Urinary biomarkers for assessment of human exposure to monomeric aryl phosphate flame retardants

Fanrong Zhao, Qiyue Kang, Xiaohua Zhang, Jiaying Liu, Jianying Hu*

Laboratory for Earth Surface Processes, College of Urban and Environmental Sciences, Peking University, Beijing 100871, China

ARTICLE INFO
Handling Editor: Heather Stapleton
Keywords: Organophosphate flame retardants Biomarkers Metabolites Temporal variability

ABSTRACT
While monomeric aryl organophosphate flame retardants (m-aryl-OPFRs) are used worldwide in a variety of consumer products, specific biomarkers for epidemiologic studies are lacking. To explore the potential of urinary hydroxylated metabolites of m-aryl-OPFRs as the biomarkers, we detected triphenyl phosphate (TPHP), 2-ethylhexyl diphenyl phosphate (EHDPP), and tricresyl phosphate (TCrP) in 259 whole blood samples and their 5-hydroxylated and 2 diester metabolites in the paired urine samples from the general population. 2-Ethyl-5-hydroxyethyl diphenyl phosphate (5-OH-EHDPP), 4-hydroxyphenyl diphenyl phosphate (4-OH-TPHP), and 3-hydroxy-4-methylphenyl di-p-tolyldiphenyl phosphate (3-OH-MDTP) were detected in >80% of urine samples after enzymatic hydrolysis of conjugates, and their concentrations showed significant positive correlations with the blood concentrations of their corresponding parent compounds, respectively. To characterize the temporal reliability, the m-aryl-OPFRs metabolites were also determined in urine samples repeated nine times from six volunteers over 3 months. Urinary 5-OH-EHDPP showed strong temporal reliability (corrected intraclass correlation coefficients [ICCs], 0.77; 95% confidence interval [CI], 0.58 to 0.90), and urinary 3-OH-MDTP (corrected ICC, 0.52; 95% CI, 0.37 to 0.87) and 4-OH-TPHP (0.56; 95% CI, 0.32 to 0.80) showed moderate-to-strong temporal reliability, while relatively weak temporal reliability was found for urinary DPHP (corrected ICC, 0.37; 95% CI, 0.12 to 0.62). This study confirmed specific, reliable, and frequently detected biomarkers for TPHP and EHDPP and developed new biomarker of TCrP for future epidemiologic research on health effects of m-aryl-OPFRs.

1. Introduction

Monomeric aryl organophosphate flame retardants (m-aryl-OPFRs), which are among the most widely used groups of organophosphate flame retardants (OPFRs), mainly include triphenyl phosphate (TPHP), 2-ethylhexyl diphenyl phosphate (EHDPP), and tricresyl phosphate (TCrP). TPHP and TCrP are used as plasticizers, lubricants, and flame retardant chemicals and in hydraulic fluids and nail polish (Andresen et al., 2004; Mendelsohn et al., 2016; van der Veen and de Boer, 2012). EHDPP is mainly used as a flame retardant and a plasticizer and in rubber, paints, textile coatings, photograph film, adhesives, pigment dispersions, and food packaging applications (Brooke et al., 2009). Humans are exposed to m-aryl-OPFRs via various pathways, and these chemicals have been widely detected in blood, milk, tissue, seminal fluid, and even in the early embryos (Ding et al., 2016; Hudec et al., 1981; Lebel and Williams, 1986; Sundkvist et al., 2015; Zhao et al., 2016; Zhao et al., 2017). Adverse health issues such as neurotoxicity, cardiotoxicity, and reproductive toxicity have been observed in animals after exposure to m-aryl-OPFRs (Camarasa and Serra-Baldrich, 1992; Matthews et al., 1993; Patisaul et al., 2013). Thus, human exposure to m-aryl-OPFRs and their adverse effects on human health have raised concerns.

