

Biomonitoring of Di-(2-Ethylhexyl) Phthalate (DEHP) Exposure in Human

Petrovičová I.

Kolena B.

Pilka T.

Constantine the Philosopher University in Nitra,
Faculty of Natural Science, Department of Zoology and Anthropology,
Nábřežie mládeže 91, 949 74, Nitra, Slovakia; ipetrovicova@ukf.sk

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Abstract

The exposure of the general population to phthalates is of increasing public health concern. Most substantial phthalate in the environment is di-(2-ethylhexyl) phthalate (DEHP); higher molecular weight is the reason for use in construction material, clothing and furnishings. By far, their largest application is to impart flexibility to polyvinyl chloride plastic (PVC). Study focuses on the biomonitoring of DEHP in adults ($n=108$; males=42, females=66; average age 39.67 ± 14.01) from Nitra, because of the potential human toxicities of phthalates as a particle of plastic material. In response to determine human exposure to phthalates, we used high performance liquid chromatography and tandem mass spectrometry analysis to estimate trace levels phthalate metabolite mono-(2-ethylhexyl) phthalate (MEHP) in human urine. The mean daily intake for DEHP ranged from 4.66 to 141.58 $\mu\text{g}/\text{kg}$ per day, in 18 subjects were higher than corresponding tolerable daily intake (TDI) for DEHP (50 $\mu\text{g}/\text{kg}$ per day) set by the European Food Safety Authority. The presence of DEHP in more than 92% of the urine samples indicating a ubiquitous exposure of people living in Nitra. This study is the result of implementation of projects: "Environmental aspects of urban area" (ITMS: 26220220110) supported by the Research & Development Operational Programme funded by the ERDF; VEGA (V1/0042/12).

Keywords: biomonitoring, MEHP, DEHP

1. Introduction

For at least three decades, scientists at CDC's Environmental Health Laboratory have been determining which environmental chemicals people have been exposed to and how much of those chemicals actually gets into their bodies. This technique is known as biomonitoring. Biomonitoring measurements are the most health-relevant assessments of exposure because they measure the amount of the chemical that actually gets into people, not the amount that may get into people (CDC, 2012).

Human biomonitoring includes monitoring of chemicals, their metabolites or specific reaction products in blood, urine, faeces, hair, saliva, breast milk or human adipose tissue, which are suitable for assessing environmental exposure, as well as diseases and disorders of bodily functions.

Progress in human biomonitoring has opened new possibilities in assessing phthalate exposures, because most of the biomarkers used in modern phthalate biomonitoring are specific metabolites generated in the human body (secondary oxidized metabolites) which are not prone to external contamination (Angerer et al., 2007).

Phthalates are alkyl diesters of phthalic acid named based on the lengths of the alkyl chains and are used to impart flexibility in plastic or as a matrix in cosmetic products. Phthalates are not covalently bound to molecules of plastic polymer and thus easily released into the environment by direct release, migration, evaporation, leaching and abrasion of and from the products they are used in (Wittassek et al., 2011). Human exposure occurs through inhalation, dermal contact, intravenous and ingestion especially with contaminated food. Food considered to be the most important source of exposure to high-molecular weight phthalates (high-MWP; metabolites >250 Da) for the general population (Schettler, 2006). After absorption, phthalates are initially metabolized to monoesters which can be metabolized further and are eventually excreted as glucuronides in urine (Silva et al., 2003). The content of phthalate metabolites in human urine represents a measure of the exposure to the respective parent phthalate that occurred within the last 24h (Koch et al., 2006).

Of the approximately 20 phthalate esters in common use, di-(2-ethylhexyl) phthalate (DEHP) constitutes approximately half the total; 1–4 million tons are produced per year (Thomas and Thomas, 1984; Huber et al. 1996; Halden, 2010). Di-(2-ethylhexyl) phthalate (DEHP) is the most substantial phthalate in the environment which belongs to the group of phthalates with long chain and high molecular weight. DEHP is used in numerous consumer products, especially those made of flexible polyvinyl chloride. Because of his toxicity the use of DEHP in teething rings, pacifiers, and toys for young children has largely been discontinued, but DEHP continues to be used in clothing, toys, food containers, medical devices (i.e. PVC infusion lines) and a variety of building, household, and automotive products (David et al., 2001; Heudorf a et al., 2007; Loff et al., 2007).

