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Original Article

Determination of Phthalate Metabolites in Human Serum and Urine as Biomarkers for Phthalate Exposure Using Column-Switching LC-MS/MS

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Objectives: Although phthalates like dibutyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) are commonly used as plasticizers and their metabolites are especially suspected of reproductive toxicity, little is known about occupational exposure to those phthalates. The aim of this study was to assess the utility of measuring the metabolite concentrations of DBP and DEHP in serum and urine samples as an indicator of occupational exposure to those phthalates.
Methods: Phthalate metabolites were analyzed by using column-switching high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS).
Results: We detected phthalate metabolites in serum and urine matrices at approximately 10-fold lower than the limit of detection of those metabolites in the same matrix by LC-MS/MS without column switching, which was sufficient to evaluate concentrations of phthalate metabolites for industrial workers and the general population.
Conclusion: The accuracy and precision of the analytical method indicate that urinary metabolite determination can be a more acceptable biomarker for studying phthalate exposure and adverse health outcomes.

Key Words: Phthalates, Dibutyl phthalate, di-2-ethylhexyl phthalate, Metabolites, Accuracy

Introduction

Dibutyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) are industrial chemicals that can act as plasticizers that impart flexibility and resilience, and which are used in enormous quantities as plasticizers in polyvinyl chloride resins, nitrocellulose lacquers, and elastomers. These phthalates also have been used as solvents for perfume, oils, textiles, lubricating agents, and safety glass [1,2]. As the phthalate plasticizers are not covalently bound to resins, they can leach, migrate, or evaporate into the workplace environment.

People can be exposed to these phthalates through ingestion, inhalation, and dermal exposure. Both a population study [3] and occupational studies [4,5] have indicated that the personal air sample levels of phthalates correlate modestly well with concentrations of urinary metabolites. This implies that inhalation exposure is a more important route of exposure than oral and dermal exposure [6].

Toxicological studies on animals have demonstrated that several phthalates cause decreased testicular and epididymal weight, lessened testes, deterioration of semen quality, and decreased fertility index [7-9]. Some phthalates are also suspected human endocrine disruptors [10,11].

Studies on occupational exposure to DBP and DEHP so far are very limited [4,5,12]. Toxicological studies of these

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phthalates have so far been researched extensively in experimental animals. The American Conference of Governmental Industrial Hygienists has classified DBP as a substance that produces ocular and respiratory irritation, reproductive effects, testicular injury on the basis of findings from experimental animal studies, and has categorized DEHP as a confirmed animal carcinogen with unknown relevance to humans.

Even though there are many analytical methods for the determination of phthalates in water and air, including high performance liquid chromatography [14-16], analysis of phthalates, such as DBP and DEHP, in various samples may present a difficult and serious problem because a higher background is often encountered. This is due to phthalate contamination in many laboratory products, plastic tubing, room air, etc. To overcome these contaminations during the analytical procedure, the metabolites analysis of phthalates and column-switching techniques are preferable. The use of column-switching techniques allows the entire extraction, including load, wash, and re-equilibration to be performed in a short time scale while the separation is in progress. This has the advantage of decreasing the risk of contamination and analytical error.

Generally, phthalates are metabolized and excreted and do not accumulate in the body [13]. Studies on biomarkers for the estimation of phthalate exposure in the general population have been performed using several analysis methods [14-16]. Recently, column-switching techniques have been combined with tandem mass spectrometry for successful biosample analysis [17].

The present study was undertaken to investigate the utility of measuring the metabolite (mono-n-isobutyl phthalate, [MBP]) concentration of DBP and the metabolite (mono-2-ethylhexyl phthalate, [MEHP]; mono-(2-ethyl-5-hydroxyhexyl phthalate, [MEHHP]; mono-(2-ethyl-5-oxohexyl) phthalate, [MEOHP]) concentrations of DEHP in serum and urine samples as an indicator of occupational exposure to those phthalates by use of column-switching high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Chemicals

MBP, MEHP, MEHHP, and MEOHP were purchased from Sigma-Aldrich (St. Louis, MO, USA). $^{13}C_4$-MBP, $^{13}C_4$-MEHP, $^{13}C_4$-MEHHP, and $^{13}C_4$-MEOHP were purchased from Cambridge Isotopes Laboratories (Cambridge, MA, USA). HPLC-grade acetonitrile, water and all other chemicals were obtained from Honeywell Burdick & Jackson (Morristown, NJ, USA).

