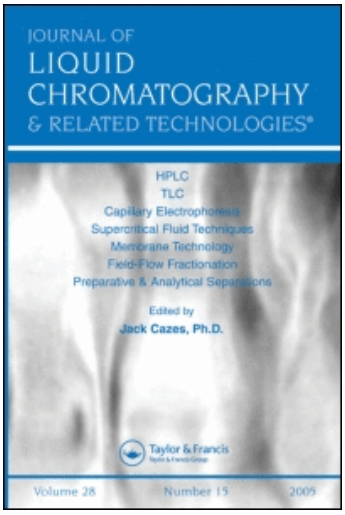

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Kayoko Kato[a]; Takayasu Yamauchi[b]; Kimio Higashiyama[b]; Hiroyuki Nakazawa[a]
[a] Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, Tokyo, Japan [b] Institute of Medicinal Chemistry, Hoshi University, Tokyo, Japan

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High Throughput Analysis of Di-(2-Ethylhexyl)phthalate Metabolites in Urine for Exposure Assessment

Kayoko Kato,[1] Takayasu Yamauchi,[2] Kimio Higashiyama,[2] and Hiroyuki Nakazawa[1,*]

[1]Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, Tokyo, Japan
[2]Institute of Medicinal Chemistry, Hoshi University, Tokyo, Japan

ABSTRACT

In order to assess exposure, we developed high throughput analysis of di-(2-ethylhexyl)phthalate (DEHP) metabolites in urine using an on-line solid phase extraction–liquid chromatography/tandem mass spectrometry (on-line SPE–HPLC–MS/MS) method. The metabolites were detected in the negative mode by select reaction monitoring at low nanogram per milliliter levels. Repeatability and reproducibility of the methods was checked by analyzing spiked urine. The results, expressed as relative standard deviation (RSD%), varied from 0.8 to 6.9. The recoveries were

*Correspondence: Hiroyuki Nakazawa, Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, 2-4-41, Ebara, Shinagawa-ku, Tokyo 142-8501, Japan; Email: nakazawa@hoshi.ac.jp.

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above 85.8%. Three metabolites of DEHP were detected with the levels from 9.5 to 320.8 ng mL^{-1} for mono-(2-ethyl-5-hydroxyhexyl)phthalate (VI), from 13.9 to 187.5 ng mL^{-1} for mono-(5carboxy-2-ethylpentyl)phthalate (IX), and from ND to 13.9 ng mL^{-1} for mono-(2-ethylhexyl)phthalate (MEHP). The concentration levels of VI and IX were detected higher than MEHP. The frequencies of VI and IX were 100%. The proposed method introduces the possibility of determining these metabolites within 8 min (including sample preparation time), while also increasing sensitivity and reducing amounts of sample and solvent required.

Key Words: DEHP metabolites; HPLC-MS/MS; Exposure assesment.

INTRODUCTION

Di-(2-ethylhexyl)phthalate (DEHP) is widely used as a plasticizer in polyvinyl chloride plastics such as medical devices,[1] cosmetics, paints, and wood finishes. A number of studies have described the toxicity of DEHP in animals[2–5] and raised concern about the potential hazard for humans. After exposure in humans, DEHP is rapidly hydrolyzed in the liver to mono-(2-ethylhexyl)phthalate (MEHP) and 2-ethylhexanoic acid,[6–8] which then undergo oxidation to form oxidative metabolites.[6,7,13] The observed toxic effects have been reported not only to be caused by DEHP itself, but also by its related metabolites.[5] The human exposure assessment for DEHP has been evaluated by the level of MEHP in urine.[9–14] However, the results show that the MEHP was detected less frequently and at lower levels than expected. It has been reported that after oral dosing of humans with ^{13}C-derivative phthalates, the excretion yield of monoester phthalate in urine was at a level of approximately 69% for dibuthyl phthalate (DBP) to monobuthyl phthalate (MBP) and 13% for DEHP to MEHP.[11]

The results show that the excretion of long chain alkyl phthalates would metabolize to more polarized compounds. Therefore, it has required development of the analysis method of the oxidative metabolites for the exact method for the exposure assessment of DEHP.

We established an on-line solid phase extraction–liquid chromatography/tandem mass spectrometry (on-line SPE–HPLC/MS/MS) method for the analysis of the oxidative metabolites of DEHP in human urine. The proposed method determined these metabolites rapidly, accompanied by increased sensitivity and a reduction in the amounts of sample and solvent required. It can also help to limit variation between personnel and to ensure the safety of analysts.

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EXPERIMENTAL

Chemicals

Mono-(2-ethylhexyl)phthalate, (99.3%), d4-MEHP (deuterated MEHP, 99.8%), and d4-MBP (deuterated MBP, 99.0%) were standards for environmental residuum, which were purchased from Hayashi Pure Chemicals Industries (Osaka, Japan). The oxidative metabolites of DEHP, mono(2-ethyl-5-hydroxyhexyl)phthalate (Metabolite VI), and mono-(5carboxy-2-ethylpentyl)phthalate Metabolite IX) were synthesized and confirmed with magnetic resonance and MS (Fig. 1). β-Glucuronidase solution (85 U mL^{-1}, from *Escherichia coli*) was purchased from Wako Pure Chemicals (Tokyo, Japan). Acetonitrile for standard preparations and methanol for SPE were used for residual pesticide analysis reagents. HPLC grade acetonitrile was used for separation. All other chemicals and solvents were obtained from commercial sources and were of the highest purity available. The water used was purified using a Milli-Q gradient A10 system (Millipore Corp., Milford, MA). The acidic buffer was a solution of 0.14 M NaH_2PO_4 and 1.0% concentrated H_3PO_4. (around pH = 2.0).

