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4 1 Identification of potential biomarkers of exposure to diundecyl phthalate
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Abstract

Diundecyl phthalate (DUP) is a high production volume chemical used as a plasticizer in polyvinyl chloride and other plastics. Specific biomarkers of DUP would be useful for human exposure assessment. To identify such biomarkers, we investigated the *in vitro* metabolism of DUP with human liver microsomes using online solid phase extraction coupled to HPLC-mass spectrometry. Using high resolution mass spectrometry, we conclusively confirmed the structures of four DUP specific metabolites: monoundecyl phthalate (MUP), mono-hydroxyundecyl phthalate (MHUP), mono-oxoundecyl phthalate (MOUP), and mono-carboxydecyl phthalate (MCDP). We also used high resolution mass spectrometry to isolate MCDP and MHUP from co-eluting isobaric metabolites of diisononyl phthalate (i.e., monocarboxyisononyl phthalate) and diisododecyl phthalate (i.e., monohydroxyisododecyl phthalate), respectively, that could not be separated with low resolution tandem mass spectrometry. To evaluate the potential usefulness of the newly identified DUP metabolites as exposure biomarkers, we analyzed 36 human urine samples by high resolution mass spectrometry. We detected MHUP and MCDP in >83% of the samples; median concentrations were 0.21 ng/mL and 0.36 ng/mL, respectively. MOUP was detected only in 14% of the samples analyzed, and MUP was not detected. All three metabolites eluted as peak clusters likely because of the presence of multiple oxidation sites and multiple isomers in DUP technical mixtures. Taken together, these findings suggest that with the appropriate mass spectrometry quantification techniques, MHUP and MCDP may serve as suitable biomarkers for assessing background exposure to DUP.

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1 Key words: biomonitoring, *in vitro*, mass spectrometry, oxidative metabolites, DUP

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1. Introduction

Diundecyl phthalate (DUP, Palatinol® 111P-1, Jayflex™ L11P-E), a high molecular weight phthalate comprised of a pair of 11-carbon esters linked to a benzene-1,2-dicarboxylic moiety, is manufactured as a mixture of primarily branched chain isomers. DUP is a high production volume chemical (OECD 2015) mainly used as a polyvinyl chloride (PVC) plasticizer in applications requiring low fog and low temperature flexibility such as wiring, cable jacketing and insulation, furniture and automobile upholstery, and flooring and wall covering (NICNAS 2008; US Consumer Product Safety Commission 2011). DUP can also be used in non-PVC polymers; in flame retardant nylon, rubbers, paints, and adhesives (NICNAS 2008; US Consumer Product Safety Commission 2011). US production of DUP and its proportion in the total phthalate production market have been slowly increasing since the implementation of chemical tracking in 1982 (US Consumer Product Safety Commission 2011).

Toxicity data for DUP are relatively limited (Barber et al., 2000; David et al., 2001; Kwack et al., 2009; Kwack et al., 2010; Saillenfait et al., 2013). Exposure to C7-11 phthalate monoester mixture did not show mutagenic potential in the L5178Y mouse lymphoma mammalian cell mutation assay, or genotoxicity in the Balb/3T3 cell transformation assay (Barber et al., 2000). In another *in vitro* study, cell-to-cell communication was inhibited by the C7-C11 phthalate monoester mixture with rat and mouse hepatocytes, but not with hamster, cynomolgus monkey, or human hepatocytes or in a human liver cell line; the same C7-11 monoester mixture did not markedly change peroxisomal beta-oxidation in hepatocytes from any species (Kamendulis et al., 2002). Exposure to DUP (500 mg/kg body weight/day) for 4 weeks in Sprague-Dawley male

