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Phthalate metabolites in urine samples from Danish children and correlations with phthalates in dust samples from their homes and daycare centers

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ABSTRACT

Around the world humans use products that contain phthalates, and human exposure to certain of these phthalates has been associated with various adverse health effects. The aim of the present study has been to determine the concentrations of the metabolites of diethyl phthalate (DEP), di(n-butyl) phthalate (DnBP), di(iso-butyl) phthalate (DiBP), butyl benzyl phthalate (BBzP) and di(2-ethylhexyl) phthalate (DEHP) in urine samples from 441 Danish children (3–6 years old). These children were subjects in the Danish *Indoor Environment and Children's Health* study. As part of each child's medical examination, a sample from his or her first morning urination was collected. These samples were subsequently analyzed for metabolites of the targeted phthalates. The measured concentrations of each metabolite were approximately log-normally distributed, and the metabolite concentrations significantly correlated with one another. Additionally, the mass fractions of DEP, DnBP, DiBP and BBzP in dust collected from the children's bedrooms and daycare centers significantly correlated with the concentrations of these phthalates' metabolites (monoethyl phthalate (MEP), mono-n-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP) and monobenzyl phthalate (MBzP), respectively) in the children's urine. Such correlations indicate that indoor exposures meaningfully contributed to the Danish children's intake of DEP, DnBP, DiBP and BBzP. This was not the case for DEHP. The urine concentrations of the phthalate metabolites measured in the present study were remarkably similar to those measured in urine samples from children living in countries distributed over four continents. These similarities reflect the globalization of children's exposure to phthalate containing products.

Introduction

Phthalates are a group of semivolatile organic compounds commonly found in indoor environments (Weschler and Nazaroff, 2008). They are used as plasticizers in a large variety of consumer products including food packaging, toys, furnishings and building materials. Lower molecular weight phthalates are also used as solvents in personal care products. The use of phthalates has dramatically increased over the past 60 years (Weschler, 2009). Humans are exposed to phthalates via several different pathways – diet, inhalation, incidental ingestion (dust and hand-to-mouth activity) and dermal absorption. Following exposure, most of the metabolized phthalates are excreted within 24 h (Koch et al., 2005a). Hence, an individual's total recent exposure to phthalates from all pathways can be estimated from the concentrations of phthalate metabolites in his/her urine.

Numerous studies over the past 15 years report concentrations of phthalate diesters in indoor environments. The levels of various phthalates in dust (mass fractions) are most frequently reported (Langer et al., 2010, and references therein), followed by concentrations in indoor air (Fromme et al., 2004; Kanazawa et al., 2010; Rudel et al., 2003). Other studies have reported concentrations of phthalate metabolites in urine samples. These include articles reporting phthalate metabolite concentrations in general populations of adults (e.g., CDC, 2012; Fromme et al., 2007a; Guo et al., 2011a,b; Göen et al., 2011; Hildenbrand et al., 2009; Högberg et al., 2008; Romero-Franco et al., 2011), in pregnant women (e.g., Casas et al., 2011; Ye et al., 2008, 2009), in pregnant women and their children (e.g., Kasper-Sonnenberg et al., 2012; Lin et al., 2011a); in young men (Frederiksen et al., 2010) and in children of various ages

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(e.g., Becker et al., 2004, 2009; Boas et al., 2010; Casas et al., 2011; CDC, 2012; Cho et al., 2010; Colacino et al., 2011; Frederiksen et al., 2011, 2012; Hsu et al., 2012; Koch et al., 2004, 2005b, 2011; Lin et al., 2011a; Teitelbaum et al., 2008; Wolff et al., 2007). The dominant source of DEHP metabolites in urine appears to be diet; for the metabolites of DEP, DnBP, DiBP and BBzP, other sources and exposure pathways appear to make meaningful contributions (Fromme et al., 2007b; Koch et al., 2013; Rudel et al., 2011; Wormuth et al., 2006).

Several studies have examined associations between metabolite levels and various health or developmental endpoints. Metabolites of DEHP and di-n-butyl phthalate (DnBP) have been negatively correlated with school-age children's vocabulary development (Cho et al., 2010). Prenatal exposures to certain phthalates have been associated with a decrease in children's mental, motor and behavioral development (Whyatt et al., 2012). Maternal exposure to phthalates may affect sex steroid hormone status in the fetus and newborn (Lin et al., 2011b). Phthalate metabolites have been associated with biomarkers for inflammation and oxidative stress (Ferguson et al., 2011), an increase in body mass index and waist circumference (Hatch et al., 2008), a decrease in thyroid hormone levels, insulin-like growth factor and growth in children (Boas et al., 2010), and delayed onset of puberty in healthy school girls (Frederiksen et al., 2012). Some of the studies cited in this paragraph have measured metabolites in maternal urine during pregnancy, while others have measured metabolites in children's urine at various stages of the child's development. The optimal time to sample for metabolites may vary with the health endpoint under investigation.

