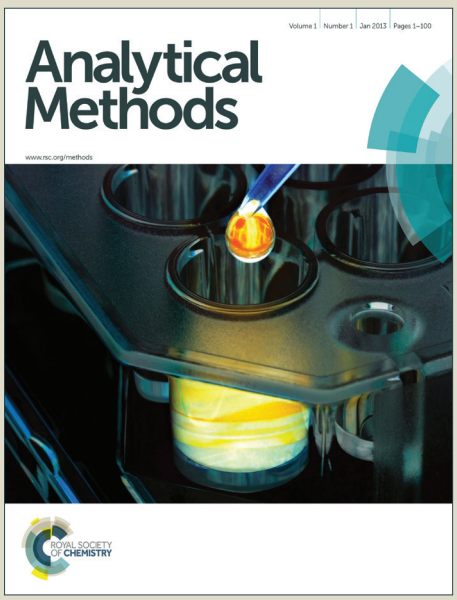
Analytical Methods

Accepted Manuscript



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Determination of parabens in human urine by liquid chromatography coupled with electrospray ionization tandem mass spectrometry

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Abstract

A simple and sensitive method was developed for the simultaneous determination of methyl, ethyl, *n*-propyl, *n*-butyl, and benzyl parabens in human urine by liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Enzymatic hydrolysis conditions were optimized to deconjugate the urinary parabens, glucuronide and sulfate conjugates. Solid phase extraction (SPE) was then used for sample clean-up. LC-ESI-MS/MS conditions for sample analysis were also optimized to achieve maximal sensitivity and accuracy. Parabens were finally separated on a C8 reversed phase column. Correlation coefficients (R^2) and recoveries ranged from 0.998 to 0.999 and 80.6% to 95.6%, respectively, and intra-day and inter-day precisions (relative standard deviation, RSD) were within 1.2−4.5% and 2.2−7.1%, respectively. Limits of detection (LODs) for methyl, ethyl, *n*-propyl, *n*-butyl, and benzyl parabens were 3, 3, 3, 3, and 1 pg, respectively. The optimized method was successfully used to determine parabens in urine samples from school students in southern China.

Keywords: Paraben, Urine, Solid phase extraction, LC- MS/MS

Introduction

Parabens are a group of alkyl esters of *p*-hydroxybenzoic acid (Fig.1). They are widely used as preservatives in cosmetic products, drugs, and processed foods for their broad-spectrum antimicrobial activities, low toxicity, low production cost, and worldwide regulatory acceptance.[1] Parabens exhibit higher antimicrobial activity, but lower water solubility as the length of the alkyl chain increases.[2] Therefore, methyl, ethyl, and *n*-propyl parabens are the most commonly used parabens.[3]

The use of parabens has caused great concern over the past decade due to their potential adverse effects in animals and humans. For example, studies have shown that parabens have weak estrogenic activity[4–6] and promote the proliferation of breast cancer cells (MCF-7 and ZR-75-1).[7–9] They have also been found in human breast tumor tissues and have been associated with the incidence of breast cancer, although the debate regarding this association is ongoing.[10] In addition, exposure to some parabens reduces sperm counts and testosterone levels in male rats and mice,[11–13] suggesting that parabens may be potentially harmful to the human reproductive system.

People are probably exposed to parabens in everyday life due to their widespread use. Parabens enter the human body mainly through inhalation, dermal contact and ingestion. Parabens can be hydrolyzed to *p*-hydroxybenzoic acid, which can be conjugated before urinary excretion,[1,14,15] but they can also be excreted as intact esters.[15] Since *p*-hydroxybenzoic acid and its conjugates in urine are not specific metabolites of all parabens and its conjugates, thus they are not optimal biomarkers of

exposure to parabens. In fact, the concentrations of total (free plus conjugated) urinary species of the parent parabens are often used as biomarkers for assessment of human paraben exposure.[16–19] When determining parabens in human urine, an enzymatic hydrolysis step is necessary to deconjugate the parabens, glucuronide and sulfate conjugates.

Current analytical methods for the determination of parabens in human samples mainly include high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS).[20-22] HPLC was often used in early studies, but is not in active use nowadays due to its poor sensitivity.[20] GC-MS has the advantage of high sensitivity, but it requires time-consuming derivatization of samples before instrumental analysis.[21] Ye et al. reported an on-line SPE-LC-MS/MS method for the determination of five parabens in human urine samples,[15] which has been used to determine human exposure to parabens in a US population.[16, 23–26] However, using this method, *n*-butyl and benzyl paraben are coeluted in the chromatogram, which may cause ion interference during MS/MS analysis and result in inaccurate quantification. In addition, the on-line SPE-LC-MS/MS method requires expensive specific instrument, hence it is unsuitable for general laboratory application. Recently, Lee et al. (2013) reported an off-line SPE and LC-MS/MS method for the determination of methyl, ethyl, *n*-propyl, and *n*-butyl parabens in human urine, however, benzyl paraben was not included.[27] Given that paraben levels in human urine samples are usually below nanograms per milliliter, a simple and sensitive method is needed for

quick and accurate assessment of paraben exposure in humans.