1 rats decreased sperm count and sperm mobility, although it did not significantly affect
2 liver or testis weights (Kwack et al., 2009). In another study, male Sprague-Dawley rats
3 administered 500 mg/kg body weight/day of DUP for two weeks showed significant
4 increases in aspartate aminotransferase and alkaline phosphatase (Kwack et al., 2010). In
5 Sprague-Dawley rats dosed with 0.5 and 1 g DUP/kg body weight/day by gavage on
6 gestation days 6–20, maternal body weight and food consumption were not affected, but,
7 compared to controls, treated fetuses showed small decreases in anogenital distance
8 (males) as well as a higher incidence of supernumerary lumbar ribs (Saillenfait et al.,
9 2013). Although health effects of DUP in humans are largely unknown, identifying
10 biomarkers of DUP would facilitate human exposure assessment, particularly because
11 exposures to phthalates and phthalate alternatives may be changing as a result, at least in
12 part, of legislative activity, industrial practices, public concerns and consumers' demands
13 (Goen et al., 2011; Health Canada 2013; Schutze et al., 2014; Silva et al., 2013;
14 Wittassek et al., 2007; Zota et al., 2014).

15 *In vitro* metabolism has been used to identify potential biomarkers of exposure to
16 select environmental chemicals (Choi et al., 2013; Roberts et al., 2012; Silva et al., 2015).
17 For the current study, we used high resolution mass spectrometry to conclusively identify
18 potential DUP specific exposure biomarkers after *in vitro* metabolism of DUP using
19 human liver microsomes. We also quantified the urinary concentrations of these DUP
20 metabolites in 36 samples collected in 2013–2015 from a convenience sample of adults
21 with no known occupational exposure to DUP.

22 **2. Materials and Methods**

2.1 Reagents and standards

DUP (>99%, CAS # 3648-20-2) and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-methyl-7-oxo-octyl phthalate (MONP), 4-methyl-7-hydroxyoctyl phthalate (MHNP), 4-methyl-carboxyheptyl phthalate (MCOP), d₄-MONP, d₄-MHNP and ¹³C₆-MCOP were purchased from ADM (Teltow, Germany). β-glucuronidase (*Escherichia coli-K12*) was purchased from Roche Biomedical (Mannheim, Germany). Acetonitrile (HPLC grade) was purchased from Burdick and Jackson (VWR, Radnor, PA). All reagents, solvents and standard materials were used without further purification.

2.2 Urine Collection

We used 36 spot urine specimens collected anonymously from U.S. adult volunteers in Atlanta, GA in 2013–2015, and stored at –70 °C until analysis. The volunteers had no documented occupational exposure to DUP. CDC’s Subjects Institutional Review Board reviewed and approved the collection of the samples. A waiver of informed consent was requested under 45 CFR 46.116(d); no personal or demographic data were available.

We also used archived urine, stored at -70 °C until analysis, that had been collected from rats dosed with diisodecyl phthalate (DiDP) (500 mg/kg body weight) as presented elsewhere (Kato et al., 2007).

2.3 In vitro metabolism of DUP

We followed a procedure similar to the approach described previously (Choi et al., 2013; Roberts et al., 2012; Silva et al., 2015). In a 30 mL Qorpak™ Clear Wide Mouth French square bottle (Fisher Scientific, Pittsburg, PA, USA), DUP standard solution (1 mL, 1.3 mg/mL) was concentrated to approximately 0.5 mL under a stream of nitrogen. Then, we added pH 7.4 phosphate buffer (0.1M, 8 mL), water (1 mL), NADPH solution (A) (500 µL, BD Gentest™, Woburn, MA, USA), NADPH solution (B) (100 µL, BD Gentest™) to generate NADPH *in situ* using an enzymatic reaction, and female pooled human liver microsomes (200 µL, BD Gentest™). The bottle was capped and the contents were gently mixed and placed in an incubator (Fisher Scientific, Hampton, NH, USA) at 37 °C for 8 h. Because of the limited water solubility of DUP and to produce *in vitro* metabolites in sufficient amounts for mass spectrometry identification, after 8 h, the solution was replenished with 500 µL NADPH solution (A), 100 µL NADPH solution (B), and 200 µL female human liver microsomes, gently mixed, and incubated for another 17 h. Aliquots of microsomal suspension (1 mL) were transferred into microcentrifuge tubes, vortex mixed, and centrifuged at 12,500 rpm for 20 min on an Avanti high performance centrifuge (Beckman Coulter Inc, Brea, CA, USA). The supernatant was transferred into autosampler vials for analysis (Silva et al., 2015). The above procedure was repeated without DUP, but with water (500 µL) for the preparation of the control samples.