Standard solutions were dissolved in acetonitrile to a concentration of 1000 ng mL^{-1} and then diluted in water prior to use. The spiked standard solution was dissolved in water at a concentration of 400 ng mL^{-1} of each deuterated standard.

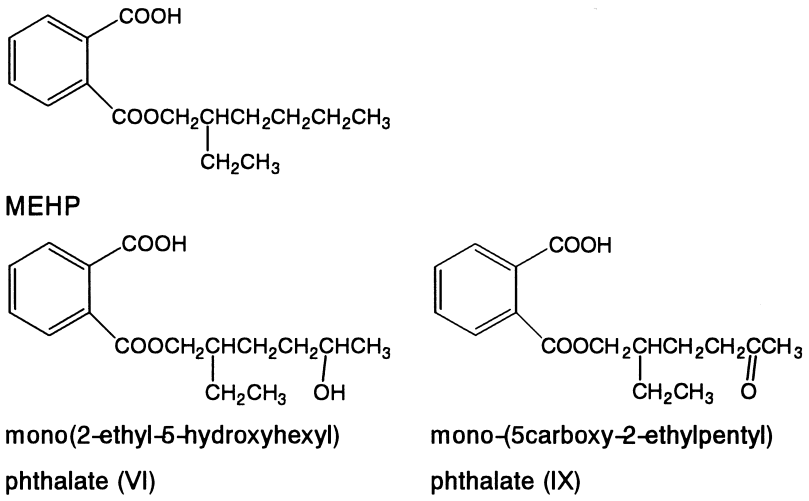

Figure 1. Chemical structures of DEHP metabolites.

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Instruments

The HPLC separation was performed using a Waters™ 616 pump (Waters, Milford, USA). The mass spectrometer was used an API 300 triple quadrupole (PE Biosystems, Concord, Canada) equipped with Turboionspray interface. The on-line SPE was performed with a Prospekt (Spark, Emmen, The Netherlands) using one six port valve, a-cartridge-switching device, and solvent delivery unit (SDU), and with a Midas (Spark) autosampler equipped with a 200 μL injection loop. Activation, conditioning, sampling, trapping, and washing were performed using SDU. The Prospekt cartridges were HySphere-C18 HD (10×2 mm, particle size 3 μm, Spark). The HPLC column was Inertsil ODS-3 (inner diameter 2.1×50 mm, particle size 5 μm, GL Sciences Inc., Tokyo, Japan).

On-line Solid Phase Extraction Procedure

The SPE portion of the method was based on the off-line[9] and the on-line method[15] for primary metabolites for phthalates. The cartridges in the cartridge holder were replaced with new ones after a single use. The SPE procedure was as follows: conditioning was performed 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^{-1}. Two hundred microliter of the sample was loaded into the cartridge with 1.0 mL acidic buffer at a flow rate of 1.0 mL min^{-1}. This was followed by a washing step with 1.0 mL H_2O at a flow rate of 1.0 mL min^{-1}. The sample was eluted with the mobile phase for HPLC separation for 3 min and the obtained elution was added to the HPLC column. Table 1 shows the time schedules for the SPE procedure, HPLC, and MS/MS.

Conditions for HPLC–MS/MS

The analytical column was connected to the six port switching valve and the elution of the HPLC column for 3 min. This process avoided contaminating the interface. A mobile phase was 0.1% acetic acid/acetonitrile (75/25) at a flow rate of 0.6 mL min^{-1} for 3 min. Then, the concentration of acetonitrile was raised to 90% immediately, and held for 2 min. Then, the HPLC column was conditioned for 3 min with acetonitrile at a concentration of 25%. The mass spectrometer was operated in the negative ion mode. The ion voltage was −4000 V, the turbo spray temperature was 400°C, and the turbo spray gas (N_2) flow was at a rate of 7.0 mL min^{-1}, the nebulizer gas (N_2), the curtain gas (N_2), and at a pressure of instrument setting 11 and 8, respectively. The precursor ions were fragmented by collision-activated dissociation with nitrogen as collision gas at a pressure of instrument setting

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Table 1. Time table for on-line SPE, HPLC, and MS/MS process.

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

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3. The other MS/MS conditions were dependent on each ion, respectively. The mass dependent parameters (Ion source parameters and triple-quadrupole parameters) were as follows: OR, RNG, RO2, RO3 for d4-MBP, VI, IX, and MEHP, as well as their internal standards, were −50, −190, 25, 30 V, −51, −185, 33, 38 V, −31, −185, 29, 34 V, and −36, −180, 33, 38 V, respectively.

