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Development of a Quantitative Analytical Method for Determining the Concentration of Human Urinary Paraben by LC-MS/MS

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Parabens, the esters of *p*-hydroxybenzoic acid, have been widely used as antimicrobial preservatives in cosmetic products, drugs, and processed foods and beverages. However, some parabens have been shown to have weak estrogenic effects through *in vivo* and *in vitro* studies. Because such widespread use has raised concerns about the potential human health risks associated with exposure to parabens, we developed a simultaneous analytical method to quantify 4 parabens (methyl, ethyl, propyl, and butyl) in human urine, by using solid-phase extraction and high-performance liquid chromatography coupled with triple quadrupole mass spectrometry. This method showed good specificity, linearity ($R^2 > 0.999$), accuracy (92.2-112.4%), precision (0.9-9.6%, CV), and recovery (95.7-102.0%). The LOQs for the 4 parabens were 1.0, 0.5, 0.2, and 0.5 ng/mL, respectively. This method could be used for quick and accurate analysis of a large number of human samples in epidemiological studies to assess the prevalence of human exposure to parabens.

Key Words : Paraben, Human urine, Triple quadrupole mass spectrometry, Simultaneous analysis

Introduction

Parabens are a group of alkyl (*e.g.*, methyl, ethyl, propyl, and butyl) esters of *p*-hydroxybenzoic acid shown in Figure 1. They have been used widely as antimicrobial preservatives, especially against molds and yeast, in cosmetic products, drugs, and processed foods and beverages for over half a century.[1] When the antimicrobial activity of parabens increases, water solubility decreases with the length of the alkyl chain.[2] Therefore, methyl and propyl parabens are the most extensively used in those fields.[3]

In our daily life, we are highly exposed to parabens because of their widespread usage. They have many benefits: parabens generally are considered safe with low or no toxic effects, they have no taste or odor and are low-cost products.[3,4] The most commonly used parabens are methyl, ethyl, propyl, and butyl paraben, which often are used in combination with one another. Moreover, they increase the activity against microbial contamination in the host products.

Some parabens have been shown to have weak estrogenic effects *in vitro* and *in vivo*.[5-11] Furthermore, studies have shown effects on the male reproductive system, resulting in the reduction of testosterone levels and mature sperm counts in rats and mice after *in utero* exposure to some parabens.[12-15] Recently, it has been discussed whether parabens could have an adverse effect on testis mitochondrial function, followed by decreased reproductive potential.[16]

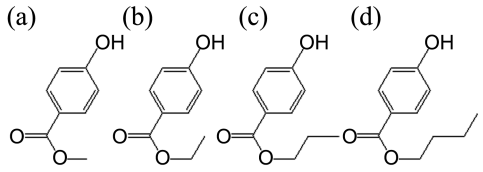

Figure 1. Structures of *p*-hydroxy benzoic acid, (a) Methyl paraben, (b) Ethyl paraben, (c) Propyl paraben, (d) Butyl paraben.

Parabens can be hydrolyzed to *p*-hydroxybenzoic acid, which can be conjugated before urinary excretion;[17,18] however, they can also be excreted as intact esters.[19] After oral exposure, parabens are metabolized by esterases in the intestine and liver, while some excretion occurs in bile and feces in addition to the urinary output.[20]

An estimation of paraben uptake can be found from human urinary measurements. Janjua *et al*. analyzed levels of free and conjugated paraben in urine from subjects by using a topical application.[21] A combination of free and conjugated (glucuronidate) butylparaben was detected, indicating a recovery of 0.3% and a maximal recovery of 0.9%. The total uptake was likely larger, as it is known that > 50% of parabens are eliminated in the unmeasured sulfate form. The systemic uptake of free and conjugated butylparaben is estimated to be up to 2%. In addition, a large percentage of the byproduct is present as free and conjugated *p*-hydroxybenzoic acid.

