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Long-term exposure assessment to phthalates: How do nail analyses compare to commonly used measurements in urine

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ABSTRACT

Phthalate esters (PEs) are easily metabolized and commonly excreted via urine within 24 h, therefore their bioaccumulation potential is thought to be rather low. In the present study, we developed a sample preparation combined with a new microextraction method to measure seven PE metabolites in nails. The use of whole nails did not result in significantly different levels compared to powdered nails, which makes the method very fast and user friendly. The method was validated using whole nails showing good accuracy, satisfactory precision and low limits of quantification (2–14 ng/g).

Although method development was the primary aim of the study, the method was also applied to real samples. PEs were measured in nails of 9 individuals collected at 2 distinct time points (15 days apart) and compared to levels in the respective urine samples (daily morning sample for 15 days). Additionally two volunteers have collected two more urine spots (afternoon and evening) per day.

Major metabolites in nails were mono (ethyl hexyl) phthalate (MEHP), monoethyl phthalate (MEP) and sum of mono-*n*-butyl and mono-isobutyl phthalate (Σ(MnBP, MiBP)) while MEP and Σ(MnBP, MiBP) were the major ones identified in urine. In urine, first void morning urine reflected higher total excretion (sum of PEs of 7.0 μg/g creatinine) for all individuals than the afternoon/evening voids.

Participants also filled a questionnaire regarding their life-style. The use of hand care products and consumption of pre-packed food was associated with di-(2-ethylhexyl) phthalate (DEHP) oxidative metabolites, while the use of medical devices with butylbenzyl phthalate (BBzP) exposure. Although the metabolism (rate) and other factors that influence the transfer of the analytes from blood or other body compartments into nails needs further investigation, nails can be used to assess exposure to PEs. From our knowledge, urine reflects the excretion of PEs on 'daily basis' while nails show less fluctuation and more stable levels.

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1. Introduction

Phthalate esters (PEs) have been widely used in a multitude of products. Depending on their number of carbon atoms in the alkyl chain, PEs are classified as short (1–6-carbon atoms) to long (>6 carbon atoms) alkyl chain PEs [1,2]. The stability, fluidity and low volatility of long-chain PEs makes them highly suitable as plasticizers (e.g. to soften plastic polymers as polyvinyl chloride (PVC)) in a wide array of products (for example, construction materials, upholstery, medical devices, food contact materials or toys), while short-chain PEs are principally used as solvents and emollients of adhesives, personal care and house cleaning products and pharmaceuticals [1,3,4].

Since PEs are not chemically bound to these products, they are easily released to the environment and enter in the human body via innumerous pathways including inhalation, absorption, ingestion or dermal contact [1].

After intake, PEs are hydrolyzed to their monoesters and may undergo further metabolism to produce (oxidative) secondary metabolites or glucuronide conjugates [5].

Many human biomonitoring (HBM) studies have been conducted to assess human exposure on PEs in several matrices including urine [3,6–20], serum [9,21], plasma [9], saliva [21], breast milk [21], semen [22] and more recently in hair [23].

Although urine is the most common matrix for measuring PE metabolites, there are two main drawbacks in using it, as the uri-

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nary concentrations are affected by urine dilution and the time point of the urine collection. Thus, some authors defend that normalizing the levels of the metabolites based on creatinine is a suitable approach to minimize errors on the measurements, to better quantify excretion and evaluate exposures [17,19,24]. In contrast, others defend that normalization is not relevant because several factors such as sex, age, body mass index (BMI), fat-free mass, and even ethnicity can have a significant influence on the renal clearance of PEs [25,26]. Instead, the urine osmolality or the specific gravity determination is suggested as an alternative for correcting the urine dilution [11,18,22]. Another influential factor that determines the concentration is the time point or period of urine collection (24 h, spot and morning urine). There are some evidences of a higher variability of the levels for both between and within-persons for 24 h urine than for first-morning or spot urine samples [11]. Therefore, some authors consider that it is of considerable importance to collect multiple urine spots [19,24] to accurately classify exposure to PEs. Still it is noteworthy that the current literature regarding the time sampling (days to two weeks) and the number of spots collected is limited [11,17,19,24].

Although there are disadvantages on sampling and quantifying PE metabolites in urine, it seems that this is a very common matrix used for assessing the exposure on PEs. Thus, in order to overcome some of the drawbacks on sampling and in particular on the PEs' exposure assessment based on urinary levels, other non-invasive matrices such as nails were recently explored [27]. Although from our understanding, exists a lack of knowledge and some skepticism in exploring the potential of this matrix for translating internal exposure, yet we believe that nails can better reflect a wider exposure range (days to months) than urine, for instance.

