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Measurement of eight urinary metabolites of di(2-ethylhexyl) phthalate as biomarkers for human exposure assessment

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Abstract

Human metabolism of di(2-ethylhexyl) phthalate (DEHP) is complex and yields mono(2-ethylhexyl) phthalate (MEHP) and numerous oxidative metabolites. The oxidative metabolites, mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) and mono(2-carboxymethyl-hexyl) phthalate (MCMHP), have been considered to be better biomarkers for DEHP exposure assessment than MEHP because urinary levels of these metabolites are generally higher than MEHP, and their measurements are not subject to contamination. The urinary levels of the above metabolites, and of three other recently identified DEHP oxidative metabolites, mono(2-ethyl-3-carboxypropyl) phthalate (MECPrP), mono-2-(1-oxoethylhexyl) phthalate (MOEHP), and mono(2-ethyl-4-carboxybutyl) phthalate (MECBP), were measured in 129 adults. MECPP, MCMHP and MEHHP were present in all the samples analysed. MEHP and the other oxidative metabolites were detected less frequently: MEOHP (99%), MECBP (88%), MECPrP (84%), MEHP (83%) and MOEHP (77%). The levels of all DEHP metabolites were highly correlated ($p < 0.0001$) with each other, confirming a common parent. The ω and ω-1 oxidative metabolites (MECPP, MCMHP, MEHHP and MEOHP) comprised 87.1% of all metabolites measured, and thus are most likely the best biomarkers for DEHP exposure assessment. The percentage of the unglucuronidated free form excreted in urine was higher for the ester linkage carboxylated DEHP metabolites compared with alcoholic and ketonic DEHP metabolites. The percentage of the unglucuronidated free form excreted in urine was higher for the DEHP metabolites with a carboxylated ester side-chain compared with alcoholic and ketonic metabolites. Further, differences were found between the DEHP metabolite profile between this adult population and that of six neonates exposed to high doses of DEHP through extensive medical treatment. In the neonates, MEHP represented 0.6% and MECPP 65.5% of the eight DEHP metabolites measured compared to 6.6% (MEHP) and 31.8% (MECPP) in the adults. Whether the observed differences reflect differences in route/duration of the exposure, age and/or health status of the individuals is presently unknown.

Keywords: *DEHP, MEHP, Di(2-ethylhexyl) phthalate, biomonitoring, phthalates*

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Introduction

Di(2-ethylhexyl) phthalate (DEHP) has numerous commercial applications (ATSDR 2002). In 1986, industries consumed an estimated 95% of DEHP as a plasticizer for polyvinyl chloride (PVC), and 5% for other uses. DEHP is a primary component in PVC plastics used in household products, toys, floor tiles, furniture upholstery, blood storage bags and medical devices (ATSDR 2002). Because of its ubiquitous presence, the potential for human exposure to DEHP is high. DEHP is not chemically bound to the plastics and can leach to the environment during the manufacturing process, product use and after disposal. The general population is exposed to DEHP mainly via ingestion or inhalation. Mono(2-ethylhexyl) phthalate (MEHP), the hydrolytic metabolite of DEHP, is found in human urine (Brock et al. 2002, Silva et al. 2004a), serum (Silva et al. 2003a), saliva (Silva et al. 2005b), breast milk (Calafat et al. 2004b) and amniotic fluid (Silva et al. 2004b). Patients, through intravenous infusion of drugs or nutrition solutions, transfusion of blood or blood products, or medical treatments such as cardiopulmonary bypass, extracorporeal membrane oxygenation, haemodialysis and peritoneal dialysis, may be at risk for exposure to DEHP doses much higher than those to which the general population is exposed (Roth et al. 1988, Plonait et al. 1993, Koch et al. 2005b). At high doses, DEHP, a liver carcinogen, alters thyroid structure and activity and also produces developmental and reproductive toxicities in rodents (Gray et al. 1982, ATSDR 2002). DEHP is known to produce decreased testicular weight (Sjoberg et al. 1986), severe testicular atrophy and reduced weight of sex organs in adult male rats by a mechanism thought to involve decreased fetal testosterone synthesis during male sexual differentiation (Parks et al. 2000).

