DIRECT ANALYSIS OF PHTHALATE ESTER BIOMARKERS IN URINE WITHOUT PRECONCENTRATION: METHOD VALIDATION AND APPLICATION

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Introduction

Phthalates are a group of high-production industrial chemicals that are nearly ubiquitous. They can be found among others in toys, food packaging, personal care products where they are mainly used as plasticizers. Due to their omnipresence humans are exposed to phthalates through different routes (Wormuth et al., 2006; Fromme et al., 2012). Phthalates are rapidly metabolized in humans to their respective monoesters, which can be further metabolized to their oxidation products. The monoesters and oxidation products can be glucuronidated, and in this way excreted via urine and faeces (Silva et al., 2004). 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 (Koch et al., 2003; Silva et al., 2004). Data on the levels 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. The biologic half-life of phthalate biomarkers is less than 24 hours (Hoppin et al., 2002; Koch et al., 2005).
Few studies have investigated urinary phthalate excretion in children and adults. 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 (Koch et al., 2003; Silva et al., 2004; Kato et al., 2005; Silva et al., 2007; Silva et al., 2008; Chen et al., 2012). 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 (Koch and Angerer, 2008). 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 variesbetween 85% for 5-oxo-MEHP and 106% for MEHP with a precision (reproducibility) not exceeding 15%. The limit of quantification is 0.5 g/L for each compound (Koch and Angerer, 2008). 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 prior preconcentration of the samples. The following compounds were measured in the urine samples: monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP), monobenzyl phthalate (MBzP), mono-(2-ethylhexyl)phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (5-OH-MEHP) andmono-(2-ethyl-5-oxohexyl) phthalate (5-oxo-MEHP). 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.

Materials and methods

Materials

The phthalate ester metabolites and isotopically labelledinternal standard compounds (^{13}C-MEP, ^{13}C-MBzP, ^{13}C-MnBP, ^{13}C-MEHP, ^{13}C-5-OH-MEHP and ^{13}C-5-oxo-MEHP)were purchased from Cambridge Isotope Laboratories (Andover, USA). Appropriate dilutions and mixtures were prepared.

An ammonium acetate buffer was prepared by dissolving ammonium acetate (99.999 %; Aldrich) 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) in 100 mL of water, followed by the addition of 1 mL of H_3PO_4.Acetonitrile and water were of ULC-MS grade (99.98 % purity) (Biosolve, Valkenswaard, The Netherlands). Glacial acetic acid (100 % purity) was from Merck. The enzyme β-glucuronidase from E. Coli K12 (concentration > 200 U/mL) was purchased from Roche.

Methods

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 hours to remove any background phthalate metabolites.

Frozen urine samples were thawed, vortexed and sonicated for 5 minutes. To 1 mL of urine sample 250 µL of an ammonium acetate buffer solution (pH 6.5) containing 5 µL of a β-glucuronidase solution as well as 50 µL of the internal standard solution were added. After homogenisation, this mixture was incubated at 37 °C for 90 minutes to induce the enzymatic cleavage of the conjugates. Finally, an aliquot of the sample (200 µL) was transferred into an injection vial, where after 10 µL was injected into the LC–MS/MS system.

The SPE-based method that was validated previously was used for comparison and is briefly described below.

Instead of transferring the solution into an injection vial as described above, 1 mL of a phosphate buffer (pH 2) was added to the sample, 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 2 mL of phosphate buffer (pH 2). Glass cartridges were used to avoid any phthalate contamination from plastic materials. The pre-treated urine sample was loaded onto the SPE cartridge, the cartridge was washed with 2 mL of formic acid (0.1 M) and 5 mL of UPLC water. The cartridge was consecutively dried under reduced atmosphericpressure during 30 s. The compounds of interest were eluted with 2 mL of acetonitrile and 2 mL of ethyl acetate. The extract was evaporated to dryness and reconstituted with 200 µL of UPLC water. An aliquot of 10 µL of the extract was injected into the LC–MS/MS system.

The instrumental analysis was performed by means of UPLC-tandem MS using a Waters H-class Acquity UPLC system. The phthalate ester metabolites were separated on an Acquity UPLC BEH phenyl column (100 mm x 2.1 mm; 1.7 µm) with a Van Guard Acquity UPLC BEH C18 precolumn (5 mm x 2.1 mm; 1.7 µm). The column temperature was kept at 40 °C. Optimum separation was obtained using a gradient elution program with a binary mobile phase of ultrapure waterand acetonitrile, both solvents acidified with 0.1 % acetic acid. The flow rate of the mobile phase was 0.4 mL/min. An aliquot of 10 µL of the urine sample (or extract in case of SPE) was injected into the LC system.