From the recent discussions and concerns about the possibility of the endocrine-disrupting effects of parabens, we have tried to develop a quantitative, analytical method with efficiency and precision for 4 parabens in human urine by using solid-phase extraction (SPE) and high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (HPLC-MS/MS). Existing reports on the

quantification of parabens in human samples have used gas chromatography-mass spectrometry (GC-MS) and on-line SPE-HPLC-MS/MS for quantitative analysis.[22-25] However, GC methods usually require a relatively large amount of sample, extensive sample cleanup, and a derivatization step, because of the relatively low volatility of these compounds. The on-line SPE-HPLC-MS/MS method requires a relatively short analysis time, but specific instrumentation is required, such as a switching pump module. In this study, we report the development and validation of a new method using HPLC coupled to a triple quadrupole mass spectrometer to measure the urinary concentration of 4 parabens. This method was successfully applied in a biomonitoring study of 4 parabens in human urine.

Experimental

Analytical Standards and Reagents. Analytical or HPLC grade acetonitrile, methanol (MeOH), and water were purchased from J. T. Baker (Center Valley, USA). Formic acid was purchased from Merck (USA). Methyl-, ethyl-, propyl-, and butyl-parabens were purchased from Fluka (USA). The internal standards methyl 4-hydroxybenzoate-2,3,5,6-d_4; ethyl 4-hydroxybenzoate-2,3,5,6-d_4; *n*-propyl 4-hydroxybenzoate-2,3,5,6-d_4; and *n*-butyl 4-hydroxybenzoate-2,3,5,6-d_4 were purchased from CDN ISOTOPES (USA). Ammonium acetate, 4-methylumbelliferyl sulfate, β-glucuronide, 4-methylumbelliferyl glucuronide, potassium chloride, sodium chloride, urea, citric acid, ascorbic acid, potassium phosphate, creatinine, sodium hydroxide, sodium bicarbonate, acetic acid, and sulfuric acid were purchased from Sigma (USA).

Preparation of Synthetic Urine. For preparation of synthetic urine, 3.8 g of potassium chloride, 8.5 g of sodium chloride, 24.5 g of urea, 1.03 g of citric acid, 0.34 g of ascorbic acid, 1.18 g of potassium phosphate, 1.4 g of creatinine, 0.64 g of sodium hydroxide, 0.47 g of sodium bicarbonate, and 0.28 mL of sulfuric acid were added in 500 mL of deionized water and stirred for 1 h. The synthetic urine was stored at −4 °C until further use.

Preparation of Standards and Quality Control Materials. The stock solutions of the analysis standards(methyl, ethyl, propyl, and butyl paraben) and the stable isotope-labeled internal standards (methyl 4-hydroxybenzoate-2,3,5,6-d_4, ethyl 4-hydroxybenzoate-2,3,5,6-d_4, *n*-propyl 4-hydroxybenzoate-2,3,5,6-d_4, and *n*-butyl 4-hydroxybenzoate-2,3,5,6-d_4) were generated by dissolving them in MeOH for final concentration 5000 μg/mL and were stored at −80 °C in a deep refrigerator until further use. Artificial urine was used to produce a standard solution for a calibration curve. The methyl paraben concentrations of the standard solutions for the calibration curve were set for 500, 250, 100, 50, 10, 1.0 ng/mL and ethyl paraben, propyl paraben, and butyl paraben concentrations were 50, 20, 10, 2, 0.5 ng/mL respectively. The stock solutions 100 μg of the analysis standards for the calibration curve were added to the artificial urine 900 μg/mL and the stock solutions 10 μg of the internal standards were added to it as well. Then, through the same preparation processes adjusted to human urine samples, the LC-MS/MS parameters for the analysis standards and internal standards were established. Quality control (QC) materials were prepared from synthetic urine. The urine was mixed uniformly and divided into 3 aliquots for QC low (QCL), QC medium (QCM), and QC high (QCH). The QCL, QCM, and QCH were enriched with different levels of native target compounds. The paraben concentrations in QCL were 3.0, 1.5, 0.6, and 1.5 ng/mL; in QCM were 125, 100, 100, and 100 ng/mL; and in QCH were 400, 200, 200, and 200 ng/mL, respectively. All QC materials were stored at −20 °C until further use.