Sample Preparation Procedure

0.8 mL of human urine was added to 100 μL of the internal standard solution and 100 μL of glucoulonidase solution. And then, those were incubated for 120 min at 37°C and were filtered over a 0.45 μm membrane filter prior to analysis. All urine samples were stored at −80°C until use.

RESULTS AND DISCUSSION

Effect of the Linearity on Urinary Matrix Influence

The linearity of each metabolite and the correlation coefficients were good when d4-MBP was used as an internal standard for metabolites VI and IX. However, the calibration curves for VI and IX produced by standards prepared using SPE methods, significantly differ from the slopes produced by direct injection standards. It is known that ion suppression may occur independently of the electrospray ionization mode since undetected matrix components may reduce the ion intensity of the analyte and affect the reproducibility and accuracy of the LC–MS/MS methods. There might also be a difference of retention on the SPE cartridge between d4-MBP and those metabolites. Therefore, we prepared the calibration curves the same as the procedure of the sample preparation method. From the results, each of the linearity of the obtained three metabolites was good and correlation coefficients were higher than 0.996, from 1.0 to 800 ng mL^{-1} (Table 2).

Validation

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 quantification (LOQ) was calculated as $10S_0$. The LOD and LOQ for the analysis of the three metabolites in human urine were at the low nanogram per mililiter level. Repeatability and reproducibility were checked by analyzing urine spiked with 25, 50, and 300 ng mL^{-1} of each analyte.

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Table 2. Selected ion and the linearity.

	Precursor/production	Retention time (min)	Linearity ($ng\,mL^{-1}$)	r^2
VI	291/121	3.4	1.0–800	0.996
IX	293/121	3.2	1.0–800	0.997
d4-MBP[a]	225/71	3.2		
MEHP	277/134	4.5	1.0–800	0.999
d4-MEHP	281/138			

[a]An internal standard for metabolite IX and VI.

The results, expressed as recoveries (%) and relative standard deviation (RSD%), varied from 85.8 to 95.3 and from 0.8 to 6.9, respectively. It was confirmed that this present method was high sensitive, reproducible, and accurate (Table 3).

Quantitative Analysis of Three Di-(2-Ethylhexyl)phthalate Metabolites in Human Urine

Seventy samples of human urine were subjected to analysis. The results are shown in Table 4 and the MRM chromatogram is shown in Fig. 2. Three metabolites of DEHP were detected with the levels from 9.5 to 320.8 $ng\,mL^{-1}$ for VI, from 13.9 to 187.5 $ng\,mL^{-1}$ for IX, and from ND to 13.9 $ng\,mL^{-1}$ for MEHP. The concentration levels of VI and IX were detected as higher than MEHP. The frequencies of VI and IX were 100%. The results showed that the exposure to DEHP should be estimated by analysis of these oxidative metabolites.

Table 3. Validation data.

	Recovery, % (RSD)				
Concentration ($ng\,mL^{-1}$)	25	50	300	LOD	LOQ
VI	85.8 (6.9)	89.4 (6.1)	89.8 (6.3)	1.0	3.5
IX	88.6 (5.9)	86.9 (6.6)	92.3 (5.8)	1.1	3.8
MEHP	90.8 (1.3)	94.2 (2.2)	95.3 (0.9)	0.7	2.1

Note: $n = 5$.

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Table 4. Concentration ($ng\,mL^{-1}$) of the three DEHP metabolites in human urine.

	VI	IX	MEHP
Men ($n = 50$)			
Mean ± SD	48.3 ± 38.5	83.5 ± 41.2	3.1 ± 4.9
Min	9.5	13.9	ND
Max	148.3	158.8	27.5
Frequency (%)	100.0	100.0	27.8
Women ($n = 20$)			
Mean ± SD	68.3 ± 26.3	53.8 ± 12.2	2.7 ± 3.4
Min	13.4	16.5	ND
Max	320.8	187.5	13.9
Frequency (%)	100.0	100.0	20.0

Note: ND < under LOQ.

CONCLUSION

The results show that exposure to DEHP should be estimated by analysis of oxidative metabolites. An online SPE–HPLC–MS/MS method enables extraction and analysis to be performed in a closed system. This has advantages over conventional offline preparation methods in that contamination by

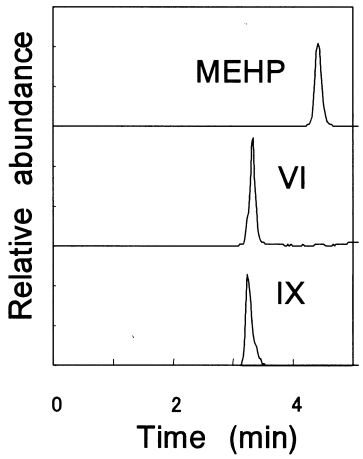

Figure 2. MRM chromatograms of human urine.

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external factors can be reduced and the safety of personnel analyzing biological samples can be enhanced. In addition, the method of automated extraction of metabolites followed by determination using a MS/MS method has made highly selective, sensitive, and short time analysis possible. The method presented will, therefore, be useful in assessing the effect of DEHP on human health.

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