The metabolism of OPFRs involves hydrolysis to the corresponding diesters and glucuronide conjugates and excretion in urine (Van den Eede et al., 2013a). As for m-aryl-OPFRs, measurement of the urinary diester metabolite diphenyl phosphate (DPHP) has been used to assess human exposure to TPHP in epidemiologic studies (Cooper et al., 2011; Van den Eede et al., 2013b), and the urinary DPHP concentration has frequently been associated with outcomes of in vitro fertilization treatment (Carignan et al., 2017). However, DPHP is the metabolite not only of TPHP but also of EHDPP and oligomeric-aryl-OPFRs such as resorcinol bis(diphenyl phosphate) (Ballesteros-Gomez et al., 2015a; Ballesteros-Gomez et al., 2015b). When DPHP is used as a biomarker of TPHP, human exposure to TPHP is likely to be overestimated due to confounding via the contribution from other aryl organophosphate flame retardants (aryl-OPFRs) in the urine samples. A need thus exists to...
develop proper urinary biomarkers to assess exposure to m-aryl-OPFRs because they co-exist in indoor dust and food (Gunderson, 1995; Liu and Mabury, 2018). Several in vitro studies have reported that EHDPP, TPHP, and TCrP can be metabolized into monohydroxylated metabolites (Ballesteros-Gomez et al., 2015a; Van den Eede et al., 2013a; Su et al., 2015; Su et al., 2014), and 2-ethyl-5-hydroxyethyl diphenyl phosphate (5-OH-EHDPP), hydroxyl triphenyl phosphate (OH-TPHP), and OH-TPHP glucuronide have been detected in human urine samples (Araki et al., 2018; Su et al., 2016). Such hydroxylation metabolism pathways present an opportunity to develop exposure biomarkers that could specifically indicate exposure to each of the m-aryl-OPFRs in future epidemiologic studies.

In this study, five hydroxylated and two diester metabolites of EHDPP, TPHP, and TCrP were analyzed in 259 urine samples from the general population to understand whether they can be frequently detected. We also detected TPHP, EHDPP and TCrP in the paired 259 whole blood samples to investigate the relationship of concentrations in urine and blood. Since m-aryl-OPFRs can be rapidly metabolized, the concentrations of urinary metabolites in spot urine samples would present recent exposure and relatively weak temporal reliability in DPHP urinary concentrations has been reported (Meeker et al., 2013). Thus, we also evaluated the temporal variability of the urinary hydroxylated metabolites to assess the feasibility of their application in epidemiologic research.

2. Materials and methods

2.1. Standards and reagents

Four hydroxylated metabolites, 2-ethyl-3-hydroxyhexyl diphenyl phosphate (3-OH-EHDPP), 5-OH-EHDPP, 3-hydroxy-4-methylphenyl di-p-tolyl phosphate (3-OH-MDTP), and 4-(hydroxymethyl) phenyl di-p-tolyl phosphate (4-OH-MDTP) and one isotope-labeled internal standard 4-hydroxyphenyl diphenyl phosphate-d15 (4-OH-TPHP-d15) was supplied by C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada) and tri-p-cresyl-d21 phosphate (TCrP-d21) was from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Triphenyl-d15 phosphate (TPHP-d15) was supplied by C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada) and tri-p-cresyl-d13 phosphate (TCrP-d13) was from Hayashi Pure Chemical Ind. Ltd. (Osaka, Japan). All solvents and other materials are described in Supplemental Material.

2.2. Study population and urine collection

Two hundred fifty-nine residents between 20 and 50 years of age were recruited from the general population; the basic study details were previously described (Zhao et al., 2016). The participants visited a mobile examination center for a physical examination and to provide blood and urine samples. All participants were asked to fast for at least 10 h overnight before collection of the fasting blood sample. Six volunteers were recruited to evaluate the temporal variability of the potential exposure biomarkers, and a random urine sample was collected on each of three repeated days in a month for 3 months. All participants signed informed consent forms at the time of enrollment. Participants were asked by trained interviewers to complete interview questionnaires about their demographic information, including age, gender, body mass index (BMI), household income, alcohol consumption, and smoking intake. Blood and urine samples were collected in the morning using amber glass vials (CNW Technologies GmbH, Germany), pre-treated at 500 °C for 6 h, and stored at −80 °C until analysis. The study was approved by the Human Ethics Committee of Peking University (IRB00001052–12058).

