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Exposure marker discovery of di-2(propylheptyl) phthalate using ultra-performance liquid chromatography-mass spectrometry and a rat model

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ABSTRACT

Di-(2-propylheptyl) phthalate (DPHP) is a plasticizer and has been suggested to be a subchronic toxicant in rats. DPHP has been approved to be used in food containers and handling by the U.S. Food and Drug Administration. The use of DPHP is still increasing, and the risk of human exposure to DPHP via food may be high. Exposure markers measured in human samples are commonly used to monitor human exposure levels. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) and a rat model were used to discover tentative DPHP exposure markers. DPHP and mono-(2-propylheptyl) phthalate (MPHP) were used as the precursors for calculating metabolite candidates using biotransformation mass changes of known enzymatic reactions. A rat model was designed to validate these metabolite candidates as tentative exposure markers. A total of 28 signals show dose–response relationships and these signals contain a few isomers. The chemical structures of 15 tentative exposure marker signals were speculated based on the product ion mass spectra from MS/MS analysis. These 15 signals included 7 chemical structures and some of them may be isomers. The different arrangement of the atoms in space of these isomers should be validated by standard compounds in the future studies. Among the 7 speculated chemical structures, 2 structures were novel tentative DPHP metabolites, and 5 structures have been previously reported in the literature. The results indicate that using UPLC-MS and a rat model can be used to identify tentative toxicant exposure markers.

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1. Introduction

Di-(2-propylheptyl) phthalate (DPHP) is intended as a plasticizer in polyvinyl chloride formulations and is a substitute for high molecular weight phthalates under scrutiny for their reproductive toxicity and suspected endocrine disrupting activity, such as di-(2-ethylhexyl)-phthalate (DEHP) and di-iso nonyl-phthalate (DINP). DPHP is used in high temperature-resistant products, such as carpet backing, car interiors, roofing membranes, and tarpaulins [1,2]. Furr et al. (2014) suggested that DPHP has not an impact on fetal testicular testosterone production [3]. Experimental evidence supported that DPHP was a subchronic toxicant [4]. For example, rats exposure to DPHP resulted in significant decreases in body weight and food consumption, significant changes in adrenal and liver histopathology, and increased incidence in soft tissue variations (such as dilated renal pelvis) [5]. Thus, DPHP may an adverse effect on human health.

The worldwide DPHP consumption increased from 196,000 metric tons (2011) to 208,000 metric tons (2012), and a consumption of 308,000 metric tons is expected in 2018 [6]. The general German population exposure to DPHP have been observed, and their detection rates increased from 3.3% in 2009 to 21.7% in 2012 [6]. The use of DPHP in food containers and handling has been approved by the U.S. Food and Drug Administration [4]. Like other phthalates, DPHP can migrate out of the polymer, because it is physically dissolved in the polymer. In addition, plasticizers have been illegally used in food as clouding agents in food and beverages in Taiwan [7]. Therefore, the risk of human exposure to DPHP via food may be high.

Exposure markers in human samples, such as blood, hair, and urine, are usually used to monitor the levels of human exposure to toxicants [6]. Toxicant metabolites are commonly used as exposure markers, as they can response human exposure levels and have specific structures related with toxicants [8]. Metabolite identification in biological matrices is more challenging, because biological matrices is extremely complex. So far, only four DPHP metabolites have been identified, including mono-(2-propylheptyl) phthalate (MPHP), mono-(propyl-6-oxo-heptyl) phthalate (oxo-MPHP), mono-(propyl-6-hydroxyheptyl) phthalate (OH-MPHP) and mono-(propyl-6-carboxyhexyl) phthalate (cx-MPHxP) [9]. Three (oxo-MPHP, OH-MPHP, and cx-MPHxP) of them have been used to assess human exposure levels [6]. In addition, one tentative DPHP metabolite (*m/z* 337.168) has been discovered recently [10].

Mass spectrometry (MS) is an analytical technique for identifying and quantifying chemicals via ionizing chemicals and sorting the ions. MS can be applied to measuring the extremely complex metabolism in organism samples. Recently, ultra performance liquid chromatography-mass spectrometry (UPLC-MS) has been develop to measure chemicals with high resolving power (150,000) and high mass accuracy (<5 ppm) [12]. UPLC-MS was used to measure the metabolisms of rat urine samples collected from rats administered different DPHP doses, and these detected metabolite signals and the corresponding raw abundances are expected to be accurate.

Several methods have been developed for identifying exposure markers. A traditional method is to predict possible metabolite structures of a toxicant via biotransformation mass changes of known enzymatic reactions, and it requires the synthesis of standard compounds and months to validate these predicted metabolites [8]. Mass spectrometry-based metabolomics data processing methods have emerged as an ideal approach for the fast identification of metabolite candidates, such as signal mining algorithm with isotope tracing, mass defect filter, and XCMS [12–14]. However, some chemical structures of these candidates cannot be speculated using MS/MS analysis, because they may not be the metabolites of the targeted compound. In contrast, predicting all possible metabolite candidates via biotransformation mass changes of known enzymatic reactions may provide a high possibility to identify the toxicant metabolites, and they have predicted chemical structures which may be more easily to be confirmed by MS/MS analysis.

The objective of this study was to identify DPHP exposure markers for human exposure assessments. Biotransformation mass changes of known enzymatic reactions were used to predict DPHP metabolite candidates. These candidates were measured in MS data of rat urine samples collected from rats administered different DPHP doses. A dose–response relationship of these candidate signals was assessed. Finally, the DPHP structure-related metabolites of these signals which show a dose–response relationship were confirmed using UPLC-MS/MS analysis.

2. Materials and methods

2.1. Biotransformation mass changes

Biotransformation mass changes of known enzymatic reactions were collected from the literature (Table 1) [15–17]. These reactions contain classical primary metabolic pathways for xenobiotic biotransformations [18,19]. DPHP is first metabolized to mono-(2-propylheptyl) phthalate (MPHP) by ester cleavage, followed by various oxidized monoester metabolites [9]. Thus, MPHP could also become the subject of further biotransformation reactions and both DPHP and MPHP were used as the precursors for predicting the DPHP metabolite candidates via biotransformation mass changes.

2.2. Chemicals and reagents

DPHP, CAS No. 53306-54-0, and D_4-mono-cyclohexyl phthalate (D_4-MCHP), CAS No. 1398066-18-6, were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Sulfatase,β-glucuronidase, acetic acid (purity $\geq$ 99.9%), formic acid (purity $\geq$ 99.9%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol (purity $\geq$ 99.9%) was purchased from Merck (Darm-stadt, Germany).

