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Assessment of human exposure to di-isodecyl phthalate using oxidative metabolites as biomarkers

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Abstract

Di-isodecyl phthalate (DiDP), primarily used as a plasticiser, is a mixture of isomers with predominantly ten-carbon branched side chains. Assessment of DiDP exposure has not been conducted before because adequate biomarkers were lacking. In 129 adult volunteers with no known exposure to DiDP, the urinary concentrations of three oxidative metabolites of DiDP: monocarboxyisononyl phthalate (MCiNP), monooxoisodecyl phthalate (MOiDP) and monohydroxyisodecyl phthalate (MHiDP), previously identified in DiDP-dosed rats, were estimated by solid-phase extraction coupled to high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) using the respective oxidative metabolites of di(2-ethylhexyl)phthalate since authentic standards of the DiDP oxidative metabolites were unavailable. Interestingly, the hydrolytic monoester of DiDP, monoisodecyl phthalate (MiDP), was not detected in any of the samples, while MCiNP, MHiDP and MOiDP were detected in 98%, 96% and 85%, respectively, of the samples tested. MCiNP was excreted predominantly in its free form, whereas MOiDP was excreted as its glucuronide. MCiNP, MHiDP and MOiDP eluted as clusters of multiple peaks from the HPLC column probably due to the presence of numerous structurally similar isomers present in commercial DiDP formulations. The urinary concentrations of these oxidative metabolites correlated significantly ($p < 0.0001$) with each other, thus confirming a common precursor. The urinary concentrations of these DiDP oxidative metabolites also correlated significantly ($p < 0.0001$) with oxidative metabolites of di-isononyl phthalate (DiNP) suggesting the potential presence of DiNP isomers in commercial DiDP or simultaneous use of DiDP and DiNP in consumer products. The concentrations presented are semiquantitative estimates and should be interpreted cautiously. Nevertheless, the higher frequency of detection and higher urinary concentrations of MCiNP, MHiDP and MOiDP than of MiDP suggest that these oxidative metabolites are better biomarkers for DiDP exposure assessment than MiDP. These data also suggest that unless oxidative metabolites are measured, the prevalence of exposure to DiDP will probably be underestimated.

Keywords: *DiDP, MiDP, di-isodecyl phthalate, monoisodecyl phthalate, oxidative metabolite, oxidative metabolism, biomonitoring*

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Introduction

Di-isodecyl phthalate (DiDP) is a phthalic acid ester with branched C9-11 isomeric side chains, predominantly C-10. DiDP is a general purpose plasticiser, used primarily to soften polyvinyl chloride (PVC) (CERHR 2000, Kavlock et al. 2002). DiDP's properties of volatility resistance, heat stability and electric insulation make it suitable as a plasticiser for heat-resistant electrical cords, leather for car interiors and PVC flooring. The typical content of DiDP in flexible PVC products is between 25% and 50% (CERHR 2000, Kavlock et al. 2002). Non-PVC applications are relatively minor, and include use in anti-corrosion and anti-fouling paints, sealing compounds, and textile inks. DiDP, like other phthalates, is not chemically bound to PVC and can be released throughout the lifecycle of the product.

Compared with other phthalates, such as di(2-ethylhexyl)phthalate (DEHP) and di-*n*-butyl phthalate (DBP), DiDP has low toxicity in animals (McKee et al. 2000). In reproductive studies with rats dosed with DiDP, no effects on fertility were noted, but reduced offspring survival and skeletal defects in the fetus (Waterman et al. 1999) were observed in the F2 generation (Exxon Chemical Company 1997). The results from several subchronic toxicity studies conducted in rats fed DiDP are suggestive of liver and kidney enlargement, reduced cytoplasmic basophilia and increased levels of peroxisomal enzymes (CERHR 2000). No information exists on the toxicity of DiDP in humans (CERHR 2000, Kavlock et al. 2002).

After exposure, DiDP is metabolised to its hydrolytic monoester, monoisodecylphthalate (MiDP), which subsequently metabolises further to form oxidative metabolites (Figure 1) with increased water solubility, which are more amenable to urinary excretion than MiDP. A similar metabolic pathway has been observed in rodents and/or humans for di-*n*-octyl phthalate (DnOP) (Silva et al. 2005), DEHP (Koch et al. 2004a, Koch et al. 2005a) and di-isononyl phthalate (DiNP) (McKee et al. 2002, Silva et al. 2006a). Although DiDP is widely used in consumer products, no DiDP human exposure assessment data are available because good biomarkers for exposure assessment to DiDP have been lacking. In DiDP-dosed rats, MiDP was present as a minor metabolite in the urine. By contrast, the oxidative metabolites, monohydroxyisodecyl phthalate (MHiDP), monocarboxyisononyl phthalate (MCiNP) and monooxodecyl phthalate (MOiDP) were the predominant metabolites (Kato et al., unpublished results). In the present study, the urinary concentrations of MHiDP, MCiNP and MOiDP (Figure 1), identified in DiDP-dosed rats previously (Figure 2), were estimated for the first time for exposure assessment of DiDP in humans. In humans at environmental exposure levels, MiDP was not detected, but urinary MHiDP, MOiDP, and MCiNP were present in most subjects studied indicating widespread exposure to DiDP.