Similar to its structural isomer di-*n*-octyl phthalate (Silva et al. 2005a), after exposure DEHP is metabolized to its hydrolytic monoester, MEHP, which then undergoes ω, ω-*n*, α or β oxidation to form several oxidative metabolites (ATSDR 2002, Barr et al. 2003, Kato et al. 2004, Koch et al. 2004a, 2005a, Silva et al. 2006). MEHP and other metabolic products are excreted in urine or faeces as free metabolites or glucuronidated conjugates (Silva et al. 2003a). Age, sex, health status, dose and route of exposure may influence the relative concentration of the oxidative metabolites in urine. Until recently, DEHP exposure assessment in humans, using biomonitoring data, relied mostly on urinary concentrations of MEHP (Blount et al. 2000b, Brock et al. 2002, Hoppin et al. 2002, Adibi et al. 2003, Duty et al. 2003a,b, 2004, 2005, Silva et al. 2004a). However, in recent studies, urinary concentrations of the DEHP oxidative metabolites mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) and mono(2-carboxymethylhexyl) phthalate (MCMHP) have been used, along with MEHP, to assess exposure to DEHP (Barr et al. 2003, CDC 2005, Kato et al. 2004, Koch et al. 2004a, 2005a). Recently, the present authors identified three additional oxidative metabolites of DEHP in human urine (Silva et al. 2006), mono(2-ethyl-3-carboxypropyl) phthalate (MECPrP), mono(2-(1-oxoethyl)hexyl) phthalate (MOEHP) and mono(2-ethyl-4-carboxybutyl) phthalate (MECBP), known to be DEHP metabolites in rodents (Albro et al. 1983, Albro 1986). The present study measured eight DEHP metabolites (Figure 1), MECPrP, MOEHP, MECBP, MEHP, MEHHP, MEOHP, MECPP and MCMHP, in urine samples from 129 adults with no known occupational exposure to DEHP. Furthermore, the DEHP metabolic profiles were studied both in this group of adults and in six neonates undergoing medical treatment in a neonatal intensive care unit.

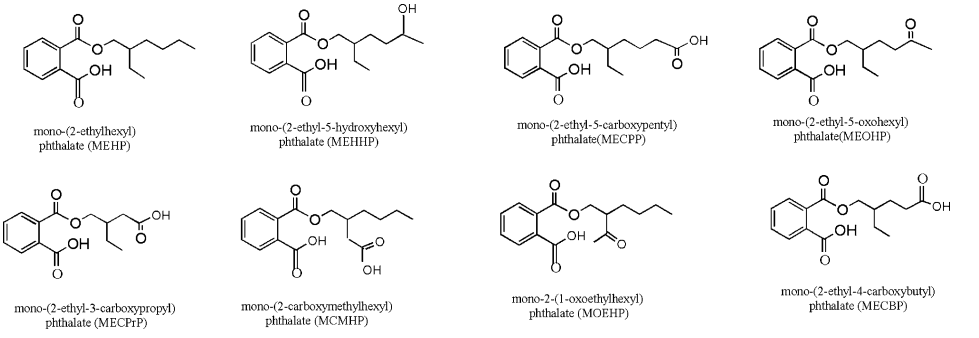

Figure 1. Di(2-ethylhexyl) phthalate (DEHP) metabolites analysed as biomarkers for exposure assessment to DEHP in humans.

Materials and methods

MEHHP, MEOHP, MEHP, $^{13}C_4$-MEHP, $^{13}C_4$-MEHHP, $^{13}C_4$-MEOHP and $^{13}C_4$-4-methyl-umbelliferone ($^{13}C_4$-MeUmb) were purchased from Cambridge Isotopes Laboratories, Inc. (Andover, MA, USA) with chemical and isotopic purities $>98\%$. MECPP and D_4-MECPP (purity $>98\%$) were generous gifts from Professor Jurgen Angerer (Erlangen, Germany). HPLC-grade acetonitrile and water were purchased from Tedia (Fairfield, OH, USA), 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 (MEHP, MEOHP, MEHHP, MECPP and MeUmb) and internal standards ($^{13}C_4$-MEHP, $^{13}C_4$-MEHHP, $^{13}C_4$-MEOHP, D_4-MECPP and $^{13}C_4$-MeUmb) were prepared in acetonitrile. D_4-MECPP was used as the internal standard for MECPP, MECPrP, MECBP and MCMHP. $^{13}C_4$-MEOHP was used as the internal standard for both MEOHP and MOEHP, and $^{13}C_4$-MEHHP and $^{13}C_4$-MEHP were used as internal standards for MEHHP and MEHP, respectively.