2.3. Animal experiments

A rat model was used for assessing dose–response relationships of these metabolite candidates calculated via the biotransformation mass changes. Experimental protocols and

Table 1 – The m/z values of DPHP metabolite candidates calculated from biotransformation mass changes of known enzymatic reactions.

OBS	Metabolic reaction					Precursor (*m/z*)	
	Original compound	product	Description	Formula change	Monoisotopic mass change	DPHP	MPHP
1	R-$CH_2C_6H_5$	R-H	debenzylation	-C_7H_6	−90.0470	355.2853	215.1288
2	R-$C(CH_3)_3$	R-H	tert-butyl dealkylation	-C_4H_8	−56.0626	389.2697	249.1132
3	R-COOH	R-H	decarboxylation	-CO_2	−43.9898	401.3425	261.1860
4	R-$CH(CH_3)_2$	R-H	isopropyl dealkylation	-C_3H_6	−42.0470	403.2854	263.1289
5	R-$CH_2CO(CH_2)_2CH_3$	R-COOH	propyl ketone to acid	-C_4H_8+O	−40.0677	405.2646	265.1081
6	R-$C(CH_3)_3$	R-OH	tert-butyl to alcohol	-C_4H_8+O	−40.0677	405.2646	265.1081
7	R-CH_2OH	R-H	hydroxymethylene loss	-CH_2O	−30.0106	415.3218	275.1653
8	R-$CH_2O(CH_2)_2CH3$	R-COOH	propyl ether to acid	-C_3H_8+O	−28.0677	417.2646	277.1081
9	R-C_2H_5	R-H	demethylation	-C_2H_4	−28.0313	417.3010	277.1445
10	R-CO-R′	R-R′	decarboxylation	-CO	−27.9949	417.3374	277.1809
11	R-$CH_2COCH_2CH_3$	R-COOH	ethyl ketone to acid	-C_3H_6+O	−26.0520	419.2803	279.1238
12	R-$CH(CH_3)_2$	R-OH	isopropyl to alcohol	-C_3H_6+O	−26.0520	419.2803	279.1238
13	R-CH_2-CH_2OH	R-CH=CH_2	alcohols dehydration	-H_2O	−18.0106	427.3218	287.1653
14	R-$CH_2OCH_2CH_3$	R-COOH	ethyl ether to acid	-C_2H_6+O	−14.0520	431.2803	291.1238
15	R-CH_3	R-H	demethylation	-CH_2	−14.0157	431.3167	291.1602
16	RCOOH	RCH_2OH	acid to alcohol	-O + H_2	−13.9793	431.3531	291.1966
17	R-$C(CH_3)_3$	R-COOH	tert-butyl to acid	-C_3H_8+O_2	−12.0728	433.2596	293.1031
18	R-CH_2COCH_3	R-COOH	methyl ketone to acid	-C_2H_4+O	−12.0364	433.2959	293.1394
19	R-CH_2CH_3	R-OH	ethyl to alcohol	-C_2H_4+O	−12.0364	433.2959	293.1394
20	R-CH_2-CH_2-CH_2-CH_2-R′	R-CH=CH-CH=CH-R	two sequential desaturation	-H_4	−4.0313	441.3010	301.1445
21	hydroxylation + dehydration		hydroxylation and dehydration	-H_2	−2.0157	443.3167	303.1602
22	R-CH_2-OH	R-CHO	first/second alcohols to aldehyde/ketone	-H_2	−2.0157	443.3167	303.1602
23	R-CH_2-CH_2-R′	R-CH=CH-R′	desaturation	-H_2	−2.0157	443.3167	303.1602
24	demethylation and methylene to ketone		demethylation and methylene to ketone	-CH_4+O	−0.0364	445.2959	305.1394
25	R-CH(OH)CH_3	R-COOH	2-ethoxyl to acid	-CH_4+O	−0.0364	445.2959	305.1394
26	R-$CH(CH_3)_2$	R-COOH	isopropyl to acid	-C_2H_6+O_2	1.9429	447.2752	307.1187
27	R-CH_3	R-OH	demethylation and hydroxylation	-CH_2+O	1.9793	447.3116	307.1551
28	R-CO-R′	R-CHOH-R′	ketone to alcohol	+H_2	2.0157	447.348	307.1915
29	RCH = CHR′	R-CH_2CH_2-R′		+H_2	2.0157	447.348	307.1915
30	R-CH_2-R′	R-(C=O)-R′	methylene to ketone	-H_2+O	13.9793	459.3116	319.1551
31	hydroxylation and desaturation		hydroxylation and desaturation	-H_2+O	13.9793	459.3116	319.1551
32	R-H	R-CH_3	methylation	+CH_2	14.0157	459.348	319.1915
33	R-CH=CH-R′	R-C(O)C-R′	alkene to epoxide	-H_2+O	13.9793	459.3116	319.1551
34	R-CH_2-R′	R-C(O)-R′	methylene to ketone	-H_2+O	13.9793	459.3116	319.1551
35	R-CH=CH-R′	R-CH_2-CHOH-R′	hydration, hydrolysis (internal)	H_2+O	18.0106	463.3429	323.1864
36	R-CH_2CH_3	R-COOH	ethyl to carboxylic acid	-C-H_4+O_2	15.9585	461.2909	321.1344
37	R-H	R-OH	hydroxylation	+O	15.9949	461.3272	321.1707
38	R-CH-CH-R′	R-CH(O)-CH-R′	aromatic ring to arene oxide	+O	15.9949	461.3272	321.1707
39	R-CH_2CH_3	R-CH-$(OH)_2$	demethylation and two hydroxylation	-C-H_2+O_2	17.9742	463.3065	323.1500
40	hydroxylation and ketone formation		hydroxylation and ketone formation	-H_2+O_2	29.9742	475.3065	335.1500
41	C_nH_m	C_nH_m-$2O_2$	quinone formation	-H_2+O_2	29.9742	475.3065	335.1500
42	R-CH_3	R-COOH	demethylation to carboxylic acid	-H_2+O_2	29.9742	475.3065	335.1500
43	RH	R-OCH_3	hydroxylation and methylation	+OCH_2	30.0106	475.3429	335.1864
44	2 × hydroxylation		2 × hydroxylation	+O_2	31.9898	477.3222	337.1657
45	R-CH=CH-R′	R-CH(OH)CH(OH)-R′	dihydroxylation (alkenes to dihydrodiols)	2 × (OH)	34.0055	479.3378	339.1813
46	3 × (RH)	3 × (ROH)	3 × hydroxylation	+O_3	47.9847	493.3171	353.1606

