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Contributed Paper

Development and Validation of a Simple High Performance Liquid Chromatography/UV Method for Simultaneous Determination of Urinary Phthalates in Human Urine

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ABSTRACT

This study aimed to develop a simple HPLC method for the simultaneous determination of four urinary phthalate (PAE) metabolites, including monomethyl PAE (MMP), monoethyl PAE (MEP), mono-n-butyl PAE (MBP) and mono(2-ethylhexyl) PAE (MEHP). The metabolites were simultaneously analyzed by a simple dilution method with acetonitrile and phosphate buffer pH 2.0 followed by filtration. No interfering peaks were observed at the retention time of the investigated analytes. Separation of four PAE metabolites was successfully achieved in less than 16.5 min with good resolution. The linear range of calibration curves for the four analytes were respectively from 10-3,000 ng/mL for MMP and MEP, 20-3,000 ng/mL for MBP and MEHP with correlation coefficient $\geq$ 0.998. Intra- and inter-day precision ranged from 0.5 to 10.2% and 2.1 to 9.9%; whereas intra- and inter-day relative errors (%bias) were less than 11.1 and 7.3%, respectively. This method was successfully applied to preliminary screen four PAEs in urine samples from Thai children and adolescents. Since, the levels of PAEs metabolites have been found to associate with health conditions including precocious puberty in children. Measurement of their metabolites can be helpful in clinical application and can provide the basis for appropriate risk management measures.

Keywords: biomarkers, high performance liquid chromatography, phthalates, phthalate metabolites, urinary metabolites

1. INTRODUCTION

Phthalates (PAEs) or acid esters are widely used as plasticizers in manufacturing plastic products and as additives in many consumer products, such as food packaging, medical devices, children's toys and cosmetics [1, 2]. PAEs are not covalently bound to the plastic matrix and show a tendency to migrate, especially when they are in contact with lipophilic substance and/or under mechanical or thermal stress into the environment, including food, water and pharmaceutical products [3].

Consequently, they can bioaccumulate in human body via ingestion, inhalation, intravenous and dermal [4-6], and can cause a serious risk to for human health [7]. This has become an extremely important issue across the globe. PAEs have been documented as suspected endocrine disruptors that may affect many biochemical processes and damage to reproductive systems in human and animal [8-10]. Several previous studies have reported increased incidences of precocious puberty, attention deficit disorder and learning disability in children, etc., associated with PAE exposure [11-13]. Because of the increased interest in assessing human exposure to PAEs and their potential harm to humans, especially in children, a reliable and sensitive bioanalytical method is needed to support preclinical and clinical studies. Among several biological samples, the measurement of urinary PAE metabolites has been considered as a valuable approach to assessing exposure to PAEs in clinical studies **[**14]**.** Since the metabolites are rapidly excreted and provide the highest concentration in urine [15]. This study aims to develop a simple method for simultaneous determination of urinary PAE metabolites which were monomethyl PAE (MMP), monoethyl PAE (MEP), mono-n-butyl PAE (MBP) and mono(2-ethylhexyl) PAE (MEHP). The simple dilution method for sample preparation was chosen to improve the simplicity of the method, and high-performance liquid chromatography (HPLC) was employed for quantitative analysis. Parameters affecting analyte recovery were optimized including solvent volume ratio. The method was validated in terms of selectivity, sensitivity, linearity, accuracy and precision. The proposed method was applied to preliminary investigate PAE metabolites in urine samples collected from Thai children and adolescents.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Reference standards of MMP and MBP (>99.5% purity) were purchased from Sigma-Aldrich (Buchs, Switzerland) and Sigma-Aldrich (Saint Louis, USA), respectively. Reference chemicals of MEP and MEHP (>98% purity) were obtained from Toronto Research Chemicals Inc. (Toronto, Canada). The deionized water was obtained from Thai Nakorn Patana (Nonthaburi, Thailand). HPLC grade of acetonitrile was supplied by Macron Fine Chemicals (Avantor Performance Materials, Center Valley, PA, USA) Analytical grade of glacial acetic acid and phosphoric acid was provided by RCI Labscan Limited (Bangkok, Thailand). A phosphate buffer solution 50 mM was prepared by dissolving sodium dihydrogen phosphate from Carlo Erba Reagents S. A. S. (Val de Reuil, France) in deionized water, and adjusted pH to 2.0 by using phosphoric acid.

