Identification of potential biomarkers of exposure to diundecyl phthalate

Manori J. Silva[*], **Trevor W. Bontke**, **Antonia M. Calafat**, and **Xiaoyun Ye**

Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, 4770 Buford Hwy, Mailstop F53, Atlanta, GA 30341, United States

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: mono-undecyl 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.*, mono-carboxyisononyl 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.

Keywords

[*]Corresponding author. ; Email: zca2@cdc.gov (M.J. Silva)..

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, 2010). DUP can also be used in non-PVC polymers; in flame retardant nylon, rubbers, paints, and adhesives (NICNAS, 2008; US Consumer Product Safety Commission, 2010). 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, 2010).

Toxicity data for DUP are relatively limited (Barber et al., 2000; David et al., 2001; Kwack et al., 2009, 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 rats decreased sperm count and sperm mobility, although it did not significantly affect liver or testis weights (Kwack et al., 2009). In another study, male Sprague-Dawley rats administered 500 mg/kg body weight/day of DUP for two weeks showed significant increases in aspartate aminotransferase and alkaline phosphatase (Kwack et al., 2010). In Sprague-Dawley rats dosed with 0.5 and 1 g DUP/kg body weight/day by gavage on gestation days 6–20, maternal body weight and food consumption were not affected, but, compared to controls, treated fetuses showed small decreases in anogenital distance (males) as well as a higher incidence of supernumerary lumbar ribs (Saillenfait et al., 2013). Although health effects of DUP in humans are largely unknown, identifying biomarkers of DUP would facilitate human exposure assessment, particularly because exposures to phthalates and phthalate alternatives may be changing as a result, at least in part, of legislative activity, industrial practices, public concerns and consumers' demands (Goen et al., 2011; Health Canada 2013; Schutze et al., 2014; Silva et al., 2013; Wittassek et al., 2007; Zota et al., 2014).

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

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), d_4-MONP, d_4-MHNP and $^{13}C_6$-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 (0.1 M, 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 in-cubated 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 μL) 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 μM, 2.1 mm × 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 QEx-active 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 μL) was spiked with the internal standard solution containing d_4-MHNP, d_4-MONP, and $^{13}C_6$-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% acetic acid in acetonitrile. The DUP metabolites were quantified using the calibration curves derived from analogous metabolites of di-isononyl phthalate (DiNP), namely MHNP, MONP, and MCOP. The limits of detection (LOD) of all analytes were set at the lowest standard concentration (0.01 ng/mL).

3. Results and discussion

The metabolism of high molecular weight phthalates, such as DUP, generally involves the formation of hydrolytic monoesters, followed by oxidation (Phase I reaction) and/or conjugation (Phase II reaction) before urinary excretion. These metabolites, in turn, can serve as biomarkers of exposure to the parent phthalate (Silva et al., 2013). We undertook the current study to identify and characterize *in vitro* and *in vivo* exposure biomarkers of DUP.

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

Fig. 1S), mono-oxoundecyl phthalate (MOUP, $m/z = 333.17075$, Fig. 1), mono-hydroxyundecyl phthalate (MHUP, $m/z = 335.18640$, Fig. 2), and mono-carboxydecyl phthalate (MCDP, $m/z = 349.16566$, Fig. 3). Interestingly, for the *in vitro* study we used reagent grade DUP, and MOUP and MHUP eluted as peak clusters most likely because oxidation occurred at different carbons on the C_{11} side chain. By contrast, because MCDP can only result from ω-oxidation in the side chain, the fact that MCDP eluted as three distinct peaks suggests that the DUP used for the *in vitro* study also contained branched isomers (Fig. 4). Similarly, we observed three closely eluting MUP peaks (Fig. 4).

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

To study environmental exposure of DUP, we measured four DUP metabolites in 36 spot urine samples collected from a convenience sample of adults with no known occupational exposure to DUP using online SPE coupled with HPLC and high resolution mass spectrometry. We detected MHUP (86%) and MCDP (83%) in most samples, whereas MOUP was detected only in 14% of the samples tested (Table 2). MHUP and MCDP were also detected at higher concentrations than MOUP (Table 2). Because MUP is relatively hydrophobic and would be further metabolized before its excretion in urine, MUP was not detected. As expected, the urinary concentrations of MHUP correlated well with MCDP ($p < 0.001$). Because high resolution mass selection excludes most isobaric chemical interferences (Be et al., 2013), we achieved well defined chromatographic peak clusters even at concentrations below the LOD of 0.01 ng/mL.