procedures and care and use of laboratory animals have been approved by the International Animal Care and Use Committee, National Cheng Kung University, Taiwan. Male Sprague Dawley rats were purchased from the Laboratory Animal Center, National Cheng Kung University, Taiwan. Before the experiment, the rats were housed in polycarbonate cages for two weeks of acclimatization. They were maintained on a 13:11-h light/dark cycle (lights off at 20:00 h) at a temperature of approximately 20 °C and 60% relative humidity with unlimited access to food (Laboratory Autoclavable Rodent Diet 5010) and purified water. After acclimatization, rats were equally divided into 5 groups for administering 5 DPHP doses (0, 150, 300, 600, and 1200 mg/kg body weight, n = 6, respectively) via oral gavage. They were housed individually in metabolism cages for collecting consecutive 24 h urine samples. The samples were stored at −80 °C until UPLC-MS analysis.

2.4. Sample preparation

Before UPLC-MS analysis, 25 μL ofβ-glucuronidase and 10 μL of sulfatase were added in the urine sample (100 μL) for hydrolyzing the metabolite conjugates. A total of 55 μL of 20% (v/v) acetic acid in deionized water was added in the sample for terminating the enzyme reaction and then centrifuged (13,500 rpm for 10 min). A total of 200 μL D_4-MINP was added in the sample. Solid-phase extraction (SPE) was performed using one-step SPE clean-up procedure [13]. C18 cartridge (HYPERSEP C18, 60,40-63, 100 mg/mL, Thermo Scientific, USA) was preconditioned with methanol, followed by 1% (v/v) acetic acid in deionized water. The supernatant was loaded on the C18 cartridge, washed with 5 mL of 1% (v/v) acetic acid in deionized water, and analytes were eluted with 1 ml of methanol. The eluate was evaporated to dry using gentle gas (nitrogen) flow and reconstituted in 200 μL of 0.1% (v/v) formic acid in deionized water before it was subjected to UPLC-MS analysis.

2.5. UPLC-MS and MS/MS

Liquid chromatography was performed on an UPLC system (Waters Acquity UPLC core system, Waters) coupled with an LTQ-Orbitrap MS system equipped with an electrospray ion source (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was done on an ACQUITY UPLC BEH C18 Column (2.1 mm × 50 mm, 1.7 μm). Mobile phase A consisted of 2% (v/v) acetonitrile and 0.1% (v/v) formic acid in deionized water, and mobile phase B consisted of 0.1% (v/v) formic acid in methanol. Elution conditions were as follows: 0-1 mins, 99% (A); 1–1.01 min, 99-50% (A); 1.01–7 mins, 50-99% (B); and 7–8.5 mins, 99% (B). The column temperature was maintained at 40 °C. LC flow rate was set at 300 μL/min and 10 μL of each sample was injected. Electrospray ionization was performed in the negative mode. The optimized parameters were as follows: spray voltage, 3.2 eV; and source temperature, 350 °C. Full-scan data were acquired in the range 80–700 Da, with a resolution of 60,000. To obtain structural information about these tentative exposure marker signals, product ion profiles of these signals in the rat urine samples (dose 1200 mg/kg body weight) were obtained by MS/MS analysis. The LC conditions for MS/MS analyses were identical to those from the UPLC-MS analysis and the collision energies were set at 25, 30, and 35 eV, respectively.

2.6. Exposure marker validation

These *m/z* values of DPHP metabolite candidates calculated via the biotransformation mass changes were further validated as tentative exposure marker signals by a rat model. The MS files of these rat urine samples administered 5 DPHP doses were imported into Progenesis QI software (Nonlinear Dynamics, Newcastle, UK) for retention time alignment and signal identification, and the abundance ratios (the ratio of the signal abundance of metabolite candidates to that of the internal standard, D_4-MCHP) of these identified signals were calculated. Spearman correlations among the five DPHP exposure doses and the abundance ratios of the *m/z* values of metabolite candidates measured in the MS data of the rat urine samples were estimated using the R software version 3.31 (R Development Core Team 2016). A tentative exposure marker signal was defined as Spearman's correlation coefficients >0.7 and p-values < 0.001.

3. Results and discussion

The goal of this study was to discover DPHP exposure markers using UPLC-MS and a rat model, and the study design was shown in Fig. 1. Firstly, the biotransformation mass changes of known enzymatic reactions were collected from the literature [15–17]. Two precursors of DPHP and MPHP were used to predict the metabolite candidates via these biotransformation mass changes. Secondly, these candidate signals were measured in the MS data of rat urine samples collected from rats administered different DPHP doses. The dose–response relationship of these candidate signals was assessed. Finally, the structures of these signals validated as tentative exposure markers were speculated based on UPLC-MS/MS analysis.

3.1. DPHP exposure marker validation

A total of 46 biotransformation mass changes of enzymatic reactions were collected from the literature [15–17] (Table 1). Classical primary metabolic pathways for xenobiotics and multistage oxidative metabolic reactions were considered in these biotransformation mass changes. However, phase II biotransformations that are conjugated reactions were not considered in these biotransformation mass changes, because the rat urine samples which were used to validate the metabolite candidates were deconjugated in the sample preparation procedure.

The *m/z* values of DPHP metabolite candidates calculated via the biotransformation mass changes of known enzymatic reactions were further validated as tentative exposure markers using a rat model. The levels of DPHP metabolites raise as rat exposure levels increase. Thus, a rat model that rats were orally administered five different DPHP doses was designed to validate these metabolite candidates. The MS data of these urine samples were obtained from UPLC-MS analysis. The dose–response relationships of these *m/z* values of

