Rapid analysis of phthalate metabolites in human urine using a liquid chromatography-tandem mass spectrometer

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Introduction

Phthalates have become one of the most wide spread contaminants in the modern world since their first application as plasticizers in the 1930s. They are present in medical devices, toys, plastic wrap for food, shower curtains, floors and walls, cosmetics and personal care products. Also, there are a large number of drugs and supplements using phthalates as coatings or binding matrices in enteric tablets for time-and pH-controlled drug release. The potential consequences of patient exposure to phthalates have raised concerns [1].

Phthalates are rapidly metabolized in humans to their respective monoesters, which can be further metabolized to their oxidation products. The metabolites can be glucuronidated, and in this way excreted via urine and feces [2]. The analysis of phthalate metabolites in human urine usually involves enzymatic hydrolysis, sample clean-up/pre-concentration using SPE, followed by LC-MS-MS analysis.

The objective of this study was to develop a rapid and sensitive liquid chromatograph tandem mass spectrometer method without pre-concentration to analyze 12 phthalate metabolite concentrations in human urine.

Materials and methods

All standards and their corresponding 13C labeled internal standards were obtained from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). The working standards were prepared in acetonitrile and stored in silanized amber vials. The calibrators were prepared in Surine® negative urine (Cerilliant, Lenexa, KS, USA).

Sample preparation procedure was as follows: 50 µL of ISTD and 200 µL of urine sample were pipetted into 2 mL silanized amber vials. 50 µL of diluted β-Glucuronidase (E.Coli K 12 from Roche Diagnostics, Laval Quebec, Canada) was then added to each vial, vortexed and capped. The vials were then placed into a pre-heated autosampler and hydrolyzed at 37 °C for 90 min. The samples were then cooled to room temperature. 700 µL of D.I. water was added into each vial. The vials were then vortexed and capped for LC-MS-MS analysis. The separation of phthalate metabolites was carried out on a 100x2.1 mm Betasil phenyl column (Thermo Scientific, Burlington, ON, Canada) using an Agilent 1260 HPLC system (Agilent technologies, Mississauga, ON, Canada) with mobile phase of 0.1% acetic acid in D.I. water (A) and 0.1% acetic acid in acetonitrile (B) with gradient elution (Table1). The injection volume was 10 µL and the column temperature was kept at 40 °C. The phthalate metabolites were detected using a 5500 Q-trap system (AB Sciex Concord, Ontario, Canada) operated in negative MRM mode. The source parameters were 20 (CUR), medium (CAD), -4500 (IS), 4500 (TEM), 50 (GAS1), 50 (GAS2) and -10 (EP). The identification and quantification of individual phthalate metabolites were based on two MRM transitions combined with the retention time.

Table 1. Mobile phase gradient table

Time	Flow rate (µL/min.)	A%	B%
0	300	75	25
3	300	75	25
5	350	75	25
10	350	67	33
17	350	70	30
19.8	350	66	34
21	350	60	40

Time	Flow rate (µL/min.)	A%	B%
23	350	45	55
25	350	25	75
28	350	15	85
30	350	10	90
30.1	350	0	100
38	400	0	100
38.1	400	75	25

Table 2. MRM transitions, MS parameters and retention time for individual metabolites