Taking into account the anatomy of the nail bed, i.e. that it is beneath the nail plate and is in contact with nerves, blood vessels and lymph, then it is possible to consider that many chemicals can get easily the contact with nail via diffusion into the nail matrix (which is the responsible tissue by producing cells that become the nail plate) [28]. Likewise in hair [29], we believe that once the compounds are incorporated into keratin of nails (and are not excreted via urine), the levels remain isolated from other tissues, therefore low (or no) fluctuation in the levels is expected and they are not affected by metabolism activities that may occur in other body regions. Thus, it is possible to evaluate past exposures especially due to the bioaccumulation of chemicals in the nail plate and low growth rate of nails.

Also advantages associated with an easier collection, transport, storage, stability and handling can make this matrix more attractive for HBM purposes [30,31]. As an example, nail clippings can eventually obtained by participants at home and mailed to the researcher, making studies in the remote areas highly feasible. Epidemiological research in large populations, children, babies or vulnerable people is also no issue [31]. For instance, the exposure assessment to diverse types of pollutants (e.g. perfluoroalkyl substances, heavy metals, etc.) [30,32,33] and more recently to PE metabolites [27,34] have shown that nails can be a reliable alternative. Making the sample preparation less time consuming and using an environmental friendly microextraction (i.e. ultrasound assisted extraction combined with dispersive liquid-liquid microextraction (US-DLLME)) can be considered as a step forward in routine HBM/analyses.

Several challenges remain regarding the assessment of PE metabolites in nails (including MEHP (mono (ethylhexyl) phthalate), 5-oxo-MEHP (mono(2-ethyl-5-oxohexyl) phthalate), 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)). This study aims at improving the following issues: 1) improvement of the sample preparation methodology (time reduction) was tested in nails by comparing levels of the metabolites between powdered and whole nail samples; 2) a microextraction method was validated using the best conditions (i.e. for whole nails); 3) the validity of the method was verified by assessing the PE metabolites in nine individuals from who two nail samples were collected 15 days apart. Also, one morning spot urine sample was sampled per day per participant. More, two participants have collected two more urine spot samples/day (one afternoon and one evening) during the sampling period to evaluate the diurnal variation in excretion levels. Finally, the levels in the two matrices were compared (short and long-term exposure) and major predictors of exposure were investigated based on personal/environmental surveys.

One of the major novelties of this study is the optimization of the US-DLLME method using whole nails for assessing human exposure to PEs, which makes the method suitable for large-scale routine analysis.

2. Materials & methods

2.1. Reagents, standards and equipment

Neat standards of phthalate metabolites, including MEHP (99.9%), MiBP (97.8%), MnBP (97.4%), MBzP (99.8%) and MEP (100%) were supplied from Accustandard Inc. (Conneticut, USA). 5-oxo-MEHP (>95%) and 5-OH-MEHP (>96.5%) were obtained from BCP instruments (Irigny, France). 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) were acquired from Cambridge Isotope Laboratories (Andover, USA) (95%). Individual stock standard solutions of the metabolites and IS were prepared on a weight basis in acetonitrile at final concentrations approximately of 20 μg/mL. Working, spike and calibration standard solutions of both native and IS were prepared in UPLC acetonitrile and in ultra-pure water.

The organic solvents trichloroethylene, acetic acid (100%) and nitric acid (65%) were purchased from Merck (Darmstadt, Germany). The organic solvents such as acetone, acetonitrile (ACN), methanol (MeOH) were of UPLC grade (Fisher Scientific, Loughborough, UK). Trifluoroacetic acid (TFA, 99%) was supplied by Sigma-Aldrich (Steinheim, Germany). The ammonium acetate buffer (NH_4Ac) was prepared by dissolving 1.93 g of ammonium acetate (99.99%, Sigma-Aldrich, Diegem, Belgium) in 200 mL ultra-pure water and acidifying the solution with acetic acid to 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 (Netherlands). The creatinine measurements were done using the 96-well plate spectrophotometer Tecan Bio M200 PRO (Mechelen, Belgium).

The ultra-pure water was provided by the Millipore S.A (Advantage A10 system, Overijse, Belgium).

Before use, all glassware was decontaminated in the oven at 450 °C (overnight) for removal of organic contamination possibly deposited on the glass surface. Also, the contact with plastic materials (e.g. septa/caps of centrifuge vials) was eliminated by sealing the septa with aluminium foil during sample preparation and extraction.

2.2. Nails and urine collection

Nine adults, five males and four females were recruited for this study in the beginning of 2015 in our institute (VITO NV, Belgium). The participants were duly informed about the purpose of the study giving their consent to participate (Ethical approval register N°. B300201316329). All volunteers collected one morning urine sample per day during 15 days of sampling period and two nail samples

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