Original Article

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

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A B S T R A C T

Di-(2-propylheptyl) phthalate (DPHP) is a plasticizer and has been suggested to be a sub-chronic 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-isononyl-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 roof membranes, and tarpaulins [1,2].

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].

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 D₄-mono-cyclohexyl phthalate (D₄-MCHP), CAS No. 1398066-18-6, were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Sulphatase, β-glucuronidase, acetic acid (purity ≥ 99.9%), formic acid (purity ≥ 99.9%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol (purity ≥ 99.9%) was purchased from Merck (Darmstadt, 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 | Original compound | product | Description | Formula change | Monoisotopic mass change | Precursor (m/z) |
|-----|--------------------|-------------------|---------|-------------|----------------|-------------------------|-----------------|
| 1   | R-CH₂C₆H₅ | R-H | debenzylation | -C₆H₅ | -90.0470 | 355.2853 | 215.1288 |
| 2   | R-C(CH₃)₃ | R-H | tert-butyl dealkylation | -C₆H₅ | -56.0626 | 389.2697 | 249.1132 |
| 3   | R-COOH | R-H | decarboxylation | -CO₂ | -43.9898 | 401.3425 | 261.1860 |
| 4   | R-CH(CH₃)₂ | R-H | isopropyl dealkylation | -C₆H₅ | -42.0470 | 403.2854 | 263.1289 |
| 5   | R-CH₂CO(CH₂)₂CH₃ | R-COOH | propyl ketone to acid | -C₆H₅+O | -40.0677 | 405.2646 | 265.1081 |
| 6   | R-C(CH₃)₃ | R-OH | tert-butyl to alcohol | -C₆H₅+O | -40.0677 | 405.2646 | 265.1081 |
| 7   | R-CH₂OH | R-H | hydroxymethylene loss | -C₆H₅ | -30.0106 | 415.3218 | 275.1653 |
| 8   | R-CH₂(OCH₂)₂CH₃ | R-COOH | propyl ether to acid | -C₆H₅ | -28.0677 | 417.2646 | 277.1081 |
| 9   | R-C₆H₅ | R-H | demethylation | -C₆H₅ | -28.0313 | 417.3010 | 277.1445 |
| 10  | R-CO-R' | R-R' | decarboxylation | -CO | -27.9949 | 417.3374 | 277.1809 |
| 11  | R-CH₂COCH₂CH₃ | R-COOH | ethyl ketone to acid | -C₆H₅+O | -26.0502 | 419.2803 | 279.1238 |
| 12  | R-CH₂(CH₃)₂ | R-OH | isopropyl to alcohol | -C₆H₅+O | -26.0502 | 419.2803 | 279.1238 |
| 13  | R-CH₂-CH₂OH | R-CH=CH₂ | alcohols dehydration | -H₂O | -18.0106 | 427.3218 | 287.1653 |
| 14  | R-CH₂OCH₂CH₃ | R-COOH | ethyl ether to acid | -C₆H₅+O | -14.0520 | 431.2803 | 291.1238 |
| 15  | R-CH₃ | R-H | demethylation | -CH₃ | -14.0157 | 431.3167 | 291.1602 |
| 16  | RC(OOH) | RCH₂OH | acid to alcohol | -O + H₂ | -13.9793 | 431.3531 | 291.166 |
| 17  | R-C(CH₃)₃ | R-COOH | tert-butyl to acid | -C₆H₄ + O₂ | -12.0728 | 433.2596 | 293.1031 |
| 18  | R-CH₂COCH₂CH₃ | R-COOH | methyl ketone to acid | -C₆H₄ + O₂ | -12.0364 | 433.2959 | 293.1394 |
| 19  | R-CH₂CH₃ | R-OH | ethyl to alcohol | -C₆H₄ + O₂ | -12.0364 | 433.2959 | 293.1394 |
| 20  | R-CH₂-CH₂-CH₂-CH₂-R' | R-CH=CH=CH=CH-R | two sequential desaturation | -H₄ | -4.0313 | 441.3010 | 301.1445 |
| 21  | hydroxylation + dehydration | | | | | | |
| 22  | R-CH₂-OH | R-CHO | first/second alcohols to aldehyde/ketone | -H₂ | -2.0157 | 443.3167 | 303.1602 |