Sample Preparation. We measured the total concentrations of methyl, ethyl, *n*-propyl, and butyl paraben by using a modification of a method used for quantifying other environmental phenols in urine.[25] Urine samples were thawed and vortexed before dividing into aliquots. A 1-mL aliquot of urine was mixed with 10 μL of internal standard solution, 10 μL of conjugation standard solution, and 50 μL of enzyme solution in a 2.0-mL tube. The deconjugation standard solution was prepared by dissolving 240 μg of 4-methylumbelliferyl glucuronide, 200 μg of 4-methylumbelliferyl sulfate, and 200 μg of $^{13}C_4$-4-methylumbelliferyl in 100 mL of MeOH. To raise the detection sensitivity, the enzyme solution was needed to deconjugate glucuronidated and sulfated parabens. The enzyme solution was prepared by dissolving 0.578 g of sulfatase (16,020 U/g solid) and 0.00307 g of β-glucuronidase (3,015,000 U/g solid) in 10 mL of 1 M ammonium acetate buffer solution (pH 5.0). Samples were incubated for 4 h at 37 °C, and then acidified with acetic acid. In this way, the detection sensitivity rose about 5-13 times more than free urine samples. The mixture was applied to Strata-X (33L, polymeric reversed phase, 30 mg/mL; Phenomenex, USA), which had been conditioned by sequential elution with 3 mL of acetonitrile and 3 mL of deionized water. Adsorbed parabens were rinsed with 5% MeOH, dried for 2 h *in vacuo* at room temperature, and then eluted with 1 mL of acetonitrile. Finally, the parabens were quantified by LC-MS/MS.

Table 1. Analyte retention time, precursor ion, product ion transition, and mass parameters for quantification of 4 parabens

Analyte	Retention time (min)	Precursor ion	Product ion	Declustering potential	Entrance potential	Collision energy	Colliosion cell exit
Methyl paraben	2.7	150.8	91.9	-62.0	-5.2	-28.9	-13.3
Ethyl paraben	4.1	164.9	91.9	-61.3	-7.0	-32.2	-14.9
Propyl paraben	6.0	178.9	91.9	-64.1	-5.5	-32.7	-14.3
Butyl paraben	7.9	193.0	91.9	-69.4	-3.8	-35.0	-13.9

