Journal of Exposure Science and Environmental Epidemiology (2010) 20, 169–175

www.nature.com/jes

Within-person variability in urinary phthalate metabolite concentrations: measurements from specimens after long-term frozen storage

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Laboratory studies show that exposure to phthalates during development can cause adverse effects, especially for males. Studies in humans would be facilitated by collection of urine during pregnancy, long-term storage, and measurement of phthalate metabolites at the time that offspring health is assessed. Our aims were to measure urinary phthalate metabolites after long-term freezer storage, to use those measurements to evaluate within-woman variability over 2- and 4-week intervals, and to determine whether the phases of the menstrual cycle affect metabolite levels. Samples were selected from daily first-morning urine specimens collected by 60 women and stored frozen since 1983–1985. Three specimens per woman were selected at approximately 2-week intervals to include both follicular and luteal phase samples. Seven metabolites of five phthalates were measured by mass spectrometry. Statistical analyses were conducted with correlation, mixed model regression, and the Wilcoxon signed rank test. Creatinine-corrected urinary phthalate metabolite concentrations measured in samples after long-term storage tended to have a similar right-skewed distribution, though with somewhat higher concentrations than those reported for recently collected US samples. The concentrations of three metabolites of di(2-ethylhexyl)phthalate in the same specimen were very highly correlated (Pearson $r = 0.85$–0.97). Reproducibility over a 4-week interval was moderate for the metabolites of diethyl phthalate and benzylbutyl phthalate (intraclass correlation coefficients, ICCs, 0.48 and 0.53, respectively), whereas five other metabolites had lower ICCs (0.21–0.37). Menstrual phase was not related to metabolite concentrations. Although the same samples have not been measured both before and after long-term storage, results suggest that the measurement of phthalate metabolites after long-term sample storage yield generally similar distributions and temporal reliability as those reported for recently collected specimens. These findings support the use of stored urine specimens collected during the relevant stage of human pregnancy to investigate the influence of phthalate exposures on later outcomes.
Journal of Exposure Science and Environmental Epidemiology (2010) **20,** 169–175; doi:10.1038/jes.2009.17; published online 11 March 2009

Keywords: *biomarker, phthalate, reproducibility, menstrual cycle, temporal variability*.

Introduction

Phthalates are used in a wide variety of industrial and consumer applications. They were developed primarily as plasticizers, with the major use in polyvinyl chloride that came onto the market nearly a century ago. Today, phthalates are found in building materials (such as flooring, roofing, paints, and adhesives), packaging materials (including food packaging), personal care products (such as cosmetics and lotions), medical devices (such as tubing and catheters), and the coatings of pills (Schettler, 2006). Leaching of phthalates from polymer products can contaminate food, water, and air as well as intravenously administered fluids (reviewed in Heudorf et al., 2007). Phthalate metabolites have been found in liver, semen, saliva, urine, blood, placenta, amniotic fluid, fetal blood, and breast milk (reviewed in Frederiksen et al., 2007). The first US national survey of phthalate exposure was based on NHANES urine specimens from 1999–2000 and showed ubiquitous exposure among US residents (Silva et al., 2004).

Health concerns regarding phthalate exposure have been raised by toxicology studies (reviewed by Heudorf et al., 2007) and human studies (reviewed by Hauser and Calafat, 2005, and more recent publications including Marsee et al., 2006; Huang et al., 2007; Kolarik et al., 2008; Matsumoto et al., 2008). Mechanisms of action differ among the phthalates, but several have anti-androgenic activity that can cause developmental toxicity, especially in males (Foster, 2006).

A difficulty in conducting human studies of phthalates is the assessment of exposure. Phthalates are rapidly

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Received 5 September 2008; accepted 23 January 2009; published online 11 March 2009

metabolized, with most excretion occurring in <24 h (Anderson et al., 2001; Koch et al., 2005). A single specimen reflects a person's chronic exposure only if daily exposures are fairly constant. Multiple measurements from the same individuals have been assessed in five studies with 11 to 50 longitudinally monitored participants. Reproducibility of creatinine-corrected phthalate metabolite levels was moderate to high between first-morning urine samples collected on consecutive days (Hoppin et al., 2002), but levels were less consistent for spot samples taken at longer intervals (Hauser et al., 2004; Fromme et al., 2007; Adibi et al., 2008; Teitelbaum et al., 2008).