2.2 Instrumentation and Chromatographic Conditions

Chromatographic analysis was carried out using a Thermo Scientific Dionex Ultimate 3000 Series HPLC system (Dionex Softron GmbH, Germering, Germany) equipped with a UV-visible variable-wavelength detector, an autosampler and a binary pump. Analytes were separated on a Purospher STAR RP-18 endcapped (Merck KGaA, Darmstadt, Germany) analytical column (250 × 4.6 mm, 5 μm). The detection wavelength was 224 nm. The mobile phase consisted of solvent A (acetonitrile/acetic acid, (99.9:0.1, v/v)) and solvent B (0.1% acetic acid in water). The gradient elution was performed as follows: 0.0-15.0 min (10-100% A) and 15.1-17.0 (100% A). The column was then equilibrated to the initial condition for 7 min before the next injection. The column temperature was controlled at 30 ± 2°C. The injection volume was 20 μL and the flow rate was maintained at 1.0 mL/min with a total run time of 17 min. The integrations were performed using Chromeleon software, version 6.80 (Dionex, Sunnyvale, CA, US).

2.3 Subjects and Urine Specimens

The study protocol was approved by the

Institutional Review Board (IRB) of Faculty of Dentistry and Faculty of Pharmacy, Mahidol University, Bangkok, Thailand (COE.No.MU-DT/PY-IRB 2017/029.3108).

Fifteen children and adolescents aged 7-18 years (7 males and 8 females) were recruited from the outpatient clinic of King Chulalongkorn Memorial Hospital during. The urine creatinine and protein levels of all subjects were within the ranges of 92.15 ± 35.22 and 10.22 ± 5.56 mg/dL, respectively. Subjects with any of following conditions were excluded: impaired kidney function, inability to urinate voluntarily, or chronic illness. Prior to enrolment all subjects and their parents signed informed consent documents. On the day of investigation, a morning urine sample was collected at least 50 mL from each subject into a PAE-free polypropylene tube and kept at -20°C until analysis.

2.4 Preparation of Stock Solutions, Calibration and Quality Control Samples

Standard stock solutions of MMP, MEP, MBP and MEHP were separately prepared in acetonitrile at a concentration of 1 mg/mL and stored at 4 °C for a maximum of 1 week. These solutions were diluted with acetonitrile to obtain appropriate working solutions (0.1-100 μg/mL) for preparing calibration and quality control (QC) samples.

Pooled blank urine was used for preparation of calibration standards and QC samples. Matrix-based calibration standards in human urine were prepared by spiking standard working solution into blank urine (200 μL) sample to yield concentrations of 10, 100, 500, 1,000, 1,500 and 3,000 ng/mL for MMP and MEP, and 20, 100, 500, 1,000, 1,500 and 3,000 ng/mL for MBP and MEHP, respectively. QC samples were prepared in the same manner as the calibration standards at three concentration levels as follows: 30, 800 and 2,000 ng/mL for MMP and MEP, and 60, 800 and 2,000 ng/mL for MBP and MEHP, respectively.

2.5 Sample Preparation

Factors influencing the analysis efficiency were optimized for the separation of the four studied metabolites, including solvent volume (40-400 μL), pH of phosphate buffer (2.0-5.0) and sonication time (0-20 min). The optimum condition was successfully obtained and used for sample preparation.

Frozen urine samples were thawed unassisted at room temperature (30 ± 2°C). A 200 μL of urine sample was transferred to a 2-mL plastic tube followed by the addition of 400 μL of acetonitrile. The mixture was diluted with phosphate buffer pH 2.0 to a total volume of 1,000 μL and sonicated for 10 min. The sample was filtered using PTFE syringe filter (13 mm diameter, 0.45 μm pore size, Membrane solution, TX, USA) and analyzed by HPLC.

2.6 Method Validation

The method was validated according to the FDA guidance on bioanalytical method validation [16]. The specificity was evaluated by analyzing blank urine from six different subjects to test for matrix interfering peaks. Absence of interfering components was accepted if the response was <20% of the lower limit of quantification (LLOQ) for all analytes. Sensitivity was achieved by determining the LLOQ of each urinary metabolite. The LLOQ was established as the lowest concentration of investigated compound used in the calibration curve with accuracy and precision of 100 ± 20%. Bias and relative standard deviation (%R.S.D.) were used as measures of accuracy and precision, respectively. Linearity was assessed by plotting peak areas of analytes versus concentrations of calibration standards. All calibration curves were required to have a correlation value (r^2) of at least 0.995. Precision and accuracy were assessed by determining QC samples in pentaplicate on three different days. Precision was measured by inter- and intra-day R.S.D. (%). The accuracy was evaluated by the deviation or bias (%) of the observed concentration from the expected concentration.

The potential for carryover was investigated by injecting blank solvent immediately after the highest concentration of calibration curve.