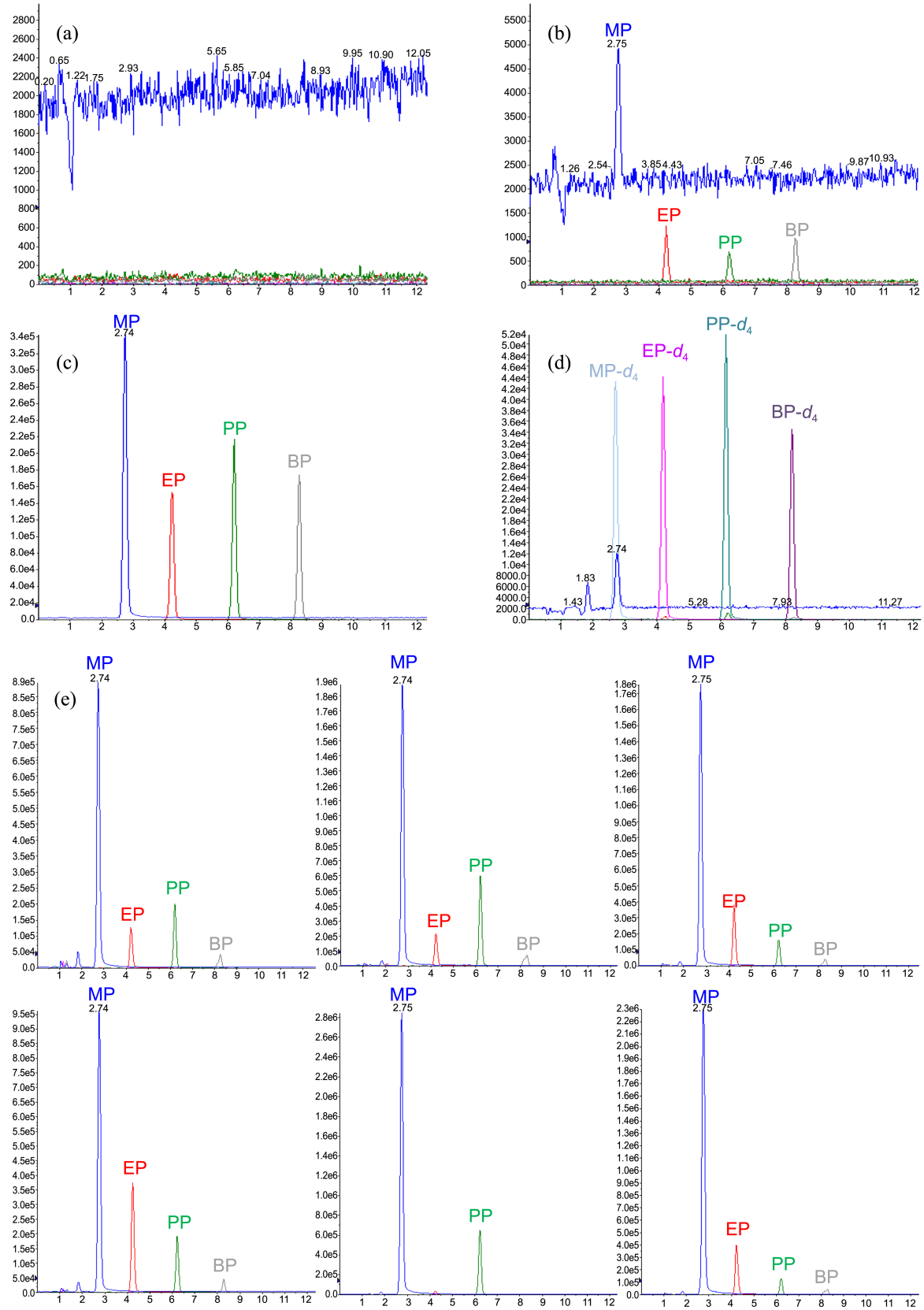

Figure 2. LC-MS/MS extracted ion chromatograms for the blank (a), LLOQ level of standard solution (b), ULOQ level of standard solution (c), internal standard solution (d), and 6 human urine samples (e). Blue line shows methyl paraben (MP), red line shows ethyl paraben (EP), green line shows propyl paraben (PP), gray line shows butyl paraben (BP), sky-blue line shows methyl 4-hydroxybenzoate-2,3,5,6-d_4 (MP-d_4), pink line shows ethyl 4-hydroxybenzoate-2,3,5,6-d_4 (EP-d_4), bluish green line shows *n*-propyl 4-hydroxybenzoate-2,3,5,6-d_4 (PP-d_4), and violet line shows *n*-butyl 4-hydroxybenzoate-2,3,5,6-d_4 (BP-d_4).

Instrument and Analytical Conditions. The chromatographic separation was carried out on a Synergi 4U Fusion-RP (75 × 2.0 mm, 80A; Phenomenex). The LC-MS/MS analysis was achieved with an Agilent 1200 series HPLC system (Palo Alto, CA, USA) connected to a G1312A binary pump, G1379B degasser, 1367B autosampler, 1336A column oven, and G1315D photo-diode-array detector, which was connected to an API 4000 (triple quadrupole mass spectrometer; Applied Biosystems, USA). A triple quadrupole mass spectrometer (API 4000 QTrap; SCIEX Toronto, Canada) with electrospray ionization (ESI) was used for negative ionization. Multiple reaction monitoring transitions (MRMs) were created for the analytes and internal standards. Data acquisition was performed using Analyst™ 1.4.2 software (Applied Biosystems, USA).