Instruments and chromatographic conditions

The analyses for measuring phthalate metabolites in urine and serum were performed on a Nanospace SI-2 high-performance liquid chromatography (Shiseido, Tokyo, Japan) with an API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA). On-line clean-up and separation of phthalate monoesters was accomplished by the switching-column technique with a pretreatment column (Shisido MF C_8, 50 × 4.6 mm, 5 μm), trap column (Imtackt Cadenza CD C_{18}, 30 × 2.0 mm, 5 μm), and analytical column (Imtackt Cadenza CD C_{18}, 75 × 2.0 mm, 3 μm). The pretreatment column temperature was 37°C, the injection volume was 10 μL, and the flow rates were 600 μL/min for the pretreatment and 200 μL/min for the analytical column. The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Column switching was performed as follows: 8.1 min for MBP, MEHHP and MEOHP trapping (mode A → mode B), 10.0 min (mode B → mode A), 14.8 min for MEHP trapping (mode A → mode B), and 15.3 min (mode B → mode A). Fig. 1 presents schemes of the column switching proce-

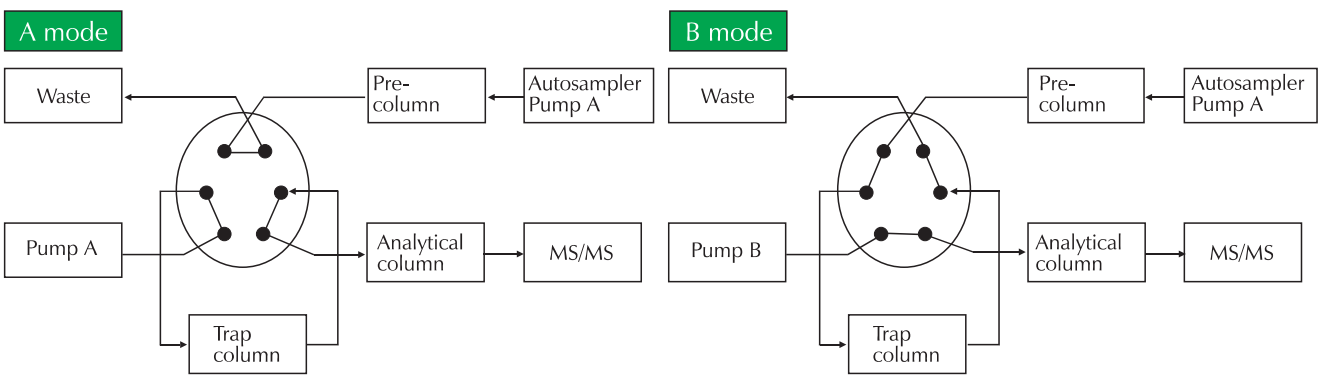

Fig. 1. Schemes of switching column procedure. 1) A mode - online clean-up, 2) B mode - concentration to trap-column, 3) A mode - separation by analytical column and injection to MS/MS detector.

dure. In mode A, the sample was loaded onto the pre-column controlled by autosampler pump A. While the pre-column was directed to waste, the sample was extracted on the pre-column. The matrices of compounds in the sample were removed while metabolites (MBP, MEHP, MEHHP, MEOHP) were retained on the pre-column. The extraction process was performed after closure of the on-line solid phase extraction. When the switching valve was changed to mode B, the pre-column was connected to a trap column. During this process all of the metabolites were retained on the trap column. After the metabolites were retained on the trap column, mode B changed into mode A again. Metabolites on the trap column transferred to an analytical column and the effluent from the analytical column was directed to the MS/MS. Determination of phthalate metabolites was accomplished by electrospray ionization in the negative mode with spray ion voltage (-4,500 V), nitrogen nebulizer gas pressure (GS1 40 psi, GS2 60 psi), nitrogen curtain gas pressure (15 psi), capillary temperature (400°C), and collisionally-activated dissociation (CAD, 7). The parent ion (m/z), product ion (m/z), declustering potential (eV), and collision energy (eV) of each phthalate monoester were as follows: 221/149/-48/-18 (MBP), 225/151/-48/-16 ($^{13}C_4$-MBP), 277/134/-55/-21 (MEHP), 281/137/-55/-22 ($^{13}C_4$-MEHP), 293/121/-60/-23 (MEHHP), 297/145/-75/-20 ($^{13}C_4$-MEHHP), 291/121/-55/-24 (MEOHP), and 295/124/-55/-24 ($^{13}C_4$-MEOHP).