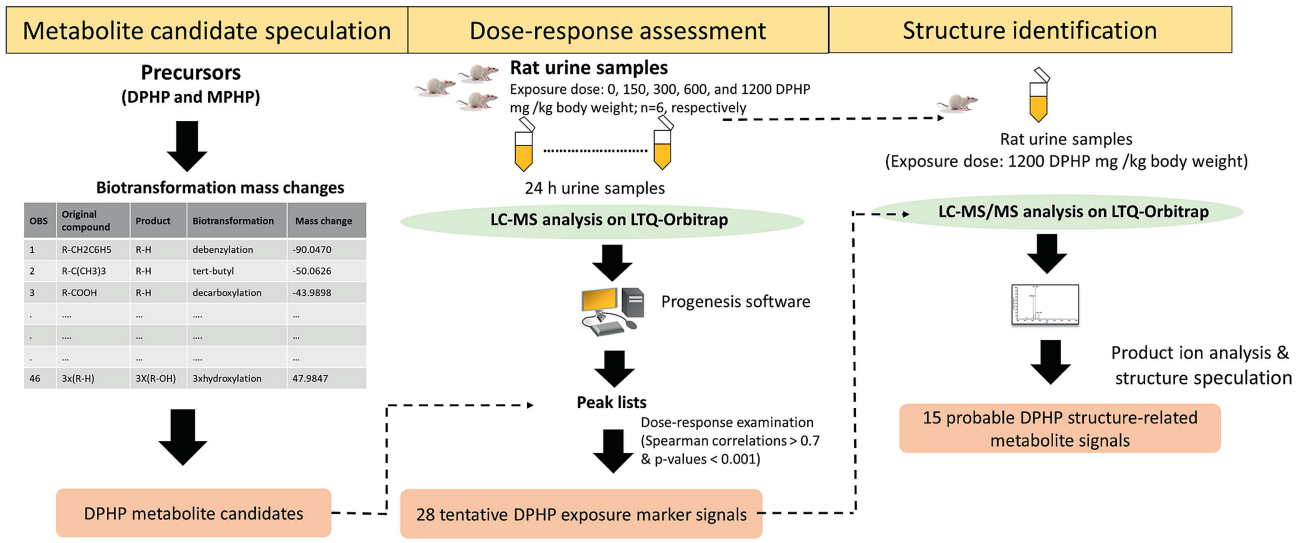

Fig. 1 – Experimental scheme of DPHP exposure maker discovery using UPLC-MS and a rat model.

metabolite candidates measured in the MS data were assessed. A candidate with higher correlation coefficient between abundance ratios and administered doses indicating a metabolite with high confidence. The candidates with Spearman correlation coefficients >0.7 and p-values < 0.001 was defined as dose–response, and these candidates were considered tentative exposure markers.

When DPHP was taken as the precursor for calculating DPHP metabolite candidates via the biotransformation mass changes, no dose–response relationships of the metabolite candidate signals measured in the MS data of the rat urine samples were observed. However, when MPHP was taken as the precursor, 28 metabolite candidate signals were validated as tentative exposure marker signals (Table 2), and their dose–response curves showed positive correlations between the abundance ratios and administered doses (Fig. 2). No metabolite candidates that were validated when DPHP was taken as the precursor seem to be reasonable, because phthalates are first metabolized to a monoester, followed by various oxidation or reduction products [12]. Based on molecular ion *m/z* information, these tentative exposure marker signals contain three known DPHP metabolites, OH-MPHP, oxo-MPHP, and cx-MPHxP [9] and one tentative DPHP metabolite [10]. These tentative exposure marker signals contain some isomers, indicating that chemical structures of these known DPHP metabolites may have isomers in urine.

The extracted ion chromatograms (EICs) of the 28 identified tentative DPHP exposure markers were obtained from the rat urine sample collected from rats administered a DPHP dose (1200 mg/kg body weight) (Fig. S1). All signals of the identified metabolites could be observed in the EICs except for M1 (Fig. S1 (1)). M1 may be a false positive exposure marker. The EICs show that some identified metabolites that were isomers with close retention times (such as M15, M16, M17, and M18) were well separated by UPLC (Table 2 and Fig. S1).

UPLC-MS was implemented to detect metabolites in rat urine samples. Although UPLC-MS can be used to measure metabolites with high accuracy, not all metabolites in biological samples can be detected. Some metabolites may not be extracted during the sample preparation (such as SPE). The physicochemical properties of metabolites cover a wide range (such as pK_a, polarity, and size), and not all metabolites can be separated well by LC [20]. The presence of matrix compounds may have impact on ionization of metabolites [3]. These limits on UPLC-MS can result in that not all DPHP metabolites were identified by our experimental design.

3.2. *MS/MS verification of the probable DPHP metabolite signals*

It is difficult to synthesize pure compounds for confirming chemical structures of new metabolite discovery. MS/MS analysis is an alternative method to confirm that these structures of signals may be actual DPHP metabolites [12]. The product ion mass spectra of the 28 tentative exposure marker signals were obtained from the MS/MS analysis of the urine samples collected from rats administered a DPHP dose (1200 mg/kg body weight). The possible structure fragments were speculated from the product ions and a reasonable chemical structure of a precursor can be confirmed based on these fragments.

The chemical structures of 15 tentative exposure marker signals were speculated based on their product ion profiles (Fig. 3 and Table 2). The other tentative exposure marker signals did not have enough information to speculate their chemical structures. The fragment ions at *m/z* 121.0306 and 157.1248 of M8 were assigned to a benzoic acid and a 5-(hydroxymethyl) ocan-2-one, respectively, so M8 was tentatively identified as mono-(propyl-6-oxo-hexyl) phthalate (oxo-MPHxP). The fragment ions at *m/z* 137.0258 and 159.1042 of M10 were assigned to a 2-hydroxybenzoic acid and a 4-(hydroymethyl) heptanoic acid, respectively, so M10 was tentatively identified as mono-(propyl-5-carboxylbutyl) phthalate (cx-MPBP). The similar fragment ions of M9 were observed, so M9 was tentatively identified as oxo-MPHxP. The fragment ions at *m/z* 121.0305 and 159.1405 of M12 were assigned to a benzoic acid and a 2-propylhexane-1,5-diol, respectively, so M12 were tentatively identified as mono-(propyl-6-hydroxyhexyl)

Table 2 – Characteristics of the tentative DPHP exposure marker signals detected in the rat urine samples.

