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Direct analysis of phthalate ester biomarkers in urine without preconcentration: Method validation and monitoring

K. Servaes*, S. Voorspoels, J. Lievens, B. Noten, K. Allaerts, H. Van De Weghe, G. Vanermen

Flemish Institute for Technological Research (VITO), Unit Environmental Analysis and Technology, Boeretang 200, 2400 Mol, Belgium

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ABSTRACT

Phthalates, which are ubiquitous in the environment, are readily metabolized in human bodies to their respective monoesters. These phthalate monoesters are non-persistent with short half-lives, which make them the ideal biomarkers of human exposure to phthalates. In this study a direct analysis method without preconcentration was developed and validated for the following phthalate ester metabolites in urine: mono-(2-ethylhexyl) phthalate, mono-(2-ethyl-5-hydroxyhexyl) phthalate, mono-(2-ethyl-5-oxohexyl)phthalate, monobenzyl phthalate, mono-isobutylphthalate, mono-*n*-butyl phthalate and monoethyl phthalate. The recovery of the phthalate ester metabolites varied between 97% and 104%. The intraday precision for the replicate analysis ($n = 10$) of a urine sample did not exceed 5% for most of the compounds. The coefficient of variance amounted to 2–3%. The limit of quantification was set equal to 0.5 μg/L for the majority of the compounds. A comparison between the direct analysis method and a foregoing solid phase extraction (SPE) of the urine sample was made. Finally, the applicability of the direct analysis method was tested in three interlaboratory comparisons.

1. Introduction

Phthalates are a group of high-production industrial chemicals that are nearly ubiquitous. They can be found among others in toys, food packaging, medical supplies, personal care products, adhesives, lubricants and vinyl flooring. In the majority of the applications phthalates are used as plasticizers to increase flexibility. Due to their omnipresence humans are exposed to phthalates through different routes: food intake, ingestion of dust, inhalation of indoor air and dermal absorption [1,2]. Phthalates are rapidly metabolized in humans to their respective monoesters, which can be further metabolized to their oxidation products, depending on the phthalate compound. The monoesters and oxidation products can be glucuronidated, and in this way excreted via urine and feces [3].

Phthalates and their metabolites are suspected of causing adverse effects on human health. In animals these compounds have shown teratogenic, carcinogenic, reproductive and developmental effects. Studies on their effects in humans are however controversial [3,4]. Data on the concentration of phthalates in humans are essential to get insight into the human exposure to phthalates. Since phthalate monoesters are non-persistent chemicals with short half-lives, these compounds are used as biomarkers of acute human exposure to phthalates [5,6]. The biologic half life of phthalate biomarkers is less than 24 h [5,6].

Few studies have investigated urinary phthalate excretion in children and adults. Comparison of the results is complicated by differences in types of samples collected (spot urine, first morning urine and 24 h urine samples) [7]. In 2011, reference values (RV_{95}), derived from actual population-based exposure levels were provided for children and adults by the German Human Biomonitoring Commission for phthalate metabolites from di-2-ethylhexyl phthalate (DEHP), di-*n*-butyl phthalate (DnBP), di-isobutyl phthalate (DiBP) and di-isononyl phthalate (DiNP) [8]. For the phthalate ester metabolites investigated in this study, these RV_{95} values for adults are the following: 20 μg/L for mono-(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP), 30 μg/L for mono-(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP), 70 μg/L for mono-*n*-butyl phthalate (MnBP) and 140 μg/L for mono-isobutyl phthalate (MiBP). For children these RV_{95} values are approximately a factor 5 higher [8]. In the Duisburg birth cohort study (Germany) 21 primary and secondary phthalate ester metabolites were investigated in 208 urine samples from 104 mothers and their school-aged children [9]. The metabolites of DiBP were most prominent in the children (104 μg/L) followed by those of DnBP (ΣD*n*BP) (57 μg/L) > metabolites of DEHP (ΣDEHP) (56 μg/L) > monoethyl phthalate (MEP) (39 μg/L) > monobenzyl phthalate (MBzP) (13 μg/L) > monomethyl phthalate (MMP) (4.9 μg/L). Also in mothers the metabolites ΣDiBP were observed in the highest concentration (67 μg/L) [9]. In a Danish study 24 h