3. RESULTS AND DISCUSSION

3.1 HPLC Operating Condition

Several preparation parameters were optimized for the analytical conditions, including pH of phosphate buffer, urine/phosphate buffer ratio and sonication time. The developed sample preparation method demonstrated maximum absorbance wavelength at 224 nm. The pH of phosphate buffer was varied from 2.0 to 5.0, and it was found that the optimum recovery was obtained at pH 2.0. The good peak shape was obtained when the ratio of urine:acetonitrile:-phosphate buffer pH 2.0 was 2:4:4. In addition, acetonitrile containing glacial acetic acid gave the better system suitability parameters. The numbers of theoretical plates were more than 2,000, and the tailing factors were always less than 1.5. Under the gradient elution condition, the total run time for all PAE metabolites were 16.5 min. The retention time of MMP, MEP, MBP and MEHP were 9.02, 10.12, 12.33 and 16.21 min, respectively.

3.2 Method Validation

The specificity of the method was evaluated as lack of endogenous interference by analyzing blank urine. Interferences were assessed by comparing chromatograms of blank urine and the urine samples spiked with the metabolite standards. No significant interfering peaks from blank samples were observed. The linear range of calibration curves for the four analytes were respectively from 10-3,000 ng/mL for MMP and MEP 20-3,000 ng/mL for MBP and MEHP, respectively. The correlation coefficient was $\geq$ 0.998 for all validation batches**.** The LLOQ for MMP, MEP, MBP and MEHP were 10, 10, 20 and 20 ng/mL with accuracy of 20.0, 18.6, 7.1 and 10.0%, respectively. Representative chromatograms of blank urine, blank urine spiked with PAEs metabolites (800 ng/mL) and real sample are shown in Figure 1. Furthermore, there was no evidence of carry-over effect for this matrix. Table 1 summarizes the intra- and inter-day accuracy and precision of the method. The results were within the acceptable ranges for bioanalytical purposes. Intra- and inter-day precision ranged from 0.5 to 10.2% and 2.1 to 9.9%; whereas intra- and inter-day relative errors (%bias) were less than 11.1 and 7.3%, respectively.

3.3 Application of Urine Sample Analysis

Currently, plastics are one of the most practical and economical ways to contain food, cosmetics and pharmaceutical products [17]. PAEs are widely used as plasticizers in the plastic products [2] which could bioaccumulate in animal and human [4-6]. It has been reported that PAEs can cause a serious risk to for human health [7]. Therefore, it is necessary to implement more in-depth research for assessing human exposure to PAEs and their potential harm to human health. Furthermore, continued monitoring efforts for PAE combinations are required to provide the basis for appropriate risk management measures [18].

The validated method was applied for the analysis of urine samples of fifteen children and adolescents in Thailand. The analytical results were shown in Table 2. It was found that MMP were detected in all urine samples; whereas MEP, MBP and MEHP were detected in 12 (80.0%), 2 (13.3%) and 10 (66.67%) samples, respectively. The mean concentration of MMP, MEP, MBP and MEHP found in the urine samples were 421.34 $\pm$ 347.91, 59.55 $\pm$ 42.07, 59.14 $\pm$ 8.28 and 25.66 $\pm$ 4.43 ng/mL with the maximum concentration of 1,240.9, 138.1, 65.0 and 34.4 ng/mL, respectively.

The developed method allows for simultaneous measurement of four urinary PAE metabolites within one single determination**.** In this study, the simple dilution method for sample preparation was chosen to improve the simplicity of the method. This dilution could reduce between-sample matrix variability that may affect analyte recovery [19]. However, as can be seen from table 2, the

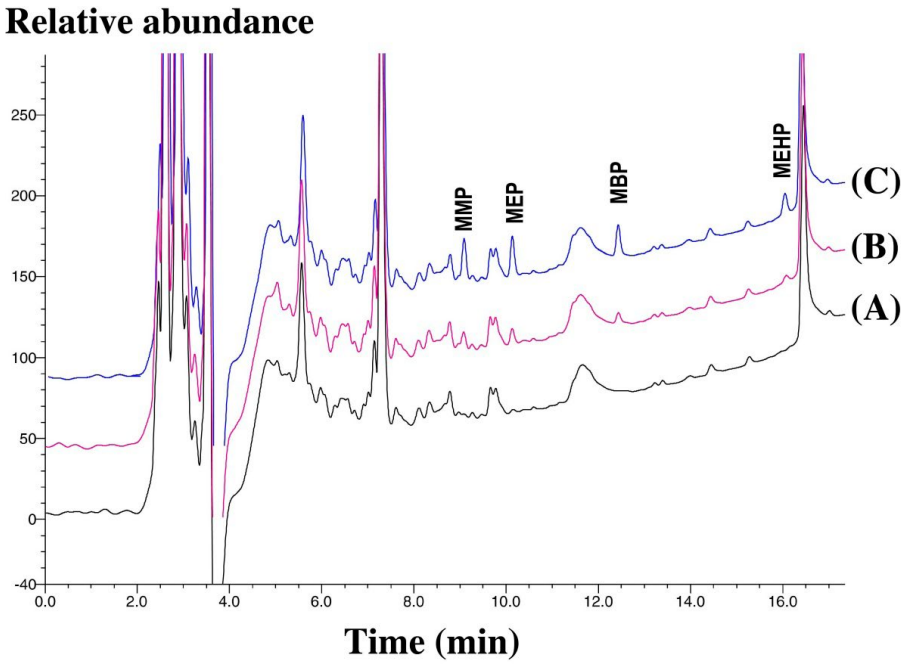

Figure 1. Representative chromatogram of blank urine (A), real urine sample (B) and blank urine spiked with PAE metabolites (C, 800 ng/mL) under the optimized condition.