ID	*m/z*	RT[a] (min)	Peak width (min)	Expected *m/z*	Mass accuracy (ppm)	Formula	Abbreviated name	Structure speculation[b]
M1	215.1289	4.90	0.44	215.1288	0.09	$C_{11}H_{20}O_4$		
M2	265.1078	2.00	0.16	265.1081	−1.37	$C_{14}H_{18}O_5$		
M3	275.1651	3.54	0.09	275.1653	−0.60	$C_{17}H_{24}O_5$		
M4	277.1444	3.53	0.09	277.1445	−0.58	$C_{16}H_{22}O_4$		
M5	279.1237	2.03	0.15	279.1238	−0.41	$C_{15}H_{20}O_5$		
M6	293.1392	3.10	0.24	293.1394	−1.01	$C_{16}H_{22}O_5$		
M7	303.1601	3.14	0.10	303.1602	−0.17	$C_{16}H_{26}O_4$		
M8	305.1392	3.21	0.33	305.1394	−0.81	$C_{16}H_{22}O_5$	oxo-MPHxP	+
M9	305.1404	2.81	0.13	305.1394	2.96	$C_{16}H_{22}O_5$	oxo-MPHxP	+
M10	307.1184	2.97	0.82	307.1187	−1.06	$C_{16}H_{20}O_6$	cx-MPBP	+
M11	307.1548	3.10	0.20	307.1551	−0.82	$C_{17}H_{24}O_5$	OH-MPHxP	+
M12	307.1549	3.53	0.59	307.1551	−0.57	$C_{17}H_{24}O_5$	OH-MPHxP	+
M13	319.1548	3.54	0.61	319.1551	−0.93	$C_{18}H_{24}O_5$	oxo-MPHP	+
M14	321.1340	3.31	0.42	321.1344	−1.28	$C_{17}H_{22}O_6$		
M15	321.1702	3.61	0.93	321.1707	−1.84	$C_{18}H_{26}O_5$		
M16	321.1705	3.85	0.23	321.1707	−0.81	$C_{18}H_{26}O_5$	OH-MPHP	+
M17	321.1705	4.09	0.15	321.1707	−0.66	$C_{18}H_{26}O_5$	OH-MPHP	+
M18	321.1706	3.72	0.10	321.1707	−0.42	$C_{18}H_{26}O_5$	OH-MPHP	+
M19	323.1493	2.76	0.25	323.1500	−2.17	$C_{17}H_{24}O_6$		
M20	323.1496	2.57	0.24	323.1500	−1.37	$C_{17}H_{24}O_6$		
M21	335.1495	3.68	0.43	335.1500	−1.44	$C_{18}H_{24}O_6$	cx-MPHxP	+
M22	335.1495	2.38	2.52	335.1500	−1.41	$C_{18}H_{24}O_6$	cx-MPHxP	+
M23	335.1496	3.57	0.12	335.1500	−1.30	$C_{18}H_{24}O_6$	cx-MPHxP	+
M24	335.1496	3.06	0.19	335.1500	−1.11	$C_{18}H_{24}O_6$	cx-MPHxP	+
M25	337.1650	3.10	0.33	337.1657	−1.92	$C_{18}H_{26}O_6$	OH-OH-MPHP	+
M26	337.1651	2.51	0.34	337.1657	−1.79	$C_{18}H_{26}O_6$	OH-OH-MPHP	+
M27	337.1651	2.88	0.25	337.1657	−1.67	$C_{18}H_{26}O_6$	OH-OH-MPHP	+
M28	353.1599	2.14	0.39	353.1606	−1.91	$C_{18}H_{26}O_7$		

[a] Retention time.
[b] Chemical structures of tentative exposure marker signals were speculated based on MS/MS analysis.

phthalate (OH-MPHxP). The fragment ions at *m/z* 121.0306 and 171.1406 of M13 were assigned to a benzoic acid and a 6-(hydroxymethyl) nonnan-2-one, respectively, so M13 was tentatively identified as oxo-MPHP. The fragment ions at *m/z* 121.0305 and 173.1562 of M16 were assigned to a benzoic acid and a 2-propylheptane-1,6-diol, respectively, so M16 was tentatively identified as OH-MPHP. The similar fragment ions were observed in M17 and M18 and they were tentatively identified as OH-MPHP. The fragment ions at *m/z* 121.0304 and 187.1352 of M24 were assigned to a benzoic acid and a 6-(hydroxymethyl) nonanoic acid, so M24 was tentatively identified as cx-MPHxP. The fragment ions at *m/z* 121.0305 and 189.1509 of M25 were assigned to a benzoic acid and a 6-(propylheptane)-1,1,7-triol, so M25 was tentatively identified as mono-(propyl-6-hydroxy-hydroxyheptyl) phthalate (OH-OH-MPHP). The similar fragment ions were also observed in M26 and M27, and they were identified as OH-OH-MPHP. These signals share a benzoic acid, which is the common product ion of DPHP metabolites (OH-MPHP and oxo-MPHP) [9], indicating that these signals were most likely the DPHP structure-related metabolite signals. Six chemical structures of the 11 signals were speculated, indicating that they contain some isomers (Fig. 4). These isomers with different arrangement of the atoms in space were not investigated in this study. However, the chemical structures of some fragment ions with a relatively high abundance (>10%) could not be speculated, such as *m/z* 197.1750 in Fig. 3(A) and *m/z* 191.0757 in Fig. 3(B). To our knowledge, these chemical structures could not be speculated based on DPHP related structures and these may not be the fragment ions of DPHP metabolites. Product ion profiles of these signals were obtained from the rat urine samples, and the urine samples contain extremely complicated compounds. Therefore, these ions may be from other compounds.

Six tentative exposure marker signals were inferred as the three known DPHP metabolites (oxo-MPHP (M13), OH-MPHP (M16, M17, and M18), and cx-MPHxP (M24)) based on their speculated structures. The chemical structures (M10, M25, and M26) have been identified by our group [10,11]. Two chemical structures (oxo-MPHxP and OH-MPHxP) that have not been reported in the literature were identified in this study.

3.3. *Proposed biotransformation of identified DPHP metabolites*

Biotransformation is essential to determine the pharmacokinetic parameters, such oral bioavailability, clearance, and the half-life of the entity within the cell. The molecular structure of a toxicant is commonly metabolized to be more hydrophilic compounds that can be readily excreted from the body via urine [8]. Biotransformation plays a role in the toxicity, because toxic metabolites may format via metabolic reactions.

