Levels of Seven Urinary Phthalate Metabolites in a Human Reference Population

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Using a novel and highly selective technique, we measured monoester metabolites of seven commonly used phthalates in urine samples from a reference population of 289 adult humans. This analytical approach allowed us to directly measure the individual phthalate metabolites responsible for the animal reproductive and developmental toxicity while avoiding contamination from the ubiquitous parent compounds. The monooesters with the highest urinary levels found were monoethyl phthalate (95th percentile, 3,750 ppb, 2,610 µg/g creatinine), monobutyl phthalate (95th percentile, 294 ppb, 162 µg/g creatinine), and monobenzyl phthalate (95th percentile, 137 ppb, 92 µg/g creatinine), reflecting exposure to diethyl phthalate, dibutyl phthalate, and benzyl butyl phthalate. Women of reproductive age (20–40 years) were found to have significantly higher levels of monobutyl phthalate, a reproductive and developmental toxicant in rodents, than other age/gender groups (p < 0.005). Current scientific and regulatory attention on phthalates has focused almost exclusively on health risks from exposure to only two phthalates, di-(2-ethylhexyl) phthalate and di-isononyl phthalate. Our findings strongly suggest that health-risk assessments for phthalate exposure in humans should include diethyl, dibutyl, and benzyl butyl phthalates.

Key words: exposure, glucuronidase, human, metabolism, phthalates, urine. Environ Health Perspect 108:979–982 (2000). [Online 1 September 2000] http://ehpnet1.niehs.nih.gov/docs/2000/108p972-982blount/abstract.html
Methods

The method used has been described previously (23). All samples were spiked with $^{13}$C$_4$-labeled phthalate monoesters and 4-methylumbellifere glucuronide. The samples were then treated with a β-glucuronidase to release the phthalate monoesters from its conjugated form. Deconjugated urine samples were extracted twice with Oasis HLB SPE (Waters Corp., M Millford, MA) as described (23) and resuspended in mobile phase. Chromatographic separation by HPLC was followed with tandem mass spectrometry on a triple quadrupole instrument (Finnigan TSQ 7000; Finnigan MAT, San Jose, CA) using atmospheric pressure chemical ionization. Retention times as well as parent-fragment ion combinations have been described previously (23). Levels of 4-methylumbellifere were monitored as quality control for the deconjugation step. Urinary creatinine was measured with an ASTRa analyzer (Beckman, Brea, CA) based on a Jaffé rate reaction, in which creatinine reacts with picrate in an alkaline solution to form a red creatinine-picrate complex (24). Results are reported as micrograms phthalate monoester per gram of urinary creatinine. Creatinine adjustment is used to correct for variations in urine volume. Method blanks, quality control samples (spiked human urine), and standards were analyzed along with unknown human urine samples.

This method has been refined to monitor and eliminate monoester contamination by the exposure to phthalate-containing plastics, prescreening reagents, and labware, and routinely monitoring method and instrument blanks. Phthalate diesters are often components of flexible PVC plastics. Therefore, products containing this polymer were not used. Although the polypropylene and glass components used were unlikely to contain significant amounts of phthalates, we prescreened representative vials, pipette tips, glassware, and sampling cups for phthalate monoester contamination. As an additional precaution, method blanks were analyzed in parallel with unknown samples. Insignificant phthalate monoester levels were found in blanks (typically < 0.5% of the mean level found in human samples). A blank containing > 5 ppb of any analyte resulted in rejection of all analytical results acquired on that day and an effort to identify the source of the contamination.

One source of contamination that had to be eliminated was diester lipase activity in the glucuronidase enzyme preparation (23). We enzyme-treated artificial urine samples (25) with and without spiked phthalate diesters to determine if the presence of phthalate diesters would cause an artifactual increase in phthalate monoester levels. When samples spiked with phthalate diesters were incubated with β-glucuronidase/sulfatase (H. elix pomata, H-1), significant amounts of the corresponding phthalate monoesters were formed (M EP, M BP, M BzP, and M EHP). These enzymes, H. pomatia β-glucuronidase/sulfatase (from two vendors), were contaminated with an enzymatic activity that hydrolyzed phthalate diesters to generate phthalate monoesters; nonspecific lipases with this activity are common (26). Given the abundance of phthalate diesters in the environment (including the laboratory), the use of the H. pomatia β-glucuronidase/sulfatase should be avoided for analysis of phthalate monoesters. Escherichia coli β-glucuronidase (K12; Roche Biomedical, Indianapolis, IN) has excellent glucuronidase activity and no measurable lipase activity on phthalate diesters. Although this enzyme lacks sulfatase activity, no phthalate conjugates other than glucuronides have been detected in human urine (27). For previously published phthalate monoester measurements, H. pomatia β-glucuronidase/sulfatase was used; therefore artifically high results may be present (3,22).