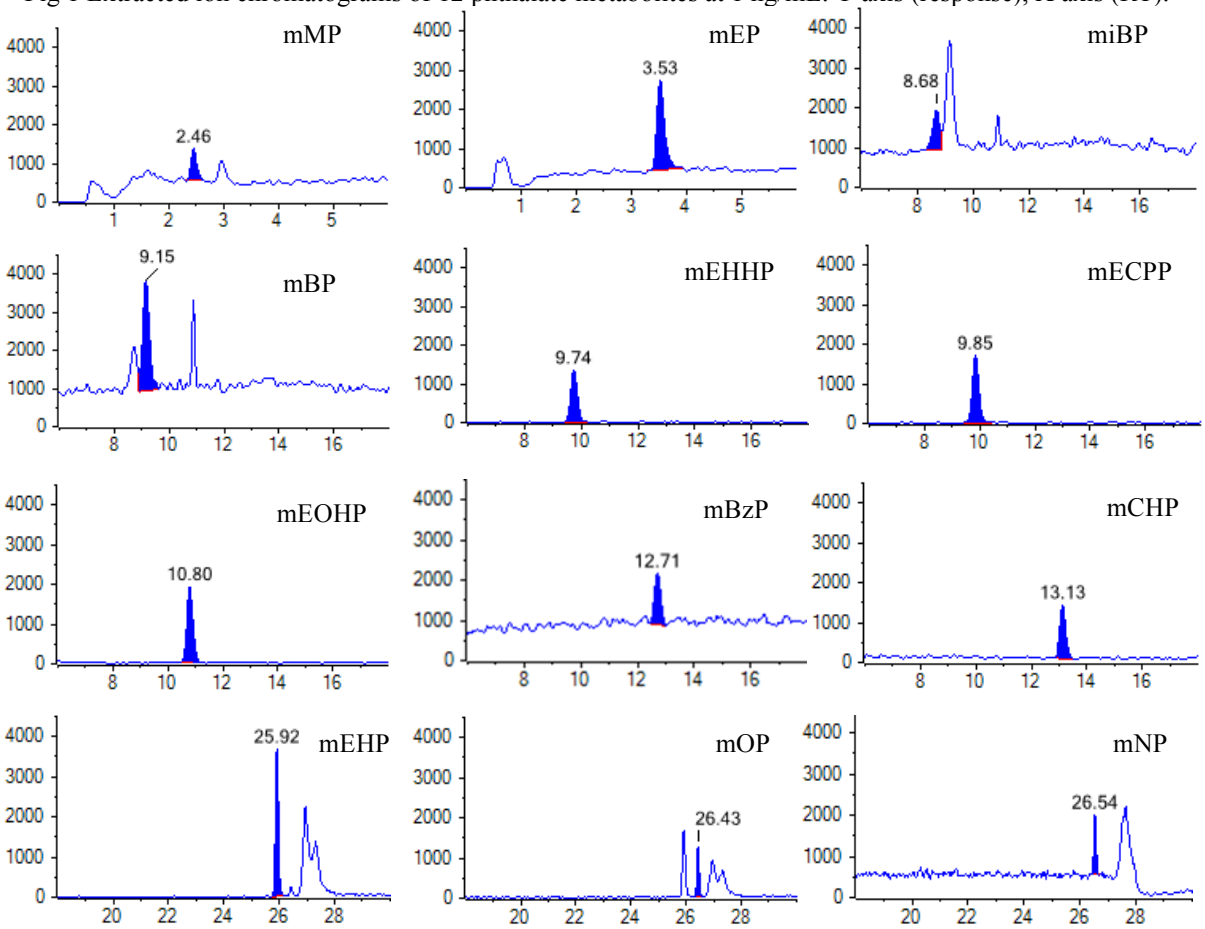
Analyte Name	Precursor ion	Product ion 1	Dwell time (msec)	DP	CE	CXP	Product ion 2	RT(min.)
mMP	179.0	77.0	50	-40	-21.0	-9.1	107.0	2.46
mEP	193.1	77.1	50	-50	-24.0	-9.2	121.0	3.53
miBP	221.1	77.1	30	-50	-23.3	-9.2	134.0	8.68
mBP	221.1	77.0	30	-50	-23.0	-9.2	177.0	9.15
mEHHP	293.1	145.0	30	-50	-19.0	-9.0	121.0	9.74
mECPP	307.1	159.0	30	-50	-16.0	-14.0	113.0	9.85
mEOHP	291.1	121.0	30	-60	-23.0	-10.0	143.0	10.80
mBzP	255.1	77.1	30	-50	-27.5	-9.0	107.0	12.71
mCHP	247.1	97.2	30	-55	-20.1	-11.1	77.1	13.13
mEHP	277.1	134.0	40	-50	-20.2	-11.0	127.1	25.92
mOP	277.1	127.0	40	-60	-22.0	-9.2	77.1	26.43
mNP	291.2	141.1	40	-60	-24.0	-11.0	77.1	26.54

Results and discussion

Figure 1 shows the extracted ion chromatograms of a spiked urine sample at 1 ng/mL. The chromatographic conditions were optimized for resolution and peak shape. It is important to separate mBP/ miBP and mEHP/mOP by LC as they are isomers and produce the same fragments. Several columns including Poroshell EC C18, Poroshell SB C18 and Betasil phenyl were evaluated during the method development. The Betasil phenyl (100x2.1mm) column provided superior separation power and reproducible results and was chosen for the method. Under optimized LC conditions, both mBP/miBP and mEHP/mOP were well resolved. ESI- MSMS parameters were optimized for sensitivity and are shown in the Materials and Methods section in Table 2.