Standard solutions and sample preparation

Working solutions of standards (MBP, MEHP, MEHHP, MEOHP) and internal standards ($^{13}C_4$-MBP, $^{13}C_4$-MEHP, $^{13}C_4$-MEHHP, $^{13}C_4$-MEOHP) were made up at a concentration of 1.0 mg/mL in acetonitrile. From the working solution of the analytes, calibration standards were prepared by spiking pooled serum and urine from 322 persons (elementary school: 60, middle and high school: 62, adult: 139, and old: 61) from the general population in Kyunggi province.

The ages of the sampled persons ranged from 8 to 65. These spot serum and urine samples were pooled, frozen at -20°C, and thawed at room temperature and vortex mixed before use. The non-spiked pooled serum and urine was used as a blank. The background concentration levels of metabolites in the pooled serum were 3.3 ng/mL (MBP), 3.9 ng/mL (MEHP), 0.3 ng/mL (MEHHP), and 0.4 ng/mL (MEOHP). The background concentration levels of metabolites in the pooled urine were 7.2 ng/mL (MBP), 6.2 ng/mL (MEHP), 17.0 ng/mL (MEHHP), and 16.6 ng/mL (MEOHP). Additionally, a blank value consisting of water was included in every analytical series. The 0.5 mL aliquots of spiked serum and urine sample were transferred to 1.5 mL glass screw-cap vials. Then, 10 μL of each internal standard, 10 μL of β-glucuronidase (Roche Diagnostics, IN, USA), and 100 μL of 1 M ammonium acetate were added to the samples. The samples were mixed gently and incubated at 37°C for 2 h. After a 10 min sonication, the samples were diluted two times with acetonitrile, and vortex mixed and centrifuged at 5,000 g for 10 min. The supernatant was filtered with a syringe filter (Nylon 0.2 μm, Whatman, Kent, UK). A 100 μL aliquot was then injected into the LC-MS/MS system for quantitative analysis. Fifty healthy male volunteers were included in this study. The study protocol was approved by the Ethics Committee of Yongin University and written informed consent was obtained from the volunteers.

Table 1. LOD and LOQ of phthalate metabolites in serum and urine samples

Matrix	Metabolites	LOD (ng/mL)	LOQ (ng/mL)	Concentration range (μg/mL)	r value	SE	Slope RSD
Serum	MBP	0.67	2.02	0-3.76	0.9985	340.7	0.027
	MEHP	0.24	0.73		0.9953	809.3	0.125
	MEHHP	0.08	0.23		0.9703	180.9	0.048
	MEOHP	0.14	0.43		0.9959	302.4	0.046
Urine	MBP	1.05	3.15	0-0.47	0.9943	1,180.3	0.062
	MEHP	0.22	0.67		0.9994	22.3	0.014
	MEHHP	0.15	0.46		0.9998	483.5	0.010
	MEOHP	0.16	0.49		0.9964	94.5	0.042

LOD: limit of detection, LOQ: limit of quantitation, r: correlation coefficient, SE: standard error, RSD: relatively standard deviation, MBP: mono-n-isobutyl phthalate, MEHP: mono-2-ethylhexyl phthalate, MEHHP: mono-(2-ethyl-5-hydroxyhexyl) phthalate, MEOHP: mono-(2-ethyl-5-oxohexyl) phthalate.

