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Are nails a valuable non-invasive alternative for estimating human exposure to phthalate esters?

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ABSTRACT

Most human biomonitoring studies conducted in the past year for assessing the human exposure to phthalate esters (PEs) employed measurements of PE metabolites in urine. Although urine is recognized as a valuable non-invasive matrix, it has also limitations regarding the short time window for exposure.

Therefore, in this pilot feasibility study we aimed to assess the human exposure to seven PE metabolites (including mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), mono-*n*-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP), monoethyl phthalate (MEP) and mono-benzyl phthalate (MBzP)) using human nails. Paired nails and urine samples from the same individuals were used for comparison. Median levels of specific PE metabolites measured in nails and in spot urine of twenty Belgian individuals ranged from $<LOQ_m$ to 146 ng/g and from 0.2 to 6.7 ng/mL (creatinine adjusted), respectively. The major PE metabolites found in nails were MEHP (average 146 ng/g), sum (MnBP, MiBP) (average 212 ng/g) and MEP (average 205 ng/g).

Significant correlations were achieved between different metabolites in nails and urine, i.e., MEHP levels in nails correlate well with sum (MnBP, MiBP) ($r=0.73$, $p<0.01$) and with MBzP ($r=0.52$, $p<0.05$) levels in urine. Moderate correlations were observed between 5-OH-MEHP and sum (MnBP, MiBP) ($r=0.62$, $p<0.01$) as well as with MEP ($r=0.56$, $p<0.05$) in both matrices. However, no significant correlation was observed for the same metabolite measured in both matrices.

Based on participant questionnaires and after performing multivariate statistics, the relevant parameters of exposure positively associated with PE metabolites in nails were the use of hand care products, weight of the individuals and sport activity hours. Based on the detected levels and aforementioned predictors, nails seem a valuable non-invasive matrix for estimating human long-term exposure to DEP, DBnP and/or DIBP and DEHP.

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1. Introduction

For more than 50 years, phthalate esters (PEs) have been part of our daily life. Due to their outstanding chemical properties, PEs have been successfully incorporated mainly as excipients, emollients or plasticizers in diverse products, such as medical devices, clothing, car upholstery, food packaging, furniture, varnishes, or personal care products (e.g. hairsprays, shampoos, deodorants, nail polish) (Koo and Lee, 2004; Wittassek et al., 2011a). As a result, their release to the environment or contact with humans is practically constant and unavoidable. The main known routes of human exposure are ingestion, dermal contact, inhalation or intravenous contact through medical devices (Hines et al., 2009; Wittassek et al., 2011b).

PEs can induce toxic effects (e.g. reproductive, developmental, and neurological, among other effects) on humans, rats and other animal species (Blount et al., 2000; Koch et al., 2012; Peck and Albro, 1982; Wittassek and Angerer, 2008). Additionally, recent restrictions were introduced for the toxic PEs (reproduction categories 1 and 2) such as DBP, BBzP and DEHP used in toys or personal care products (EU, 2005; EU, 2009) and for DEHP or DBP in food contact materials (e.g. seals, caps) (Ventrice et al., 2013; Wittassek et al., 2011b). Still, no restrictions were yet foreseen for other PEs pointed out as toxic to humans (e.g. short chain PEs) (Ventrice et al., 2013).

As a result, human biomonitoring (HBM) has gained a lot of attention, especially to identify exposure and to perform risk assessment. Thus, the detection of PEs in humans is mainly demonstrated by their primary and/or secondary metabolites

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(hydrolytic or oxidative). For PEs, the first detoxification step consists in the conversion of diesters into monoesters that can be transformed into even more bioactive substances, i.e. more toxic to humans (Ventrice et al., 2013).

PE metabolites are considered as valid biomarkers of internal exposure due to its good representativeness in our body and strong correlation with the parent diester. Until now, urine has been the matrix of choice for measuring PE metabolites, due to all advantages associated to the ease of sampling, stability, storage, and fast screening of (short term) exposure (Barr et al., 2003; Blount et al., 2000; Braun et al., 2013; Frederiksen et al., 2010; Hines et al., 2009; Koch et al., 2013; Preuss et al., 2005; Romero-Franco et al., 2011). Within 24 h after exposure, most of the metabolized PEs are excreted. For example, 67% of the DEHP (diethyl hexyl phthalate) is eliminated after 24 h via its oxidative metabolites (e.g. mono-(2-ethyl-5-carboxypentyl) phthalate (5-cx-MEPP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), and mono-(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP). Still, a small percentage (3.8% of DEHP) can be eliminated after 48 h (Preuss et al., 2005; Ventrice et al., 2013).

Nevertheless, there are also disadvantages associated to the use of urine, namely related to the creatinine content (e.g. highly dependent on the time collection) (Barr et al., 2004).

In this sense, is important to explore alternative matrices and more recently few studies have shown the potential of other non-invasive matrices (e.g. hair or saliva) (Hines et al., 2009; Hsu et al., 2015; Silva et al., 2005) to assess the human exposure to PEs, integrating larger detection windows of exposure. Particularly, five major DEHP metabolites were determined in human hair Chang et al. (2013), considering that MEHP is a relevant metabolite of internal exposure (levels in hair ranged from 15 to 96 ng/g). Yet, in previous HBM studies using urine, MEHP was not considered an important biomarker of human internal exposure (Preuss et al., 2005).