2.3. Sample preparation and analysis

Urine samples were analyzed according to the previous paper (Van den Eede et al., 2013b) with some modification. Briefly, urine (0.5 mL) was spiked with 20 μL of surrogate standard mixture (50 ng/mL for each in acetonitrile) after thawing. Samples were adjusted to pH 6.5 by sodium acetate buffer (pH 5, 1 M) and then treated with 20 μL β-glucuronidase/aryl sulfatase enzyme solution (1000 units per mL in sodium acetate buffer) since metabolites would be excreted as conjugate forms in urine (Su et al., 2015). Samples were then incubated at 37 °C for 120 min and then loaded on Oasis WAX cartridges (Milford, MA, USA). Oasis WAX cartridges were pre-washed using 6 mL of 5% NH4OH in MeOH and equilibrated with 3 mL of sodium acetate buffer (pH5). The urine was diluted by 1.0 mL of sodium acetate buffer (pH5) and loaded to the well. After washing with 3 mL of 30% MeOH in water, 3 mL of 5% NH4OH in MeOH was used to elute the analytes from the WAX cartridges. The extracts were collected and evaporated to dryness, and then re-dissolved in 100 μL of 15% MeOH in water for UPLC-MS/MS analysis.

For measuring m-aryl-OPFRs metabolites in blood, each blood sample (0.5 mL) was spiked with 20 μL of an internal standard solution containing 10 ng/mL of 4-OH-TPHP-d10 and DPHP-d10 in acetonitrile. Ethyl acetate (2 mL) was added to the sample, which was shaken for 20 min on an orbital shaker and centrifuged at 4000 rpm for 10 min. Extraction from the residue was repeated twice, and the organic layers were combined, concentrated to near dryness under a gentle stream of nitrogen, and redissolved in 500 μL sodium acetate buffer (pH5, 1 M) and then treated with 20 μL β-glucuronidase/aryl sulfatase enzyme solution. After enzymatic treatment at 37 °C for 120 min, WAX-SPE cleanup processes was further performed using the same processes as that for the analysis of urinary m-aryl-OPFR metabolites. Detailed information about the UPLC-ESI-MS/MS analysis of m-aryl-OPFRs metabolites is shown in the Supplementary Material.

The analysis of m-aryl-OPFRs in blood was performed using our previously developed method (Zhao et al., 2016). The creatinine concentration in urine was measured using an enzymatic reaction on a Roche Hitachi chemistry analyzer (Roche Hitachi, Basel, Switzerland).

2.4. Quantification and quality control

In the analysis of urine samples, matrix-spiked recoveries were estimated via the analytical procedure by spiking 3-OH-EHDPP, 5-OH-EHDPP, 4-OH-TPHP, 3-OH-MDTP, and DPHP, and DCp in 0.5, 2.5, and 12.5 ng/mL into six urine sample matrices. The recoveries ranged from 76% ± 9% to 88% ± 4%, 81% ± 6% to 91% ± 5%, and 78% ± 5% to 92% ± 5% at the low, medium, and high concentrations, respectively, and the matrix effects ranged from −14.2% to −8.3% (Table S1). DPHP-d10 and 4-OH-TPHP-d10 were used as the surrogates for quantification of DPHP and DCrP and the five hydroxylated metabolites in samples, respectively. A procedural blank was analyzed in each batch of 10 samples to correct the sample values. The procedural blanks were prepared from 0.5 mL of ultrapure water, followed by passing through the entire analytical procedure to determine background values. We detected background DPHP and DCrP in the procedural blanks at 0.033 ± 0.015 ng/mL and 0.056 ± 0.021 ng/mL, respectively; therefore, the limits of quantification (LOQs) were set at 10 times the standard deviation of the procedural blanks, and the final concentrations of these compounds were blank-corrected. The LOQs for other chemicals were calculated based on signal-to-noise ratios of 10 in matrix-spiked samples. The LOQs of the m-aryl-OPFR...
metabolites in urine ranged from 0.001 to 0.21 ng/mL (Table S1).

For the analysis of blood samples, three parent m-aryl-OPFRs were spiked into the blood matrices at concentrations of 1, 5, and 10 ng/mL, and 7 m-aryl-OPFR metabolites were spiked at concentrations of 0.1, 0.5, and 5 ng/mL to estimate the matrix-spiked recoveries. The recoveries ranged from 80% ± 10% to 85% ± 8%, 83% ± 2% to 87% ± 4%, and 89% ± 6% to 92% ± 4% for m-aryl-OPFR metabolites at the low, medium, and high concentrations, respectively, and the matrix effects ranged from −12.1% to 1.9%. The LOQs of the m-aryl-OPFRs and their metabolites in blood were 0.10 to 0.14 ng/mL and 0.001 to 0.12 ng/mL, respectively (Table S2).