Determination of limit of detection (LOD) and limit of quantitation (LOQ)

Six low-level calibration standards including a blank were prepared. The linear regression equation of the relation between the analyte amount and the detector response, and the standard error (Sy) of this regression equation were obtained, and the LOD and LOQ were calculated through a method suggested by the National Institute for Occupational Safety and Health [18].

Determination of recovery

The recovery was tested at three different levels (low, medium, and high concentration) as previously reported [19-21]. In those studies, the ranges of the mean concentration for DEHP urine metabolites (MEHP, MEHHP, MEOHP) and DBP urine metabolite (MBP) in the general population were 4.23-182 ng/mL and 17.7-20.2 ng/mL, respectively. In the study of the Korean general population [22], the geometric mean concentration range of DBP and DEHP metabolites in urine and serum were 11.0-31.1 ng/mL, and 0.4-5.7 ng/mL, respectively. We decided to use the three different levels of low to high concentrations from those studies. Each set of six spiked samples was prepared at the three concentration levels. The recovery was calculated as the percentage recovery.

Determination of precision of the analytical procedure

The precision of the analytical procedure was measured as the pooled coefficient of variations (CVs) determined from replicate analysis of standards within the range of low to high concentrations. The homogeneity of the CVs over this range of concentrations was ascertained using Bartlett's test [23].

Results

LOD and LOQ

The retention times of MBP, MEHP, MEHHP, and MEOHP were 14.92 min, 7.05 min, 17.36 min, 14.19 min, and 14.50 min, respectively. The relative standard deviations of the retention times were < 0.60%. As presented in Table 1, the LODs in

Table 2. Recovery of phthalate metabolites in serum

Metabolite	Spiking level, ng/sample	Recovery, %	
		Mean ± SD	Range
MBP	2	120.5 ± 11.0	107.5-134.5
	4	115.9 ± 16.0	96.5-135.3
	8	126.5 ± 9.4	110.9-137.5
	Total	123.0 ± 12.4	96.5-137.5
MEHP	2	104.8 ± 19.8	72.5-124.5
	4	114.7 ± 13.0	96.5-130.5
	8	103.5 ± 10.0	91.6-119.4
	Total	107.7 ± 14.9	72.5-130.5
MEHHP	2	100.6 ± 9.7	85.5-112.0
	4	112.7 ± 5.0	107.3-120.8
	8	111.0 ± 8.7	101.9-124.3
	Total	108.1 ± 9.3	85.5-124.3
MEOHP	2	88.1 ± 9.1	72.5-98.0
	4	95.1 ± 9.8	86.8-110.0
	8	95.4 ± 3.5	89.5-99.6
	Total	92.9 ± 8.3	72.5-110.0

SD: standard deviation, MBP: mono-n-isobutyl phthalate, MEHP: mono-2-ethylhexyl phthalate, MEHHP: mono-(2-ethyl-5-hydroxyhexyl) phthalate, MEOHP: mono-(2-ethyl-5-oxohexyl) phthalate.
Number of samples per level, n = 6.

Table 3. Recovery of phthalate metabolites in urine

Metabolite	Spiking level, ng/sample	Recovery, %	
		Mean ± SD	Range
MBP	8	103.0 ± 4.4	96.5-109.0
	16	99.2 ± 4.0	94.6-106.0
	32	97.9 ± 4.5	89.7-101.8
	Total	100.0 ± 4.6	89.7-106.0
MEHP	4	96.8 ± 3.3	90.8-100.2
	8	99.1 ± 4.2	93.3-105.5
	16	97.0 ± 4.7	89.7-101.9
	Total	97.6 ± 4.0	89.7-105.5
MEHHP	12	98.5 ± 5.0	93.0-104.6
	24	103.9 ± 4.8	98.7-112.2
	48	104.3 ± 3.3	99.0-108.9
	Total	102.1 ± 5.0	93.0-112.2
MEOHP	65	102.4 ± 5.9	97.2-112.8
	130	97.9 ± 6.7	87.4-104.7
	260	98.3 ± 4.8	91.0-103.7
	Total	99.5 ± 5.9	87.4-112.8

SD: standard deviation, MBP: mono-n-isobutyl phthalate, MEHP: mono-2-ethylhexyl phthalate, MEHHP: mono-(2-ethyl-5-hydroxyhexyl) phthalate, MEOHP: mono-(2-ethyl-5-oxohexyl) phthalate.
Number of samples per level, n = 6.