Measuring phthalate metabolites in archived samples could facilitate cost-efficient studies of phthalate exposure and adverse human health effects. To explore the feasibility of such studies, we investigated the reproducibility of phthalate metabolite levels for 60 women from urine sample taken 2 and 4 weeks apart and archived in freezer storage since 1983–1985. Specimens were also selected to allow comparisons between the follicular and luteal phases of the women's menstrual cycles.

Methods

Study Subjects and Urine Sample Selection

The participants in the Early Pregnancy Study were 221 volunteers who enrolled at the time they discontinued birth control to become pregnant (Wilcox et al., 1988). Women agreed to collect daily first-morning urine samples for up to 6 months during their attempt to conceive. Specimen collection took place from 1982 to 1986. Urine was collected in 30-ml wide-mouth polypropylene jars with screw tops. Samples were stored without preservatives in the participants' home freezers, with weekly pickup and transport to a central storage unit where they were kept at -20°C. Specimens were analyzed for reproductive hormones and were then transferred to long-term storage vials (first in glass and later in polypropylene). Thus, specimens had been thawed and refrozen at least twice before the phthalate measurement.

Sixty women were selected who had adequate quantities of urine from two sequential ovulatory menstrual cycles before any pregnancy. Most of the women were white (94%), and their ages ranged between 21 and 42 years (mean = 29, SD = 4). Day of ovulation was estimated from an algorithm based on urinary estrogen and progesterone metabolites (Baird et al., 1991). For each woman, three samples were selected based on menstrual cycle phase. For 56 women, two follicular phase samples and one luteal phase sample were selected, and for 4 women, one follicular phase sample and two luteal phase samples were selected. The three samples were designated in chronological order as Time 1, 2, and 3, and in most cases, the Time 2 sample was from the luteal phase. For most women, these samples were collected 2 weeks apart ($\text{mean}_{T1-T2} = 16.6$ days, SD = 6.8; $\text{mean}_{T2-T3} = 15.3$ days, SD = 8.5; $\text{mean}_{T1-T3} = 31.8$ days, SD = 11.2). For 20 of the 180 collection days selected, we prepared two replicate samples as blind replicates. Thus, a total of 200 samples were analyzed. Specimens were shipped with dry ice by overnight freight to AXYS Laboratory (BC, Canada).

Measurement of Phthalates and Creatinine

The seven measured phthalate metabolites and their abbreviations are listed in Table 1. Mono-*n*-butyl (MnBP) includes both mono-*n*-butyl phthalate and mono-isobutyl phthalate. The combination of free and conjugated metabolite were measured. Deconjugation was performed with β-glucuronidase at 37°C. A 4-methylumbelliferyl glucuronide solution was used for monitoring the deconjugation efficiency. For isotope-dilution methodology, isotope-labeled internal standards were added, and samples were then extracted and cleaned using a Waters Oasis HLB 200 mg, 5 ml, solid phase extraction glass cartridge. The extract was then spiked with labeled recovery standards to calculate internal standard recovery. Analysis of sample extracts for phthalates was conducted using Waters 2695 HPLC coupled with a triple quadrapole mass spectrometer (Micromass Quattro Ultima MS/MS, LC column: Sunfire C18 3.5 μm, 4.6 × 30 mm analytical column, injection volume 20 μl). The LC/MS/MS conditions and quantification reference for each target

Table 1. Names and abbreviations for phthalates and metabolites.

