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Detection of Phthalate Metabolites in Human Amniotic Fluid

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Diesters of phthalic acid, commonly known as phthalates, are ubiquitous industrial chemicals used in consumer products (e.g., soaps, shampoos, and cosmetics), plastics, paints, enteric coatings in some medications, and pesticide formulations (ATSDR 2002; ATSDR 2001; ATSDR 1995). Because of the extensive use of these products, humans are at potential risk for exposure to phthalates. Some phthalates and their metabolic products are known to function as antiandrogens during the prenatal period and cause reproductive and developmental toxicities in animals. Specifically, in utero or lactational exposure to di(2-ethylhexyl)phthalate (DEHP) and dibutyl phthalate (DBP) alters reproductive development in male rat pups (Moore et al. 2001; Mylchreest et al. 1998). In addition, DEHP produces testicular toxicity in young male rats (ATSDR 2002).

Upon exposure, phthalates are rapidly hydrolyzed to their monoesters, which may be further biotransformed to oxidative metabolites; all of these metabolites can be glucuronidated and excreted in the urine and feces (ATSDR 2002; ATSDR 2001; ATSDR 1995). The relatively polar and low molecular weight phthalates (e.g., diethyl phthalate [DEP] and DBP) are primarily metabolized to their monoesters and excreted (ATSDR 2001; ATSDR 1995). The higher molecular weight phthalates (e.g., DEHP) are hydrolyzed to their monoester and then further metabolized in a multi step oxidative pathway to oxidative metabolites (ATSDR 2002), which are the major metabolites detected in the urine (ATSDR 2002). Recent data suggest that DBP and benzylbutyl phthalate (BzBP) are mostly excreted in urine as their corresponding glucuronidated monoesters, monobutyl phthalate glucuronide (mBP-glu) and monobenzyl phthalate glucuronide (mBzP-glu), respectively, while DEP is mostly excreted as free monoethyl phthalate (mEP) (Silva et al. 2003a) and DEHP is excreted as the glucuronidated form of its oxidative metabolites (Kato et al. In press).

Human exposure to phthalates is widespread (CDC 2003). Because of the potential adverse health impact of phthalates during the early stages of human development, concern is that fetal exposure to phthalates may adversely affect development of the reproductive system. However, no data exist on fetal exposure to phthalates in humans, and data on exposure among young children are limited (Brock et al. 2002).

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Accurate exposure assessment is an essential and fundamental requirement of the human risk assessment process. Evaluation of *in utero* exposure to environmental chemicals in humans has traditionally been limited to umbilical cord blood, placenta or accompanying fluid collected at birth, although the presence of man-made chemicals in amniotic fluid has been reported (Foster et al. 2000). Amniotic fluid, which is formed from metabolized fetal cells and fetal urination, surrounds the fetus and protects it from injury and temperature changes. Beginning with the 11th week of gestation, the fetus starts producing urine that enters the amniotic fluid, which in turn is swallowed by the fetus and re-absorbed by the gastrointestinal and respiratory tracts. Because of the non persistent nature of phthalates, amniotic fluid may offer a more relevant reflection of *in utero* exposure than either umbilical cord blood or maternal serum collected during delivery. Sex differentiation of the external genitalia in humans occurs during the early weeks of gestation (Moore et al. 1998). Amniotic fluid obtained by routine amniocentesis, normally performed at 16-20 weeks of gestation, may be a useful matrix for the assessment of fetal exposure during a period of reproductive differentiation and organogenesis.

As part of our ongoing efforts to assess human fetal exposure to phthalates, we measured the levels of 10 phthalate metabolites in amniotic fluid samples obtained during routine amniocentesis from 54 anonymous donors. We found quantifiable levels of three phthalate monoesters in a significant percentage of the human amniotic fluid samples examined, thus suggesting *in utero* fetal exposure to phthalates.

MATERIALS AND METHODS

The amniotic fluid samples were obtained during routine amniocentesis. The samples obtained for analysis were residual specimens; no demographic or personal information, including term into pregnancy or medical history, was available for any of the women.