The UPLC system was coupled to a Waters Xevo TQ-S tandem mass spectrometer, which was operated in the negative electrospray ionization mode (ESI-).MS parameters were optimized through infusion experiments in order to obtain maximum sensitivity. Out of these results characteristic precursor and product ions were selected for detection in the multiple reaction monitoring (MRM) mode. The precursor/product ion transitions are listed in Figure 1.

Positive identification of the phthalate ester metabolites was based on LC retention time match and their specific MRM transitions. Quantification of the individual compounds was done with the internal standard method by adding isotopically labelled analogues to the sample. The relative response factors of the compounds in relation to

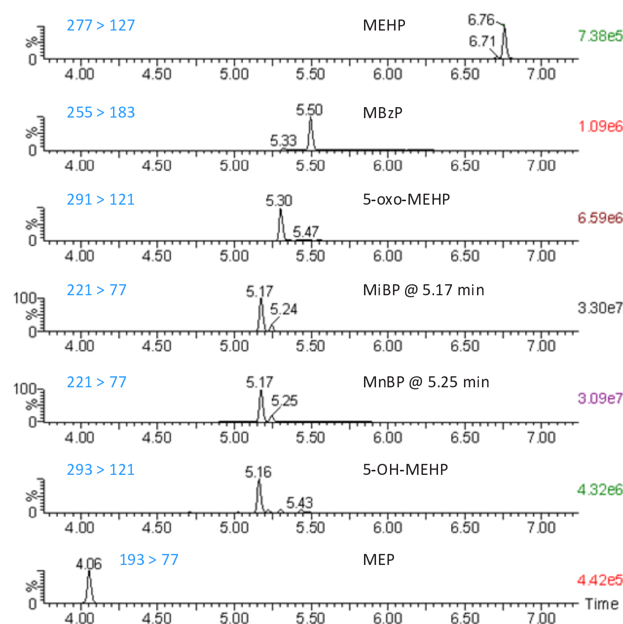

Figure 1.LC-MS/MS chromatogram of a urine sample in negative ionization mode (ESI-).

the corresponding internal standard were calculated. Thus, the reported concentrations are corrected for recovery.

Results and discussion

The linearity of the method was determined for each compound by injecting 6 standard solutions in the concentration range 1-250 µg/L. The concentration of the ^{13}C-labeled internal standards in these solutions was constant (approximately 10 µg/L). Within the working range, the calibration curve fulfilled the requirements for a linear fit. Linear calibration curves were constructed with squared regression coefficient (R^2) higher than 0.995. The accuracy of the direct analysis LC-MS/MS method for phthalate ester metabolites in urine was determined by fortifying different urine samples at a concentration level of 25 µg/L. The recovery was calculated as the ratio between the experimentally observed concentration and the nominal concentration. The recovery of the phthalate ester metabolites by the direct analysis method varied around 95-100 % (Table 1). The precision (reproducibility) of the recovery, expressed as % relative standard deviation, was lower than 15 %. Compared to the analytical procedure applying a SPE extraction the accuracy for some phthalate ester metabolites was even better with the direct analysis method. For example, the average recovery for 5-OH-MEHP amounted to 78 % with the SPE extraction compared to 97 % with the direct analysis method. In general, the average accuracy with the SPE extraction amounted to 78-102 % with a precision between 13 and 19 %.

Table 1. Performance characteristics for the direct analysis LC-MS/MS method for phthalate ester metabolites in urine.

	Accuracy (%)[a]	**Precision (repeatability)**[b] **(%)**	**CV %**[c]	**LOQ (µg/L)**[d]
MEP	104 (12)	2.6	5.5	0.50
MBzP	104 (12)	5.7	3.8	0.20
MiBP	103 (12)	6.0	2.8	0.50
MnBP	103(12)	8.4	2.2	0.50
MEHP	101 (7)	2.2	2.7	0.50
5-OH-MEHP	97 (12)	2.0	3.5	0.10
5-oxo-MEHP	97 (10)	2.5	3.5	0.10

[a] % RSD, expressing the variation of the recovery of different fortified urine samples, is given between parentheses.

[b] Precision in terms of repeatability, determined by the replicate analysis (n = 10) of 1 urine sample.

[c] Precision in terms of reproducibility for the duplicate analysis of different urine samples.

[d] Concentrations in the procedure blanks of different measurement sequences have been taken into account when calculating the limits of quantification (LOQs).

For an assessment of precision in terms of repeatability a urine sample was repeatedly analysed (n=10). Based on the measured concentrations in the sample, the method relative standard deviation (% RSD) was calculated (Table 1). For all phthalate ester metabolites the % RSD values were smaller than 10 % and for most compounds did not even exceed 5 %.