Phthalate name	Abbreviation	Phthalate metabolite	Abbreviation
Dimethyl phthalate	DMP	Mono-methyl phthalate	MMP
Diethyl phthalate	DEP	Mono-ethyl phthalate	MEP
Dibutyl phthalates	DBP	Mono-*n*-butyl phthalate and mono-isobutyl phthalate	MnBP
Benzylbutyl phthalate	BzBP	Mono-benzyl phthalate	MBzP
Di-2-ethylhexyl phthalate	DEHP	Mono-(2-ethylhexyl) phthalate	MEHP
Di-2-ethylhexyl phthalate	DEHP	Mono-(2-ethyl-5-hydroxyhexyl) phthalate	MEHHP
Di-2-ethylhexyl phthalate	DEHP	Mono-(2-ethyl-5-oxohexyl) phthalate	MEOHP
Di-2-ethylhexyl phthalate	DEHP SUM	MEHP + MEHHP + MEOHP	DEHP SUM

analyte are listed in Appendix. The mass spectrometer was run at unit mass resolution in the Multiple Reaction Monitoring mode. Resulting measurements are produced by the manufacturer's MassLynx v.4.0 software. On the basis of spiked recovery standards, a "specimen detection limit" was determined for each sample by converting the area equivalents corresponding to three times the height of the chromatographic noise to a concentration (in the same way that peak areas are converted to concentrations). The method detection limit of each assay was calculated as the greater of two concentrations: (1) the lowest calibration standard converted to a sample equivalent concentration or (2) the sample-specific detection limit. All were <1 ng/ml.

Samples were analyzed in batches including a procedural blank, two spiked reference samples (one low and one high-level concentration spike), and a reference sample in duplicate using laboratory stock urine for inter- and intrabatch comparisons. All intraassay coefficients of variation (CV) were $<6\%$, and interassay CVs ranged from 11% to 13% except for MEP (23%) and MEHP (19%), based on these stock urine specimens. The intraassay CVs calculated based on our blind replicates ranged from 10% to 15% except for MEP (18%). No blind interassay CV was calculated because there were not enough replicates distributed among batches. Creatinine was assessed by the Jaffe assay (Taussky, 1954).

Statistical Analyses

We described the distribution of urinary phthalate metabolite values for the 180 samples and for each of the three sampling times using percentiles and geometric means. For analyses, specimens with phthalate levels below the specimen-specific detection limit (SDL) were imputed by assigning a value equal to the SDL divided by the square root of 2 (Hormung and Reed, 1990). Descriptive analyses were conducted for both unadjusted and creatinine-adjusted metabolite levels (ng/ml and ng/mg creatinine, respectively). The distributions were right-skewed, so the natural logarithms of the metabolite concentrations were used in statistical analyses for which a normal distribution is optimal. Pearson correlations were calculated between each of the three pairwise comparisons (Times 1 and 2, Times 2 and 3, and Times 1 and 3). We estimated the effect of collection year with mixed model regression, menstrual phase with the nonparametric Wilcoxon signed rank test after calculating the geometric mean of the two samples from the same menstrual phase, and reproducibility using the intraclass correlation coefficient (ICC) based on all three measurements per woman. ICCs and 95% confidence intervals (Shrout and Fleiss, 1979) were calculated using a SAS macro written by Steinley and Wood (Psychology Department, University of Missouri-Columbia, 2000). Statistical significance was based on a two-sided P-value of 0.05.

Results

All seven phthalate metabolites were detected in over 96% of the urine samples stored from the early 1980s. Table 2 shows the distribution of unadjusted concentrations of the seven phthalate metabolites. The distributions at each of the three sampling times (Time 1, Time 2, and Time 3) were all very similar (data not shown). Table 3 shows the creatinine-adjusted distributions, which were very similar to the unadjusted distributions. The geometric means from NHANES data are included for comparison (CDC, 2005). The geometric means for the 1980s data were higher for most metabolites, but lower for MEP compared with the recent NHANES data.

Of the seven urinary phthalates, three are metabolic products of DEHP and would be expected to be highly correlated. Table 4 shows the Pearson correlations among the seven urinary phthalates from the same urine sample. Correlations among MEHP, MEHHP, and MEOHP (all metabolites of DEHP) are shown in bold. As expected,

Table 2. Urinary phthalate metabolite concentrations (ng/ml), geometric means and standard deviations with selected percentiles based on unadjusted concentrations, $n=180$ samples from 60 women, Early Pregnancy Study, 1982–1986.