2.4 Identification of *in vivo* and *in vitro* DUP metabolites

The HPLC gradient for separation of DUP metabolites and the on-line solid phase extraction (SPE) procedure were adapted from methods published elsewhere (Silva et al.,

2007). Briefly, *in vitro* metabolites in the supernatant of the human liver microsomal homogenate (500 μ L) obtained after incubating with DUP were extracted using on-line SPE on a Chromolith RP-18 pre-column (Merck KGaA, Darmstadt, Germany) and resolved on a Betasil phenyl HPLC column (3 μ M, 2.1mm $\times$ 25 mm, ThermoFisher Scientific, San Jose, CA, USA) using a water/acetonitrile gradient. The mass spectral analysis was initially performed on a TSQ Vantage AM triple quadrupole mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA). All ions on Q1 were scanned from $m/z = 150$ to $m/z = 480$ in electrospray ionization (ESI)-negative ion mode. ESI Q1 full scan produced multiple peaks at m/z of 319, 333, 335 and 349. For *in vivo* metabolite identification, human and rat urine (1 mL) were treated with β -glucuronidase before separation and detection of the DUP biomarkers (Silva et al., 2007). The identity of the DUP metabolites was confirmed by high resolution mass spectrometry using parallel reaction monitoring (PRM) on a QExactive Plus mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA) in negative mode to obtain accurate m/z of precursors without applying collision energy [CE]) and products of the DUP metabolites after applying CE (Table 1).

2.5 Quantification of DUP metabolites in human urine

After vortex mixing and aliquoting, human urine (100 μ L) was spiked with the internal standard solution containing d₄-MHNP, d₄-MONP, and ¹³C₆-MCOP. The target metabolites were extracted after hydrolysis with β -glucuronidase, chromatographically resolved by HPLC, and detected by high resolution mass spectrometry in PRM mode after applying CE (Table 1). The mobile phases were 0.1% acetic acid in water and 0.1%

1 acetic acid in acetonitrile. The DUP metabolites were quantified using the calibration
2 curves derived from analogous metabolites of di-isononyl phthalate (DiNP), namely
3 MHNP, MONP, and MCOP. The limits of detection (LOD) of all analytes were set at the
4 lowest standard concentration (0.01 ng/mL).

6 **3. Results and Discussion**

7 The metabolism of high molecular weight phthalates, such as DUP, generally
8 involves the formation of hydrolytic monoesters, followed by oxidation (Phase I reaction)
9 and/or conjugation (Phase II reaction) before urinary excretion. These metabolites, in
10 turn, can serve as biomarkers of exposure to the parent phthalate (Koch et al., 2004; Silva
11 et al., 2006). We undertook the current study to identify and characterize *in vitro* and *in*
12 *vivo* exposure biomarkers of DUP.

13 Full scan mass spectra in negative ion mode from $m/z=150$ to $m/z=480$ of the
14 supernatant of the human liver microsomes incubated with DUP revealed four unique
15 peaks with $m/z=319, 333, 335$ and 349 . We did not observe these peaks in the
16 microsomal suspension when incubated without DUP suggesting that the $m/z=319, 333,$
17 335 and 349 peaks corresponded to *in vitro* metabolites of DUP. First, we analyzed
18 fragmentation patterns through product ion scans and tentatively identified the m/z
19 specific for three oxidative ($m/z=333, 335, 349$) and one hydrolytic ($m/z=319$) DUP
20 metabolites. Then, we determined the accurate masses of these metabolites and their mass
21 fragments by high resolution mass spectrometry and conclusively identified the
22 metabolites as monoundecyl phthalate (MUP, $m/z=319.1915$, Figure 1S), mono-
23 oxoundecyl phthalate (MOUP, $m/z=333.17075$, Figure 1), monohydroxyundecyl