Experimental procedures

Mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(3,7-dimethyloctyl) phthalate (MiDP), $^{13}C_4$-MEHHP, $^{13}C_4$-MEOHP, $^{13}C_4$-MiDP, and $^{13}C_4$-4-methyl-umbelliferone ($^{13}C_4$-MeUmb) were purchased from Cambridge Isotopes Laboratories, Inc. (Andover, MA, USA). Mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) and D_4-MECPP were gifts from Prof. Jurgen Angerer (Gilsing et al. 2005). High-performance liquid chromatography (HPLC)-grade acetonitrile and water were purchased from Tedia

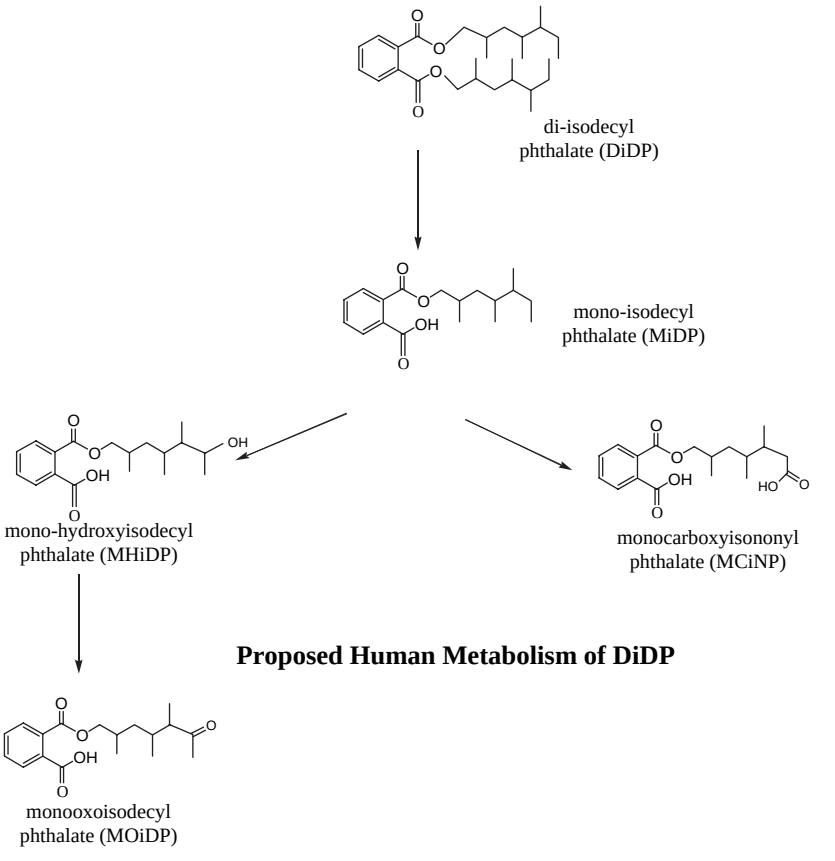

Figure 1. Di-isodecyl phthalate (DiDP) metabolites analysed as biomarkers for exposure assessment to DiDP in humans. Figure shows only one isomer from each metabolite class.

(Fairfield, OH), and MeUmb and its glucuronide (MeUmb-glu) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). β-Glucuronidase (*Escherichia coli*-K12) was purchased from Roche Biomedical (Mannheim, Germany). Stock solutions of standards (MEOHP, MEHHP, MECPP, MiDP and MeUmb) and internal standards ($^{13}C_4$-MiDP, $^{13}C_4$-MEHHP, $^{13}C_4$-MEOHP, D_4-MECPP and $^{13}C_4$-MeUmb) were prepared in acetonitrile.