2.5. Statistical analysis

Data analysis was performed with SPSS (v22.0, IBM, Armonk, NY). When the concentrations were below the corresponding LOQs, the values of the LOQs divided by 2 were used for calculation and statistical analysis. To account for urine dilution, the concentrations of OPFR metabolites were adjusted for urinary creatinine concentration. For m-aryl-OPFR metabolites in urine as well as m-aryl-OPFRs in blood with detection frequencies of > 70%, their distributions were right-skewed, and thus we used the log-transformation of these variables for statistical analyses. Pearson correlation coefficients ($r_p$) were calculated to assess pairwise correlation for the log-transformed concentrations of the m-aryl-OPFR metabolites in urine samples and to assess the association between the log-transformed levels of the m-aryl-OPFR metabolites in urine and m-aryl-OPFR in blood. An alpha level of 0.05 was chosen for all tests, and $p$ values of < 0.05 were considered to indicate statistical significance in all statistical models.

Intraclass correlation coefficients (ICCs) and their 95% confidence intervals (CIs) were calculated to assess between-subjects and within-subject variability in the urinary biomarker concentrations using mixed random-effects models.

3. Results

Our analysis included 259 subjects (118 men and 141 women). The participants’ average age (± standard deviation) was 31.8 ± 6.8 years (range, 20 to 58 years). Most participants had a BMI between 15.7 and 36.9 kg/m² (median, 21.2 kg/m²). The volunteers recruited for the variability sub-study had BMIs between 17.1 and 22.5 kg/m², and their ages ranged from 22 to 29 years (26.2 ± 2.6 years), which was a relatively narrow range.