Phthalate metabolite	Nondetectable (*N*)	Geometric mean (95%CI)	SD	Min	5th	25th	50th	75th	95th	Max
MMP	4	24.1 (21.1–27.6)	2.5	3.0	5.5	15.3	23.4	38.4	90.6	8210.0
MEP	0	115.6 (97.0–137.7)	3.3	9.3	19.0	49.0	103.5	250.0	982.5	3140.0
MnBP	0	78.1 (68.3–89.3)	2.5	6.3	16.2	43.6	74.0	134.0	379.5	758.0
MBzP	0	34.8 (30.2–40.0)	2.6	1.7	7.4	19.8	33.2	66.6	170.0	794.0
MEHP	5	8.3 (7.1–9.6)	2.8	0.6	1.3	4.3	7.7	17.5	39.7	136.0
MEHHP	1	33.4 (28.8–38.7)	2.7	0.8	7.6	18.2	34.4	53.6	164.0	1060.0
MEOHP	1	35.2 (30.3–40.8)	2.7	1.4	8.0	18.8	36.6	58.5	175.5	593.0
DEHP SUM[a]	1	78.8 (68.3–91.1)	2.7	3.6	18.4	42.4	78.8	129.0	375.5	1527.7

[a]Sum of MEHP, MEHHP, and MEOHP concentrations (ng/ml); The summed molar concentrations (nmol/ml) are geometric mean, 0.27 (0.23–0.31); min, 0.012; 5th, 0.063; 25th, 0.15; 50th, 0.27; 75th 0.45, 95th 1.3; max, 5.2.

Table 3. Urinary phthalate metabolite concentrations (ng/mg creatinine), geometric means and standard deviations with selected percentiles based on 180 creatinine-adjusted samples from 60 women, Early Pregnancy Study, 1982–1986.

Phthalate metabolite	Nondetectable (*N*)	Geometric mean	SD	Min	5th	25th	50th	75th	95th	Max
MMP	4	24.7 (21.8–27.9) 1.2 (1.1–1.4)[a]	2.3	6.2	9.0	15.4	21.5	34.6	79.5	7672.9
MEP	0	118.4 (101.4–138.2) 187.0 (165–211)[b]	2.9	13.6	26.6	52.1	109.1	219.1	814.4	2899.2
MnBP	0	80.0 (72.0–89.0) 28.6 (25.3–32.3)[b]	2.1	11.9	27.1	51.6	72.2	124.3	291.1	779.0
MBzP	0	35.6 (32.0–39.6) 15.3 (13.8–16.8)[b]	2.1	5.1	11.3	22.8	36.0	53.7	120.4	327.7
MEHP	5	8.5 (7.4–9.7) 3.4 (3.1–3.6)[b]	2.5	0.5	2.2	4.9	8.2	14.8	44.5	91.5
MEHHP	1	34.2 (30.4–38.5) 19.7 (17.3–22.5)[a]	2.2	4.1	10.7	20.6	31.5	47.1	141.8	716.2
MEOHP	1	36.1 (31.9–40.8) 13.5 (11.9–15.3)[a]	2.3	3.0	9.5	22.1	36.8	53.5	163.1	670.1
DEHP SUM[c]	1	80.8 (71.9–90.8)	2.2	7.6	26.2	50.7	77.0	113.5	349.5	266.0

[a]NHANES females 01-02 data CDC, 2005, comparison data provided when NHANES 99-00 not available.
[b]NHANES females 99-00 data CDC, 2005, MnBP includes both mono-*n*-butyl phthalate and mono-isobutyl phthalate in both our samples and in the NHANES samples. The 99-00 NHANES data are provided for comparison because it is closer in time to our samples.
[c]Sum of MEHP, MEHHP, and MEOHP concentrations (ng/mg creatinine); the summed molar concentrations (nmol/mg creatinine) are geometric mean, 0.28 (0.25–0.31); min, 0.026; 5th, 0.09; 25th, 0.17; 50th, 0.26; 75th 0.39; 95th, 1.2; max, 4.3.