* Corresponding author at: Flemish Institute for Technological Research (VITO), Industrial Innovation, Boeretang 200, 2400 Mol, Belgium. Tel.: +32 14 33 56 15; fax: +32 14 31 94 72.
E-mail address: kelly.servaes@vito.be (K. Servaes).

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urine samples were collected from 129 healthy children and adolescents [7]. The median concentrations of MEP, MBzP and the sums of the two monobutyl phthalate isoforms (ΣMBP($i+n$)), metabolites of DEHP (ΣDEHPm) and of DiNP (ΣDiNPm) were 29, 17, 111, 107 and 31 μg/L, respectively. Recent US data (NHANES 2007–2008) show that lower levels of D*n*BP, DiBP, DiNP and mono-(2-ethylhexyl) phthalate (MEHP) metabolites were detected in US children and adults compared to the German and Danish study [10]. However, considerably higher MEP and MBzP concentrations were observed. The MEP concentrations were 68.7 μg/L and 128 μg/L in children and adults, respectively, while the concentration of MBzP in children and adults amounted to 24.2 μg/L and 9.90 μg/L, respectively [10].

Various analytical methods have been reported in literature for the determination of phthalate ester metabolites in urine. The majority of these methods apply a liquid chromatographic (LC) separation followed by a mass spectrometric detection (MS or MS/MS). These LC–MS or LC–MS/MS methods are combined with a preconcentration step by means of a SPE extraction. This SPE extraction can occur offline or online [3,4,11–14]. In the framework of the European FP7 project COPHES (Consortium to Perform Human Biomonitoring on a European Scale) the analytical method developed by the German Research Foundation has been indicated as the reference method for the determination of phthalate ester metabolites in urine [15]. This method allows the determination of MEHP, 5-OH-MEHP and 5-oxo-MEHP in urine by means of SPE-LC–MS/MS. The sample processing and the subsequent chromatographic separation are carried out online. The compounds of interest are enriched on a Restricted Access Material (RAM) phase and separated from most of the matrix. The average accuracy varies between 85% for 5-oxo-MEHP and 106% for MEHP with a precision (reproducibility) not exceeding 15%. The limit of quantification is 0.5 μg/L for each compound [15].

These SPE LC–MS/MS methods are however time-consuming and limited by their high analysis cost. Due to the increasing concern on endocrine disrupting compounds and the need to assess human exposure to these compounds, a sensitive, low cost and efficient analysis method is essential.

In the present study, an analytical procedure was developed for the direct analysis of phthalate ester biomarkers in urine, without any foregoing preconcentration of the samples. The following compounds were measured in the urine samples: MEHP, 5-OH-MEHP, 5-oxo-MEHP, MnBP, MBzP, MEP and MiBP (Fig. 1). Performance characteristics (accuracy, precision, limits of detection and quantification) were determined for the direct analysis of the compounds of interest in urine. A comparison was made between SPE and direct analysis. The direct analysis method was applied in three interlaboratory comparisons for toxicological analyses in biological matrices. The results are discussed.

2. Materials and methods

2.1. Chemicals and reagents

The phthalate ester metabolites were provided as a solution (100 μg/mL) in acetonitrile, except for MBzP, for which the solution (100 μg/mL) was prepared in methyl-*tert*-butylether (MTBE). These solutions were from Cambridge Isotope Laboratories (Andover, USA). All compounds had a chemical purity of 95%.

