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Short communication

Determination of three phthalate metabolites in human urine using on-line solid-phase extraction–liquid chromatography–tandem mass spectrometry

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Abstract

An on-line solid-phase extraction–liquid chromatography–tandem mass spectrometry (on-line SPE–HPLC–MS/MS) method was developed for the analysis of metabolites of three phthalate esters in human urine at the low nanogram per milliliter level. The recoveries were above 84.3% and relative standard deviations varied from 0.8 to 4.8%. The compounds along with their deuterated internal standards were detected in the negative ion mode by selective reaction monitoring and the accuracy of the method was improved by isotope dilution. Monobutyl phthalate was detected with median level of 22.5 ng/ml. The median levels for monobenzyl phthalate and monoethylhexyl phthalate were less than the limit of quantitation (LOQ). The on-line SPE–HPLC–MS/MS method allowed the possibility of determining these metabolites within a short time, with increased sensitivity and by using decreased amounts of sample and solvent.

Keywords: On-line solid-phase extraction; Phthalate monoester

1. Introduction

Dialkyl phthalate esters (phthalates) are widely used as plasticizers in the manufacture of polyvinyl chloride to improve flexibility and as additives to hold color and fragrance in a variety of products. Because phthalates do not chemically bind to the products, they tend to show up in the environment [1–3] (e.g. in food, air and water) during the production process, use, and disposal. The toxicity of several phthalates has been evaluated in animals [4–7]. The results indicate that native phthalates as well as their primary metabolites are toxic. Phthalates are ubiquitous compounds, they can be ingested and inhaled [8], resulting in human exposure and therefore raising public health concerns. Phthalates are eliminated in the urine as their monoester [9–14]. An off-line method of analysis was developted by Blont et al. [10] and used for epidemiologic research. Assessment of exposure to monoester phthalates

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requires increased throughput assays for quantitative determination of the monoester phthalates. We developed an on-line solid-phase extraction–liquid chromatography–tandem mass spectrometry (on-line SPE–HPLC–MS/MS) method for the analysis of three metabolites of phthalate esters in human urine at low nanogram per milliliter levels.

The on-line SPE method is an excellent tool for determining these compounds in a short time with increased sensitivity and requires reduced amounts of sample and solvent. This method also helps to decrease the variability of the analysis and increase the safety of analysts.

2. Experimental

2.1. Chemicals and standard solutions

Mono butyl phthalate (MBP, 99.4%), mono benzyl phthalate (MBzP, 99.4%), mono ethylhexyl phthalate (MEHP, 99.3%), and their deuterated d4-MBP (99.0%), d4-MBzP (99.0%), and d4-MEHP (99.8%) (standards for environmental residuum) were purchased from Hayashi Pure Chemicals Industries (Osaka, Japan). β-Glucuronidase solution (85 U/ml, from *Escherichia coli*) was purchased from Waco Pure Chemicals (Tokyo, Japan). Acetonitrile for standard preparation and methanol for SPE were used for residual pesticide analysis reagents. High-performance liquid chromatography (HPLC)-grade acetonitrile was used for separation. All other chemicals and solvents were obtained from commercial sources and were of the highest purity available. Water used was purified using a Milli-Q gradient A10 system (Millipore, Milford, MA, USA)

Standard solutions were dissolved in acetonitrile to a concentration of 1000 ng/ml and then diluted in water prior to use. The internal standard solution (spiked standard solution) was dissolved in water at a concentration of 400 ng/ml of each deuterated standard. The acidic buffer was a solution of 0.14 *M* NaH_2PO_4 and 1.0% of concentrated H_3PO_4 (pH 2.0).

2.2. Instruments

The HPLC separation was performed using a Waters™ 616 pump (Waters, Milford, MA, USA). The mass spectrometer used was an API 300 triple quadrupole (PE Biosystems, Concord, Canada) equipped with a Turboionspray interface. The on-line experiments were performed with a Prospekt (Spark, Emmen, The Netherlands) using single six port valve, a-cartridge-switching device, a solvent delivery unit (SDU), and a Midas (Spark) autosampler equipped with a 200-μl injection loop. Activation, conditioning, sampling, trapping, and washing were performed using the SDU. The prospect cartridges were HySphere-C_{18} HD (10×2 mm, particle size 3 μm, Spark). The HPLC column was an Inertsil ODS-3 (2.1×50 mm I.D., particle size 5 μm, GL Sciences, Tokyo, Japan).