The DiDP oxidative metabolites evaluated in the present study were identified in the urine of 75-day-old female Sprague-Dawley rats administered with a commercial DiDP formulation (300 mg kg^{-1}, CAS 68515-49-1 or 26761-40-0) by gavage. For each dosed rat, the urine samples were collected 24 h before and after DiDP administration. The DiDP oxidative metabolites were tentatively identified by full scan negative ion electrospray ionisation (ESI) mass spectra; compounds with similar molecular weights were further studied using accurate mass spectrum mode to confirm their identities (Kato et al., unpublished results). For the present study, human urine was analysed for the DiDP oxidative metabolite specific fragments at the same retention times observed in the urine of DiDP-dosed rats. The full scan mass spectra of DiDP oxidative metabolites of the urine samples from these rats (Figure 2) were identical to the full scan mass spectrum of DiDP oxidative metabolites from an individual with relatively

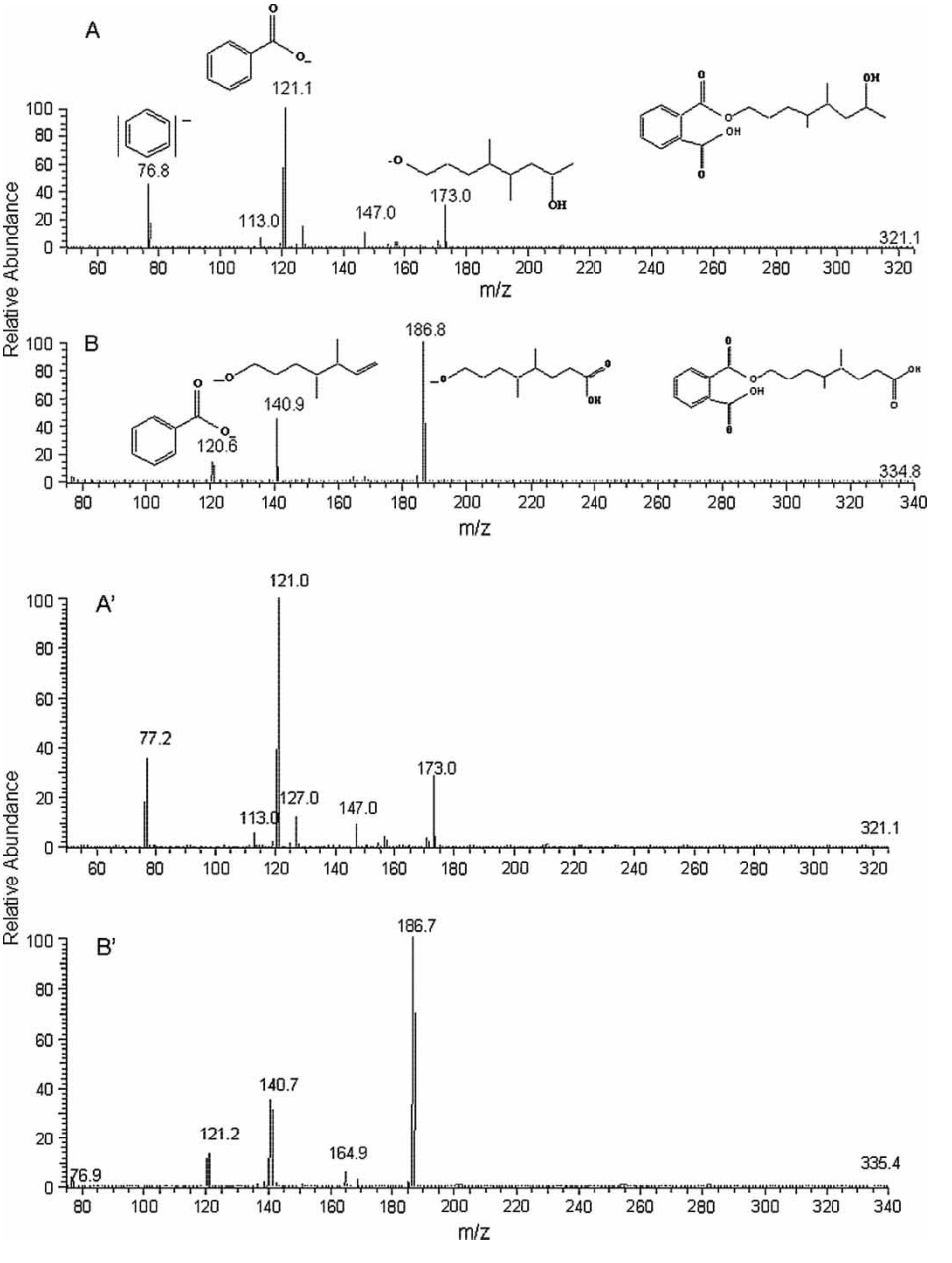

Figure 2. The full scan mass spectral fragmentation of isomers of monohydroxyisodecyl phthalate (MHiDP) (A) and monocarboxyisononyl phthalate (MCiNP) (B) in urine from rats dosed with di-isodecyl phthalate (DiDP). Similar mass spectral fragmentation patterns were observed for MHiDP (A') and MCiNP (B') in human urine. Structures shown are for only one of the potential isomers.

high urinary concentrations of DiDP oxidative metabolites (Figure 2) suggesting the presence of the same DiDP metabolic products in human urine.