The analytical method for measuring phthalate metabolites in urine was adapted from the authors' previously developed methods (Blount et al. 2000a, Silva et al. 2003b, 2004c). Briefly, the urine samples (1 ml) were spiked with an internal standard solution containing $^{13}C_4$-MEHP, $^{13}C_4$-MEOHP, $^{13}C_4$-MEHHP, D_4-MECPP and $^{13}C_4$-MeUmb, and with a standard solution of MeUmb-glu. Phthalate metabolites were extracted by automated solid-phase extraction (SPE) using a commercial SPE system (Zymark Corporation, Hopkinton, MA, USA) after enzymatic hydrolysis with β-glucuronidase (0.8 μg ml^{-1}, 50 μl) at 37°C for 90 min to deconjugate completely the phthalate metabolites from their glucuronidated form MeUmb was measured to evaluate the completion of the deglucuronidation reaction. The phthalate metabolites in the urine extract were chromatographically resolved by high-performance liquid chromatography (HPLC) using a Surveyor HPLC system (ThermoFinnigan, San Jose, CA, USA) equipped with a Betasil phenyl HPLC column (3 μm, 100 × 2.1 mm; ThermoHypersil-Keystone, Bellefonte, PA, USA) using a non-linear water:acetonitrile solvent gradient. The metabolites were detected by negative-ion electrospray ionization mass spectrometry using a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer. For the analysis of unconjugated metabolites, treatment with β-glucuronidase was eliminated. The limits of detection (LODs) were 0.9 ng ml^{-1} (MEHP) and 0.25 ng ml^{-1} (MEOHP, MEHHP, and MECPP,

MCMHP, MECPrP and MECBP). For MCMHP, MECPrP and MECBP, an LOD = 0.25 ng ml^{-1} was used because these three metabolites were quantified using either the MECPP or the MEOHP calibration curves.

Statistical analysis of the data was performed using Statistical Analysis System (SAS) software (SAS Institute, Cary, NC, USA). Samples with values below the LODs were assigned a concentration equal to the LOD divided by the square root of 2 for the statistical analyses. Statistical significance was set at $p < 0.05$.

Subjects

The urine samples analysed were collected from a demographically diverse group of 129 US male and female adults with no documented exposure to DEHP. No personal information from the subjects was available. The samples were collected between 08.00 and 17.00 hours during 2003 and 2004 and were not first-morning voids. Urine samples used for comparison were collected from six patients in a neonatal intensive care unit after obtaining informed consent. Details about enrolment of the study subjects have been described elsewhere (Calafat et al. 2004a).

Results and discussion

The complicated metabolism of DEHP is relatively well studied in rodents (Albro et al. 1984, 1987, Albro 1986, Albro & Lavenhar 1989). In both rodents and humans, initial hydrolysis of DEHP produces MEHP, which can undergo a series of oxidation reactions that result in oxidative metabolic products (ATSDR 2002, Koch et al. 2004a, 2005a). Human toxicokinetic studies suggest that about 75% of the DEHP oral dose is excreted in urine within 48 h of exposure as MEHP and four oxidative metabolites, MEHHP, MEOHP, MECPP and MCMHP (Koch et al. 2004a, 2005a). The present study measured the urinary concentrations of these five DEHP metabolites in 129 samples from adult anonymous donors (Figure 1). We also measured the urinary concentrations of three other DEHP metabolites, MECBP, MECPrP and MOEHP, identified previously in rats and mice that had been administered DEHP (Albro et al. 1983, Albro 1986) and in humans (Silva et al. 2006). The urinary concentrations of these eight DEHP metabolites varied widely (Figure 2), ranging from 0.6 to 298.5 ng ml^{-1} (MECPP), from 0.5 to 93.5 ng ml^{-1} (MCMHP), from 0.3 to 367.5 ng ml^{-1} (MEHHP), from <LOD to 175.5 ng ml^{-1} (MEOHP), from <LOD to 85.2 ng ml^{-1} (MEHP), from <LOD to 13.9 ng ml^{-1} (MOEHP), from <LOD to 33.4 ng ml^{-1} (MECBP) and from <LOD to 29.9 ng ml^{-1} (MECPrP) (Table I). MEHP, MEHHP, MEOHP, MECPP and MCMHP comprised 93.6% of the eight DEHP metabolites monitored in urine. MECPP and MCMHP, two metabolites with a carboxylic acid group in the 8-carbon alkyl side-chain, comprised 42% of the eight metabolites measured (Figure 3). The levels of MECPrP and MECBP, two carboxylate metabolites with 7- and 6-carbon alkyl side-chain, respectively, were relatively low compared with other oxidative products. However, measuring all the metabolites may be important because of potential differences in biological activity of each metabolite that warrant further research.