Data from Koch et al. (2006) and Fromme et al. (2007) and others studies suggested that the major source of exposure to long-chain phthalates such as DEHP is foodstuff. In all age groups ingestion of food was assessed to be the most dominant pathway for exposure to DEHP, more than 90% for children, teenagers and adults and approximately 50% in infants and toddlers (reviewed by Wittassek et al., 2011).

DEHP is extensively metabolized after all routes of uptake and eliminated in the urine. In a first and fast step DEHP is cleaved into primary metabolite monoester MEHP which is again extensively metabolized by different oxidation reactions to secondary metabolites mono(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP) and mono(2-ethyl-5-oxohexyl) phthalate (5oxo-MEHP) (Koch et al., 2005). These oxidative metabolites are excreted in at least three times higher concentrations than MEHP in the general population (Kato et al., 2004).

DEHP is a known reproductive and developmental toxicant in animals exerting its toxicity already in utero. Some effects are malformed reproductive organs (hypospadias, cryptorchidism), decreased anogenital distance, mating, pregnancy and fertility, retained nipples at bird and reduced reproductive organ weights, disrupt testicular germ cell organization in foetus and spermatogonial stem cells function in a transgenerational manner (Kavlock et al., 2002; Akingbemi et al., 2004; Borch a et al., 2005; Foster, 2006; Swan et al., 2006; Doyle et al., 2013). DEHP is also a considered a human endocrine disruptor affecting the reduction in sperm motility and chromatin damage (Hauser et al. 2007), disruption of fetal germ cell development (Lambrot et al., 2009), disruption of Leydig cell development and testosterone levels (Desdoits-Lethimonier et al., 2012), reduction in anogenital distance (AGD) (Swan et al., 2005; Marsee et al., 2006) and have the potential to alter androgen-responsive brain development in humans (Mendiola et al., 2012.)

Typical human exposure is estimated to be 4–30 µg DEHP/kg/day, but some individuals have substantially greater exposure resulting from DEHP-plasticized medical devices such as blood bags, hemodialysis tubing and membranes, autophoresis equipment, and nasogastric feeding tubes (Loff et al., 2007). The aim of our study was biomonitoring of this hazardous toxicant on general population in Nitra, Slovak Republic.

2. Material and methods

The cohort consists from 108 probands with age range 19-69 years. Probands' participation in this study was entirely voluntary and also had the possibility to withdraw their participation at anytime during the study. Informed consent was required to be interviewed by the researcher, to provide samples of urine, complete questionnaires and allow the researchers to take measurements and also to process their medical and personal records and data. The anthropometric data was collected using standard anthropological methods; body height (by A 319 TRYSTOM, Ltd., Pasteurova 15, 772 00 Olomouc Czech Republic), waist girth and hip girth (by spreading calliper P-374 TRYSTOM, Ltd. Pasteurova 15, 772 00 Olomouc Czech Republic). Body-mass index (BMI) was estimated and classified by WHO (1995). Waist-to-height ratio (WHTR) was calculated:

$$\text{WHTR} = \frac{\text{waist circumference (cm)}}{\text{height}^2 (\text{cm}^2)}$$

Waist to hip ratio (WHR) was estimated by dividing the waist circumference by the hip circumference (WHO, 1986). Body weight, body fat percentage, muscle mass percentage, and visceral fat level were estimated by The Omron BF510 (Kyoto, Japan) by bio-electrical impedance analysis, using a 50 kHz current source with electrodes on each hand and foot. The FMI indices are concept equivalent to BMI, as shown in the following definition (Schutz et al., 2002).