The analytical method used was adapted from our previously developed methods for measuring phthalate metabolites in urine (Silva et al. 2004). Briefly, the urine

Table I. The precursor/product ion combinations used for estimating the concentrations of di-isodecyl phthalate (DiDP) metabolites.

DiDP metabolite	Precursor/production combination used for quantification	Phthalate metabolite/isotope-labeled metabolite used in the calibration
MCiNP	335/187	MECPP/D_4-MECPP
MHiDP	321/121	MEHHP/^{13}C-MEHHP
MOiDP	319/121	MEOHP/^{13}C-MEOHP
MiDP	305/261	MiDP/^{13}C-MiDP

samples (1 ml) were spiked with an internal standard solution containing $^{13}C_4$-MEOHP, $^{13}C_4$-MEHHP, D_4-MECPP, $^{13}C_4$-MiDP and $^{13}C_4$- MeUmb. MeUmb-glu was added to evaluate the completion of the deglucuronidation reaction. The samples were incubated with β-glucuronidase at 37°C for 90 min and the deconjugated phthalate metabolites were extracted by automated solid-phase extraction (SPE) using a commercial SPE system (Zymark Corporation, Hopkinton, MA, USA). The metabolites in the urine extract were then chromatographically resolved by HPLC using a Surveyor HPLC system (ThermoFinnigan, San Jose, CA, USA) equipped with a Betasil phenyl HPLC column (3 μm, 100 mm × 2.1 mm, ThermoHypersil-Keystone, Bellefonte, PA, USA) using a non-linear water: acetonitrile solvent gradient. The metabolites were detected by negative ion ESI tandem mass spectrometry (Table I) using a ThermoFinnigan TSQ Quantum triple quadruple mass spectrometer. For the analysis of unconjugated metabolites, treatment with β-glucuronidase was eliminated. The standards for MOiDP, MHiDP and MCiNP were not available to construct the calibration curves. Because of their structural similarities, recoveries of MOiDP, MHiDP and MCiNP were expected to be comparable to those of MEOHP, MEHHP and MECPP, respectively. Therefore the concentrations of MOiDP, MHiDP and MCiNP from the chromatographic peaks relevant to mass spectral transitions 319/221, 321/121 and 335/187, respectively, were estimated from the calibration curves constructed using MEOHP, MEHHP, and MECPP, respectively (Table I). $^{13}C_4$-MEOHP was used as the internal standard for MOiDP, D_4-MECPP was used as the internal standard for MCiNP, and $^{13}C_4$-MEHHP was used as the internal standard for MHiDP. The area under the whole peak encompassing all isomers was integrated for quantification. The limits of detection (LOD) of MEHHP, MEOHP and MECPP were used for MHiDP, MOiDP and MCiNP, respectively. LODs were 0.3 ng ml^{-1} (MiDP) and 0.25 ng ml^{-1} (MHiDP, MOiDP and MCiNP). Aliquots of the same urine samples from the same subjects were used for measurement of metabolites of DEHP, DiNP and DiDP.

Statistical analysis of the data was performed using Statistical Analysis System (SAS) software (SAS Institute, Cary, NC, USA). Results with values below the LOD were assigned a concentration equal to the LOD divided by the square root of two for the statistical analyses. Statistical significance was set at $p < 0.05$.

Subjects

The urine samples analysed for this study were collected during 2003 and 2004 from a demographically diverse group of 129 US adults of both sexes with no documented exposure to DEHP, DiNP or DiDP (Silva et al. 2006b, 2006c). No personal

information from the subjects was available. The samples were collected between 08.00 and 17.00 and were not necessarily first-morning voids.