3.1. Urinary concentrations of biomarkers of m-aryl-OPFRs

Each of the five hydroxylated m-aryl-OPFR metabolites were detected in the urine samples after enzymatic hydrolysis of conjugates, and two diester metabolites were also analyzed for comparison. Typical reaction monitoring chromatograms are shown in Fig. S1. Of the seven OPFR metabolites, the urinary DPHP concentration was the highest, with a geometric mean (GM) of 0.40 ng/mg creatinine (< LOQ to 356.06 ng/mg creatinine), followed by 4-OH-TPHP, with a GM concentration of 0.09 (< LOQ to 26.1) ng/mg creatinine; their detection frequencies were both 80% (Table 1). The GM concentration of DPHP was about two fold lower than that (0.90 ng/mg creatinine) based on U.S. National Health and Nutrition Examination Survey (Ospina et al., 2018). The median uncorrected concentration of DPHP was 1.08 ng/mL, slightly lower than that (1.6 ng/mL) observed in pregnant women from central North Carolina (Hoffman et al., 2014), but much higher than that (0.27 ng/mL) observed in men from Massachusetts (Meeker et al., 2013). The urinary GM concentration of 5-OH-EHDPP (0.08 ng/mg creatinine; range, < LOQ to 35.06 ng/mg creatinine), followed by 4-OH-TPHP, with a GM concentration of 0.09 (< LOQ to 26.1) ng/mg creatinine; their detection frequencies were both 80% (Table 1). The GM concentration of 5-OH-EHDPP was much lower than that (0.90 ng/mg creatinine) based on U.S. National Health and Nutrition Examination Survey (Ospina et al., 2018). The median uncorrected concentration of DPHP was 1.08 ng/mL, slightly lower than that (1.6 ng/mL) observed in pregnant women from central North Carolina (Hoffman et al., 2014), but much higher than that (0.27 ng/mL) observed in men from Massachusetts (Meeker et al., 2013). The urinary GM concentration of 5-OH-EHDPP (0.08 ng/mg creatinine; range, < LOQ to 35.06 ng/mg creatinine), followed by 4-OH-TPHP, with a GM concentration of 0.09 (< LOQ to 26.1) ng/mg creatinine; their detection frequencies were both 80% (Table 1). The GM concentration of 5-OH-EHDPP was much lower than that (0.90 ng/mg creatinine) based on U.S. National Health and Nutrition Examination Survey (Ospina et al., 2018). The median uncorrected concentration of DPHP was 1.08 ng/mL, slightly lower than that (1.6 ng/mL) observed in pregnant women from central North Carolina (Hoffman et al., 2014), but much higher than that (0.27 ng/mL) observed in men from Massachusetts (Meeker et al., 2013). The urinary GM concentration of 5-OH-EHDPP (0.08 ng/mg creatinine; range, < LOQ to 35.06 ng/mg creatinine), followed by 4-OH-TPHP, with a GM concentration of 0.09 (< LOQ to 26.1) ng/mg creatinine; their detection frequencies were both 80% (Table 1). The GM concentration of 5-OH-EHDPP was much lower than that (0.90 ng/mg creatinine) based on U.S. National Health and Nutrition Examination Survey (Ospina et al., 2018). The median uncorrected concentration of DPHP was 1.08 ng/mL, slightly lower than that (1.6 ng/mL) observed in pregnant women from central North Carolina (Hoffman et al., 2014), but much higher than that (0.27 ng/mL) observed in men from Massachusetts (Meeker et al., 2013). The urinary GM concentration of 5-OH-EHDPP (0.08 ng/mg creatinine; range, < LOQ to 35.06 ng/mg creatinine), followed by 4-OH-TPHP, with a GM concentration of 0.09 (< LOQ to 26.1) ng/mg creatinine; their detection frequencies were both 80% (Table 1). The GM concentration of 5-OH-EHDPP was much lower than that (0.90 ng/mg creatinine) based on U.S. National Health and Nutrition Examination Survey (Ospina et al., 2018). The median uncorrected concentration of DPHP was 1.08 ng/mL, slightly lower than that (1.6 ng/mL) observed in pregnant women from central North Carolina (Hoffman et al., 2014), but much higher than that (0.27 ng/mL) observed in men from Massachusetts (Meeker et al., 2013). The urinary GM concentration of 5-OH-EHDPP (0.08 ng/mg creatinine; range, < LOQ to 35.06 ng/mg creatinine), followed by 4-OH-TPHP, with a GM concentration of 0.09 (< LOQ to 26.1) ng/mg creatinine; their detection frequencies were both 80% (Table 1). The GM concentration of 5-OH-EHDPP was much lower than that (0.90 ng/mg creatinine) based on U.S. National Health and Nutrition Examination Survey (Ospina et al., 2018). The median uncorrected concentration of DPHP was 1.08 ng/mL, slightly lower than that (1.6 ng/mL) observed in pregnant women from central North Carolina (Hoffman et al., 2014), but much higher than that (0.27 ng/mL) observed in men from Massachusetts (Meeker et al., 2013). The urinary GM concentration of 5-OH-EHDPP (0.08 ng/mg creatinine; range, < LOQ to 35.06 ng/mg creatinine), followed by 4-OH-TPHP, with a GM concentration of 0.09 (< LOQ to 26.1) ng/mg creatinine; their detection frequencies were both 80% (Table 1). The GM concentration of 5-OH-EHDPP was much lower than that (0.90 ng/mg creatinine) based on U.S. National Health and Nutrition Examination Survey (Ospina et al., 2018). The median uncorrected concentration of DPHP was 1.08 ng/mL, slightly lower than that (1.6 ng/mL) observed in pregnant women from central North Carolina (Hoffman et al., 2014), but much higher than that (0.27 ng/mL) observed in men from Massachusetts (Meeker et al., 2013).
3.2. Associations among urinary biomarkers

Unlike specific biomarkers such as 4-OH-TPHP for TPHP and 5-OH-EHDPP for EHDPP, DPHP has multiple precursors, including TPHP and EHDPP. Significant correlations were observed between 4-OH-TPHP and DPHP levels in urine (r = 0.451; p < 0.001) and between the urinary concentrations of 5-OH-EHDPP and DPHP (r = 0.220; p = 0.003), which suggests that TPHP and EHDPP both contributed to the urinary DPHP levels in our participants. The correlation between the concentrations of 5-OH-EHDPP and 4-OH-TPHP was weakly positive but not statistically significant (r = 0.148; p = 0.067; Table S3). Significant correlations were observed between 5-OH-EHDPP and 4-OH-MDTP (r = 0.200; p = 0.002) and between 5-OH-EHDPP and 3-OH-MDTP (r = 0.180; p = 0.011), which suggests common exposure sources for TCrP and EHDPP. No significant correlation was observed between urinary 4-OH-TPHP and 3-OH-MDTP (r = 0.021; p = 0.778) or between 4-OH-TPHP and 4-OH-MDTP (r = 0.053; p = 0.499).