| 23  | R-Ch₂-Ch₂-R' | R-CH=CH=R' | desaturation | -H₂ | -2.0157 | 443.3167 | 303.1602 |
| 24  | demethylation and methylene to ketone | | | | | | |
| 25  | R-CH(O)CH₃ | R-COOH | 2-ethoxy to acid | -C₆H₄+O₂ | -0.0364 | 445.2959 | 305.1394 |
| 26  | R-CH₂(CH₃)₂ | R-COOH | isopropyl to acid | -C₆H₄+O₂ | 1.9429 | 447.2752 | 307.1187 |
| 27  | R-CH₃ | R-OH | demethylation and hydroxylation | -C₆H₄ | 1.9793 | 447.3116 | 307.1551 |
| 28  | R-CO-R' | R-COHOH-R' | ketone to alcohol | +H₂ | 2.0157 | 447.348 | 307.1915 |
| 29  | RCH=CH-R' | R-CH₂CH₂-R' | methyl ketone | +H₂ | 2.0157 | 447.348 | 307.1915 |
| 30  | R-CH₂R' | R-CH₂OH-R' | | | | | |
| 31  | hydroxylation and desaturation | | | | | | |
| 32  | R-H | R-CH₂ | methylation | +CH₂ | 14.0157 | 459.348 | 319.1915 |
| 33  | R-CH=CH-R' | R-C(O)-R' | alkene to epoxide | -H₂O | 13.9793 | 459.3116 | 319.1551 |
| 34  | R-CH₂-R | R-C(O)-R' | methylene to ketone | -H₂O | 13.9793 | 459.3116 | 319.1551 |
| 35  | R-CH=CH-R | R-CH₂CH₂OH-R' | hydration, hydrolysis (internal) | +H₂ | 18.0106 | 463.3429 | 323.1864 |
| 36  | R-CH₂CH₃ | R-COOH | ethyl to carboxylic acid | -C₆H₄ + O₂ | 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-CH₂OCH₂-R' | aromatic ring to arene oxide | +O | 15.9949 | 461.3272 | 321.1707 |
| 39  | R-CH₂CH₃ | R-CH₂(O)-H₂ | demethylation and two hydroxylation | -C₆H₄+O₂ | 17.9742 | 463.3065 | 323.1500 |
| 40  | hydroxylation and ketone formation | | | | | | |
| 41  | C₆H₆ | C₆H₄=2O₂ | quinone formation | -H₂O | 29.9742 | 475.3065 | 335.1500 |
| 42  | R-CH₃ | R-COOH | demethylation to carboxylic acid | -H₂O | 29.9742 | 475.3065 | 335.1500 |
| 43  | RH | R-OCH₂ | hydroxylation and methylation | +OCH₂ | 30.0106 | 475.3429 | 335.1864 |
| 44  | 2 x hydroxylation | 2 x hydroxylation | | +O | 31.9898 | 477.3222 | 337.1657 |
| 45  | R-CH=CH-R' | R-CH₂OHHOH-CH(OH)-R' | dihydroxylation (alkenes to dihydrodiols) | 2 x (OH) | 34.0055 | 479.3378 | 339.1813 |
| 46  | 3 x (RH) | 3 x (ROH) | 3 x hydroxylation | +O | 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 of β-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 D4-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 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, D4-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
metabolite candidates measured in the MS data were assessed. A candidate with a 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 pKa, 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-(hydroxymethyl) 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)
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) nonan-2-one, respectively, so M13 was tentatively identified as oxo-MPHxP. The fragment ions at m/z 69.0504 and 121.0304 of M16 were assigned to a benzoic acid and a 2-propylheptane-1,6-diol, respectively, so M16 was tentatively identified as OH-MPHxP. 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-MPHxP and oxo-MPHxP) [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-MPHxP (M13), OH-MPHxP (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