The Danish *Indoor Environment and Children's Health (IECH)* study was a multiyear investigation of potential associations between different indoor environmental factors and children's health, especially allergies and asthma (Clausen et al., 2012). The study was performed in two steps. First, questionnaires were distributed to 17,500 families on the Danish island of Fyn that had children between the ages of 1 and 5 years. Based on responses from this initial survey, 500 children between 3 and 6 years of age – 200 cases with asthma and/or allergy and 300 randomly selected bases were chosen for the next stage. During the second stage, detailed investigations of the children's living environments were performed, including collection of settled dust samples from their bedrooms and daycare centers. The children also received a detailed examination by a medical doctor, at which time urine samples were collected from 441 of the children. A summary of the background, design and methods used in the IECH study is presented in Clausen et al. (2012).

In the present study we present results from the urine analysis. A total of eight phthalate metabolites were measured – five primary metabolites: monoethyl phthalate (MEP) from DEP, mono-n-butyl phthalate (MnBP) from DnBP and BBzP, mono-isobutyl phthalate (MiBP) from DiBP, monobenzyl phthalate (MBzP) from BBzP, mono(2-ethylhexyl) phthalate (MEHP) from DEHP; and three secondary metabolites: mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxohexyl phthalate (MEOHP) and mono-2-ethyl-5-carboxypentyl phthalate (MECPP), each from DEHP. Although slightly higher levels of phthalate metabolites were found among bases, none of the differences were statistically significant. Detailed analyses of associations between phthalate metabolites in the children's urine and their health status have been reported elsewhere (Callesen et al., submitted for publication). Estimates of daily intakes of the phthalate diesters based on the metabolite levels measured in the urine samples, as well as a detailed analysis of the various exposure pathways for these children will be presented in another paper (Bekö et al., 2013). In the current paper we proceed to compare our findings with those of other studies whose subjects were children. We also present correlations between phthalate levels in dust (Langer et al., 2010) and the concentrations of metabolites in corresponding urine samples (each child's urine measurement paired with measurements of dust samples from their home and daycare environment).

Methods

Ethics statement

The study was approved by The Regional Scientific Ethical Committee for Southern Denmark (Case # S-20070108).

Samples

The dust samples were analyzed for five phthalates – diethyl phthalate (DEP), di-n-butyl phthalate (DnBP), di-isobutyl phthalate (DiBP), butyl benzyl phthalate (BBzP) and di(2-ethylhexyl) phthalate (DEHP), as well as other semi-volatile organic compounds (Langer et al., 2010). The urine samples were analyzed for the major metabolites of these five phthalates. The urine samples were collected between August 2008 and April 2009, while the dust sampling within the children's homes and daycare facilities occurred between March and May 2008. The average time lag between dust and urine sampling for all children was 7.3 months (median 7.1 months). The children were between 3 and 6 years old at the time of sampling. The first morning urine was collected in sterile urine sample kits distributed from the children's center at Odense University Hospital. The samples were stored at 5 °C until the family attended the clinical examination later on the same day, at which point it was labeled and stored at – 23 °C. At the conclusion of the clinical examinations of all children, the samples were packed in dry-ice and transported to the laboratory in Sweden.

Reagents

Almost all of the labeled ($^{13}C_4$) and unlabeled metabolite standards were purchased from Cambridge Isotope Laboratories (CIL), USA as 0.1 mg/mLsolutions in acetonitrile. The unlabeled MBzP standard from CIL was a 0.1 mg/mLsolution in methyl tert-butyl ether. The ring-D4-labeledMBzP was purchased neat from QMX Laboratories Limited, UK; stock solutions (0.1 mg/mL) were subsequently prepared in house. $^{13}C_4$-MiBPwas not commercially available; $^{13}C_4$-MnBP was used for quantification of MiBP. β-Glucuronidase (EC3.2.1.31 from *E. coli* K12) was purchased from Roche Diagnostics GmbH, Germany. Water (Milli-Q grade) was prepared "in-house", formic acid (ACS grade) and acetonitrile (HPLC grade) were obtained from Merck and Fisher Scientific, respectively. Isolute C18 200 mg solid phase extraction (SPE) columns were purchased from Biotage, Sweden.

Sample preparation

The children's urine samples were thawed and 250 μL aliquots were taken for subsequent analysis. Phthalate metabolites in the urine samples were quantified following methods similar to those previously described by Silva et al. (2003, 2007) with one major exception. Rather than adding all seven $^{13}C_4$-labeled metabolites to each of the urine aliquots, only $^{13}C_4$-labeled MEPand MEHP were added to each aliquot. Then the native phthalate metabolites present in the children's urine were deconjugated from their glucuronated form by incubation with β-glucuronidase for 2 h at 37 °C. After cooling to room temperature, the enzyme reaction was quenched by the addition of 125 μL of formic acid and diluted with 1 mL of 5% acetonitrile. The analytes in the enzyme treated children's urine samples, calibration standards and blanks were separated from their urinary matrix using solid phase extraction