As expected, because all eight metabolites result from DEHP, the urinary levels were highly correlated with each other (Figure 4 and Table II), similar to previous

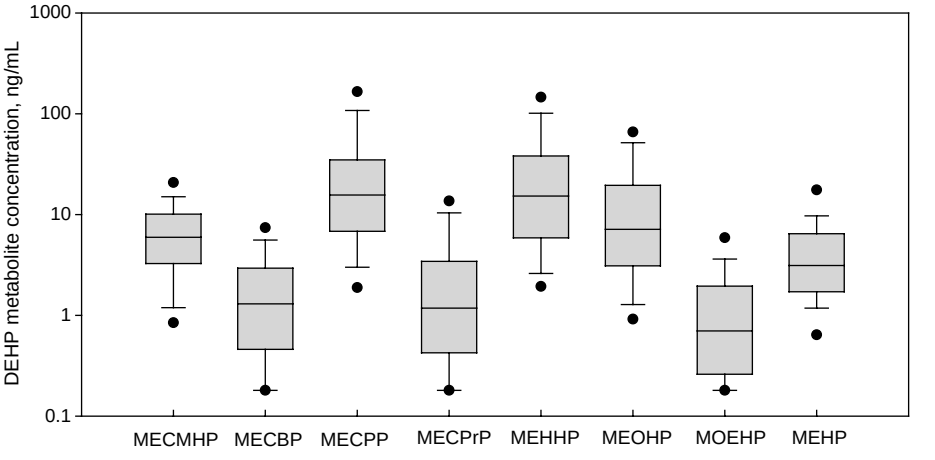

Figure 2. Levels of eight DEHP metabolites in a group of 129 US adults. Circles indicate the 5th and 95th percentiles; horizontal bars inside the boxes indicate the medians; whiskers above and below are the 10th and 90th percentiles; and box boundaries are the 25th and 75th percentiles. For concentrations <LOD, LOD/$\sqrt{2}$ was used.

findings regarding MEHHP and MEOHP (Barr et al. 2003, Kato et al. 2004, Koch et al. 2004a). Although highly significant ($p < 0.0001$), the correlations between MEHP and the oxidative metabolites were not as good (Pearson correlation coefficient, r, ranged from 0.46 to 0.62) as among oxidative metabolites (r between 0.58 and 0.99). Contribution to MEHP levels from the ubiquitously present DEHP in the environment via abiotic hydrolysis may explain the relatively poor correlations between MEHP and the other oxidative metabolites.

The degree of conjugation of the DEHP metabolites in the 129 adults was also estimated by measuring the concentrations of the free metabolite species after conducting the analyses with and without enzymatic hydrolysis of the phthalate conjugates. MECPP, MCMHP, MECBP and MECPrP, the carboxylic acid metabolites, were excreted only partially glucuronidated (Figure 5A); MEHHP, MEOHP and MOEHP, the alcohol and ketone metabolites, were excreted mainly glucuronidated. The distribution of free MOEHP was similar to that of its structural isomer MEOHP (Figure 5B). MECPrP, the most hydrophilic carboxylic acid metabolite, had the lowest median frequency of glucuronidation (about 20%). The increased water solubility of MCMHP, MECPrP, MECBP and MECPP due to the additional carboxylate moiety in the side-chain may explain the relatively low degree of glucuronidation for these metabolites.

Urinary levels of the conjugated and total species correlated well (Figure 6). The concentration of the conjugated species did not plateau even at the highest levels of the total species, indicating that saturation or inhibition of the enzyme catalysing the glucuronidation reaction did not occur at environmental exposure levels.

The DEHP urinary metabolic profile was examined in 32 samples from six neonatal intensive care unit patients exposed to DEHP doses much higher than the doses to which the general population had been exposed (Calafat et al. 2004a, Silva et al. 2004a, CDC 2005) and the DEHP urinary metabolic profile from the neonatal patients was compared with the profile of the 129 adults with no known occupational exposure to DEHP. Although all eight DEHP metabolites were present in both

Table I. Urinary levels (ng ml^{-1}) of eight DEHP metabolites in a group of 129 US adults.

Urinary DEHP metabolite[a]	*n*	Percentile						Geometric mean[b]	Minimum	Maximum	Frequency of detection (%)
		5th	25th	50th	75th	90th	95th				
MEHP											
total	129	0.9	1.7	3.1	6.3	9.7	17.0	3.3	<LOD	85.2	83
free	82	<LOD	<LOD	0.8	1.1	1.5	2.2	0.9	<LOD	13.8	38
MCMHP											
total	129	0.9	3.3	5.9	10.0	15.0	20.7	5.2	0.5	93.5	100
free	82	0.3	0.9	2.5	5.1	11.6	16.3	2.2	<LOD	35.2	96
MECPP											
total	129	1.9	7.0	15.6	34.5	107.9	159.3	16.2	0.6	298.5	100
free	82	1.3	3.8	7.0	13.7	22.8	48.1	7.4	0.6	153.7	100
MEHHP											
total	129	2.0	5.9	15.3	37.9	101.2	120.8	15.1	0.3	367.5	100
free	82	<LOD	<LOD	1.0	2.8	6.7	12.9	1.0	<LOD	221.8	70
MEOHP											
total	129	0.9	3.1	7.1	19.4	51.6	62.4	7.8	<LOD	175.5	99
free	82	<LOD	<LOD	0.9	2.0	4.2	5.8	1.0	<LOD	117.6	87
MOEHP											
total	129	<LOD	0.3	0.7	2.0	3.6	4.7	0.7	<LOD	13.9	77
free	82	<LOD	<LOD	<LOD	<LOD	0.5	0.6	<LOD	<LOD	7.3	17
MECBP											
total	129	<LOD	0.5	1.3	2.9	5.6	7.1	1.2	<LOD	33.4	88
free	82	<LOD	<LOD	0.4	0.9	1.6	3.0	0.5	<LOD	19.2	63
MECPrP											
total	129	<LOD	0.4	1.2	3.2	10.4	13.1	1.3	<LOD	29.9	84
free	82	<LOD	0.3	0.8	2.2	3.8	7.5	0.9	<LOD	27.0	78