$$\text{FMI} = \frac{\text{fat mass (kg)}}{\text{height}^2 (\text{m}^2)}$$

2.1 Urine collection and analyses

Urine samples from all volunteers were stored in transport box at 2-6 °C. In laboratory were all samples stored in deep freeze at the temperature -73°C until analysis. Mono-2-ethylhexyl phthalate (MEHP) was measured in urine specimens by high performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS) (Infinity 1260, Agilent Technologies, Schweiz). Urine analysis was made according to analytical method described by Silva et al. (2004, 2007) with use of manual solid phase extraction (SPE). Analytical standards were purchased from Cambridge isotope laboratories (MA, USA). Briefly, 1ml of urine was thawed buffered with ammonium acetate and spiked with isotope labelled phthalate standards, β-glucuronidase enzyme (Roche, Germany) and incubated (37°C). After deconjugation were samples diluted with phosphate buffer (NaH₂PO₄ in H₃PO₄) and loaded on SPE cartridges (ABS Elut Nexus, Agilent). Cartridges were conditioned with acetonitrile followed by phosphate buffer before extraction. To remove hydrophilic compound were SPE cartridges flushed by formic acid and HPLC grade water. Elution of analytes was performed by acetonitrile and ethylacetate. Eluate was dried by nitrogen gas and reconstituted with 200µl of H₂O. For HPLC purposed was used Agilent 1260 liquid chromatograph equipped with ZORBAX Eclipse plus phenyl-hexyl column. Separation was done using non-linear gradient program (Table 1). Agilent 6410 triplequad with electro-spray ionization was used for mass specific detection of phthalate metabolites. Instrumental settings were as follows: spray ion voltage (-3800 V), nitrogen nebulizer gas pressure (8 psi), nitrogen curtain gas pressure (7 psi), capillary temperature (430°C), and collision gas (nitrogen) pressure (1.5 mTorr). Precursor and product ions, collision energies, retention times and limits of detection (LOD) are showed in Table 2.

Table 1. Gradient program for HPLC separation

Time, min	0	4	6	8
A%	80	60	40	10
B%	20	40	60	90

Flow rate (0.3 ml.min⁻¹); mobile phase A (0.1% acetic acid in HPLC grade water) and mobile phase B (0.1% acetic acid in acetonitrile)

Table 2. Phthalate monoesters: chromatographic and mass spectrometric parameters.

Compound Name	Precursor Ion	Product Ion	Fragmentor (V)	Collision Energy (V)	RT (min)	LOD, ng.ml ⁻¹
MEHP-C4	281,1	137,1	90	14	14,7	
MEHP	277,1	133,9	90	14	14,7	0.81

We converted those excretion values of the phthalate metabolites to total daily intake (TDI) values for the parent phthalate applying the equation according to Koch et al. (2003).

$$\text{Total daily intake } (\mu\text{g}/\text{kg}/\text{day}) = \frac{\text{ME}(\mu\text{g}/\text{g}) \times \text{CE}(\text{mg}/\text{kg}/\text{day})}{\text{Fue} \times 1,000(\text{mg}/\text{g})} \times \frac{\text{MWd}}{\text{MWm}}$$

ME- urinary concentration of monoester per gram creatinine

CE- creatinine excretion rate normalized by body weight

Fue- molar fraction of the urinary excreted monoester related to parent diesters

MWd- molecular weight of phthalate diesters

MWm- molecular weight of phthalate monesters

3. Results

The cohort consist from adults (n=108) of which were 42 males and 66 females from Nitra region, Slovak Republic. The mean (± SD) age of study participants were 38.29 ± 13.60 years for female and 41.83 ± 14.52 years for male, BMI was 24.5 ± 5.05 kg/m for female and 26.26 ± 3.36 for male respectively (see in Table 3).