Table 1. Accuracy and precision data for the phthalate metabolites in urine.

Analyte	Added (ng/mL)	Intra-day (*n*=5)			Inter-day (*n*=15)		
		Found (ng/mL)	%R.S.D.	%Bias	Found (ng/mL)	%R.S.D.	%Bias
MMP	30	31.6 ± 2.4	7.6	5.5	29.8 ± 2.5	8.5	-0.6
	800	733.3 ± 10.6	1.5	-8.3	741.7 ± 61.7	8.3	-7.3
	2,000	2,126.9 ± 46.7	2.2	6.4	1,983.3 ± 196.8	9.9	-0.8
MEP	30	29.6 ± 3.0	10.2	-1.4	30.8 ± 2.7	8.9	2.8
	800	869.7 ± 14.1	1.6	-8.7	784.7 ± 74.5	9.5	-1.9
	2,000	2,222.6 ± 40.2	1.8	11.1	2,032.3 ± 199.8	9.8	1.6
MBP	60	57.9 ± 1.2	2.0	-7.0	59.2 ± 1.7	2.8	-1.3
	800	771.5 ± 12.4	1.6	-3.6	780.3 ± 16.1	2.1	-2.5
	2,000	2,008.4 ± 51.8	2.6	0.4	2,026.4 ± 93.6	4.6	1.3
MEHP	60	59.3 ± 2.6	4.4	-2.4	59.2 ± 2.4	4	-1.3
	800	863.0 ± 3.9	0.5	7.9	785.7 ± 56.8	7.2	-1.8
	2,000	2,096.4 ± 40.4	1.9	4.8	2,005.3 ± 74.5	3.7	0.3

Table 2. Concentration of four PAE metabolites in urine samples obtained from Thai children and adolescents (n=15).

Analyte	Positive detection (%)	Found (ng/mL)[a]	Range (ng/mL)	Median concentration (ng/mL)
MMP	15 (100.00)	421.34 ± 347.91	53.94 - 1,240.87	340.87
MEP	12 (80.00)	59.55 ± 42.07	16.19 - 138.12	50.69
MBP	2 (13.33)	59.14 ± 8.28	53.29 - 65.00	59.14
MEHP	10 (66.67)	25.66 ± 4.43	20.53 - 34.40	25.77

[a] Data expressed as Mean ± S.D.

detected ranges of four PAEs metabolites were below the MOQ of this method. This indicated that the urine samples should be concentrated before analysis of both metabolites. The level of target analytes might be influenced by many factors including PAEs exposure and by their metabolism. Particularly, MBP and MEHP were totally excreted in conjugated form via urine [20]. Additional deconjugation step to the proposed method is suggested for further application. In addition, the analytes in urine samples could be concentrated by solid phase extraction (SPE). Currently, LC–MS/MS methods combined with off-line or on-line solid-phase extractions are considered as a high-throughput simultaneous quantitation of PAE metabolites. However, SPE requires multi-step sample preparation and relatively high cost procedure [21].

Since it has been reported that the levels of PAEs metabolites might be associated with health conditions including precocious puberty in children [11-13], assessment of the metabolites can be helpful in clinical application and environmental epidemiology studies. In this study, the preliminary clinical results demonstrated that the MMP levels were higher than those of other studied metabolites. The developed method could be further applied for the screening step for the association between MMP levels with health conditions. However, an accurate and sensitive method should be further developed in order to monitor different types of PAEs and their metabolites.

4. Conclusions

This study focused on the assessment of PAE metabolites in human urine. A simple and sensitive HPLC method for simultaneous quantification of four urinary PAEs metabolites was established and validated in this study. The results demonstrated that the developed method met all requirements in the FDA guidance for bioanalytical method, suggesting its applicability for analysis of clinical samples. The method was applied in preliminary assessment of four urinary PAE metabolites in urine samples obtained from Thai children and adolescents. MMP demonstrated the highest level of the metabolites found in the urine samples. However, the detected levels of all studied metabolites were below the MQC of the method. Therefore, a sensitive method should be further developed in order to monitor different types of PAEs and their metabolites.

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Conflicts of interest statement

The authors state that no conflicts of interest exist.

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