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

| ID | m/z  | RTa (min) | Peak width (min) | Expected m/z | Mass accuracy (ppm) | Formula | Abbreviated name | Structure speculationb |
|----|------|-----------|-----------------|--------------|---------------------|---------|-----------------|-----------------------|
| M1 | 215 | 215.1289  | 4.90            | 0.44         | 215.1288            | 0.09    | C11H20O4         |                       |
| M2 | 265 | 265.1078  | 2.00            | 0.16         | 265.1081            | −1.37   | C12H20O4         |                       |
| M3 | 275 | 275.1651  | 3.54            | 0.09         | 275.1653            | −0.60   | C12H20O4         |                       |
| M4 | 277 | 277.1444  | 3.53            | 0.09         | 277.1445            | −0.58   | C12H20O4         |                       |
| M5 | 279 | 279.1237  | 2.03            | 0.15         | 279.1238            | −0.41   | C12H20O4         |                       |
| M6 | 293 | 293.1392  | 3.10            | 0.24         | 293.1394            | −1.01   | C12H20O4         |                       |
| M7 | 303 | 303.1601  | 3.14            | 0.10         | 303.1602            | −0.17   | C12H20O4         |                       |
| M8 | 305 | 305.1392  | 3.21            | 0.33         | 305.1394            | −0.81   | C12H20O4         |                       |
| M9 | 305 | 305.1404  | 2.81            | 0.13         | 305.1394            | 2.96    | C12H20O4         |                       |
| M10| 307 | 307.1184  | 2.97            | 0.82         | 307.1187            | −1.06   | C12H20O4         |                       |
| M11| 307 | 307.1548  | 3.10            | 0.20         | 307.1551            | −0.82   | C12H20O4         |                       |
| M12| 307 | 307.1549  | 3.53            | 0.59         | 307.1551            | −0.57   | C12H20O4         |                       |
| M13| 319 | 319.1548  | 3.54            | 0.61         | 319.1551            | −0.93   | C12H20O4         |                       |
| M14| 321 | 321.1340  | 3.31            | 0.42         | 321.1344            | −1.28   | C12H20O4         |                       |
| M15| 321 | 321.1702  | 3.61            | 0.93         | 321.1707            | −1.84   | C12H20O4         |                       |
| M16| 321 | 321.1705  | 3.85            | 0.23         | 321.1707            | −0.81   | C12H20O4         | OH-MPHP               |
| M17| 321 | 321.1705  | 4.09            | 0.15         | 321.1707            | −0.66   | C12H20O4         | OH-MPHP               |
| M18| 321 | 321.1706  | 3.72            | 0.10         | 321.1707            | −0.42   | C12H20O4         | OH-MPHP               |
| M19| 323 | 323.1493  | 2.76            | 0.25         | 323.1500            | −2.17   | C12H20O4         |                       |
| M20| 323 | 323.1496  | 2.57            | 0.24         | 323.1500            | −1.37   | C12H20O4         |                       |
| M21| 335 | 335.1495  | 3.68            | 0.43         | 335.1500            | −1.44   | C12H20O4         |                       |
| M22| 335 | 335.1495  | 2.38            | 2.52         | 335.1500            | −1.41   | C12H20O4         |                       |
| M23| 335 | 335.1496  | 3.57            | 0.12         | 335.1500            | −1.30   | C12H20O4         |                       |
| M24| 335 | 335.1496  | 3.06            | 0.19         | 335.1500            | −1.11   | C12H20O4         |                       |
| M25| 337 | 337.1650  | 3.10            | 0.33         | 337.1657            | −1.92   | C12H20O4         | OH-OH-MPHP            |
| M26| 337 | 337.1651  | 2.51            | 0.34         | 337.1657            | −1.79   | C12H20O4         | OH-OH-MPHP            |
| M27| 337 | 337.1651  | 2.88            | 0.25         | 337.1657            | −1.67   | C12H20O4         | OH-OH-MPHP            |
| M28| 353 | 353.1599  | 2.14            | 0.39         | 353.1606            | −1.91   | C12H20O4         |                       |

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a Retention time.
b Chemical structures of tentative exposure marker signals were speculated based on MS/MS analysis.
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.
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-MPHP, 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 ($5.46E+06\sim 2.34E+06$) (Fig. S2). The peak abundance ratios of the signals identified as OH-MPHP (M16, M17, and M18), and oxo-MPHP (M13) ($3.01E+05\sim 3.27E+04$) were clearly lower than those of M23 and M24 but were clearly higher than those of the signals identified as oxo-MPHP and OH-MPHP (1.44E+04\sim 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-MPHPxP, 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 re-
actions were used to predict the m/z values of DPHP metabolite
candidates. These m/z values were validated using urine sam-
ple 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 iso-
mers in these signals. Among the 6 speculated chemical
structures, 2 structures (oxo-MPHxP, and OH-MPHxP) were
novel tentative DPHP metabolites, and 2 structures (oxo-MPHxP,
and OH-MPHxP) were previously reported in the literature. These signals that were
speculated as OH-OH-MPHP had more higher abundance ratios
than those of the known DPHP metabolites and were suggested
to be suitable DPHP exposure makers for human exposure as-
sessments. 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
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