[a]D_4-MECPP was used as the internal standard for MECPP, MECPrP, MECBP and MCMHP. $^{13}C_4$-MEOHP was used as the internal standard for MEOHP and MOEHP. $^{13}C_4$-MEHHP and $^{13}C_4$-MEHP were used as internal standards for MEHHP and MEHP, respectively.
[b]LOD/v2 was used if the concentration was below the LOD. LOD was 0.25 ng ml^{-1} for all metabolites, except MEHP, for which it was 0.9 ng ml^{-1}.

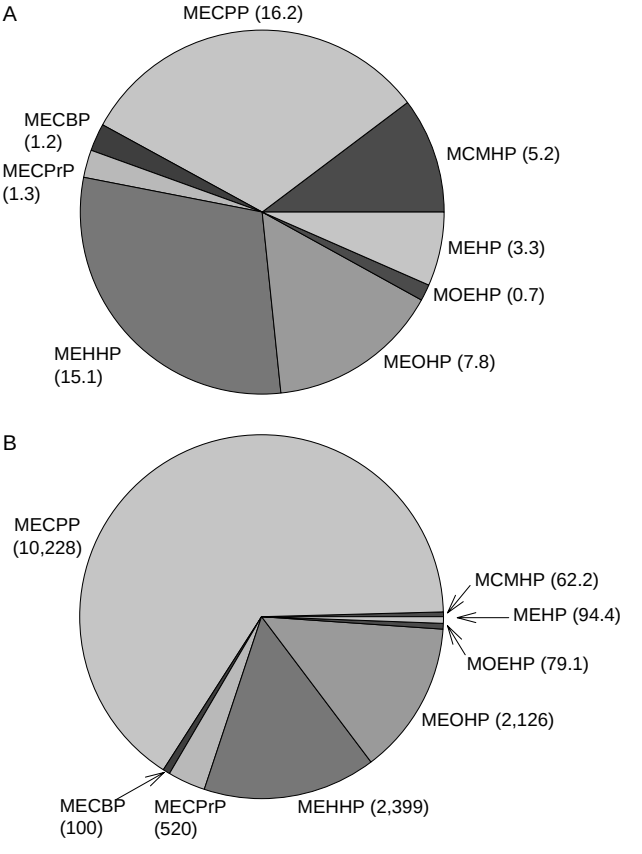

Figure 3. Urinary metabolic profile of eight DEHP metabolites in (A) 129 US adults with no known occupational exposure to DEHP and (B) six neonatal intensive care unit patients for whom 32 samples were collected. Samples analysed are not necessarily the same samples reported in Calafat et al. (2004a). Geometric mean concentrations (ng ml^{-1}) are given in parenthesis.

population groups, the distribution of metabolites differed (Figure 3). In adults, the geometric mean level of MECPP represented 31.8%, MEHHP 29.7%, MEOHP 15.3%, MCMHP 10.3% and MEHP 6.6% of all eight DEHP metabolites measured. MECBP, MECPrP and MOEHP represented only 2.4, 2.5 and 1.5%, respectively (Figure 3). In the neonates, the geometric mean level of MECPP comprised 65.5% of the eight DEHP metabolites measured; MCMHP, MECBP and MOEHP comprised less than 0.6%, MEHP was only 0.6%, and MEHHP, MEOHP and MECPrP represented 15.4, 13.6 and 3.3%, respectively (Figure 3).

