP. While the content of BDCIPP in kidney (0.98 ± 0.18 μg, 6% of total contents) was not negligible, because of BDCIPP were mainly excreted through kidney. Present results showed that hair contributed most to the body burden of both TDCIPP and BDCIPP, muscle contributed secondly to the body burden of TDCIPP, while liver and kidney were target organs for body burden of BDCIPP.

3.3. LC-Q-TOF/MS detection of metabolites derived from TDCIPP

Although previous studies have identified the potential metabolites of TDCIPP in microsomes or cells *in vitro*, few studies have identified metabolites of TDCIPP *in vivo*. In the present study, metabolites of TDCIPP in mice samples (liver, kidney, muscle, hair, feces and urine) were identified using high resolution mass spectrometry Q-TOF by full mass in ESI positive and negative mode. Full mass scan was used over a mass range of m/z 50–800 Da with a 0.3 s scan time, and in data-dependent acquisition (DDA) mode mass spectrometry.

Except for the detection of TDCIPP and BDCIPP in hair and urine samples and MDCIPP in urine samples in control group, none of the metabolites was detectable in the control group. Twelve major metabolites, including bis (1,3-dichloro-2-propyl) phosphate (BDCIPP), 1,3-dichloro-2-propyl phosphate (MDCIPP), hydroxylated TDCIPP, hydroxylated BDCIPP and other potential metabolites, have been found in mice samples using the extracted ion method. Metabolites of TDCIPP in urine samples were shown as an example in Fig. S1 and Figs. S2 and SI which were the icon chromatograms and mass spectral (MS^2) of all metabolites, respectively. Five of the metabolites were identified in previous studies (BDCIPP, MDCIPP, TDCIPP-M1, TDCIPP-M2, TDCIPP-M5) (Lynn et al., 1981; Nomeir et al., 1981; Wang et al., 2017a; Van den Eede et al., 2013a). Five novel metabolites (TDCIPP-M3, M4, M6, M7, M10) in urine samples were also identified, with chromatographic retention times (RT) of

Table 2
Metabolites of TDCIPP in mice samples.

No.	Name	Formula	Structure	Mass (g/mol)	m/z[a]	RT in urine (min)	Liver	Kidney	Muscle	Hair	Feces	Urine
1	**BDCIPP** Bis (1,3-dichloro-2-propyl) phosphate	$C_6H_{11}Cl_4O_4P$		317.9149	318.9227	15.75	✓	✓	✓	✓	✓	✓
2	**MDCIPP** 1,3dichloro-2-propyl phosphate	$C_3H_7C_{l2}O_4P$		207.9459	208.9537	15.35	×	×	×	✓	✓	✓
3	**TDCIPP-M1**	$C_9H1_4Cl_5O_6P$		423.8971	424.9049	18.72	×	×	×	✓	×	✓
4	**TDCIPP-M2**	$C_9H_{16}Cl_5O_5P$		409.9178	410.9256	17.47	×	×	×	×	×	✓
5	**TDCIPP-M3**	$C_9H_{15}Cl_4O_6P$		389.9360	390.9438	18.42	×	×	×	×	×	✓
6	**TDCIPP-M4**	$C_6H_{10}Cl_3O_6P$		313.9281	314.9354	9.28	×	×	×	×	×	✓
7	**TDCIPP-M5**	$C_6H_{12}Cl_3O_5P$		299.9488	300.9566	9.11	×	×	×	✓	✓	✓
8	**TDCIPP-M6**	$C_6H_{10}Cl_3O_4P$		281.9382	282.9461	15.75	×	×	×	×	×	✓
9	**TDCIPP-M7**	$C_6H_{11}Cl_2O_4P$	–	247.9772	248.9850	15.04	×	×	×	×	×	✓
10	**TDCIPP-M8**	$C_6H_9Cl_2O_4P$		245.9616	244.9538 [a]	15.35	×	×	×	×	×	✓
11	**TDCIPP-M9**	$C_3H_6ClO_4P$		171.9692	170.9614[a]	7.55	×	×	×	×	×	✓
12	**TDCIPP-M10**	$C_3H_5O_4P$	–	135.9925	137.0004	15.75	×	×	×	×	×	✓

Note.
[a] All m/z of metabolites were showed in positive mode except for TDCIPP-M8 and TDCIPP-M9, which were only found in negative mode.