The biotransformation of six speculated chemical structures of the tentative DPHP metabolite signals were proposed

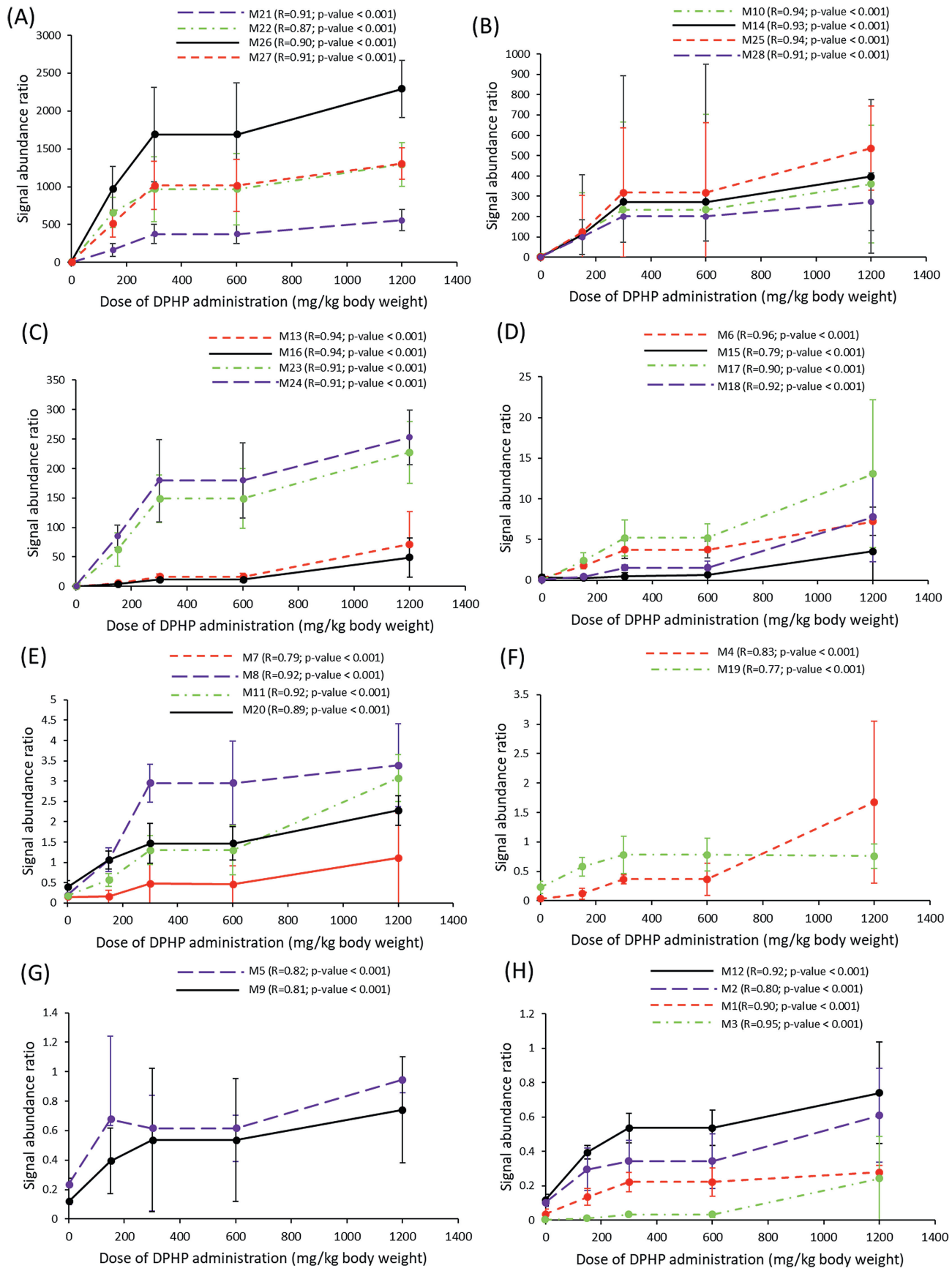

Fig. 2 – Dose–response curves of tentative DPHP exposure marker signals in rat urine samples collected from rat exposure to 0, 150, 300, 600, and 1200 mg/kg body weight (n = 6, respectively).

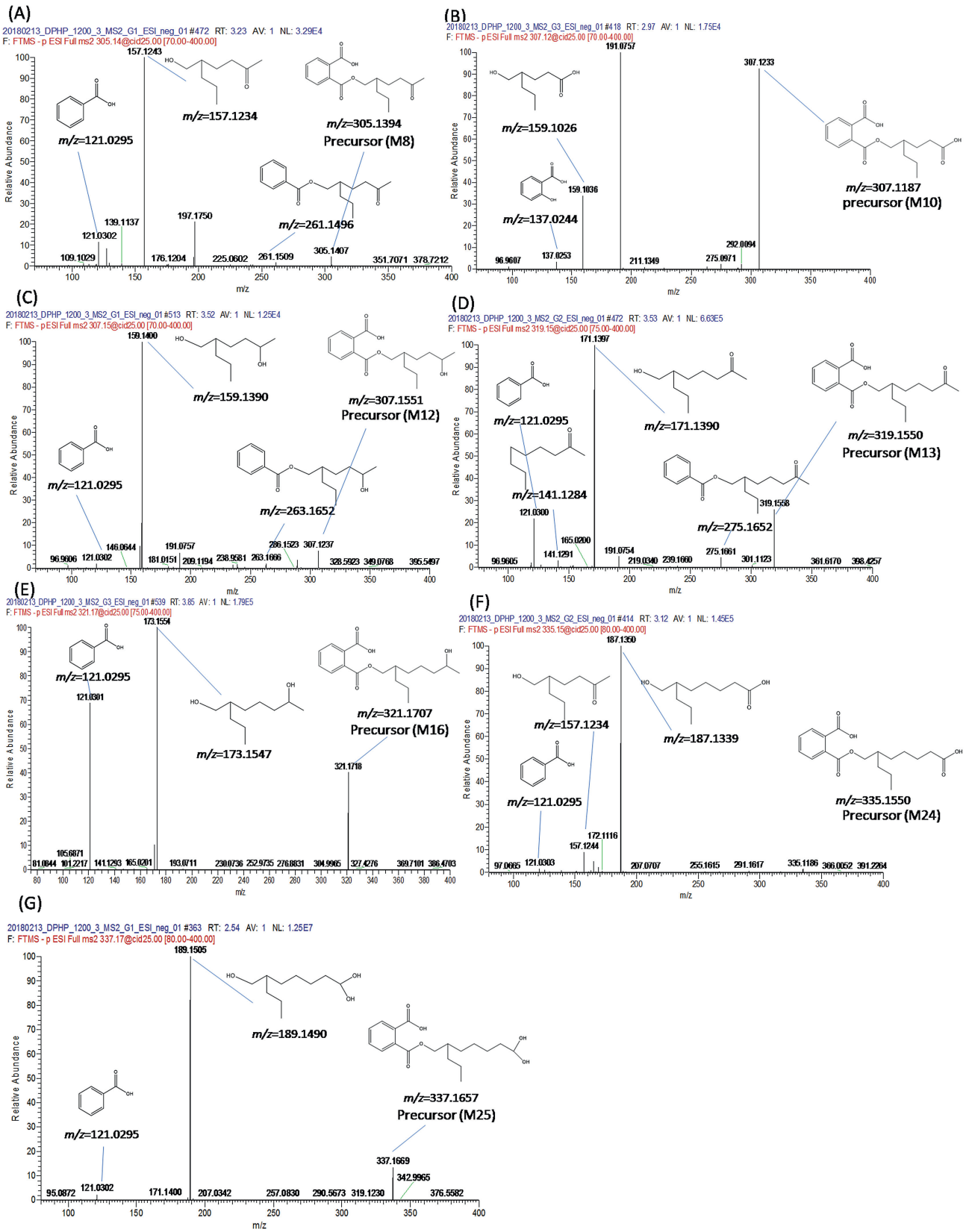

Fig. 3 – MS/MS product ion profiles of validated tentative exposure marker signals.