Several factors may have contributed to the different distribution of metabolites in these two populations, including the route and duration of the exposure, and the age and health status of the individuals. Intravenous exposure to DEHP in the neonates was not relevant among the adults who were most likely exposed to DEHP by ingestion or inhalation. Furthermore, the neonates likely were exposed to doses of DEHP at much higher levels than estimated environmental doses more or less continuously for at least 2 weeks before the samples were collected (Calafat et al. 2004a). The adults were most likely exposed to intermittent environmental doses of DEHP. In addition, the adults were healthy, and the neonates were critically ill. It is

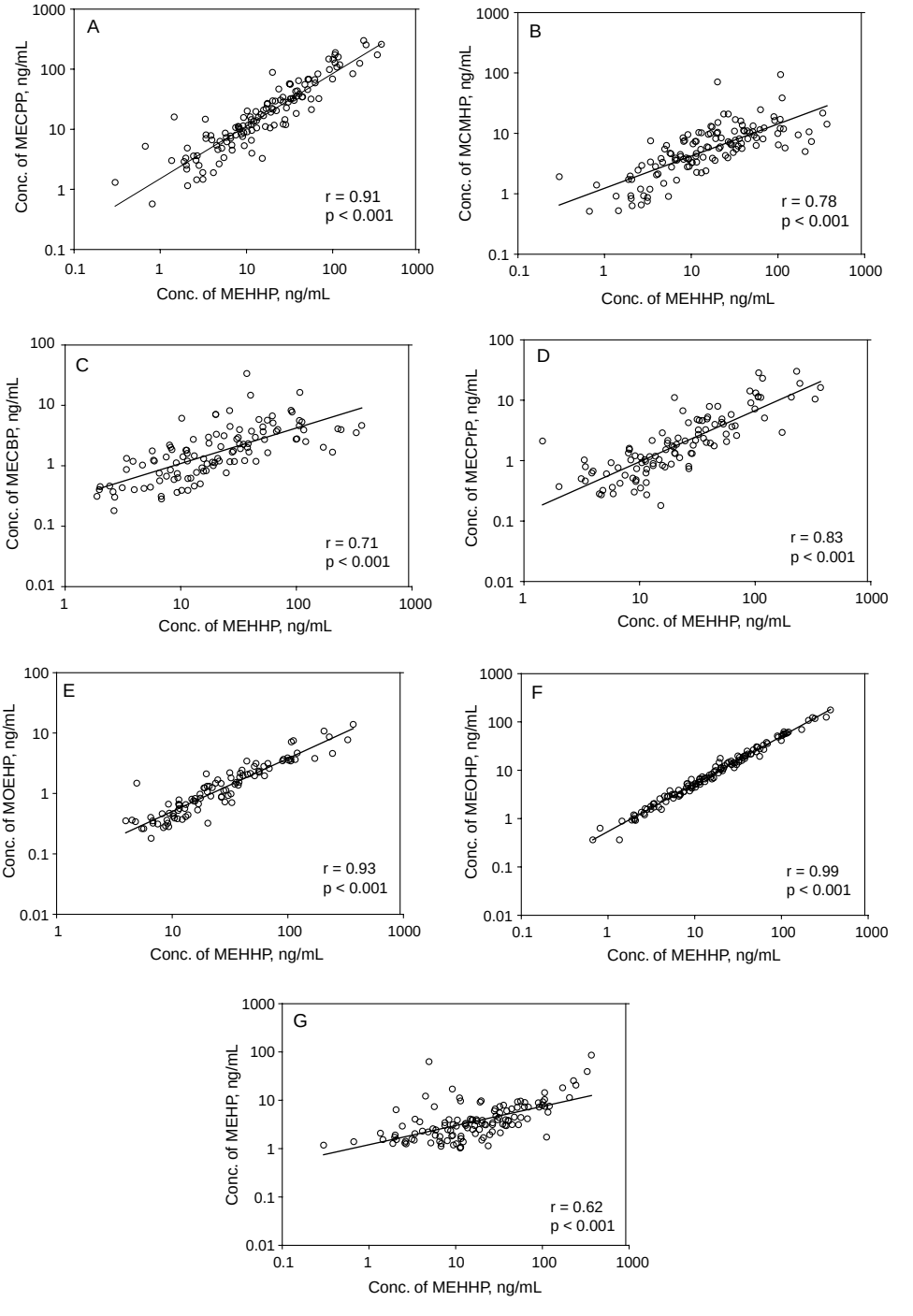

Figure 4. Correlation analyses of urinary MECPP, MCMHP, MECBP, MECPrP, MOEHP and MEHP versus MEHHP. *r*, Pearson correlation coefficient. The levels <LOD were excluded in the analysis.

Table II. Pearson correlation analysis of eight urinary DEHP metabolites in a group of 129 US adults.