Simultaneous exposure to DiDP (C_{10} side chain), DUP, and diisododecyl phthalate (DiDoP, C_{12} side chain) in humans would produce urinary metabolites isobaric to some DUP metabolites [e.g., MHUP ($m/z = 335.186$) and monocarboxynonyl phthalate (MCNP, $m/z = 335.150$); and MCDP ($m/z = 349.166$) and mono-hydroxydodecyl phthalate (MHDoP, $m/z = 349.202$)] (Fig. 5). Because all of these metabolites have similar chemical characteristics and elute as peak clusters, full chromatographic separation is difficult to achieve (Fig. 5). However, high resolution mass spectrometry adequately separated DUP metabolites in the presence of isobaric DiDP and DiDoP metabolites. We observed a higher contribution from the isobaric DiDP metabolite (MCNP) to MHUP than of the isobaric DiDoP metabolite (MHDoP) to MCDP, perhaps because human exposure to DiDP is higher than the exposure to DiDoP. Because the relative abundance of the specific fragments is different in isobars, when using low resolution tandem mass spectrometry for quantifying MHUP and MCDP,

isobaric interferences can be minimized by selecting appropriate fragment (s) for product ion scan.

In summary, we identified four DUP specific *in vitro* metabolites, MUP, MHUP, MOUP, and MCDP. Our data also suggest that MHUP and MCDP could serve as suitable biomarkers for assessing DUP exposure in humans. High resolution mass spectrometry would provide the best choice for quantification, due to low LOD and because these metabolites may partially co-elute with isobaric metabolites of DiDP or DiDoP. Therefore, if using the more common low resolution tandem mass spectrometry biomonitoring approach to detect DUP biomarkers, attention should be given to achieve adequate chromatographic separation to remove isobaric interferences or to select appropriate fragments to distinguish co-eluting isobaric metabolites from DiDP and DiDoP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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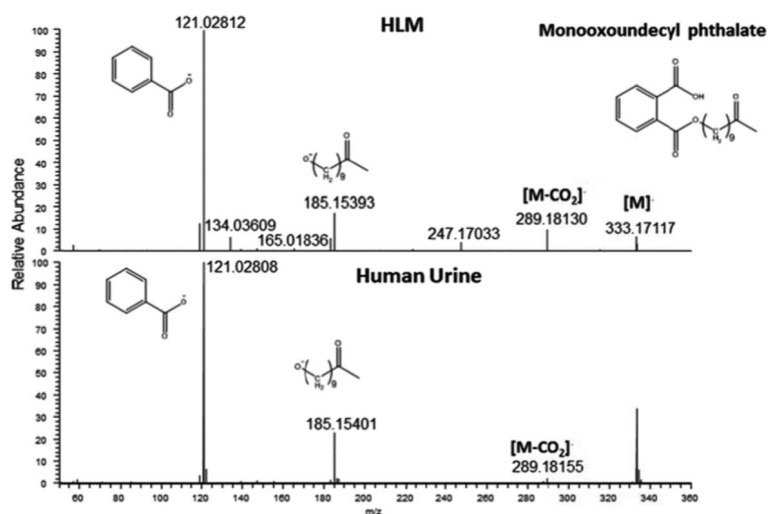
Fig. 1.
High resolution mass spectrometric fragmentation of mono-oxoundecyl phthalate identified in human liver microsomes (HLM) after *in vitro* metabolism of DUP (top) and in human urine (bottom).
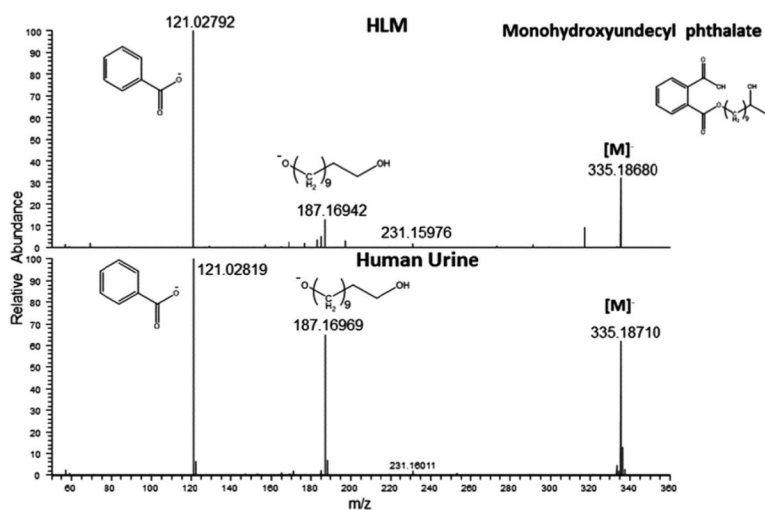
Fig. 2.
High resolution mass spectrometric fragmentation of monohydroxyundecyl phthalate
identified in human liver microsome (HLM) homogenate after *in vitro* metabolism of DUP
(top) and in human urine (bottom). Different ratios of fragments resulted due to structural
differences in isomers of DUP used in *in vitro* study (mainly linear) and in commercial
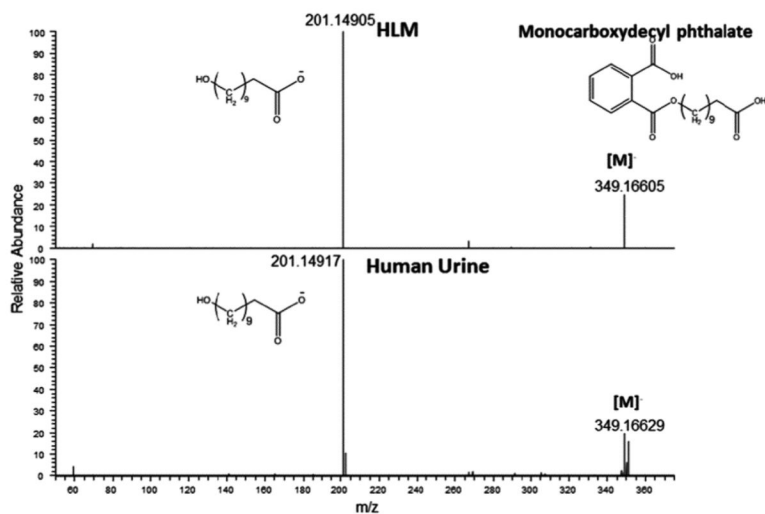formulations (mainly branched).