Table 4. Pearson correlation among phthalates measured in the same urine sample[a], $n = 180$ samples from 60 women, Early Pregnancy Study, 1982–1986.

	MMP	MEP	MnBP	MBzP	MEHP	MEHHP	MEOHP
MMP	1	0.51	0.51	0.65	0.41	0.50	0.54
MEP		1	0.51	0.42	0.37	0.46	0.45
MnBP			1	0.75	0.56	0.68	0.71
MBzP				1	0.57	0.69	0.74
MEHP					1	**0.85**	**0.86**
MEHHP						1	**0.97**
MEOHP							1

[a]Phthalate metabolite concentrations were creatinine adjusted and log transformed.

Table 5. Pearson correlation coefficients and reproducibility estimates for urinary phthalate measurements based on three specimens collected at approximately 2-week intervals[a], Early Pregnancy Study, 1982–1986.

Phthalate metabolite	Correlation coefficients			Reproducibility	
	$r_{time1,\ time2}$	$r_{time2,\ time3}$	$r_{time1,\ time3}$	ICC	95%CI
MMP	0.38	0.20	0.30	0.23	0.11, 0.38
MEP	0.54	0.48	0.40	0.48	0.36, 0.60
MnBP	0.21	0.48	0.36	0.34	0.22, 0.49
MBzP	0.47	0.62	0.51	0.53	0.42, 0.65
MEHP	0.33	0.39	0.40	0.37	0.25, 0.51
MEHHP	0.15	0.38	0.14	0.21	0.08, 0.35
MEOHP	0.28	0.45	0.27	0.33	0.20, 0.47
DEHP SUM[b]	0.22	0.39	0.21	0.26	0.14, 0.41

[a]Phthalate metabolite concentrations were creatinine adjusted and log transformed.
[b]Sum of molar concentrations of MEHP, MEHHP, MEOHP.

these three metabolites are highly correlated (≥ 0.85). There was also a moderate correlation of these three with the other four metabolites and moderate correlations among the other four. The correlation between MnBP and MBzP was especially strong (0.75). Spearman correlations showed similar patterns, as did correlations between concentrations unadjusted for creatinine (data not shown).

The Pearson correlations between urinary phthalate levels for the 60 women across sampling times are shown in Table 5. There was only one metabolite (MEP) for which correlations between samples taken 2 weeks apart were substantially higher than for samples taken 4 weeks apart. There were two metabolites (MEP and MBzP) with ICCs > 0.4. For the other metabolites, samples taken closer in time were not generally more highly correlated, and these metabolites had relatively low ICCs (0.21 for MEHHP up to 0.37 for MEHP). The correlations for data unadjusted for creatinine show the same patterns (data not shown). None of the metabolites was significantly associated with phase of the menstrual cycle (follicular or luteal). Nor was year of sample collection (1983, 1984, or 1985) a significant predictor, except for DEHP metabolites. All three of those showed

significantly higher levels in 1984 samples than in either 1983 or 1985 samples.

Discussion

Phthalates have been used commercially for nearly a century. Recent annual global production was estimated at three million metric tons (Bizzari et al., 2000). Concerns increased with reports of anti-androgenic effects of several metabolites and adverse effects on sexual development in exposed laboratory animals (Foster, 2006). Adverse health outcomes also have been reported for humans (Hauser and Calafat, 2005; Marsee et al., 2006; Huang et al., 2007; Kolarik et al., 2008; Matsumoto et al., 2008). The ubiquity of human exposure was shown in the 1999–2000 NHANES Study, which found detectable phthalate levels in nearly all urine samples (Silva et al., 2004).

Our samples are from 13 to 17 years before the NHANES Study, and nearly all of these samples also had detectable levels. The mean concentrations in our samples tended to be higher than for the NHANES samples for all metabolites except MEP, but the relative ranking of the metabolites changed little between sampling periods.