The preparation of the standards and the analytical method for measuring phthalate monoesters in amniotic fluid were analogous to the procedure described previously for urine (Silva et al. 2003b). Briefly, the phthalate metabolites were extracted from the amniotic fluid using solid phase extraction, separated by reverse phase high performance liquid chromatography, and detected by negative ion atmospheric pressure chemical ionization-tandem mass spectrometry on a ThermoFinnigan Surveyor liquid chromatograph coupled with a ThermoFinnigan TSQ Quantum triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA). All samples were spiked with $^{13}C_4$-labeled phthalate monoesters and with a solution of 4-methylumbelliferone glucuronide to monitor β-glucuronidase enzyme activity; β-glucuronidase enzyme (*Escherichia coli*-K12; Roche Biomedicals, Mannheim, Germany) was used to hydrolyze the glucuronidated phthalate metabolites. Because urine and amniotic fluid are similar in their composition, we used quality control

(QC) materials prepared from spiked human urine. Reagent blanks, QCs, and standards were analyzed along with unknown amniotic fluid samples.

RESULTS AND DISCUSSION

Our preliminary research detected, identified and measured metabolites of phthalates in amniotic fluid. Because *in utero* exposure to phthalates may result in adverse endocrine, reproductive, and developmental health effects in humans, the exposure assessment is important. The levels of chemicals in amniotic fluid may provide a biologically relevant reflection of fetal exposure. In this study, we attempted to measure the concentrations of 10 phthalate metabolites, monomethyl phthalate (mMP); mEP; mBP; mBzP; monocyclohexyl phthalate (mCHP); monoisononyl phthalate (mNP); monooctyl phthalate (mOP); and three metabolites of DEHP, mEHP, mono-2-ethyl-5-hydroxyl (mEHHP), and mono-2-ethyl-5-oxohexyl (mEOHP) in 54 human amniotic fluid samples obtained during routine amniocentesis. Three phthalate metabolites, mEP, mBP, and mEHP, were detected together in 18.5% of the amniotic fluid samples. mBP was detected at levels above the limit of detection (>LOD) in 50 (92.6%) of the samples, while mEP and mEHP were present in 21 (39%) and 13 (24%) of the samples at levels >LOD, respectively (Table 1). mBzP was present in one (1.9%) sample at concentrations > LOD. mMP, mEHHP, mEOHP, mCHP, mNP, and mOP were not measured at concentrations above their LOD. Our findings of the presence of mEP, mBP, and mEHP in amniotic fluid suggest that the developing fetus is exposed to potentially endocrine modulating phthalates during gestation.

Table 1. Distribution of mEP, mBP and mEHP (in ng/mL) in amniotic fluid samples from 54 pregnant women.[a]

Phthalate Metabolite	Min	Max	Percentile					
			10th	25th	50th	75th	90th	95th
mEP	<LOD	9.0	<LOD	<LOD	<LOD	4.8	7.5	8.1
mBP	<LOD	263.9	2.5	3.3	5.8	8.5	14.2	15.9
mEHP	<LOD	2.8	<LOD	<LOD	<LOD	0.8	1.7	2.6

[a] The limits of detection (LOD) for mEP, mBP and mEHP are 1.2 ng/mL, 0.97 ng/mL and 0.86 ng/mL, respectively.

We compared the amniotic fluid median levels of mBP, mEHP, and mEP from this study with the median urinary levels of children and adults from the recent National Health and Nutrition Examination Survey (NHANES) 1999-2000 and with the serum levels of a non-representative subset of participants from NHANES 1999-2000 (Table 2). The median levels in the urine samples of the NHANES 1999-2000 population (aged 6-11 years) were highest in mEP and decreased in the order mEP, mBP, mBzP, and mEHP; the concentrations in the amniotic fluid were significantly lower than the median urinary levels for children and adults from NHANES 1999-2000 (Table 2) (CDC 2003), and the concentration order decreased from mBP, mEP, mEHP, and mBzP (which was detected in only one sample). mEP, mBP and mEHP,