Fig. 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).
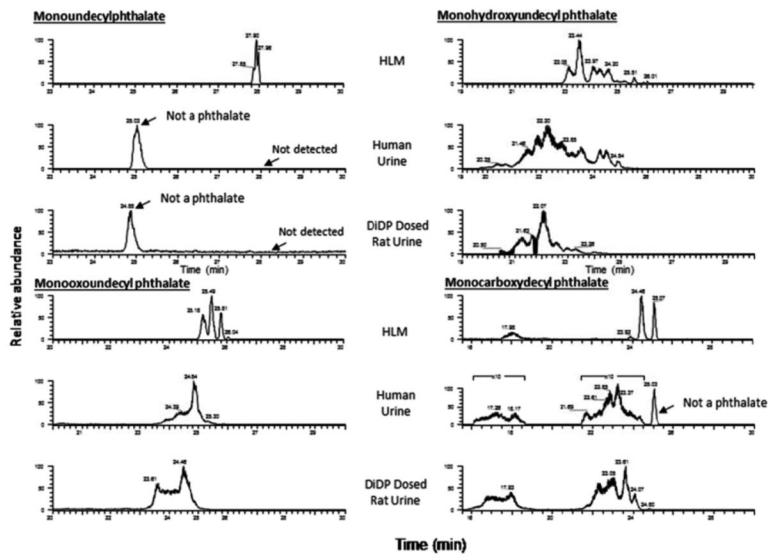
Fig. 4.
Chromatographic separation of DUP metabolites detected in human liver microsomal (HLM) suspension after incubation with DUP, in human urine, and in DiDP dosed rat urine.
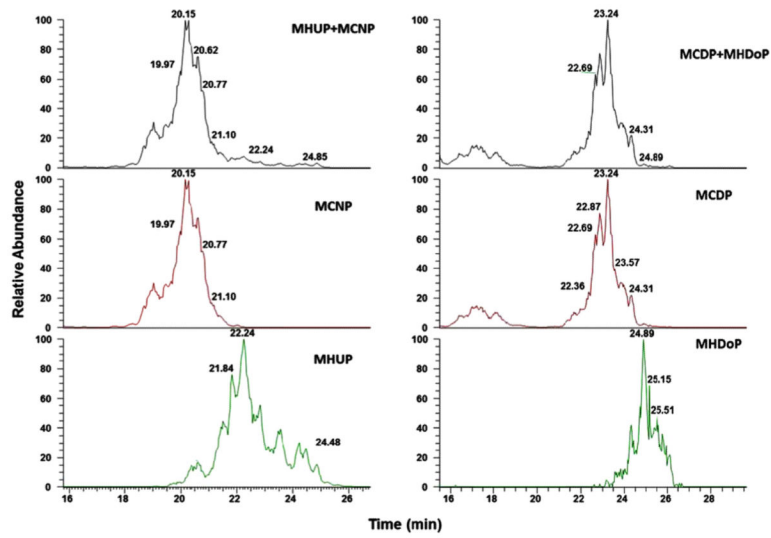
Fig. 5.
Interfering isobars MCNP ($m/z = 335$) and MHDoP ($m/z = 349$), from the metabolites of DiDP and DDoP to DUP metabolites, MHUP ($m/z = 335$) and MCDP($m/z = 349$).

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

Mono-oxoundecyl phthalate

Monohydroxyundecyl phthalate

Monocarboxydecyl phthalate
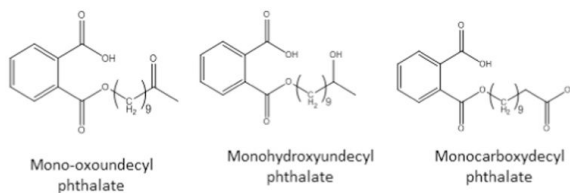
The structure shown is for one isomer only.

[*] NCE–normalized collision energy

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**
50th	<LOD[c]	<LOD[c]	0.21	0.36
75th	<LOD[c]	<LOD[c]	0.64	0.67
90th	<LOD[c]	0.20	13.7	11.5
95th	<LOD[c]	0.48	29.2	12.8

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

[b]Estimated concentrations based on the calibration curves of DiNP metabolite analogs

[c]LOD=0.01 ng/mL