Results

Several DiDP oxidative metabolites, including MHiDP, MOiDP and MCiNP, were identified as the major urinary metabolites of DiDP in rats dosed with DiDP (Figure 2), and in human urine (Figure 2). MHiDP, MOiDP and MCiNP were eluted as clusters, because of the presence of multiple isomers. The urinary concentrations of the oxidative metabolites and of MiDP were estimated in a group of 129 adults with no documented exposure to DiDP. MCiNP was detected in 98% of the samples analysed at concentrations ranging from <0.25 ng ml^{-1} to 335 ng ml^{-1}; MHiDP was detected in 96% of the samples at concentrations ranging from <0.25 ng ml^{-1} to 589 ng ml^{-1}, and MOiDP was detected in 85% of the samples at concentrations ranging from <0.25 ng ml^{-1} to 127 ng ml^{-1}. MiDP, the hydrolytic monoester of DiDP, was not detected at concentrations above 0.3 ng ml^{-1} in any of the samples. The geometric mean concentrations were 5.1 ng ml^{-1} (MCiNP), 1.4 ng ml^{-1} (MOiDP) and 5.2 ng ml^{-1} (MHiDP) (Table II). The urinary concentrations of these oxidative metabolites were highly correlated with each other ($p < 0.0001$; Figure 3, Table III). Furthermore, the urinary concentrations of oxidative metabolites of DiDP were also significantly correlated ($p < 0.001$) with monocarboxyisooctyl phthalate (MCiOP), monohydroxy isononyl phthalate (MHiNP) and monooxoisononyl phthalate (MOiNP); the oxidative metabolites of DiNP (Silva et al. 2006c). In this group of

Table II. Urinary concentrations (ng ml^{-1}) of di-isodecyl phthalate (DiDP) metabolites in a group of 129 US adults.

		Percentile							
DiDP metabolite[a]	n[b]	10th	25th	50th	75th	90th	95th	Geometric mean[c]	Frequency of detection (%)
MCiNP									
Total	129	0.9	1.9	4.4	9.6	43.0	104.4	5.1	98
Free	82	1.1	1.8	3.4	6.3	20.9	35.55	3.9	98
MHiDP									
Total	129	0.7	2.2	4.9	13.9	45.8	70.6	5.2	96
Free	82	0.3	0.4	0.6	1.4	3.5	13.8	0.8	90
MOiDP									
Total	129	<LOD	0.7	1.2	2.7	8.1	15.0	1.4	85
Free	82	<LOD	<LOD	<LOD	0.7	1.2	2.1	ND	41
MiDP									
Total	129	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0
Free	82	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0

[a]Concentrations were estimated using the calibration curves of MECPP/D_4-MECPP for MCINP, MEOHP/$^{13}C_4$-MEOHP for MOiDP, and MEHHP/$^{13}C_4$-MEHHP and MiDP/$^{13}C_4$-MiDP for MHiDP and MiDP, respectively. Geometric means were calculated if the frequency of detection was $\geq 60\%$.
[b]Free phthalate levels were measured only in 82 samples.
[c]LOD/$\sqrt{2}$ was used if the concentration was below the LOD. LODS were 0.25 ng ml^{-1} (MHiDP, MOiDP and MCiNP) and 0.3 ng ml^{-1} (MiDP).

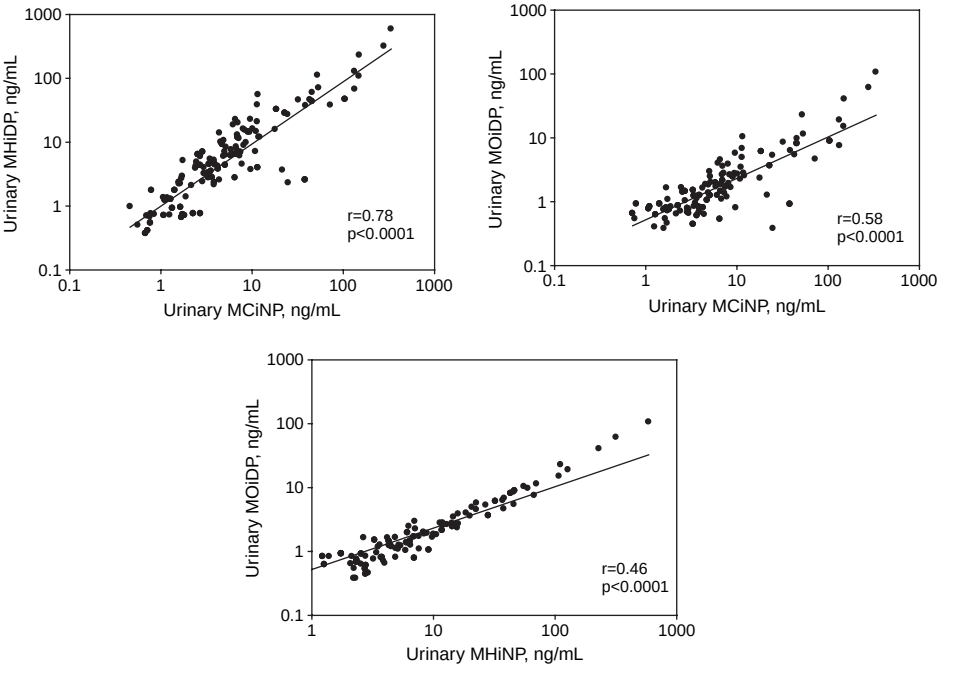

Figure 3. Correlation analyses of urinary monocarboxyisononyl phthalate (MCiNP), monohydroxyisodecyl phthalate (MHiDP) and monooxodecyl phthalate (MOiDP). Urinary concentrations below the LOD were excluded for the analysis. *r*, Pearson correlation coefficient.