18.42, 9.28, 15.75, 15.04 and 15.75 min in positive mode, respectively (Fig. S1a). Two novel metabolites (TDCIPP-M8, M9) were identified in negative mode, with retention times of 15.35 and 7.55 min (Fig. S1b). The masses, exact chemical formulas and retention times of TDCIPP metabolites were summarized in Table 2.

In the liver, kidney, and muscle samples of mice, BDCIPP was detected as the sole metabolite of TDCIPP, indicating that BDCIPP was the primary metabolite enriched in tissues, as shown in Table 2. More abundant metabolites were detected in urine than other matrices. Eleven metabolites (MDCIPP, TDCIPP-M1~M10) were identified in urine samples, four kinds of TDCIPP metabolites (BDCIPP, MDCIPP, TDCIPP-M1, M5) were detected in hair samples, which had the second most metabolites except for urine. TDCIPP-M1 was considered to be the product of oxidative dehalogenation and reoxidation of TDCIPP (Van den Eede et al., 2013a), and TDCIPP-M5 was considered as a dehalogenation and hydroxylation product of BDCIPP. Metabolites in mice feces were similar to those in hair, including BDCIPP, MDCIPP and TDCIPP-M5, suggesting that dechlorination and oxidation of TDCIPP were important in the biotransformation pathways of TDCIPP *in vivo*.

Among all the twelve metabolites, BDCIPP, MDCIPP, TDCIPP-M1, TDCIPP-M2 and TDCIPP-M5 were identified in HLM, human liver S9 fractions and zebrafish (Van den Eede et al., 2013a; Wang et al., 2017a). The structures of seven novel metabolites (TDCIPP-M3, M4, M6~M10) identified in the present study were not validated because the exact position of the substituent cannot be confirmed by mass spectrometry (Fig. 5). Previous studies have identified two hydroxylated biomarkers of triphenyl phosphate (TPHP), OH-TPHP and $(OH)_2$-TPHP (Su et al., 2014), and they also found that TPHP metabolized to *p*- and *m*-hydroxy-TPHP before phase II conjugated with glucuronic acid (Su et al., 2015, 2016). Therefore, intermediates may be included in the metabolites we have identified. TDCIPP-M6, M8 and M9 might be the dehydrogenation after TDCIPP dehalogenation, and their unstable double bonds indicated that they may be intermediate products. Oxidative dehalogenation and hydroxylation were important pathways in the biotransformation of PFRs, resulted in TDCIPP-M2 and TDCIPP-M5, respectively. TDCIPP-M2 was a dechlorination product of TDCIPP (Fig. 5) (Wang et al., 2017a). TDCIPP-M1 could be a carboxylic acid product resulted from oxidative dehalogenation and a further oxidation of TDCIPP (Van den Eede et al., 2013a). In addition, the aldehyde in TDCIPP-M1 could also be transformed to alcohol by dehydrogenase, then formed TDCIPP-M2 (Fig. 5). The glutathione conjugated product was identified in rats *in vivo* (Lynn et al., 1981; Nomeir et al., 1981; Sasaki et al., 1984) and in human liver S9 fractions (Van den Eede et al., 2013a). In addition, a metabolite of sulfate