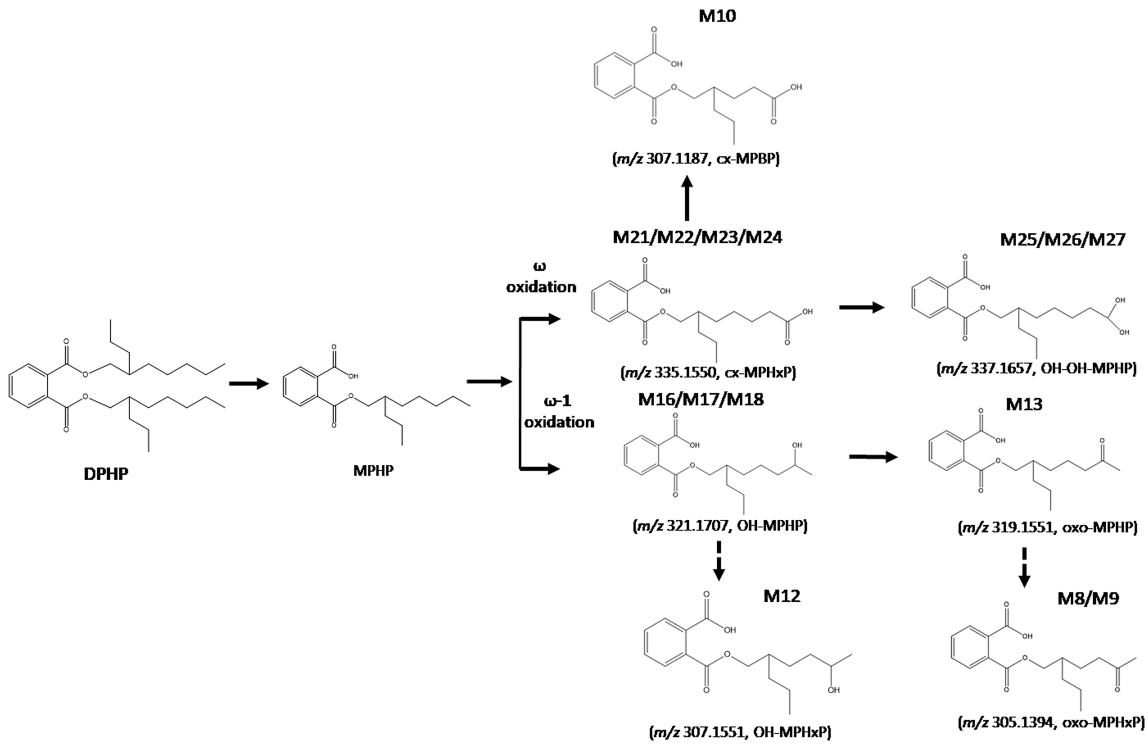

Fig. 4 – Proposed biotransformation of tentative DPHP metabolites identified in this study. Dash lines indicate newly identified tentative DPHP metabolite signals.

(Fig. 4). Based on the known biotransformation of DPHP metabolites, DPHP is first metabolized to MPHP, followed by various oxidized monoester metabolites (OH-MPHP and cx-MPHxP) [6]. OH-MPHP and cx-MPHxP may be dehydrogenated to oxo-MPHP and OH-OH-MPHP, respectively. Three newly identified tentative DPHP metabolites (OH-MPHxP, oxo-MPHxP, and cx-MPBP) may be the side-chain breakdown products of OH-MPHP, oxo-MPHP, and cx-MPHxP, respectively.

3.4. *DPHP exposure marker rankings*

The levels of toxicant metabolites excreted from the body via urine are usually different. A metabolite with a higher level in urine indicates that it can be detected more easily even when humans are exposed to a low level of toxicant. Thus, this metabolite may be a more suitable marker for human exposure assessments.

The peak abundance ratios of 28 tentative exposure markers were measured in the six urine samples that rats administered 1200 DPHP mg/kg body weight. Although the levels of these signals in rat urine samples cannot be obtained, peak abundance ratios respond to relative levels of these signals. The signals (M26 and M27) identified as OH-OH-MPHP had the highest peak abundance ratio ($\geq$ 5.49E+06), and the signals identified as cx-MPHxP (M21, M22, M23, and M24), OH-OH-MPHP (M25), and cx-MPBP (M10) had a slightly lower peak abundance ratio (5.46E+06–2.34E+06) (Fig. S2). The peak abundance ratios of the signals identified as OH-MPHP (M16, M17, and M18), and oxo-MPHP (M13) (3.01E+05–3.27E+04) were clearly lower than those of M23 and M24 but were clearly higher than those of the signals identified as oxo-MPHxP and OH-MPHxP (1.44E+04–2.61E+03). The level of cx-MPHxP in blood of rats on single oral administration was the highest among the three known DPHP metabolites [21] and this result is similar to that of our study. These results indicate that OH-OH-MPHP that had a highest abundance ratio may be a suitable exposure maker for human exposure assessments.

Our group have used two metabolomics data-screening approaches-the signal mining algorithm with isotope tracing and the mass defect filter for identifying DPHP metabolite signals from *in vitro* DPHP incubation samples [10]. The two approaches identified 17 tentative exposure marker signals, including 4 known DPHP metabolites (MPHP, oxo-MPHP, OH-MPHP, and cx-MPHxP) and one novel tentative DPHP metabolites (OH-OH-MPHP). In this study, the 4 known DPHP metabolites (oxo-MPHP, OH-MPHP, cx-MPHxP, and OH-OH-MPHP) and 3 novel tentative DPHP metabolites (oxo-MPHxP, OH-MPHxP, and cx-MPBP) were identified using UPLC-MS and a rat model. One known DPHP metabolite (MPHP) was not identified because the level of MPHP is very low in rat urine [10]. Also, MPHP was not used as exposure markers to assess human DPHP exposure levels, because of its low level in human urine [6]. UPLC-MS and a rat model seem to be suitable to identify urinary toxicant metabolites.