129 adults, MCiNP was excreted mostly in its free form while MOiDP and MHiDP were excreted predominantly glucuronidated (Figure 4).

Discussion

The metabolism of DiDP, unlike that of DEHP (Albro et al. 1983, Koch et al. 2004a, Silva et al. 2006b, 2006d), another high-molecular-weight phthalate plasticiser, is not well studied in animals or in humans. Although DiDP is widely used in consumer products with annual production reaching 135 metric tons in 1998 (CERHR 2000),

Table III. Pearson correlation analysis of di-isodecyl phthalate (DiDP)[a] and di-isononyl phthalate (DiNP)[b] metabolites in a group of 129 US adults.

DiDP/DiNP metabolite	NCiNP[a]	MHiDP[a]	MOiDP[a]	MCiOP[b]	MHiNP[b]	MOiNP[b]
MCiNP[a]						
r	1.00	0.78	0.58	0.90	0.53	0.39
p	—	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
MHiDP[a]						
r	0.78	1.00	0.46	0.86	0.41	0.47
p	<0.0001	—	<0.0001	<0.0001	<0.0001	<0.0001
MOiDP[a]						
r	0.58	0.46	1.00	0.66	0.83	0.73
p	<0.0001	<0.0001	—	<0.0001	<0.0001	<0.0001

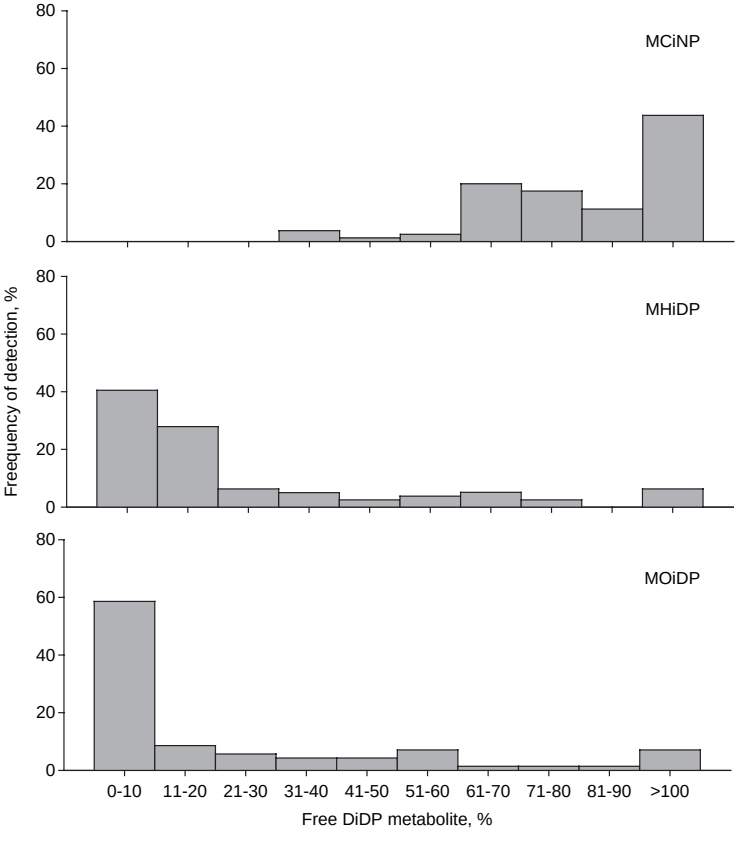

Figure 4. Frequency of detection of free urinary di-isodecyl phthalate (DiDP) metabolites.

no studies have been performed to assess human exposure because, to date, no adequate biomarkers were available for biomonitoring of DiDP. Discovery of sensitive biomarkers plays a major role in epidemiological investigations to study health effects of chronic exposure to environmental chemicals such as DiDP.