The method was fully validated. All the analytes were linear up to 100 ng/mL. Percent CV for precision studies were all below 10% except mMP, which was 17%. The recovery, accuracy, LOD and LOQ are shown in Table 3. Recovery and accuracy for all analytes were acceptable.

Fig 1 Extracted ion chromatograms of 12 phthalate metabolites at 1 ng/mL. Y axis (response), X axis (RT).



Most published methods for the analysis of phthalate metabolites in human urine involve online or automated SPE for sample clean-up and pre-concentration. However, online or automated SPE requires special equipment. This method is a dilute and shoot method. It is simple and quick without compromising the sensitivity or specificity. With optimized LC and MS-MS conditions, the LOD and LOQ are comparable to those using SPE for pre-concentration. Our lab successfully passed the G-EQUAS German External Quality Assessment Scheme using the method to analyze 7 phthalate metabolites in urine. The method was applied to quantify mBP and mECPP metabolites of DBP in urine from patients taking drugs with or without a DBP coating. In addition, ten other phthalate metabolites were analyzed. Table 4 shows some of the patients' results. Significant high levels of mBP in patients taking drugs with a DBP coating was observed, while the elevation of mECPP were as substantial as that of mBP. Ten other phthalate metabolites were also found in patients' samples except mNP, which was not found in any of the samples.

In conclusion, we developed a simple and quick method to analyze 12 phthalate metabolites in human urine. The method is sensitive and reliable, and is suitable for clinical use.

Table 3. Validation results

Name	Recovery %	Accuracy %	LOD ng/mL	LOQ ng/mL	|ME| %
mMP	75.2	105	1.0	1.0	105
mEP	78.3	75.9	1.0	1.0	103
miBP	95.6	97.7	0.1	0.2	105
mBP	107	125	0.1	0.2	134
mECPP	91.5	102	0.1	0.1	104
mEHHP	103	95.1	0.1	0.1	106
mEOHP	107	96.0	0.1	0.1	102
mCHP	111	101	0.1	0.1	105
mBzP	110	100	0.1	0.1	107
mOP	107	104	0.1	0.1	108
mEHP	105	108	0.2	0.5	105
mNP	113	98.6	0.1	0.2	106

Table 4. Patient sample results (ng/mL)

ID	mMP	mEP	mBP	miBP	mECPP	mEHHP	mEOHP	mCHP	mBzP	mOP	mEHP	mNP
A	1.5	15.6	19.2	23.8	12.5	5.7	4.4	n.d.	6.9	0.3	2.7	n.d.
B	1.0	7.6	5.1	5.0	2.5	1.8	1.4	n.d.	1.2	n.d.	0.5	n.d.
C	1.7	53.1	4.6	6.8	8.4	3.7	4.3	n.d.	1.2	n.d.	1.1	n.d.
D	0.1	83.0	3.5	3.8	3.2	2.1	1.5	n.d.	0.9	n.d.	0.5	n.d.
E	0.9	38.7	6.2	6.4	6.4	5.0	5.1	n.d.	1.7	n.d.	1.7	n.d.
F	2.3	98.3	$1.9e^4$	12.7	10.5	3.3	2.9	0.2	5.6	n.d.	1.4	n.d.
G	5.4	540.3	$5.8e^3$	17.5	13.4	5.2	5.3	2.5	4.9	n.d.	1.4	n.d.
H	16.2	151.7	$4.8e^4$	99.7	63.2	26.3	29.9	0.3	6.1	n.d.	5.6	n.d.
I	32.4	$1.1e^3$	$5.1e^4$	74.9	52.7	23.2	20.7	0.6	90.8	1.2	8.9	n.d.
J	6.3	250.3	$2.9e^4$	203.0	48.1	31.4	16.4	n.d.	12.0	0.1	2.3	n.d.

A to E: patients taking drugs without DBP coating; F to J: patients taking drugs with DBP coating.
n.d.: not detected

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References

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2. CDC method number: 6306.04