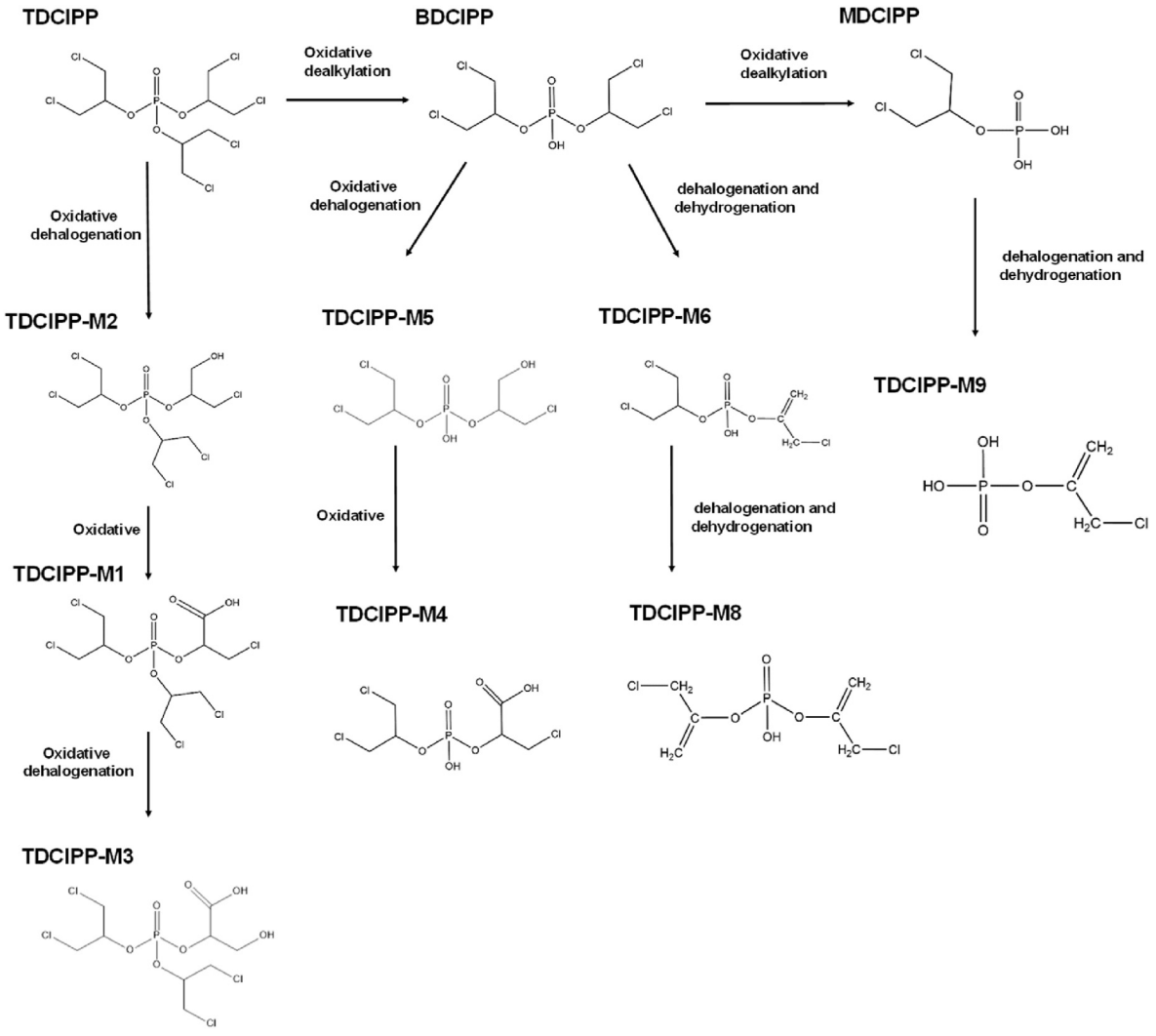


Fig. 5. TDCIPP biotransformation pathways in mice. Note: Metabolites were numbered in order of their relative molecular mass.

conjugates of hydroxylation was also identified in zebrafish (Wang et al., 2017a). However, neither of them was found in present study.

4. Conclusions

In present study, the tissue distribution of TDCIPP and its primary metabolite BDCIPP in different matrices from mice was investigated. The accumulation of TDCIPP and BDCIPP showed different profiles in tissues and excreta samples. The distribution of TDCIPP and BDCIPP in mice is tissue-specific, TDCIPP tends to accumulate in muscle, while BDCIPP tends to enrich in kidney and liver. BDCIPP is the predominant compound in urine and feces. Hair contributed most to the body burden of both TDCIPP and BDCIPP, and could serve as a reliable biomarker for both TDCIPP and BDCIPP. Twelve potential metabolites of TDCIPP were identified in various matrices and seven of them were firstly identified, mostly in urine samples of mice, which may contain metabolic intermediates. The main reactions involved in TDCIPP metabolic pathways were oxidative dealkylation (BDCIPP, MDCIPP), oxidative dehalogenation (TDCIPP-M2, M3, M5) plus reoxidation (TDCIPP-M1, M4), dehalogenation with dehydrogenation (TDCIPP-M6, M8, M9).

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ting Zhu: Methodology, Writing - original draft. **Xiao-Bo Zheng:** Methodology, Writing - review & editing. **Xiao Yan:** Validation, Project administration. **Bin Tang:** Software, Formal analysis. **Jing Zheng:** Conceptualization, Funding acquisition, Supervision. **Xiao-Jun Luo:** Writing - review & editing, Visualization. **Chun-You Zhu:** Software, Resources. **Yun-Jiang Yu:** Resources, Funding acquisition. **Bi-Xian Mai:** Writing - review & editing, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114595.

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