Table 3. Baseline anthropometric characteristic of study subjects by gender

	Mean ± SD		-95%		+95%	
	Female (n=66)	Male (n=42)	Female (n=66)	Male (n=42)	Female (n=66)	Male (n=42)
BMI	24.5 ± 5.05	26.26 ± 3.36	23.26	25.21	25.74	27.3
FMI	8.65 ± 3.74	5.99 ± 2.21	7.73	5.3	9.57	6.68
WHTR	0.5 ± 0.09	0.52 ± 0.06	0.48	0.5	0.53	0.54
WHR	0.83 ± 0.09	0.92 ± 0.08	0.8	0.9	0.85	0.95

Urinary mean concentration and distributions by minimum, median and maximum of MEHP and estimated DEHP by gender were calculated (Table 4). Mean concentration values for urine metabolite MEHP were higher in both genders than their median values, indicative of the high level in the upper quantiles. LOD value for MEHP was 0.81 µg.L⁻¹.

Table 4. Distribution of phthalate metabolite concentrations in subjects by gender (µg.L⁻¹) and estimated total daily intake TDI (µg/kg/day) based on (Koch et al. 2003)

		Mean ± SD	Min	Median	Max
All	MEHP(µg.L ⁻¹)	28.9 ± 21.25	4.54	23.49	117.77
	DEHP(µg/kg/day)	33.04 ± 24.71	4.66	28.41	141.57
Female	MEHP(µg.L ⁻¹)	28.51 ± 21.55	4.54	25.82	117.77
	DEHP(µg/kg/day)	29.35 ± 22.21	4.66	26.44	122.08
Male	MEHP(µg.L ⁻¹)	29.52 ± 20.74	7.04	21.73	108.46
	DEHP(µg/kg/day)	38.82 ± 27.21	9.19	28.57	141.58

LOD 0.81 µg.L⁻¹

Among the 108 urine samples, MEHP, metabolite of di-(2-ethylhexyl) phthalate, was detected in 93 % (n=100) of samples of which were 60 % female and 40% male.

16.67 percent of the all subjects (18 out of 108 samples) within our cohort of the general population are exceeding TDI (50 µg/kg/day) set by European Food Safety Authority (EFSA, 2005). 71.30 percent of the subjects (77 out of 108 samples) had values higher than the reference dose (RfD) of 20 micrograms/kg body weight/day set by the U.S. Environmental Protection Agency (EPA) (Table 5).

Table 5. Presence of urine metabolite in subjects and exceeded daily phthalate intake levels established by EFSA (2005) and US EPA

		Presence				
		MEHP	TDI (EFSA)		DEHP	
n (%)		>50 µg/kg/day n (%)	Mean ± SD µg/kg/day	>20 µg/kg/day n (%)	Mean ± SD µg/kg/day	
All	n=108	100 (92.59)	18 (16.67)	75.55 ± 25.54	77 (71.30)	42.11 ± 23.43
Female	n=66	60 (90.91)	7 (10.61)	77.4 ± 23.95	51 (85.00)	38.65 ± 19.52
Male	n=42	40 (95.24)	11 (26.19)	74.38 ± 26.43	26 (61.90)	48.90 ± 28.43

Concerning the monoester determination, median, mean, SD, minimum and maximum values for MEHP were higher in the men compared to the female group. Mann-Whitney U tests (Wilcoxon rank-sum) confirmed that the two data sets were not significantly different (P = 0,816 for MEHP) (Table 6).

Table 6. Comparison between excretion of the MEHP in the female (n = 66) and male (n=42) set of our study

	Mean ± SD		Median		Min		Max		P value
	♀	♂	♀	♂	♀	♂	♀	♂	
MEHP ($\mu\text{g}\cdot\text{L}^{-1}$)	28.51 ± 21.55	29.52 ± 20.74	25.82	21.73	4.54	7.4	117.77	108.46	0,8156

We also grouped our cohort according to questions asked in the extensive questionnaire to search for correlations with excreted amounts of metabolite. We found no significant coherence within eating habits or body care habits, drinking habits, lifestyle, or medication and medical history.