Results and Discussion

Analytical Condition Optimization. We initially tried to detect the ionized form of methyl, ethyl, propyl, and butyl paraben by HPLC coupled with triple quadrupole mass spectrometry. We used synthetic urine made in our laboratory, since the commercial urine being used by us contained parabens. The chromatographic separation was carried out on a reverse-phase Synergi 4U Fusion-RP column (75 × 2.0 mm, 80A; Phenomenex, USA). The mobile phase elution was as follows: 0.1% acetic acid in water (A) and 0.1% acetic acid with acetonitrile (B) with 30% solvent B gradient from 0-10 min, 30-90% solvent B for 10-13 min, 90% solvent B for 13-16 min, 90-30% solvent B for 16-16.1 min, and 30% solvent B for 16.1-22 min. The flow rate was 300 μL/min and the injection volume was 5 μL with a 35 °C column oven temperature. The LC-MS/MS analysis was achieved in negative ion ESI mode. The ESI settings were as follows: collision gas-20, curtain gas-20, ion source gas 1-30, ion source gas 2-30, and ion spray voltage of −4000. Unit resolution was used for both Q1 and Q3 quadrupoles. Ionization parameters and collision cell parameters were optimized separately for each analyte listed in Table 1.

Analysis Method Validation. The specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, and recovery of the method were determined by validation guidelines of Korea Food and Drug Administration (KFDA) on biological samples.

Specificity of 4 Parabens. The specificity is the capability to distinguish among mixed chemicals. The specificity of 4 parabens was evaluated by the processed blank sample, standard solution containing parabens as LLOQ (lower limit of quantification) and ULOQ (upper limit of quantification) for setting LOQ, internal standard solution in artificial urine, and 6 human urine samples to meet the validation guidelines of KFDA. The specificity of each paraben was clearly identified as shown in Figure 2. As a result, this method was considered to be extremely suitable for a simultaneous analysis of 4 parabens.

Linearity. Calibration curves were obtained from the standards spiked with synthetic urine. The calibration range used for the methyl paraben analysis was 1.0-500 ng/mL. The calibration curve was linear across the calibration range without special weighting or curve treatment and the equation was y = 55079x + 179745. The calibration determination (R^2) was 0.9995. The calibration range used for the ethyl, propyl, and butyl paraben analyses were 0.5-250 ng/mL and the calibration curve was linear across the calibration range. The equation for ethyl paraben was y = 47910x + 77194 and the calibration coefficient (R^2) was 0.9992. The equation for propyl paraben was y = 66368x + 62187 and the calibration coefficient (R^2) was 0.9995. The equation for butyl paraben was y = 58389x + 47411 and the calibration coefficient (R^2) was 0.9996. These results demonstrated adequate linearity in the stated concentration range.

Limit of Detection and Limit Of Quantification. LOD and LOQ were calculated as $3S_o$ and $10S_o$ where S_o is the standard deviation as the concentration approaches zero. S_o was determined from 5 repeated measurements of low-level standards prepared in synthetic urine. The calculated LODs ranged from 0.08 ng/mL to 0.30 ng/mL and the calculated LOQs ranged from 0.2 ng/mL to 1.0 ng/mL, as shown in Table 2. Compared with Centers for Disease Control and Prevention (CDC) in USA, a specialized institute for human

Table 2. Limit of detection and limit of quantification levels of 4 parabens

Analyte	LOD (ng/mL)	LOQ (ng/mL)
Methyl paraben	0.30	1.0
Ethyl paraben	0.18	0.5
Propyl paraben	0.08	0.2
Butyl paraben	0.16	0.5

Table 3. Accuracy (%)[a] at 3 concentrations of QC samples (5 replicates daily on 3 different days)