3.3. Associations of urinary biomarkers with blood m-aryl-OPFRs

Although hydroxylated metabolites of m-aryl-OPFR were not detected in blood specimens, the detection frequencies of EHDPP, TPHP, and TCrP were 100%, 100%, and 70%, and their GM concentrations were 1.24 ng/mL (range, 0.38 to 3.30 ng/mL), 0.43 ng/mL (range, 0.15 to 1.51 ng/mL), and 0.03 ng/mL (range, < LOQ to 0.48 ng/mL), respectively (Table 2). DPHP was detected in only 13.9% of blood samples with GM concentrations of 0.003 (range, < LOQ to 3.31) ng/mL, which occupied 9% of the TPHP molar concentrations in blood samples. The urinary concentrations of 5-OH-EHDPP and 4-OH-TPHP showed positive correlations with the blood concentrations of EHDPP (r = 0.311; p < 0.001) and TPHP (r = 0.270; p < 0.001), respectively (Fig. 2). However, no significant associations were found between the blood concentrations of TPHP or EHDPP and the urinary concentrations of their common diester metabolite, DPHP (r = 0.070; p = 0.268 for TPHP and r = 0.016; p = 0.609 for EHDPP; Fig. S2). Significant positive correlations were also observed between the urinary concentrations of 3-OH-MDTP and the blood concentrations of TCrP (r = 0.297; p < 0.001) and between the urinary concentrations of 4-OH-MDTP and the blood concentrations of TCrP (r = 0.157; p = 0.007).

3.4. Variability assessment of new biomarkers

To assess the feasibility of 5-OH-EHDPP, 4-OH-TPHP, and 3-OH-MDTP as biomarkers of human exposure to m-aryl-OPFR, the variability in 5-OH-EHDPP, 4-OH-TPHP, 3-OH-MDTP, and DPHP concentrations were assessed in nine repeated urine samples over the course of 3 months. One volunteer had noticeably higher concentrations of 5-OH-EHDPP, 4-OH-TPHP, 3-OH-MDTP, and DPHP. The rank order of urine concentrations of 5-OH-EHDPP among the volunteers remained rather consistent over time (Fig. 3A), whereas it showed much more overlap for 4-OH-TPHP, 3-OH-MDTP, and DPHP (Fig. 3B, C and D). To evaluate their temporal reliabilities, ICCs and 95% CIs were calculated on the basis of the concentrations of 5-OH-EHDPP, 4-OH-TPHP, and 3-OH-MDTP over 3 months. Strong temporal reliability was observed for 5-OH-EHDPP (uncorrected ICC, 0.75; 95% CI, 0.55 to 0.95; Table 3). The creatinine-uncorrected ICCs were 0.52 (95% CI, 0.30 to 0.74) for 3-OH-MDTP and 0.48 (95% CI, 0.29 to 0.66) for 4-OH-TPHP. The uncorrected ICC of DPHP (0.32) was relatively low (95% CI, 0.11 to 0.53). The creatinine-corrected ICCs of 5-OH-EHDPP, 4-OH-TPHP, 3-OH-MDTP, and DPHP were 0.77 (95% CI, 0.58 to 0.90), 0.56 (95% CI, 0.32 to 0.80), 0.62 (95% CI, 0.37 to 0.87), and 0.37 (95% CI, 0.12 to 0.62), respectively, which were slightly higher than the uncorrected values.

4. Discussion

Validation of a biomarker requires demonstration of its specificity and ubiquity in samples. Studies have shown that DPHP is not a specific biomarker for TPHP exposure (Ballesteros-Gomez et al., 2015a; Ballesteros-Gomez et al., 2015b). On the basis of toxicokinetic investigations (Ballesteros-Gomez et al., 2015a; Van den Eede et al., 2013a), hydroxylated m-aryl-OPFRs have been reported as the metabolites of m-aryl-OPFRs and have undergone structural characterization for more specific indication of the human exposure of m-aryl-OPFRs. Furthermore, DPHP is ubiquitous in the environment, so background contamination of DPHP led to an LOQ 3 to 150 times higher than those of hydroxylated m-aryl-OPFRs. As a result, the detection frequencies of several hydroxylated m-aryl-OPFRs, including 5-OH-EHDPP, 4-OH-MDTP, 3-OH-MDTP, and 4-OH-TPHP, were even same or higher than that of DPHP in urine samples, even though their urinary concentrations were lower than that of DPHP.