As far as we know, the use of other non-invasive samples, instead of urine, has not been widely explored in HBM for internal exposure assessment to PEs, therefore a lack of knowledge in this field still exists.

Thus, the aim of the present pilot study was to demonstrate the feasibility of nails as an alternative matrix for assessing the human exposure on PEs. Measurement of nails has a multitude of advantages: ease of sampling in different populations (adults, babies, elderly and/or sick people) and without restriction of collection at any time of the day; sampling of sufficiently low amounts (few mg per analysis); reproducibility of measurements over time and translation of a long exposure period (weeks to months). In the present study, the aim was to determine the levels of seven PE metabolites, including the mono-(2-ethylhexyl) phthalate, mono (2-ethyl-5-oxohexyl) phthalate, mono(2-ethyl-5-hydroxyhexyl) phthalate, mono-*n*-butyl phthalate, mono-isobutyl phthalate, monoethyl phthalate and mono-benzyl phthalate, in paired nails and urine samples collected from twenty Belgian individuals. A new approach for correcting the dilution effects of PE metabolites based on the individual and on the average of creatinine levels measured in all individuals is proposed.

Statistical analysis was performed for better understanding of the results in both matrices and in order to describe the relative importance of each matrix in human exposure to PEs. Participant questionnaires, univariate and multivariate statistical analysis was were performed in order to discriminate (personal and environmental) variables that could explain the levels in nails.

2. Materials and methods

2.1. Standards and chemicals

Seven PE metabolites, including MEHP (99.9%), MiBP (97.8%), MnBP (97.4%), MBzP (99.8%), MEP (100%) (supplied by Accustandard Inc., Connecticut, USA) and two MEHP oxidative metabolites (5-oxo-MEHP (> 95%) and 5-OH-MEHP (> 96.5%) were provided by BCP instruments (Irigny, France)) were investigated. Mass-labelled internal standard (IS) solutions for all metabolites ($^{13}C_4$-MEHP, $^{13}C_4$-5-oxo-MEHP, $^{13}C_4$-5-OH-MEHP, d_4-MiBP, $^{13}C_4$-MnBP, $^{13}C_4$-MBzP and $^{13}C_4$-MEP) (95%) were supplied by Cambridge Isotope Laboratories (Andover, USA) All solutions (stock, working and spike solutions) were prepared on a weight basis in acetonitrile or in ultra-pure water (i.e. only spike solutions).

The organic solvents, such as trichloroethylene, nitric acid (65%) and the acetic acid (100%) were obtained from Merck (Darmstadt, Germany). The organic solvents, such as acetone, acetonitrile (ACN) and methanol (MeOH) were of UPLC grade (Fisher Scientific, Loughborough, UK). Trifluoroacetic acid (TFA, 99%) was supplied by Sigma-Aldrich (Steinheim, Germany). Ammonium acetate buffer (NH_4Ac) was prepared by dissolving 1.93 g of ammonium acetate (99.99%, Sigma-Aldrich, Diegem, Belgium) in 100 mL ultra-pure water and acidifying the solution drop by drop with acetic acid until reach pH 6.5. The enzyme β-glucuronidase (E. coli K12) was supplied by Roche Applied Sciences (Mannheim, Germany). The creatinine urinary colorimetric kit was supplied by Sanbio (The Netherlands). The 96-well plate spectrophotometer for creatinine measurements was provided by Tecan Bio M200 PRO (Mechelen, Belgium). The ultra-pure water was provided by the Advantage A10 system (Millipore S. A, Overijse, Belgium).

2.2. Nails and urine collection

Finger nail and urine samples were collected among twenty volunteers at our institute (VITO). The sampling had a duration of three weeks during the second trimester of 2015. All participants were duly informed about the purpose of this study giving their consent to participate (Ethical approval register No. B300201316329).

On the last day of the sampling, the participants filled a short survey recording their personal and lifestyle information during the past three weeks. Information included the gender, body weight, use of nail polish and hand creams, time spent in transports/car and at home, frequency of sport exercise, habits of eating with hands (e.g. snacks, chips, fruit), eating pre-packed food, heating/storing food in plastic containers (frequency), habits of ventilating the house. At that time, the volunteers also collected a first morning urine sample. In the end, in total twenty nails and sixteen urine samples were collected by the participants.

Before the analysis, all glassware was washed and heated in the oven at 450 °C (overnight) to remove possible organic contamination. Afterwards, the materials were stored and wrapped in aluminum foil. The use (and contact) of plastic materials was minimized (septa and caps of vials were covered with aluminum foil prior to use to avoid dust contamination). The polypropylene bottles used for urine collection were prior rinsed with HNO_3 solution (10%, *v*/*v*) followed by methanol.

Before the extraction, the nails were rinsed twice with acetone in order to remove residues of nail polish. Then the samples were grinded and homogenized in a mixer mill (30 min, 20 Hz).

2.3. PE metabolites extraction from nails and urine

Thirty mg of powdered nails were weighed into glass vials and

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