2.3. SPE procedure

The SPE portion of the method was based on the off-line method [10]. The cartridges in the cartridge holder were replaced with new ones after each use. The SPE procedure was as follows: conditioning with 4 ml methanol, followed by 4.0 ml H_2O and 4.0 ml acidic buffer at a flow-rate of 4.0 ml/min. The 200 μl sample of deconjugated urine was loaded into the cartridge using 1.0 ml acidic buffer at a flow-rate of 1.0 ml/min, followed by a washing step with 1.0 ml H_2O at a flow-rate of 1.0 ml/min. The sample was eluted with the mobile phase for HPLC separation for 3 min and the obtained elute was added to the HPLC column. Table 1 shows the time schedules for the SPE procedure, HPLC, and MS/MS.

Table 1
The time schedules for the SPE procedure, HPLC and MS/MS

Time (min)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SPE	Conditioning			INJ	Wash	Elution				Conditioning			INJ	Wash	Elution	
HPLC						Separation					Conditioning				Separation	
MS/MS									Analysis							

2.4. Conditions for HPLC–MS/MS

The analytical column was connected to the second six-port switching valve of the Prospekt and the valve was set to the waste position to discard the elution for 3 min. This process avoided contamination of the interface of the mass spectrometry from protein, etc. The mobile phase was 0.1% acetic acid–acetonitrile (75:25 v/v) at a flow-rate of 0.6 ml/min for 3 min. The concentration of acetonitrile was immediately increased to 90% (step-wise method), and maintained for 2 min. The HPLC column was then conditioned for 3 min with 25%. The mass spectrometer was operated in the negative ion mode. Mass spectrometry parameters were optimized with the syringe filled with standard solutions. Main spectrometer parameters were as follows: The ion voltage was −4000 V, the turbo spray temperature was 400 °C, the turbo spray gas (N_2) flow-rate was 7.0 ml/min, the nebulizer gas (N_2) and the curtain gas (N_2) were at a pressure of instrument setting 8 and 3, respectively. The precursor ions were fragmented by collision-activated dissociation with nitrogen as collision gas at a pressure of instrument setting 3. The mass dependent parameters (ion source parameters and triple-quadrupole parameters) were as follows: OR, RNG, RO1, RO2 for MBP, MBzP, and MEHP as well as their internal standards were −50, −190, 25, 30 V, −50, −170, 25, 30 V, and −36, −180, 33, 38 V, respectively.

2.5. Sample preparation

Human urine (0.8 ml) was decanted into a glass tube to which 100 μl of the internal standard solution and 100 μl glucuronidase solution were added. These were then incubated for 120 min at 37 °C. The samples were filtered through a 0.45-μm membrane filter prior to analysis. All urine samples were stored at −80 °C until use.

3. Results and discussion

3.1. Investigation of matrix effects

Ion suppression might occur independently of the electrospray ionization mode and the undetected matrix components might reduce the ion intensity of the analyte and affect the reproducibility and accuracy of the LC–MS/MS methods. Any such urine matrix effect on the calibration curve, however, was evaluated by analyzing the standards prepared in water vs. those spiked into human urine. Standards spiked into urine produced calibration curves with slopes not significantly different from the slopes produced by direct injection of standards. Therefore, no interfering matrix effect was observed for the range of analyte concentrations measured, and calibration curves were produced using data collected by analyzing standards and internal standards prepared in water. The linearity of these compounds was checked from 0.5 to 800 ng/ml. There was good linearity and the correlation coefficients were higher than 0.997 (Table 2).

Table 2
Selected ion and the linear

	Precursor/product ion	Retention time (min)	Linear (ng/ml)	r^2
MBP	221/71	3.2	0.5–800	0.999
d4-MBP	225/71			
MBzP	255/183	3.5	1.0–800	0.997
d4-MBzP	259/187			
DEHP	277/134	4.5	0.5–800	0.999
d4-DEHP	281/137			

3.2. On-line SPE process

The elution profiles from the SPE cartridge are shown in Fig. 1. At a longer elution volume from the