1 phthalate (MHUP, $m/z=335.18640$, Figure 2), and mono-carboxydecyl phthalate (MCDP,
2 $m/z=349.16566$, Figure 3). Interestingly, for the *in vitro* study we used reagent grade
3 DUP, and MOUP and MHUP eluted as peak clusters most likely because oxidation
4 occurred at different carbons on the C₁₁ side chain. By contrast, because MCDP can only
5 result from ω -oxidation in the side chain, the fact that MCDP eluted as three distinct
6 peaks suggests that the DUP used for the *in vitro* study also contained branched isomers
7 (Figure 4). Similarly, we observed three closely eluting MUP peaks (Figure 4).

8 We also detected these metabolites in human urine (Figure 4). Both *in vivo* and *in*
9 *vitro* metabolites produced similar mass fragments (Figures 1-3, S1). However, *in vitro*
10 metabolites resulting from incubation of DUP retained longer on the HPLC column than
11 the *in vivo* metabolites present in the human urine (Figure 4), perhaps reflecting
12 differences in metabolite structures (e.g., fraction of branched vs. linear isomers). Using
13 the same analytical method, we evaluated the elution of DUP metabolites in archived
14 urine samples from rats dosed with a commercial DiDP, a product that also contained
15 branched DUP isomers (European Chemicals Bureau 2003). The DUP metabolites in the
16 rat urine eluted at similar retention times and with similar peak shape as the DUP
17 metabolites detected in human urine. Of interest, in both human and rat urine, MCDP
18 eluted as a broad peak cluster suggesting that the DUP used in commercial products
19 contains mainly branched isomers with numerous potential ω -oxidation sites.

20 To study environmental exposure of DUP, we measured four DUP metabolites in
21 36 urine samples collected from a convenience sample of adults with no known
22 occupational exposure to DUP using online SPE coupled with HPLC and high resolution
23 mass spectrometry. We detected MHUP (86%) and MCDP (83%) in most samples,

1 whereas MOUP was detected only in 14% of the samples tested (Table 2). MHUP and
2 MCDP were also detected at higher concentrations than MOUP (Table 2). Because MUP
3 is relatively hydrophobic and would be further metabolized before its excretion in urine,
4 MUP was not detected. As expected, the urinary concentrations of MHUP correlated well
5 with MCDP ($p < 0.001$). Because high resolution mass selection excludes most isobaric
6 chemical interferences (Be et al., 2013), we achieved well defined chromatographic peak
7 clusters even at concentrations below the LOD of 0.01 ng/mL.

8 Simultaneous exposure to DiDP (C_{10} side chain), DUP, and diisododecyl
9 phthalate (DiDoP, C_{12} side chain) in humans would produce urinary metabolites isobaric
10 to some DUP metabolites [e.g., MHUP ($m/z = 335.186$) and monocarboxynonyl phthalate
11 (MCNP, $m/z = 335.150$); and MCDP ($m/z = 349.166$) and monohydroxydodecyl phthalate
12 (MHDoP, $m/z = 349.202$)] (Figure 5). Because all of these metabolites have similar
13 chemical characteristics and elute as peak clusters, full chromatographic separation is
14 difficult to achieve (Figure 5). However, high resolution mass spectrometry adequately
15 separated DUP metabolites in the presence of isobaric DiDP and DiDoP metabolites. We
16 observed a higher contribution from the isobaric DiDP metabolite (MCNP) to MHUP
17 than of the isobaric DiDoP metabolite (MHDoP) to MCDP, perhaps because human
18 exposure to DiDP is higher than the exposure to DiDoP. Because the relative abundance
19 of the specific fragments is different in isobars, when using low resolution tandem mass
20 spectrometry for quantifying MHUP and MCDP, isobaric interferences can be minimized
21 by selecting appropriate fragment(s) for product ion scan.