Other data also suggest that exposure to specific phthalates may have been higher in the early 1980s than now. A study using stored samples from Germany has suggested declines in several phthalate metabolite levels between 1988 and 2003, concomitant with production declines in Western Europe (Wittassek et al., 2007). Subsequent analysis of the German data indicate that the yearly MEHP estimates were highly correlated with yearly industrial production in Germany (Helm, 2007). Similar data for the United States are not available, and there have been more restrictions on the use of phthalates in Europe than in the United States.

Phthalate metabolites are considered chemically stable (Barr et al., 2005; Hoppin et al., 2005; Wittassek et al., 2007), but there are no data comparing measurements taken on the same urine specimens >20 years apart. Our results support their long-term stability. First, the concentrations tended to be higher than more recent estimates, which would be unlikely if there was substantial degradation. Second, the three metabolites from DEHP were highly correlated within a sample. These high correlations among three chemically distinct metabolites after 22–24 years of storage suggest stability. Finally, the reproducibility between samples taken at 2- or 4-week intervals are similar to those reported for samples taken over a 6-week interval after short-term storage (Adibi et al., 2008).

Although the phthalates with short chains are predominantly metabolized to the monoester, the longer chain phthalates such as DEHP have more varied metabolic products with the monoester representing a minor excretion product (reviewed by Frederiksen et al., 2007). This is reflected in the higher levels of MEHHP and MEOHP compared with MEHP in our data, again supporting the stability of urinary metabolites over time and the validity of our measurements after long-term storage.

Phthalate metabolites measured from the same sample were significantly correlated. This has been reported earlier (Silva et al., 2004; Main, 2008). Although the metabolism of longer chain phthalates can result in some overlap of metabolites produced, in most cases each monoester derives from a separate parent compound. The correlation may arise because similar consumer products may be manufactured with different phthalates and the same product may contain more than one phthalate (Koo and Lee, 2004). For example, people who use high levels of personal care products and fragrances are likely to be exposed to several different phthalates, some at relatively high levels. This is of special concern because phthalate mixtures can have additive biologic effects (Howdeshell et al., 2008).

For the phthalates we evaluated, urinary excretion predominates and most are excreted within 24 h (Anderson et al., 2001; Koch et al., 2005). A single sample would not be expected to be a useful biomarker of chronic exposure unless exposure is relatively constant over time. Our sample of 60 women is the largest group to be studied with multiple specimens from the same individuals. Our measurements suggest that there was relatively stable exposure to DEP and BzBP over a 4-week interval, but less stable exposure to DMP, DBP, and DEHP. Five prior studies have examined reproducibility of phthalate levels over time based on more recently collected samples. Reproducibility was high for first-morning voids from consecutive days (ICCs ranged from 0.53 to 0.80, $n=46$ women (Hoppin et al., 2002), but lower for spot samples taken over 8-day intervals (ICCs ranged from 0.21 to 0.57, $n=50$ men and women (Fromme et al., 2007), 6-week intervals (ICCs ranged from 0.30 to 0.66, $n=28$ pregnant women (Adibi et al., 2008)), 3-month intervals (ICCs ranged from 0.28 to 0.52, $n=11$ men (Hauser et al., 2004), or 6-month intervals (ICCs were all below 0.30 except for MBP which was 0.35 and MBzP which was 0.62, $n=29$ children (Teitelbaum et al., 2008)).

MBzP, the metabolite with the highest reproducibility in our study (ICC $=0.53$), was the only metabolite to show at least moderate reproducibility (ICC >0.4) in all prior studies. This metabolite derives from BzBP whose primary use is in vinyl tiles but is also used in materials that are used to process food such as food conveyor belts. Food contamination is considered the primary source for humans (NTP-CERHR-BBP, 2000). Health concerns arise regarding MBzP because of its anti-androgenic effects and potential adverse effects on development (Gray et al., 2000). The reproducibility results from our study and others indicate that a single urinary measure of MBzP would likely serve as a useful biomarker of chronic exposure over several months.

In conclusion, though the same samples have not been repeatedly measured over many years, our data support the hypothesis that phthalates remain stable after long-term storage. This opens the possibility of using stored samples from pregnant women to estimate prenatal phthalate exposure, thus facilitating study of developmental effects in their offspring. However, for most phthalates, sample collection must be close to the developmental stage of interest because exposure is likely to change during pregnancy.