the major phthalate metabolites detected in the amniotic fluid samples analyzed for this study, were also the major phthalate metabolites detected in serum samples from a non representative multiethnic population (Silva et al. 2003a). mBP was found at the highest levels both in serum (Silva et al. 2003a) and in the amniotic fluid samples analyzed for this study, but the median levels of mEP, mBP, and mEHP in amniotic fluid were significantly lower than the median serum levels (Table 2). In a previous study, we found that the DEHP oxidative metabolites, mEHHP and mEOHP, were present in urine at higher concentrations than mEHP (Barr et al. 2003); however, in the amniotic fluid we did not find these oxidative metabolites.

Table 2. Phthalate metabolite median levels in people (ng/mL).

Analyte	Amniotic fluid (N = 54)	Serum[a]	Urine (NHANES 1999-2000) Children[b] (N = 328)	Urine (NHANES 1999-2000) Adults[c]
mEP	<LOD	4.1	74.7	180
mBP	5.8	14.4	40.0	23.0
mEHP	<LOD	5.4	4.9	3.0

[a]Non-representative subset of NHANES 1999-2000 participants, 6 years old and older. N = 93 (mEP), N = 149 (mBP), N = 141 (mEHP). [b]Children 6 to 11 years old. [c]Adults 20 years old and older. N=1456 (mEP), N = 1461 (mBP and mEHP).

The presence of mEP, mBP, and mEHP in the amniotic fluid suggests that these phthalate monoesters can cross the placental barrier after maternal exposure to DEP, DBP and DEHP, respectively, thus exposing the growing fetus to these potentially toxic or endocrine modulating chemicals. An alternative explanation, although less likely, for the presence of these monoesters in the amniotic fluid is that the phthalate diesters pass through the placenta, and the fetus metabolizes the phthalate diesters to the monoesters. Evidence not favoring this alternative mechanism is the presence in the amniotic fluid of the monoester of DEHP, mEHP, but the absence of two hydrolysis/oxidative metabolites of DEHP, mEHHP and mEOHP; this suggests the inability of the developing fetal liver to biotransform phthalates, at least to the oxidative metabolites. However, we cannot rule out the possibility of mEHP being present in the amniotic fluid because of DEHP contamination of the samples during collection and storage followed by the production of mEHP by lipase enzyme activity in the amniotic fluid or by hydrolysis during sample work-up. Because of the less chance of contamination by DEP and DBP and the higher mEP and mBP levels in the amniotic fluid samples compared to mEHP, we do not think that contamination is a viable explanation for the presence of mEP and mBP in amniotic fluid.

We also wanted to examine the percentage of phthalate metabolites in their free form versus their presence as glucuronides. Glucuronidation facilitates urinary excretion of phthalates and, because the putative biologically active species of phthalates is the free metabolite, glucuronidation reduces their potential biological activity. Because

most uridinediphosphate–glucoronosyltransferase isoenzymes involved in the glucuronidation reactions are not expressed until after birth (Coughtrie et al. 1988), the rate of clearance of the phthalate monoesters may be slower in the fetus. Unfortunately, because of the limited amount of amniotic fluid, we could not determine the percentages of free and conjugated metabolites. Additional studies to establish the percentages of prenatal glucuronidation are warranted.

In summary, we found measurable concentrations of mEP, mBP, and mEHP in human amniotic fluid samples, suggesting that *in utero* exposure to phthalates occurs. The levels of these phthalate metabolites in amniotic fluid are significantly lower than the urine and serum levels found in other general populations. Nevertheless, the detection of these phthalate metabolites in amniotic fluid demonstrates the presence of phthalates in the human fetal environment in the early stages of the second trimester of pregnancy when reproductive differentiation in humans occurs. Because of the potential reproductive and developmental toxicities of phthalates in humans, additional studies are needed to fully understand exposure to phthalates in pregnant women and developing fetuses and to evaluate the postnatal effects on the development of human fetuses exposed to amniotic fluid containing phthalates.

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