Phthalates with eight or more carbon side alkyl chains are known to metabolise extensively before excreting in urine (Albro et al. 1983, Koch et al. 2004a, 2005b, Silva et al. 2006b, 2006d). In human population studies, the urinary concentrations of these oxidative metabolites are substantially higher than the concentrations of the corresponding hydrolytic monoesters (Barr et al. 2003, Becker et al. 2004, Calafat et al. 2004, Kato et al. 2004, Koch et al. 2003, 2004a, 2004b). Therefore, oxidative metabolites can serve as better biomarkers for exposure assessment to high-molecular-weight phthalates than the hydrolytic monoesters.

In rats, similar to DiNP (McKee et al. 2002, Silva et al. 2006a), DnOP (Silva et al. 2005), and DEHP (Albro et al. 1983), several DiDP oxidative metabolites, including MHiDP, MOiDP and MCiNP, were identified as the major urinary metabolites of DiDP (Kato et al., unpublished results). In the present study, to establish whether these oxidative metabolites were also good biomarkers of exposure to DiDP in humans, the urinary concentrations of these metabolites and of MiDP were estimated in a group of 129 adults with no known exposure to DiDP (Table II). MiDP, the

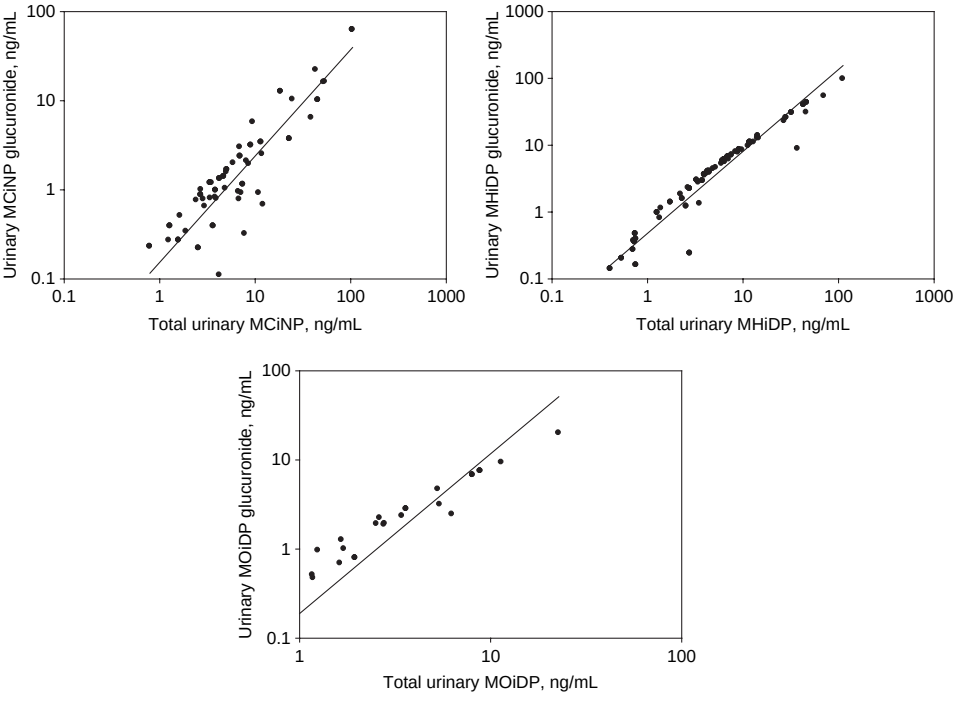

Figure 5. Correlation analysis of the glucuronide conjugate and the total (free and glucuronidated) di-isodecyl phthalate (DiDP) metabolites. Urinary concentrations <LOD were eliminated from the graphical representations.

hydrolytic monoester of DiDP was not detected at concentrations above 0.3 ng ml^{-1} in any of the samples. By contrast, MCiNP was detected in 98% of the samples analysed at concentrations ranging from <0.25 ng ml^{-1} to 334.5 ng ml^{-1}; MHiDP was detected in 96% of the samples at concentrations ranging from <0.25 ng ml^{-1} to 589 ng ml^{-1}, and MOiDP was detected in 85% of the samples at concentrations ranging from <0.25 ng ml^{-1} to 127.3 ng ml^{-1} (Table II). Considering the similar LODs for all four analytes, the higher frequency of detection and urinary concentrations of the oxidative metabolites than of MiDP suggest that these oxidative metabolites are better biomarkers for DiDP exposure assessment in humans than MiDP. In addition, the highly correlated ($p < 0.0001$; Figure 3) urinary concentrations of these oxidative metabolites with each other suggest a common parent. Of interest, the significant correlation of oxidative metabolites of DiDP ($p < 0.001$) with some oxidative metabolites of DiNP suggests either the presence of DiNP in DiDP commercial formulations or the simultaneous use of both DiDP and DiNP in product applications.