serum were 0.67 ng/mL (MBP), 0.24 ng/mL (MEHP), 0.08 ng/mL (MEHHP), and 0.14 ng/mL (MEOHP); the LODs in urine were 1.05 ng/mL (MBP), 0.22 ng/mL (MEHP), 0.15 ng/mL (MEHHP), and 0.16 ng/mL (MEOHP). The LOQs in serum were 2.02 ng/mL (MBP), 0.73 ng/mL (MEHP), 0.23 ng/mL (MEHHP), and 0.43 ng/mL (MEOHP); the LOQs in urine were 3.15 ng/mL (MBP), 0.67 ng/mL (MEHP), 0.46 ng/mL (MEHHP), and 0.49 ng/mL (MEOHP).

Recovery

Table 2 shows the results of the recovery test performed under three different serum metabolite levels (2-8 ng/sample). The recovery of MBP, MEHP, MEHHP, and MEOHP were 123.0 ± 12.4% (range: 96.5-137.5%), 107.7 ± 14.9% (72.5-130.5%), 108.1 ± 9.3% (range: 85.5-124.3%), and 92.9 ± 8.3% (range: 72.5-110.0%), respectively. The results of the recovery test for metabolites in urine samples are summarized in Table 3. The total mean recovery of each metabolite under the three different levels was 97.6-102.1%.

Precision of analytical procedure

Table 4 shows the precision of the column-switching LC-MS/MS method for analysis of the metabolites in serum and urine sample at three different concentration levels. There were no significant differences between the variances with 95% confidence based on Bartlett's test. As presented in Table 4, the pooled CVs for all three levels of the serum samples were 0.071 (MBP), 0.130 (MEHP), 0.078 (MEHHP), and 0.079 (MEOHP); the pooled CVs in urine samples were 0.035 (MBP), 0.030 (MEHP), 0.050 (MEHHP), and 0.044 (MEOHP). The pooled CVs of urine metabolites at the three levels were significantly lower than those of the serum metabolites ($p = 0.018$, Fig. 2), demonstrating reproducibility of this method over various concentrations in urine metabolites was much better than those of the serum samples.

Table 4. Precision of the column-switching HPLC-MS-MS method

Metabolites	Serum		Urine	
	Level of metabolite, ng/sample	CV	Level of metabolite, ng/sample	CV
MBP	2	0.075	8	0.051
	4	0.073	16	0.034
	8	0.064	32	0.010
	Pooled	0.071	Pooled	0.035
MEHP	2	0.091	4	0.035
	4	0.158	8	0.036
	8	0.132	16	0.016
	Pooled	0.130	Pooled	0.030
MEHHP	2	0.096	12	0.067
	4	0.083	24	0.035
	8	0.050	48	0.042
	Pooled	0.078	Pooled	0.050
MEOHP	2	0.096	65	0.071
	4	0.077	130	0.018
	8	0.061	260	0.022
	Pooled	0.079	Pooled	0.044

HPLC-MS-MS: high-performance liquid chromatography tandem mass spectrometric, CV: coefficient variation, MBP: mono-n-isobutyl phthalate, MEHP: mono-2-ethylhexyl phthalate, MEHHP: mono-(2-ethyl-5-hydroxyhexyl) phthalate, MEOHP: mono-(2-ethyl-5-oxohexyl) phthalate.
Number of samples at each level, n = 6.