Results and Discussion

Our study provides an assessment of human exposure to phthalates. Phthalate monoester levels in human urine vary widely (Table 1); urinary creatinine adjustment reduces this variation somewhat (Table 2). In this reference population, the phthalate monoesters with the highest urinary levels found are M EP (16,200 ppb, 6,790 µg/g creatinine), M BP (4,670 ppb, 2,760 µg/g creatinine), and M BzP (1,020 ppb, 540 µg/g creatinine), which reflect exposure to diethyl phthalate (DEP), DBP, and BzBP. DEP and DBP are used extensively in products with volatile components such as perfumes, nail polishes, and hair sprays, possibly leading to inhalation and efficient absorption through the lungs. Dermal absorption also occurs at a significant rate for phthalates with short side chains such as DEP, DBP, and BzBP (28).

The highest levels of M EHP in this study (67 ppb, 192 µg/g creatinine) agree with levels found previously in urine from occupationally exposed subjects (22). The median M EHP levels for this general reference population were 70-fold lower than the highest values. In urine, the more lipophilic phthalate monoesters, such as M EHP and M INP, were generally found at lower levels than other monoesters. The relatively low median M EHP and M INP levels suggest that either low exposures to DEH P and DIN P, storage in adipose tissue, or metabolism and excretion through another pathway. Limited data suggest that DEH P is partially excreted in the feces (1); because of similar lipophilicity, DIN P may also be excreted facely. Further complicating assessment, DIN P consists of a mixture of phthalate isomers that yield a large number of monoester metabolites. Only one of these metabolites, mono-3-methyl-5-dimethylhexyl phthalate, was measured in urine and was assumed to be representative of the presence of other DIN P metabolites. In any event, these data on monoesters indicate that the internal dose of M EP, M BP, and M BzP is probably much higher than that of M INP and M EHP.

**Table 1. Total urinary phthalate monoester concentrations (nanograms monoester per milliliter urine).**

| Phthalate | Percentile | 5th | 25th | 50th | 75th | 95th | Mean | Geometric mean |
|-----------|------------|-----|------|------|------|------|------|----------------|
| Ethyl     | <LOD       | 26.1| 119  | 305  | 1,10 | 3,750| 16,200| 345            |
| Benzyl    | 1.4        | 4.2 | 11.0 | 21.2 | 42.2 | 137  | 1,020 | 22.6           |
| Butyl     | 2.2        | 6.9 | 19.4 | 41.0 | 82.3 | 294  | 4,670 | 41.5           |
| Cyclohexyl| <LOD       | <LOD| <LOD | <LOD | <LOD | <LOD | <LOD | <LOD           |
| 2-Ethylhexyl| <LOD    | <LOD| 1.4  | 2.7  | 7.0  | 21.5 | 66.6  | 1.5            |
| Isononyl  | <LOD       | <LOD| <LOD | <LOD | <LOD | <LOD | <LOD | <LOD           |
| Octyl     | <LOD       | <LOD| <LOD | <LOD | <LOD | <LOD | <LOD | 30.5           |

**Table 2. Total urinary phthalate monoester concentrations (micrograms monoester per gram urinary creatinine).**

| Phthalate | Percentile | 5th | 25th | 50th | 75th | 95th | Mean | Geometric mean |
|-----------|------------|-----|------|------|------|------|------|----------------|
| Ethyl     | <LOD       | 30.2| 133  | 280  | 704  | 2,610| 6,790 | 345            |
| Benzyl    | 2.1        | 5.0 | 10.8 | 19.5 | 36.9 | 91.9 | 544  | 20.2           |
| Butyl     | 1.6        | 9.3 | 19.4 | 33.4 | 60.1 | 162  | 2,760 | 36.9           |
| Cyclohexyl| <LOD       | <LOD| <LOD | <LOD | <LOD | <LOD | <LOD | 0.3            |
| 2-Ethylhexyl| <LOD     | <LOD| 1.3  | 2.7  | 5.2  | 15.2 | 192  | 3.0            |
| Isononyl  | <LOD       | <LOD| <LOD | <LOD | <LOD | <LOD | <LOD | 0.8            |
| Octyl     | <LOD       | <LOD| <LOD | <LOD | <LOD | <LOD | <LOD | 0.5            |