4. Discussions

The use of phthalates in the production of a wide spectrum of everyday products caused that this group of chemicals has become an essential part of the human environment with potential pathogenic impact on human health. In many experimental works carried out on animal models have been confirmed several adverse effects on reproduction, especially the male sexual system, but also respiratory, digestive and endocrine system. Due to their low acute toxicity, short half-life and long-term exposure is still find it difficult to evaluate their impact on human health despite of use advanced analytical methods (Pilka, Kolena, Petrovičová, 2012).

The level of internal exposure MEHP of subjects in the present study differs from previously published results for the American reference population by Blount et al. (2000) and CDC (2003). In our study MEHP median values are 23.49 $\mu\text{g}\cdot\text{L}^{-1}$ and 95th percentile 77.54 $\mu\text{g}\cdot\text{L}^{-1}$ higher compared to the USA study (CDC, 2003) with median 3.2 $\mu\text{g}\cdot\text{L}^{-1}$ and 95th percentile 37.9 $\mu\text{g}\cdot\text{L}^{-1}$. The differences in internal exposure between our and The American reference population are probably caused by different degrees of external exposure and also by differences in sampling location and time. Unlike the samples of our cohort, they were not first-morning urine but collected at different times throughout the day and the whole year.

Higher values detected in our study indicate increased exposure to DEHP through our study despite the fact that only between 2-7% of the dose is excreted as the simple monoester i.e. primary metabolite MEHP (German Federal Environment Agency, 2011), while the rest is further metabolized to produce a number of oxidative metabolites.

DEHP is strongly suspected to be a developmental and reproductive toxicant (Kavlock et al., 2002) and also endocrine disruptor (Swan et al., 2005). Koch et al. (2003) argues that they are not aware of any other environmental contaminant for which the TDI and RfD are exceeded to such an extent within the general population. The tolerable daily intake (TDI) value settled by the European Food Safety Authority is for DEHP 50 micrograms/kg body weight/day. 16.6 percent of the subjects (18 out of 108 samples) within our cohort of the general population are exceeding this value and 71 percent of the subjects (77 out of 108 samples) had values higher than the reference dose (RfD) of 20 micrograms/kg body weight/day of the U.S. Environmental Protection Agency (EPA). In comparison study of Koch et al. (2003) on German population found 20 percent of the subjects from general population are exceeding (TDI) value but it was caused by the difference value for TDI settled by the EU Scientific Committee for Toxicity, Ecotoxicity and the Environment (CSTEE) (37 micrograms/kg body weight/day). On the contrary only 31% of the subjects had values higher than the reference dose (RfD). This can be explained by the consecutive substitutions of hazardous DEHP with DiNP/DiDP in Western Europe and mainly in United States (Wittassek et al., 2007; Silva et al., 2004), furthermore fact that estimation of creatinine excretion in our work was done by semiquantitative methods caused inaccuracy in value of TDI. In comparison with USA where is DEHP substituted for his toxicity by DiNP and DnOP, Koch et al. (2006) observed a sharp increase in urinary DEHP metabolite concentrations after the plateletpheresis procedures (intravenous DEHP exposure) in German study.

Hatch et al. (2008) studied associations between urinary phthalate metabolite concentration and BMI and WHR. They found differences in effect between females and males specifically MEHP was inversely related to BMI in adolescent girls. In the present study we found no important associations between MEHP and anthropometric measurements considering to gender which is consistent with Stahlhut et al. (2007) who found that MEHP showed substantially weaker associations in comparison with the oxidative metabolites MEHHP and MEOHP. This could be due effects of endocrine disruptors, such as phthalates, depending upon endogenous hormone levels, which vary dramatically by age and gender.

Our study has two important limitations. Single spot-urine measurements of MEHP were used to estimate exposure. MEHP are rapidly metabolized and excreted, and a single exposure measurement may not reflect long-term

exposure. The second limitation was estimation of creatinine excretion by semiquantitative method that could affect the estimated values of TDI.

But despite this, our results prove that the general population from Nitra is exposed to DEHP to a higher extent in comparison with populations in other similar studies. This is of great importance for public health since DEHP was not only the most important and ubiquitous phthalate in Europe over the last years, but also the phthalate with the greatest endocrine disrupting potency.

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