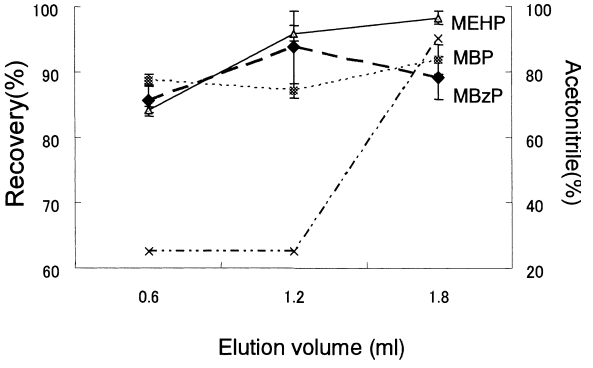

Fig. 1. Optimization of elution time from SPME cartridge data. Flow rate: 0.6 ml/min. Eluate: mobile phase for HPLC separation.

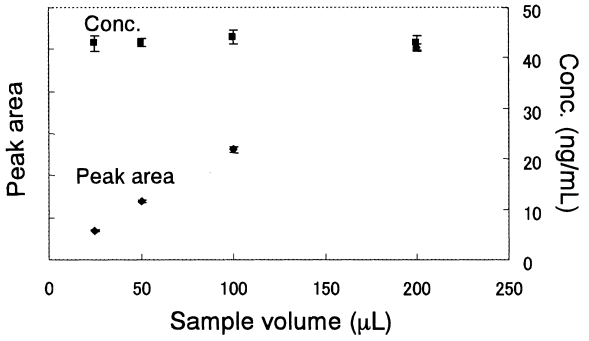

Fig. 2. Study of sample volumes using MEHP.

cartridge, the MEHP peak area increased and the recovery of MEHP to approximately 96%. Therefore, we used a 3-min elution time. The study of sample volume is shown in Fig. 2. The MEHP peak area increased with increasing sample volume, and the concentration values of each sample volume were reproducible without residual matter. Comparable results were obtained with the other monoesters. Microanalysis was then performed with a sample volume of 200 μl.

The limit of detection (LOD) was calculated as $3S_0$, where S_0 is the value of the standard deviation when the concentration approaches zero. The limit of quantitation (LOQ) was calculated as $10S_0$. The LOD for the analysis of three metabolites in human urine was at the low nanogram per milliliter level. Repeatability and reproducibility were checked by analyzing urine spiked with 10, 50, and 300 ng/ml of each analyte. The recoveries of three analytes were more than 84.3% and relative standard deviation (RSD, %) varied from 0.8 to 4.8. It was confirmed that the present method was highly sensitive, reproducible and accurate (Table 3).

Table 4
Concentration of the three mono phthalates in human urine

	MBP	MBzP	MEHP
Man ($n=50$)			
Mean±SD	15.6±19.6	2.9±3.8	3.0±4.9
Min	4.5	ND	ND
Max	130.5	8.8	27.5
Frequency (%)	100.0	31.4	27.0
Women ($n=20$)			
Mean±SD	27.1±16.3	2.9±3.7	2.4±3.5
Min	13.4	ND	ND
Max	85.8	7.5	13.9
Frequency (%)	100.0	25.0	20.0
Median	22.5	ND	ND
Mean	20.9±19.1	2.6±3.6	2.8±4.6

ND<under LOQ (ng/ml).

3.3. Application of the method

Seventy human urine samples were subjected to the analysis. The results are shown in Table 4. MBP was detected with median levels of 22.5 ng/ml. The median of MBzP and MEHP were less than LOD.

4. Conclusion

The present method of on-line SPE–HPLC–MS/MS enables extraction and analysis to be performed in a closed system. This has advantages over conventional off-line preparation methods in that contamination by external factors is reduced and the safety of the personnel analyzing biologic samples is enhanced. In addition, the present method allows for highly selective, sensitive and rapid analysis of three monoester phthalates. The present method will also

Table 3
Validation data, LOD and LOQ

Concentration (ng/ml)	Recovery %, (RSD %), $n=5$			LOD	LOQ
	10	50	300		
MBP	88.5(2.2)	91.8(2.3)	90.8(3.8)	1.3	4.3
MBzP	84.3(4.8)	89.0(3.3)	89.3(2.7)	1.7	5.7
MEHP	94.9(1.5)	96.5(1.9)	96.2(0.8)	0.7	2.3

be useful for assessing the effect of phthalates on human health.

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