Abbreviations: LOD, limit of detection; M ax, maximum; Min, minimum.
Glucuronidation has been hypothesized to mitigate phthalate monoster toxicity in rodents (29). To assess the degree of phthalate glucuronidation, we analyzed a subset of 73 samples with and without β-glucuronidase treatment. Low urinary levels of monoster before deglucuronidation suggest that M BP and M BzP were present predominately as the glucuronide form. However, a small portion (5%) of the reference population had substantially higher percentages of unconjugated M BP than the rest of the population (67% above the next lowest value). These differences in M BP glucuronidation could be caused by enzymatic saturation due to a large recent dose of parent diester phthalate. Arguing against simple saturation, urinary M BP levels did not correlate with the ratio of M BP/M BP-glucuronide. Although based on limited data, this observation underscores the potential variability in the human metabolism of phthalate monosters and thereby variable monoster exposure and toxicity.

For analytes found in > 75% of subjects (M EH P, M EP, M BzP, and M BP), we examined log-transformed creatinine-adjusted levels by analysis of variance models to assess effects from selected demographic factors: age (four groups), sex, race (four groups), socioeconomic status (two groups), urban/rural residence, and education (two groups). All comparison cells described contain > 50 people. Significant differences in creatinine-adjusted values between groups were not caused by differences in urinary creatinine levels. Because of the large number of statistical tests performed on our data, the effects of demographic factors on urinary phthalate levels should be considered for generating hypotheses only.

After adjustment for the effects of other factors, creatinine-adjusted M EP levels increased on average by 1.7% (p < 0.02) for every yearly increase in age, conversely, creatinine-adjusted M BzP levels decreased by 1% (p < 0.04) for the same increase in age. Subjects who had completed ≤ 12 years of formal education had higher levels of M BPzP (22 µg/g creatinine) than subjects with more formal education (16 µg/g creatinine; p < 0.01). Further statistical testing indicated that M BP levels in non-H-Ispanic whites also differed between the two education groups (≤ 12 years: 53 µg/g creatinine; > 12 years: 28 µg/g creatinine; p < 0.001), whereas M BP levels in non-H-Ispanic blacks and Hispanics were comparable regardless of education (33 µg/g creatinine). Further statistical testing also showed that rural females had significantly higher urinary levels of M BPzP (26 µg/g creatinine) than rural males (14 µg/g creatinine; p < 0.001), urban females (19 µg/g creatinine) than urban males (18 µg/g creatinine; p < 0.02).

Of concern, women of childbearing age (20–40 years) had significantly higher urinary levels of M BP (46.9 µg/g creatinine) than other sex/age groups (31.4 µg/g creatinine; p = 0.003). Furthermore, six of the eight highest M BP levels were found in these women. Creatinine adjustment did not account for this effect. Nine of the highest 10 noncreatinine-adjusted values were found in women of reproductive age. Ten subjects had urinary M BP > 300 µg/g creatinine, including three women of reproductive age with levels > 2,000 µg/g creatinine (Figure 1). A similar analysis for urinary M BzP did not indicate a difference, but a more detailed look at the rural women with high levels of M BzP suggests that rural women of childbearing age have higher levels (31.6 µg/g creatinine) than older (41–60 years) rural women (21.1 µg/g creatinine). The small study size and nonrepresentative nature of this sample population limits the applicability of these statistical associations. However, these findings do indicate the possibility of significant demographic variations in exposure and/or metabolism.

From a public health perspective, these data provide evidence that phthalate exposure is both higher and more common than previously suspected. Exposure data for phthalates is critically important for human risk assessment, especially among potentially susceptible populations. Although DEHP and DnNPh are produced in the largest quantities, these reference range data indicate a substantial internal human dose of D BzP, D EP, and BzBP. M BP and M BzP are of particular concern because of their developmental and reproductive toxicity in animals (12–15). Therefore, assessments of health risk from exposures to phthalates should include exposures to D BzP, D EP, and BzBP.
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