Thus we can conclude that the direct analysis of phthalate ester metabolites in urine without sample preconcentration is characterized by a good accuracy and precision, in terms of both repeatability and reproducibility.

It is well known that LC-MS/MS analysis is prone to ion suppression due to the presence of interfering matrix components. One of the possibilities to correct for this matrix suppression is the use of internal standards. Depending on the phthalate ester metabolite, the recovery of the corresponding internal standard varied between 20 % and 84 % for the direct analysis method, which is lower than for the SPE-method(between 43 % and 126 %). Nevertheless, due to proper use of internal standards accurate and precise determination is possible.

The LOD and LOQ were defined as the concentration that would give a signal-to-noise ratio of 3 and 10, respectively. Although the instrumental analysis allows the determination of phthalate ester metabolites in a concentration below 50 ng/L, the LOQs are strongly dependent on the feasible blank concentrations. Therefore, the observed concentrations in the procedural blanks have also been taken into account in the determination of LOQ values (Table 2). The LOQ was set equal to 0.50 µg/L for the majority of the compounds. Only for 5-OH-MEHP and 5-oxo-MEHP (0.10 µg/L) as well as MBzP (0.20 µg/L) lower LOQs were achievable due to the

lower blank concentrations of these compounds. A SPE preconcentration step resulted in LOQ values of below 1 µg/L. Except for MnBP a higher limit of quantification was set, i.e. 10 µg/L, when applying SPE extraction due to the higher blank concentrations. Thus, no compromise has to be made regarding LOQ values when applying direct analysis or a foregoing SPE preconcentration. The LOQ values of both methods are in the same order of magnitude.

A further comparison between the two methods was made by analysing two urine samples applying both the direct analysis method (without preconcentration) and the SPE extraction. For both analytical methods the urine samples were measured 4 times under reproducibility conditions. For the direct analysis method the phthalate ester metabolites in both urine samples were determined with a precision (reproducibility) between 5 % and 10 %. For the SPE analytical procedure a precision (reproducibility) of approximately 10 % was obtained for the majority of the compounds. The results of the direct analysis and the SPE extraction are in good agreement. Two urine samples (sample A and B) were analysed with both the direct analysis method and the reference method with SPE extraction. The results of the SPE extraction were plotted in function of the direct analysis method for each compound. So, each point inFigure 2 represents the concentration of a phthalate ester metabolite obtained via SPE extraction and the direct analysis method. A linear fit was obtained for both urine samples with squared regression coefficients(R^2) exceeding 0.995 and slopes approaching 1. Thus, the direct analysis method is fit-for-purpose and a good alternative for the analysis of phthalate ester metabolites in urine samples.

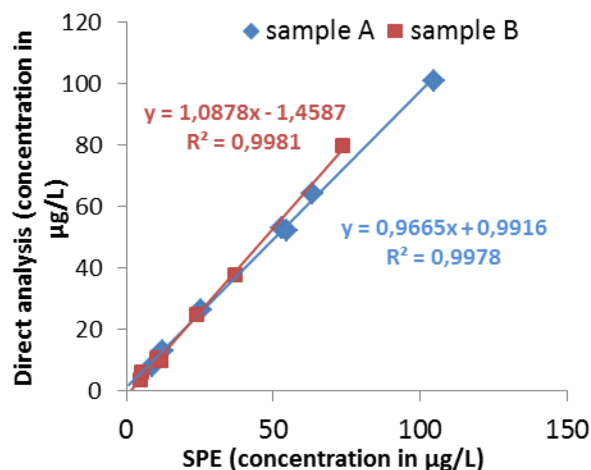

Figure 2. Comparison between direct analysis and SPE extraction for the analysis of phthalate ester metabolites in urine.

The developed direct analysis method was further validated by participating in three interlaboratory comparisons. These interlaboratory comparisons were part of an External Quality Assessment Scheme (EQUAS), organized by the Consortium to Perform Human Biomonitoring on a European Scale (COPHES), i.e. EQUAS-48 (2011), EQUAS-49 and EQUAS-50 (2012). In each interlaboratory comparison two urine samples were analysed for 5-OH-MEHP, 5-oxo-MEHP, MnBP, MiBP and MBzP. In all three comparisons the deviation from the reference value was lower than 15 % (one exception). As indicated by the performance characteristics, also the results of these interlaboratory comparisons demonstrate that the direct analysis method without preconcentration is a good alternative for the analytical procedures applying SPE, thereby giving accurate and precise results.

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