Acknowledgements

The field manager of the North Carolina Early Pregnancy Study was Joy Pierce, and D. Robert McConnaughey manages the study data files. This research was funded by the intramural program at the National Institute of Environmental Health Sciences, National Institutes of Health, HHS. We thank AXYS Analytical Services Ltd from Sidney, BC Canada, for their assistance with this project including their detailed description of the analytic methods. An earlier version of the manuscript was reviewed by Drs. Freya Kamel and Yang Cao.

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Appendix: Urinary Phthalate Measurement Information

Table 1. Analytes, ions, and quantification references.

Target analyte	Parent ion mass	Daughter ion mass	Quantified against
Phthalate metabolite analysis			
Monomethyl phthalate (mMP)	179	107	$^{13}C_4$-Monomethyl phthalate
Monoethyl phthalate (mEP)	193	121	$^{13}C_4$-Monoethyl phthalate
Mono-*n*-butyl phthalate (mBP)	221	77	$^{13}C_4$-Mono-*n*-butyl phthalate
Monobenzyl phthalate (mBzP)	255	183	$^{13}C_4$-Monobenzyl phthalate
Mono-2-ethylhexyl phthalate (mEHP)	277	134	$^{13}C_4$-Mono-2-ethylhexyl phthalate
Mono-(2-ethyl-5-oxohexyl) phthalate (DEHP Metabolite VI) (mEOHP)	291	121	$^{13}C_4$-Mono-(2-ethyl-5-oxohexyl) phthalate
Mono-(2-ethyl-5-hydroxyhexyl) phthalate (DEHP Metabolite IX) (mEHHP)	293	121	$^{13}C_4$-Mono-(2-ethyl-5-hydroxyhexyl) phthalate
Surrogate standard			
$^{13}C_4$-Monomethyl phthalate	183	109	$^{13}C_4$-Mono-*n*-octyl phthalate
$^{13}C_4$-Monoethyl phthalate	197	124	$^{13}C_4$-Mono-*n*-octyl phthalate
$^{13}C_4$-Mono-*n*-butyl phthalate	225	79	$^{13}C_4$-Mono-*n*-octyl phthalate
$^{13}C_4$-Monobenzyl phthalate	259	186	$^{13}C_4$-Mono-*n*-octyl phthalate
$^{13}C_4$-Mono-2-ethylhexyl phthalate	281	137	$^{13}C_4$-Mono-*n*-octyl phthalate
$^{13}C_4$-Mono-(2-ethyl-5-oxohexyl) phthalate	295	124	$^{13}C_4$-Mono-*n*-octyl phthalate
$^{13}C_4$-Mono-(2-ethyl-5-hydroxyhexyl) phthalate	297	124	$^{13}C_4$-Mono-*n*-octyl phthalate
$^{13}C_4$-4-methylumbelliferone	179	150	$^{13}C_4$-Mono-*n*-octyl phthalate
Recovery standard			
$^{13}C_4$-Mono-*n*-octyl phthalate	281	127	External standard
Deconjugation recovery standard			
4-methylumbelliferone	175	147	$^{13}C_4$-4-methylumbelliferone

Table 2. LC-MS/MS operating conditions for analysis.

LC gradient program		LC flow rate program	Gradient curve	General LC conditions	
Time	Flow mixture	(ml/min)		Column temp (°C)	40
0.00	60%A 40% B	0.2	1	Flow rate (ml/min)	0.150–0.200
0.50	60% A 40% B	0.2	1	Max pressure (bar)	300.0
3.0	30%A 70%B	0.2	6	MS conditions	
3.5	30%A 70%B	0.2	6	Source temp (°C)	100
8	100%B	0.2	6	Desolvation temp (°C)	300
12	100%B	0.2	6	Capillary voltage	2.90 kV
12.5	60%A 40%B	0.2	2	Hexapole1	26.6 V
17	60%A 40%B	0.2	2		