---

**Table 1**

Urinary concentrations (ng/mg creatinine) of m-aryl-OPFR metabolites.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Detection frequency</th>
<th>GM ± GSD</th>
<th>Range</th>
<th>25%</th>
<th>Median</th>
<th>75%</th>
<th>95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-OH-EHDPP</td>
<td>93%</td>
<td>0.08 ± 0.46</td>
<td>&lt; LOQ - 65.5</td>
<td>0.04</td>
<td>0.09</td>
<td>0.18</td>
<td>0.40</td>
</tr>
<tr>
<td>4-OH-MDTP</td>
<td>93%</td>
<td>0.07 ± 0.33</td>
<td>&lt; LOQ - 4.66</td>
<td>0.02</td>
<td>0.08</td>
<td>0.24</td>
<td>0.53</td>
</tr>
<tr>
<td>3-OH-MDTP</td>
<td>90%</td>
<td>0.05 ± 0.27</td>
<td>&lt; LOQ - 4.38</td>
<td>0.02</td>
<td>0.05</td>
<td>0.08</td>
<td>0.16</td>
</tr>
<tr>
<td>DPHP</td>
<td>80%</td>
<td>0.40 ± 23.42</td>
<td>&lt; LOQ - 356</td>
<td>0.17</td>
<td>0.95</td>
<td>2.81</td>
<td>12.0</td>
</tr>
<tr>
<td>4-OH-TPHP</td>
<td>80%</td>
<td>0.09 ± 1.70</td>
<td>&lt; LOQ - 26.1</td>
<td>0.06</td>
<td>0.16</td>
<td>0.42</td>
<td>1.2</td>
</tr>
<tr>
<td>3-OH-EHDPP</td>
<td>23%</td>
<td>0.002 ± 0.034</td>
<td>&lt; LOQ - 32.2</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>0.08</td>
</tr>
<tr>
<td>DCP</td>
<td>21%</td>
<td>0.004 ± 1.965</td>
<td>&lt; LOQ - 25.9</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>1.7</td>
</tr>
</tbody>
</table>

* LOQ means limits of quantification. For 5-OH-EHDPP and 3-OH-EHDPP, LOQ is 0.0002 ng/mg creatinine; for 4-OH-MDTP, LOQ is 0.0006 ng/mg creatinine; for 3-OH-MDTP, LOQ is 0.0007 ng/mg creatinine; for 4-OH-TPHP, LOQ is 0.005 ng/mg creatinine; for DPHP, LOQ is 0.012 ng/mg creatinine; for DCP, LOQ is 0.019 ng/mg creatinine.

**Table 2**

Blood concentrations (ng/mL) of m-aryl-OPFRs (n = 259).

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Detection frequency</th>
<th>GM ± GSD</th>
<th>Range</th>
<th>25%</th>
<th>Median</th>
<th>75%</th>
<th>95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHDPP</td>
<td>100%</td>
<td>1.24 ± 0.43</td>
<td>0.38-3.30</td>
<td>1.01</td>
<td>1.23</td>
<td>1.49</td>
<td>2.05</td>
</tr>
<tr>
<td>TPHP</td>
<td>100%</td>
<td>0.43 ± 0.20</td>
<td>0.15-1.51</td>
<td>0.34</td>
<td>0.43</td>
<td>0.54</td>
<td>0.88</td>
</tr>
<tr>
<td>TCrP</td>
<td>70%</td>
<td>0.03 ± 0.09</td>
<td>&lt; LOQ - 0.48</td>
<td>&lt; LOQ</td>
<td>0.10</td>
<td>0.14</td>
<td>0.25</td>
</tr>
<tr>
<td>DPHP</td>
<td>9%</td>
<td>0.005 ± 0.021</td>
<td>&lt; LOQ - 3.31</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>1.74</td>
</tr>
<tr>
<td>DCP</td>
<td>4%</td>
<td>0.001 ± 0.011</td>
<td>&lt; LOQ - 2.21</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
</tr>
</tbody>
</table>