DEHP metabolite	MCMHP	MECBP	MECPP	MECPrP	MEHHP	MEOHP	MOEHP	MEHP
MCMHP								
r	1.00	0.77	0.77	0.63	0.76	0.78	0.58	0.46
p	–	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
MECBP								
r	0.77	1.00	0.79	0.71	0.71	0.72	0.62	0.49
p	<0.0001	–	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
MECPP								
r	0.77	0.79	1.00	0.94	0.91	0.92	0.87	0.60
p	<0.0001	<0.0001	–	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
MECPrP								
r	0.63	0.71	0.94	1.00		0.85	0.84	0.52
p	<0.0001	<0.0001	<0.0001	–	<0.0001	<0.0001	<0.0001	<0.0001
MEHHP								
r	0.76	0.71	0.91	0.83	1.00	0.99	0.93	0.62
p	<0.0001	<0.0001	<0.0001	<0.0001	–	<0.0001	<0.0001	<0.0001
MEOHP								
r	0.78	0.72	0.92	0.83	0.99	1.00	0.94	0.61
p	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	–	<0.0001	<0.0001
MOEHP								
r	0.58	0.62	0.87	0.84	0.93	0.94	1.00	0.56
p	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	–	<0.0001
MEHP								
r	0.46	0.49	0.60	0.52	0.62	0.61	0.56	1.00
p	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	–

unknown whether any medications given to these infants could have modulated the metabolism of DEHP or if the metabolism in these premature infants differed considerably from that of older children or adults. The metabolic profiles shown in Figure 3 were obtained from a single urine sample collected from the 129 adults (A) and replicate measurements of 32 urine samples collected from six neonates (B). Therefore, the neonates' DEHP urinary metabolic profile reflects both inter- and intra-individual variability, while the profile for the adults included only inter-individual variability. However, the metabolic profiles obtained when only one sample for each neonate was used were similar to the profile shown in Figure 3B.

Additional studies are needed to investigate further the differences in metabolic profiles among neonates and adults observed in this study. Recent studies suggest that the metabolism of DEHP differs with age (Becker et al. 2004, CDC 2005). A decrease in the urinary concentrations of DEHP oxidative metabolites MEHHP and MEOHP with age has been reported in a group of German children 3–14 years of age (Becker et al. 2004). In another study in Germany, median urinary levels of MEHHP and MEOHP were higher in 36 children (2–6 years old) than in 19 of their teachers and parents, while median MEHP levels were higher in the adults than in the children (Koch et al. 2004b). Among the general US populations, the median MEHP urinary concentrations in children 6–11 years of age is higher than in adolescents and adults (Silva et al. 2004a). More importantly, in a representative population of 2782 people of