22 In summary, we identified four DUP specific *in vitro* metabolites, MUP, MHUP,
23 MOUP, and MCDP. Our data also suggest that MHUP and MCDP could serve as suitable

1 biomarkers for assessing DUP exposure in humans. High resolution mass spectrometry
2 would provide the best choice for quantification, because these metabolites may partially
3 co-elute with isobaric metabolites of DiDP or DiDoP. Therefore, if using the more
4 common low resolution tandem mass spectrometry biomonitoring approach to detect
5 DUP biomarkers, attention should be given to achieve adequate chromatographic
6 separation to remove isobaric interferences or to select appropriate fragments to
7 distinguish co-eluting isobaric metabolites from DiDP and DiDoP.

9 **4. Acknowledgement**

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14 Dr. Earl Gray and Johnathan Furr for conducting the rat dosing experiments.

16 **5. Disclaimer**

17 The findings and conclusions in this report are those of the authors and do not necessarily
18 represent the official position of the Centers for Disease Control and Prevention. The
19 authors declare they have no competing financial interests.

Table 1. QExactive high resolution mass spectrometric parameters for measuring the metabolites of diundecyl phthalate.

DUP metabolite	m/z		NCE*
	Precursor	Product	
Mono-oxoundecyl phthalate (MOUP)	333.1708	121.030	29
Mono-hydroxyundecyl phthalate (MHUP)	335.1864	187.170	25
Mono-carboxydecyl phthalate (MCDP)	349.1657	201.150	12

NCE*-normalized collision energy

Mono-oxoundecyl
phthalate

Monohydroxyundecyl
phthalate

Monocarboxydecyl
phthalate

The structure shown is for one isomer only.

Table 2. Urinary concentrations of diundecyl phthalate metabolites in a convenience sample of US adults^a (N=36)

Percentile	Urinary diundecyl phthalate metabolite concentrations ^b , ng/mL			
	MUP	MOUP	MHUP	MCDP
50 th	<LOD ^c	<LOD ^c	0.21	0.36
75 th	<LOD ^c	<LOD ^c	0.64	0.67
90 th	<LOD ^c	0.20	13.7	11.5
95 th	<LOD ^c	0.48	29.2	12.8

^aSpot urine samples were collected in 2013–2015 from a group of US adults with no known occupational exposure to DUP.

^bEstimated concentrations based on the calibration curves of DiNP metabolite analogs

^cLOD=0.01ng/mL

Figure 1: High resolution mass spectrometric fragmentation of mono-oxoundecyl phthalate identified in human liver microsomes (HLM) after *in vitro* metabolism of DUP (top) and in human urine (bottom).

Figure 2: High resolution mass spectrometric fragmentation of monohydroxyundecyl phthalate identified in human liver microsomes (HLM) homogenate after *in vitro* metabolism of DUP (top) and in human urine (bottom). Different ratios of fragments resulted due to structural differences in isomers of DUP used in *in vitro* study (mainly linear) and in commercial formulations (mainly branched).

Figure 3: High resolution mass spectrometric fragmentation of monocarboxydecyl phthalate identified in human liver microsomes (HLM) after *in vitro* metabolism of DUP (top) and in human urine (bottom).

Figure 4. Chromatographic separation of DUP metabolites detected in human liver microsomal (HLM) suspension after incubation with DUP, in human urine, and in DiDP dosed rat urine.

Figure 5. Interfering isobars MCNP ($m/z=335$) and MHDOP ($m/z=349$), from the metabolites of DiDP and DDoP to DUP metabolites, MHUP ($m/z=335$) and MCDP($m/z=349$).

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95 th	<LOD ^c	0.48	29.2	12.8

^aSpot urine samples were collected in 2013–2015 from a group of US adults with no known occupational exposure to DUP.
^bEstimated concentrations based on the calibration curves of DiNP metabolite analogs
^cLOD=0.01ng/mL