4. Conclusions

DPHP may have an adverse effect on human health. The risk of human exposure to DPHP may increase, because the use of DPHP is still increasing. UPLC-MS and a rat model were used for tentative DPHP exposure marker discovery. A total of 46

biotransformation mass changes of known enzymatic reactions were used to predict the *m/z* values of DPHP metabolite candidates. These *m/z* values were validated using urine samples collected from rats administered different DPHP doses. In total, 28 signals in rat urine samples were validated as tentative exposure marker signals. The chemical structures of 15 signals were speculated based on MS/MS analysis and these signals contain 7 chemical structures, indicating there were some isomers in these signals. Among the 6 speculated chemical structures, 2 structures (oxo-MPHxP, and OH-MPHxP) were novel tentative DPHP metabolites, and 4 structures have been previously reported in the literature. These signals that were speculated as OH-OH-MPHP had more higher abundance ratios than these of the known DPHP metabolites and were suggested to be suitable DPHP exposure makers for human exposure assessments. However, these signals contain a few isomers and their different arrangement of the atoms in space should be confirmed using standard compounds in the future studies. The results indicate that UPLC-MS and a rat model can be applied to effectively identifying tentative toxicant metabolites.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jfda.2018.11.002.

REFERENCES

[1] Wittassek M, Angerer J. Phthalates: metabolism and exposure. Int J Androl 2008;31:131–6 [In English].
[2] NICNAS. 1,2-Benzenedicarboxylic acid, bis(2-propylheptyl) ester (Palatinol 10-P). National Industrial Chemicals Notification and Assessment Scheme; 2003. File No: STD/1054.
[3] Furr JR, Lambright CS, Wilson VS, Foster PM, Gray LE. A short-term in vivo screen using fetal testosterone production, a key event in the phthalate adverse outcome pathway, to predict disruption of sexual differentiation. Toxicol Sci 2014;140:403–24 [In English].
[4] CPSC. Toxicity review of di(2-propylheptyl) phthalate. US Consumer Product Safety Commission; 2011. CPSC-D-06-0006.
[5] CPSC. Review of exposure and toxicity data for phthalate substitutes. Consumer Product Safety Commission; 2010.
[6] Schutze A, Gries W, Kolossa-Gehring M, Apel P, Schroter-Kermani C, Fiddicke U, et al. Bis-(2-propylheptyl)phthalate (DPHP) metabolites emerging in 24 h urine samples from the German Environmental Specimen Bank (1999-2012). Int J Hyg Environ Health 2015;218:559–63 [In English].
[7] Juan YY, Wei YC, Hung SC. In: Taipei times. Taiwan: Taipei Times; 2011. p. 2.
[8] Schutze A, Otter R, Modick H, Langsch A, Bruning T, Koch HM. Additional oxidized and alkyl chain breakdown metabolites of the plasticizer DINCH in urine after oral dosage to human volunteers. Arch Toxicol 2017;91:179–88 [In English].
[9] Leng G, Koch HM, Gries W, Schutze A, Langsch A, Bruning T, et al. Urinary metabolite excretion after oral dosage of bis(2-propylheptyl) phthalate (DPHP) to five male volunteers – characterization of suitable biomarkers for human biomonitoring. Toxicol Lett 2014;231:282–8 [In English].
[10] Shih CL, Liao PM, Hsu JY, Chung Y-N, Zgoda VG, Liao PC. Identification of urinary biomarkers of exposure to di-(2-propylheptyl) phthalate using high-resolution mass spectrometry and two data-screening approaches. Chemosphere 2018;193:170–7 [In English].
[11] Shih CL, Wu HY, Liao PM, Hsu JY, Tsao CY, Zgoda VG, et al., Profiling and comparison of toxicant metabolites hair and urine using a mass spectrometry-based metabolomic data processing method, Anal Chim Acta, In press, [In English].
[12] Hsu JY, Hsu JF, Chen YR, Shih CL, Hsu YS, Chen YJ, et al. Urinary exposure marker discovery for toxicants using ultra-high pressure liquid chromatography coupled with Orbitrap high resolution mass spectrometry and three untargeted metabolomics approaches. Anal Chim Acta 2016;939:73–83 [In English].
[13] Hsu JF, Peng LW, Li YJ, Lin LC, Liao PC. Identification of di-isononyl phthalate metabolites for exposure marker discovery using in vitro/in vivo metabolism and signal mining strategy with LC-MS data. Anal Chem 2011;83:8725–31 [In English].
[14] Tautenhahn R, Patti GJ, Rinehart D, Siuzdak G. XCMS online: a web-based platform to process untargeted metabolomic data. Anal Chem 2012;84:5035–9 [In English].
[15] Anari MR, Sanchez RI, Bakhtiar R, Franklin RB, Baillie TA. Integration of knowledge-based metabolic predictions with liquid chromatography data-dependent tandem mass spectrometry for drug metabolism studies: application to studies on the biotransformation of Indinavir. Anal Chem 2004;76:823–32.
[16] Zhang HY, Zhang DL, Ray K, Zhu MS. Mass defect filter technique and its applications to drug metabolite identification by high-resolution mass spectrometry. J Mass Spectrom 2009;44:999–1016 [In English].
[17] Ma SG, Chowdhury SK. Analytical strategies for assessment of human metabolites in preclinical safety testing. Anal Chem 2011;83:5028–36 [In English].
[18] Williams RT. Detoxification Mechanisms: the metabolism and detoxication of drugs, toxic substances and other organic compounds. 2nd ed. New York: John Willey & Sons, Inc; 1959.
[19] Testa B, Jenner P. Drug metabolism: clinical and biological aspects. 1st ed. New York: Marcel Dekker; 1976.
[20] Scalbert A, Brennan L, Fiehn O, Hankemeier T, Kristal BS, van Ommen B, et al. Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. Metabolomics 2009;5:435–58 [In English].
[21] Klein D, Kessler W, Semder B, Putz C, Lichtmannegger J, Otter R, et al. Di-(2-propylheptyl) phthalate (DPHP) and its metabolites in blood of rats upon single oral administration of DPHP. Toxicol Lett 2016;259:80–6 [In English].