Isotopically labeled compounds, used as internal standards, were ^{13}C-MEP, ^{13}C-MBzP, ^{13}C-MnBP, ^{13}C-MEHP, ^{13}C-5-OH-MEHP and ^{13}C-5-oxo-MEHP, all purchased from Cambridge Isotope Laboratories (Andover, USA) (purity 95%). These compounds were delivered as solutions (100 μg/mL) in acetonitrile, except for ^{13}C-MBzP (solution in MTBE).

Individual stock standard solutions of the phthalate ester metabolites and internal standards were prepared on a weight basis in acetonitrile, in an equivalent concentration of approximately 20 μg/mL. Working standard solutions, containing a mixture of either all native compounds or internal standards, were prepared in HPLC water by appropriate dilution of the individual stock solutions. The concentration of the phthalate ester metabolites in the working standard solutions ranged from 0.1 ng/mL to 450 ng/mL. The concentration of the internal standards in the working standard solutions amounted to approximately 50 μg/L. Prior to injection, the working standard solutions were diluted by adding 50 μL of the internal standard solution to 150 μL of the standard solutions of the native compounds.

An ammonium acetate buffer was prepared by dissolving ammonium acetate (99.999%; Aldrich, Sigma–Aldrich, Diegem, Belgium) in 200 mL of water and acidifying this solution with concentrated acetic acid to pH 6.5. A phosphate buffer solution of pH 2 was prepared by dissolving $NaH_2PO_4 \cdot H_2O$ (> 99.5%; Fluka, Buchs, Switzerland) in 100 mL of water, followed by the addition of 1 mL of H_3PO_4.

Acetonitrile, formic acid and water were of ULC–MS grade (99.98% purity) (Biosolve, Valkenswaard, The Netherlands). Ethyl acetate (LC–MS grade, min. 99.9%) was purchased from Biosolve (Valkenswaard, The Netherlands). Glacial acetic acid (100% purity) was from Merck (Darmstadt, Germany). The enzyme β-glucuronidase from *E. coli* K12 (concentration > 200 U/mL) was purchased from Roche Applied Sciences (Mannheim, Germany). Since phthalates are omnipresent in the laboratory, special precautions have been taken to avoid sample contamination. Thereto, all glassware was heated at 450 °C for at least 4 h to remove any background phthalate metabolites.

2.2. Sampling and sample storage

For the method development and validation pooled urine samples from volunteers were used. These urine samples were collected in 250 mL polypropylene containers with lids, both free of any phthalates (VWR International, Leuven, Belgium). According to the EU Pilot Study Protocol and Additional Biomarkers Protocol (FP7-244237), these recipients and lids were cleaned with 10% nitric acid in purified water solution in order to eliminate any background contamination. The vessels and lids were immersed in the HNO_3 tank for at least 3 h. Thereafter the vessels and lids were put in a first tank with purified water and shaken for 2–3 min. Then, the vessels and lids were changed to a second tank with purified water and again shaken for 2–3 min. The vessels and lids were taken out and put face down in a clean filter paper in order to dry. Once the drying was finished, the lids were screwed to the resulting nitric acid pre-treated vessels.

The urine samples were stored at −18 °C in the dark until analysis.

2.3. SPE extraction

Frozen urine samples were thawed, vortexed and sonicated for 5 min. 1 mL of the urine sample was brought into a test tube. 250 μL of an ammonium acetate buffer (pH 6.5) containing 5 μL of β-glucuronidase (from *E. coli* K12) solution was added, as well as 50 μL of the internal standard solution. The concentration of the internal standards amounted to 2.5 ng in the sample. The test tube was closed and the solution was carefully mixed. To induce the enzymatic cleavage of the conjugates, the solution was incubated at 37 °C for 90 min. After addition of 1 mL of a phosphate buffer (pH 2) the sample was vortexed and consequently concentrated by SPE. Thereto, a glass Oasis HLB cartridge (50 cc, 200 mg) (Waters, Milford, MA, USA) was conditioned with 2 mL of acetonitrile and