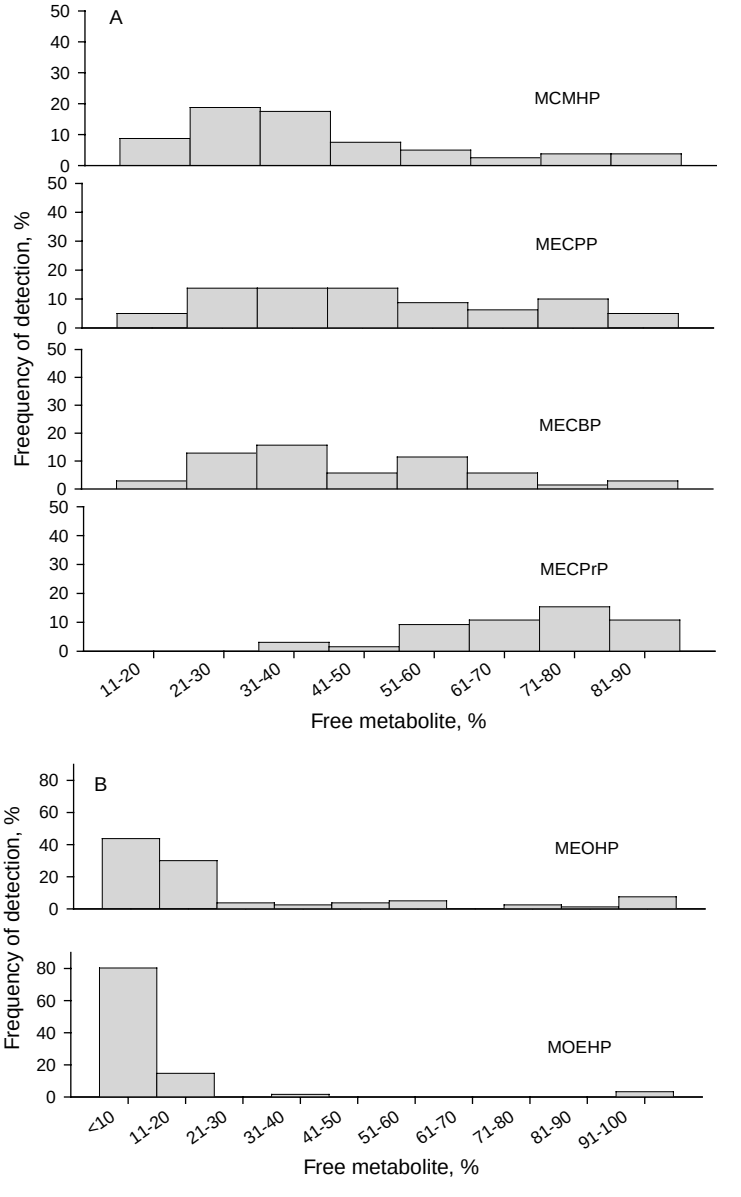

Figure 5. Frequency of detection of free urinary carboxylic acid metabolites (A) and ketone metabolites (B) of DEHP. Levels <10 and >90% were eliminated from (A) for clarity.

6 years of age and older in the USA, children also had higher urinary median concentrations of the DEHP oxidative metabolites MEHHP and MEOHP than adolescents and adults (CDC 2005). In this representative US population, the concentrations of MEHP, MEHHP and MEOHP (other oxidative metabolites were not measured) were comparable with the ones found in the 129 adults evaluated for the current study, but much lower, as expected, than those found in the critically ill infants.

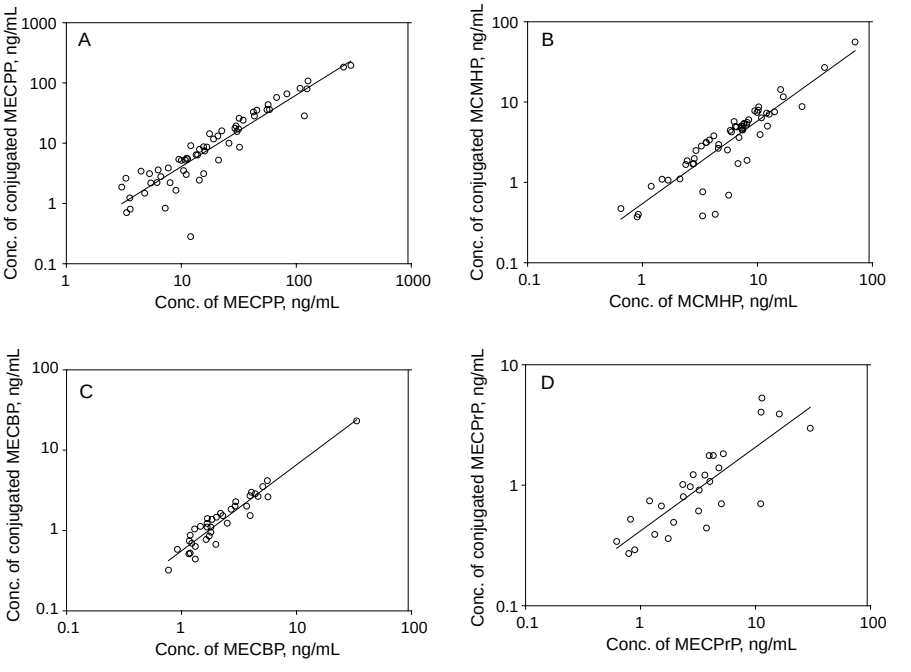

Figure 6. Correlation analysis of the glucuronide conjugated DEHP metabolites and the total (free and glucuronidated). Levels <LOD were eliminated in the graphical representations.

Conclusion

In summary, the urinary levels of eight DEHP metabolites in 129 adult anonymous volunteers and 32 urine samples from six patients from a neonatal intensive care unit were measured. As expected, the urinary concentrations of the metabolites were highly correlated with each other, confirming a common parent. The most abundant urinary metabolites and, most likely, the best biomarkers for DEHP exposure assessment were the ω and ω-1 oxidative metabolites MECPP, MCMHP, MEHHP and MEOHP, comprising 87.1% of all metabolites measured. Although the metabolites resulting from α and β oxidations, MECPrP and MECBP, were also detected in most samples, their urinary levels were much lower than those of the ω and ω-1 oxidative metabolites. The alcohol and ketone metabolites MEHHP, MEOHP and MOEHP were excreted in urine predominantly as glucuronide conjugates. For the carboxylic acid metabolites MECPP, MCMHP, MECPrP and MECBP, the degree of glucuronidation was low. It was also observed that the DEHP metabolite profile in a group of critically ill premature neonates undergoing intensive medical interventions differs significantly from the profile of healthy adults. These differences in metabolic profiles and the lack of data on the bioactivity of most DEHP metabolic products warrant measurement of multiple metabolites for the most accurate exposure assessment to DEHP.

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