In this group of adults, MCiNP was excreted mostly in its free form while MOiDP and MHiDP were excreted predominantly glucuronidated (Figure 4). The increased hydrophilicity of the carboxylic acid oxidative metabolite compared with that of the alcohol or ketone metabolites may have reduced the need for glucuronidation to facilitate urinary excretion. Similarly, at these environmental exposure levels no saturation of the enzyme uridine 5'-diphosphoglucuronyl transferase which catalyses the glucuronidation reaction, was observed. As a result, the urinary concentration of

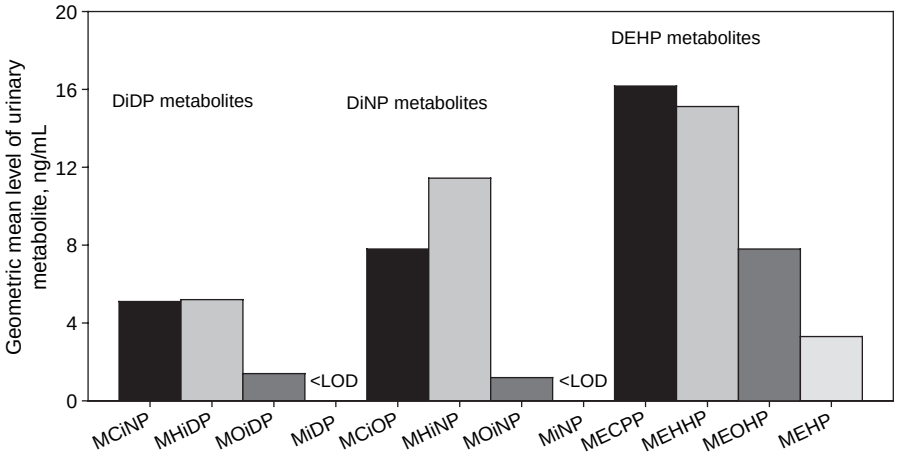

Figure 6. Median urinary concentrations of di-isodecyl phthalate (DiDP), di-isononyl phthalate (DiNP) and di(2-ethylhexyl)phthalate (DEHP) metabolites in a group of 129 US adults. For concentrations < LOD, LOD/$\sqrt{2}$ was used. Except for the DEHP metabolites, no authentic standards were available and the concentrations reported are semiquantitative estimates (see text).

the glucuronidated form of the metabolite increased with increasing concentrations of the corresponding total metabolite (Figure 5).

In this study population, the concentrations of the DiDP oxidative metabolites were lower when compared with the concentrations of analogous DiNP and DEHP oxidative metabolites (Figure 6) (Silva et al. 2006b, 2006c). Given the physicochemical similarities between DiDP, DiNP and DEHP, and assuming that the metabolism of these phthalates is comparable, lower levels of DiDP metabolites may mean that the general population exposure to DiDP is lower than that to DiNP or DEHP. However, because the concentrations of the DiDP and DiNP oxidative metabolites were semiquantitative estimates and the elimination half-lives of the DiDP oxidative metabolites or the excretion factors of DiDP and DiNP metabolites are unknown, no definite conclusions on the extent of exposure can be drawn from these data and they should be interpreted cautiously. Additional research is needed to determine the elimination toxicokinetics of the oxidative metabolites of DiDP and other high-molecular-weight phthalates.

Conclusion

For the first time, we present the usefulness of three oxidative metabolites of DiDP, namely MCiNP, MHiDP, and MOiDP as biomarkers for exposure assessment of DiDP in humans. We estimated the urinary concentrations of these oxidative metabolites and calculated the concentration of the hydrolytic monoester, MiDP, in 129 anonymous adults. Although MiDP was not detected in any of the samples tested, the oxidative metabolites were present in most of them suggesting that human exposure to DiDP is prevalent. These data along with the fact that oxidative metabolites cannot be formed as a result of contamination with the parent diester during sampling or analysis suggest that for DiDP, as for other high-molecular-weight phthalates, oxidative metabolites might be the most suitable biomarkers of exposure. In addition, for DEHP, the elimination half-life of its oxidative metabolites is longer

than that for mono(2-ethylhexyl)phthalate, its hydrolytic metabolite (Koch et al. 2004a, 2005a), suggesting that oxidative metabolites may be used to assess exposure in a wider time window than the hydrolytic metabolites. Additional research is needed to determine the elimination toxicokinetics of the oxidative metabolites of DiDP, their potential toxicity, and to obtain reference ranges for the concentrations of these metabolites in a representative sample of the general population.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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