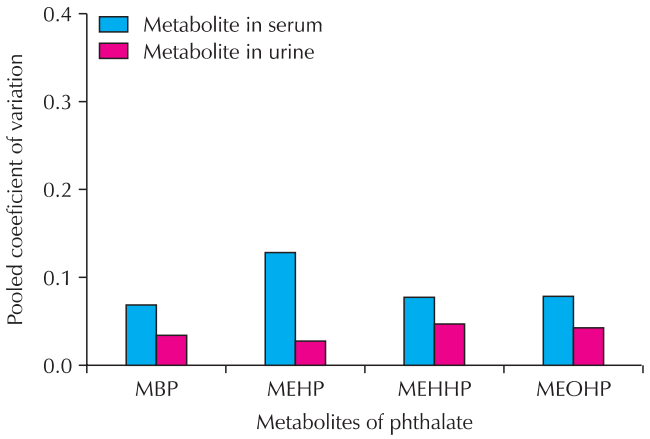

Fig. 2. Distribution of precision results in analyzing the metabolites in serum and urin samples at three different levels. MBP: mono-n-isobutyl phthalate, MEHP: mono-2-ethylhexyl phthalate, MEHHP: mono-(2-ethyl-5-hydroxyhexyl) phthalate, MEOHP: mono-(2-ethyl-5-oxohexyl) phthalate.

Discussion

Two goals are routine in analytical method development and validation. The first goal is the circumvention of extensive clean-up and concentration steps that could negatively affect method performance. The second goal is to achieve fast runtimes that allow efficient use of the analytical instrument. The column switching techniques applied in this study met these requirements in a state-of-the art technique that permitted the effective subsequent transfer, separation, and quantification on the analytical column with LC-MS/MS.

Humans are exposed to phthalates by multiple routes, with the most likely route varying depending on if one is in the general population or a worker. In the case of the general population, exposure can be oral through phthalate-contaminated food, water, and other liquids, or dermal by cosmetics and other personal care products. But, the main exposure of workers is inhalation and dermal. In contrast, almost all rodent toxicological studies to date have relied on oral exposure. Therefore, these rodent studies may not reflect toxicity of phthalates to humans who are exposed via other routes.

Traditional epidemiological methods of exposure assessment, such as questionnaires, medical records, and air monitoring that are used for multiple exposure routes, are of limited usefulness in determining individual exposure. Instead, biomarkers of exposure are preferred. But the occupational biological exposure standard for phthalates (DBP, and DEHP) is not yet established.

Phthalates and their metabolites have been measured in many body fluids and matrices, including urine, serum, saliva, seminal fluid, breast milk, amniotic fluid, and even placenta [24]. But urine and serum are the preferred matrices for phthalate determination in humans [25,26]. However, little published data exists on adverse workplace health outcomes and phthalate exposure assessment using biomarkers.

The LODs (0.08-0.67 ng/mL) of serum metabolites for DBP and DEHP were slightly lower than those (0.15-1.05 ng/mL) of urine metabolites. The LODs of MEHP, MEHHP, and MEOHP in serum and urine matrices by this analytical method are approximately 10-fold lower than the LODs of those metabolites in the same matrices by LC-MS/MS without column switching [15,27]. These levels are sufficient to evaluate concentrations of phthalate metabolites for not only industrial workers but also for the general population [5,19].

Because of the rapid metabolism of phthalates, urinary metabolite levels are typically higher than serum metabolite levels, as well as those of any other matrix. So, we carried out recovery testing of the metabolites in the representative concentration levels of each matrix. The minimum recovery of metabolites from urinary samples in this analytical method should be greater than 87%. But the minimum recovery of serum metabolites (MEHP, MEOHP) should be lower than 75%. Also, the recovery range (72-138%) of phthalate metabolites in serum was much greater than the recovery range (87-113%) of urinary phthalate metabolites, meaning that determination of urinary metabolites should be more accurate. As shown in Table 4 and Fig. 1, the precision of analytical procedure for urinary metabolites determination was more acceptable, with this method demonstrating very good repeatability.

In conclusion, our results show that the column switching LC-MS/MS method for the determination of phthalate metabolites is a very sensitive, accurate, and time saving analytical method. Also, for measurement with more acceptable accuracy and precision, urinary phthalate metabolite measurements as biomarkers may be more relevant in studies of phthalate exposure and adverse health outcomes.

The limitation of this study was that the urine and serum samples that came from the general population were used. Those samples could not sufficiently represent the samples from occupationally exposed workers. However, we thought the phthalate metabolites level of occupationally exposed workers could be higher than those of the general population. And even though we used samples from the general population, many of those in the general population had various occupational jobs. We thought that this analytical method could be used in the determination of phthalate metabolites for occupationally exposed workers.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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