Analytes	Concentration (ng/mL)	Intrabatch (n = 5) (%)	Interbatch (n = 3) (%)
Methyl paraben	3	98.8	97.3
	125	112.4	98.4
	400	108.9	105.4
	AVE	106.7 ± 7.1	100.4 ± 4.3
Ethyl paraben	1.5	99.4	96.7
	100	106.1	95.3
	200	110.0	96.0
	AVE	105.2 ± 5.4	96.2 ± 0.7
Propyl paraben	0.6	101.5	96.3
	100	106.2	92.2
	200	103.0	94.7
	AVE	103.6 ± 2.4	94.4 ± 2.0
Butyl paraben	1.5	104.1	102.0
	100	107.6	96.8
	200	93.4	101.5
	AVE	101.7 ± 7.4	100.1 ± 2.9

[a]Accuracy = Measured concentration/Spiked concentration

sample analysis, in that LODs of CDC ranged from 0.2 ng/mL to 1.0 ng/mL, our analytical method was considered to be much better than others.

Accuracy and Precision. The intrabatch and interbatch accuracy at 3 concentrations is shown in Table 3. The method showed reproducibility with an intrabatch accuracy (expressed as percent of nominal value, %) ranging from 93.4% to 112.4% and an interbatch accuracy ranging from 92.2% to 102.0%. These results demonstrated the satisfactory accuracy of the present method.

The intrabatch and interbatch precision at the same concentration is shown in Table 4. The intrabatch precision (expressed as percent relative standard deviation, RSD, %) ranged from 1.0% to 2.3%, and the method showed reproducibility with interbatch precision ranging from 1.3% to 9.6%. These results demonstrated the high precision of the present method.

Recovery. The method recovery was determined by comparing the peak areas obtained from the extracted samples by solid-phase extraction and the standard solution. The extraction recovery was evaluated by analyzing 5 replicates and is shown in Table 5, with all measured recovery values within the acceptable range.

Table 4. Precision (CV, %)[a] at 3 concentrations of QC samples (5 replicates daily on 3 different days)

Analytes	Concentration (ng/mL)	Intrabatch (n = 5) (%)	Interbatch (n = 3) (%)
Methyl paraben	3	1.8	7.3
	125	1.6	4.6
	400	2.1	2.7
	AVE	1.8 ± 0.2	4.9 ± 2.3
Ethyl paraben	1.5	0.9	6.2
	100	2.0	3.9
	200	1.0	3.9
	AVE	1.3 ± 0.6	4.7 ± 1.3
Propyl paraben	0.6	1.6	7.9
	100	2.2	9.0
	200	1.5	1.3
	AVE	1.8 ± 0.3	6.1 ± 4.1
Butyl paraben	1.5	1.7	9.6
	100	2.0	4.3
	200	2.3	6.5
	AVE	2.0 ± 0.3	6.8 ± 2.6

[a]CV = STDEV of measured concentration/Mean of measured concentration × 100

Table 5. Recovery rate of 4 parabens

Analytes	Concentration (ng/mL)	Recovery (%)	RSD (%)
Methyl paraben	3	99.8	6.1
	125	100.9	3.7
	400	100.7	4.9
	AVE	100.5 ± 0.6	
Ethyl paraben	1.5	97.1	10.6
	100	100.2	2.4
	200	99.9	0.9
	AVE	99.0 ± 1.7	
Propyl paraben	0.6	95.7	12.0
	100	101.0	2.2
	200	97.1	1.6
	AVE	97.9 ± 2.7	
Butyl paraben	1.5	102.0	8.8
	100	97.8	3.4
	200	98.4	6.2
	AVE	99.4 ± 2.3	

Conclusion

A simultaneous quantitative analytical method for 4 parabens in human urine was developed and validated by using LC-MS/MS connected to a triple quadrupole analyzer. This is the first method of its kind in Korea that showed successful results in selectivity ($R^2 > 0.999$), linearity (92.2-112.4%), accuracy (92.2-112.4%), precision (0.9-9.6% as CV), and recovery (95.7-102.0%). The LOQs for the 4 parabens were 1.0, 0.5, 0.2, and 0.5 ng/mL, respectively. Finally using this method, we successfully analyzed human urine samples (n = 1,021) and got a satisfactory result. This method is adequate enough to be used as a reference method and will be a useful tool in human biomonitoring and other studies associated with parabens.

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