Levels of phthalate metabolites and bisphenol A in urine samples from Czech mothers and newborns

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Introduction

Esters of phthalic acid, also known as phthalates, belong to a group of additive plasticizers that are not bound to the material by a strong chemical bond. Bisphenol A (BPA) is used in the production of polycarbonate materials as well as the polymerization inhibitor during PVC production [1].However, these compounds are easily released from the materials into the environment as contaminants via processes, such as evaporation, leaching or mechanical damage [2,3,4]. Because of the ubiquitous presence in the environment, humans can be exposed to phthalates and BPA through various pathways such as ingestion, inhalation or absorption through the skin. Many scientific studies indicate that children are more exposed to phthalates and BPA than adults, simply because they are more often in a direct contact with objects containing these substances and, when very young, likely to put such objects into their mouths [5].

When phthalates enter the human body, they are rapidly metabolized by cytochrome P450 enzymes and monoesters and their oxidized products (alcohols, ketones and carboxylic acids) are formed. Both monoesters and oxidation products leave the body in either free form or conjugated with glucuronic acid [6]. BPA is in the body almost immediately transformed into a glucuronide or sulphate conjugate. These conjugates are subsequently excreted from the body in urine [1].

Phthalate metabolites and BPA have adverse effects on human health. They have the ability to disrupt the effects of steroid enzymes and thyroid hormones, as well as to act as androgens, estrogens or antiestrogens [6,7]. Furthermore, there is evidence to suggest that they affect the regulation of biological processes, such as the proliferation of adipocytes or glucose homeostasis, both of which may be associated with a higher incidence of obesity and diabetes [1,8].

Because of the adverse effects of these substances on human health it is important to monitor their levels in biological samples. Generally, the first step in sample preparation is the enzymatic hydrolysis of conjugated forms of the target compounds using enzyme β-glucuronidase. Then, to isolate the phthalate metabolites and BPA, solid phase extraction (SPE) is used, with the sorbent typically being C_{18} or hydrophilic-lipophilic balanced sorbent (HLB). But this method can be often time consuming and expensive. Subsequently, identification and quantification of the target compounds is most commonly performed using ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC–MS/MS) [9,10,11,12,13].

The aim of this study was to test, modify and validate a quick and easy sample preparation method [4] for the analysis of the urine samples collected from mothers and their newborn children living in two localities of the Czech Republic

(Karvina and Ceske Budejovice) differing in atmospheric contamination to assess exposition of this part of the Czech population to BPA and phthalates.

Materials and methods

The Standard Reference Material® (SRM) 3673 (Organic Contaminants in Non-Smokers´ Urine) used for the method evaluation and validation was supplied by the US National Institute of Standards and Technology (NIST, Gaithersburg, Maryland, USA). This material contains certified levels of 8 phthalate metabolites (monoehtyl phthalate (MEP), mono-*iso*-butyl phthalate (MiBP), mono-*n*-butyl phthalate (MBP), monobenzyl phthalate (MBzP), mono(2-ethylhexyl) phthalate (MEHP), mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP)) and bisphenol A (BPA). Certified standards of phthalate metabolites represented by MEP, MiBP, MBP, MBzP and MEHP were purchased from AccuStandard®, Inc. (USA). MEHHP, MEOHP, MECPP and isotopically labelled compounds MEP-d4, MBP-d4 and MEHP-d4 were purchased from Toronto Research Chemicals, Inc. (USA). BPA was purchased from Sigma-Aldrich® (USA).

The real urine samples were obtained within the cooperation with the Institute of Experimental Medicine AS CR in Prague. The samples were collected in the Ceske Budejovice Hospital, Department of Obstetrics and Department of Neonatology and in the Karvina Hospital, Department of Obstetrics and Department of Neonatology. The study was approved by the Ethics Committee of both hospitals and the Institute of Experimental Medicine AS CR in Prague. Each mother signed a written consent. All samples were stored in the freezer (-20 °C) prior to analysis.

In this study, we used a fast and simple sample preparation technique for the determination of phthalate metabolites and BPA in human urine [4] with some additional changes. After the enzymatic hydrolysis (90 min, 37 °C, pH 5) this technique requires only dilution of the urine sample with an organic solvent. An ultra-high performance liquid chromatograph (UHPLC) (Acquity UPLC®, Waters, USA) coupled to a tandem mass spectrometer (MS/MS) (Xevo TQ-S, Waters, USA) was used to identify and quantify the target analytes. The method validation was carried out on SRM 3673 in 6 repetitions. Blank sample which contained deionized water instead of urine sample was also prepared.

Results and discussion

Published method [4] was slightly modified. Instead of 50 µL of β-glucuronidase solution with acetate buffer (0.28 units/100 µL urine, pH 6.5), we used 20 µL of β-glucuronidase and 40 µL of acetate buffer (pH 5). Then, the organic solvent was added after the enzymatic hydrolysis and instead of acetonitrile we used methanol. Further, the method was performed as published.

The performance characteristics of the analytical method obtained by the analysis of the SRM 3673 are shown in **table I**.

Table I: Performance characteristics of the analytical method (n=6)

Analyte	Certified concentration [ng/mL]	Measured concentration [ng/mL]	LOQ [ng/mL]	REC [%]	RSD [%]
MEP	80.0	85.0	0.2	107	3
MiBP	5.2	5.7	0.2	116	8
MBP	11.2	10.4	0.2	93	6
MBzP	5.7	6.5	0.2	113	5
MEHP	4.3	5.9	0.2	137	6
MEOHP	12.2	11.8	0.7	97	8
MEHHP	22.3	24.7	0.7	110	7
MECPP	30.1	18.1	0.4	60	5
BPA	2.0	n.d.	0.7	-	-

n.d. – not detected, LOQ – limit of quantification, REC – recovery, RSD – relative standard deviation

Measured concentrations for the target analytes were in a good agreement with those certified in the SRM 3673 (REC 60-137%, RDS < 8%). Only BPA wasn't detected in any of the measured samples. We believe that this problem was caused by the presence of the matrix interference on the quantification and confirmation transition of this compound. To avoid this interference, we tested different chromatographic conditions (mobile phase, gradient and chromatographic column). Any of the tested conditions did not appear to remove this interference. Thus, the prepared urine samples will be measured with UHPLC coupled to high resolution mass spectrometer (HRMS).
The validated method was subsequently used for the analysis of 200 real urine samples collected from mothers and their newborn children living in two localities of the Czech Republic. Between the most detected compounds belonged MEP, MiBP, MBP and MBzP in concentration levels up to hundreds of ng/mL urine which corresponds to the levels published in the literature.

Conclusion

We validated a fast and simple method for the preparation of the urine samples for the determination of phthalate metabolites with identification and quantification of the target compounds using UHPLC–MS/MS. The obtained limits of quantification were comparable with those reported in the literature and the measured concentrations for the target analytes were in a good agreement with those certified in the SRM 3673.
The validated method was subsequently used for evaluation of the levels of phthalate metabolites in 200 urine samples collected from mothers and their newborn children from two localities of the Czech Republic – Karvina and Ceske Budejovice.

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