* LOQ means limits of quantification. For TCrP, LOQ is 0.10 ng/mL; for DPHP, LOQ is 0.12 ng/mL; for DCP, LOQ is 0.08 ng/mL.
Because it is easier to use the concentration in blood to interpret the toxico
tokinetics of a chemical (Kato et al., 2004), the blood concentration is preferred to evaluate adverse e
ff

fects from chemical exposure. We explored the relationship between the concentrations of m-aryl-OPFRs in blood and their metabolites in urine. Although no significant associations were found between the blood concentrations of TPHP or EHDPP and the urinary concentrations of their common diester metabolite, DPHP, the sum concentrations of blood TPHP and EHDPP were correlated with the urinary concentrations of DPHP, which suggests that TPHP and EHDPP were both main sources of DPHP. Given that DPHP is not a specific metabolite of TPHP or EHDPP, it is not a proper biomarker for assessment of the exposure and subsequent health effects of these two precursor triesters in the population. In contrast, positive correlations were observed between urinary levels of 5-OH-EHDPP and blood levels of EHDPP, between urinary levels of 4-OH-TPHP and blood

Table 3

<table>
<thead>
<tr>
<th>Urinary biomarker</th>
<th>Uncorrected ICCs (95% CIs)</th>
<th>Creatinine-corrected ICCs (95% CIs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-OH-EHDPP</td>
<td>0.75 (0.55–0.95)</td>
<td>0.77 (0.58–0.90)</td>
</tr>
<tr>
<td>4-OH-TPHP</td>
<td>0.48 (0.29–0.66)</td>
<td>0.56 (0.32–0.80)</td>
</tr>
<tr>
<td>3-OH-MDTP</td>
<td>0.52 (0.30–0.74)</td>
<td>0.62 (0.37–0.87)</td>
</tr>
<tr>
<td>DPHP</td>
<td>0.32 (0.11–0.53)</td>
<td>0.37 (0.12–0.62)</td>
</tr>
</tbody>
</table>

levels of TPHP, and between urinary levels of 3-OH-MDTP and blood levels of TCrP (Fig. 2), which indicates that the urinary levels of the hydroxylated m-aryl-OPFRs 5-OH-EHDPP, 4-OH-TPHP, and 3-OH-MDTP can be used as urinary biomarkers for evaluation of exposure to EHDPP, TPHP, and TCrP and for further estimation of their potential

Fig. 2. Correlation between the log-transformed concentrations of urinary m-aryl-OPFR metabolites (ng/mg creatinine) and blood m-aryl-OPFRs (ng/mL) in 259 participants.

Fig. 3. Concentrations (ng/mg creatinine) in urine from repeated samples collected from six volunteers over a 3-month period. Colors represent individual volunteers.
health effects in epidemiological studies.

The concentrations of urinary metabolites in spot urine samples would present recent exposure. When a single urine sample is used to define individual exposure of nonpersistent environmental contaminants in an epidemiologic study, the temporal reliability of the urinary biomarkers is of concern. The ICC ranged from zero to one, with values near zero indicating poor temporal reliability and values near one indicating high temporal reliability (Rosner, 2000). The variability of urinary 5-OH-EHDP concentration is relatively small (Fig. 3A), and the ICC reached 0.77, indicating stable sources. While relatively high variability was observed for 4-OH-TPHP and 3-OH-MDTP compared with 5-OH-EHDP (Fig. 3B, C), their ICCs were higher than 0.5, showing moderate-to-strong temporal reliability. The ICC of DPHP was quite consistent with that (uncorrected ICC, 0.36; 95% CI, 0.18 to 0.60) reported previously by Meeker et al. (2013). The relatively weak temporal reliability of urinary DPHP may be due to inconsistent changes in the exposure levels of its multiple precursors and short half-lives of DPHP and its parent compounds. The relatively weak temporal reliability of urinary DPHP may be due to inconsistent changes in the exposure levels of its multiple precursors and short half-life of DPHP.

Our results should be interpreted within the context of several limitations. The age distribution of the volunteers recruited for the study might affect the variability sub-study was narrow, which may limit our ability to generalize the results to a broader population. In this study, the correlations between concentrations of blood and urine samples were used to validate the robustness of the urinary biomarkers. Considering these results, we for the first time developed specific, reliable, and frequently detected biomarkers for assessment of human exposure to OPFRs, and identified that 5-OH-EHDP is a stable biomarker for EHDP exposure assessment. 4-OH-TPHP was confirmed as the more selective biomarkers for TPHP exposure assessment than the previously used biomarker DPHP due to its specificity, low level of background contamination, and relatively higher temporal reliability. This study provides biomarkers for future epidemiological research on m-aryl-OPFRs.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China [21737001, 21577001 and 41821005].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.01.022.

References