In this study, we developed a new method for the simultaneous determination of five parabens (methyl, ethyl, *n*-propyl, *n*-butyl, and benzyl) in human urine samples by liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Samples were pretreated with enzymatic hydrolysis followed by solid phase extraction (SPE) before analysis. Experimental conditions for sample pretreatment and analysis were optimized to achieve maximal sensitivity and accuracy. The optimized method was used to determine parabens in urine samples from school students in southern China.

Experimental

Chemicals and Solvents

Methyl, ethyl, *n*-propyl, *n*-butyl, and benzyl parabens were purchased from Dr. Ehrenstorfer (Augsburg, Germany). β-Glucuronidase (124400 U/mL) and sulfatase (36010 U/mL) were from Sigma (St. Louis, MO, USA). Formic acid, methanol, and acetonitrile were from Merck (Darmstadt, Germany). SPE cartridges including Oasis HLB, MCX, and MAX (500 mg, 6 mL) were purchased from Waters (Milford, MA, USA) and C18 cartridges (ENVI, 500 mg, 3 mL) were from Supelco (Bellefonte, PA, USA).

Sample hydrolysis and Extraction

During the urine collection, having provided an informed consent, each volunteer was interviewed by a trained recruiter using a questionnaire including the information about their name, gender, age, dietary habits, health status, and cigarette and alcohol

consumption. Paraben conjugates in urine samples were hydrolyzed by β-glucuronidase/sulfatase and samples were subsequently extracted by SPE. Urine samples (4 mL) were transferred to glass tubes. The pH of each sample was adjusted to 5.0 with 0.1 M HCl followed by the addition of 1.5 mL of 0.5 M acetate buffer (pH 5.0). β-Glucuronidase/sulfatase (20 µL) was added to each sample solution and samples were hydrolyzed by incubation with shaking at 37ºC for 16 h (overnight) in the dark. Hydrolyzed samples were subsequently subjected to SPE.

A C18 SPE cartridge was preconditioned with 5 mL methanol and then 10 mL deionized water. A hydrolyzed urine sample prepared as previously stated was loaded onto the preconditioned cartridge at a flow rate less than 1.0 mL/min. The cartridge was then washed with 4 mL of deionized water followed by 4 mL of 30% methanol to remove matrix interferences. When the cartridge was completely dry, parabens were eluted with 8 mL methanol and the eluate was concentrated to 400 µL with a gentle stream of nitrogen. The concentrated eluate was filtered through a 0.22-µm filter and stored at −20ºC until LC-MS/MS analysis.

Liquid chromatography

Liquid chromatography was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, a quaternary pump, and an autosampler. Samples (10 µL) were separated on a ZORBAX Eclipse Plus C8 column (150 × 4.6 mm, 5.0 µm, Agilent Technologies) using a gradient of methanol (A), acetonitrile (B), and water with 0.5‰ formic acid (C). The gradient program started with a composition of 60:10:30 A/B/C (V/V) for

10 min, changed to 58:10:32 A/B/C in 18 min, then to 60:40:0 A/B/C in 20 min, held for 5 min, and returned to the initial composition of 60:10:30 A/B/C in 3 min. The column was washed with 60:10:30 A/B/C for 12 min before the next injection. The follow rate was fixed at 0.32 mL/min and the column was run at 25ºC. The gradient profile details are shown in Table 1.

Mass spectrometry

Mass spectroscopic analysis of samples was performed on an API 4000 triple quadruple mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface. Electrospray ionization was operated in negative mode. Q1 and Q3 were both operated with unit resolution. The source temperature was 450°C and the ionization voltage was −4500 V. The parabens were quantified in multiple reactions monitoring (MRM) mode with a dwell time of 200 ms. Optimized parameters for MS/MS analysis of each analyte are listed in Table 2.

Results and Discussion

Optimization of sample hydrolysis and cleanup

Enzymatic hydrolysis

Parabens are excreted mainly as glucuronide and sulfate conjugates in urine, therefore a deconjugation step is necessary for the accurate determination of urinary parabens. Deconjugation efficiency mainly depends on the type and amount of enzymes used and the time and temperature of the hydrolytic reaction. Given that deconjugation is most effective with β-glucuronidase/sulfatase from *Helix pomatia* and at a reaction

temperature of 37°C,[15, 28] we optimized only the amount of enzyme and time of reaction for paraben deconjugation.

In general, we found that the hydrolysis rate increased with increasing amount of enzyme. To determine the optimum amount of enzyme, 4 mL pooled urine samples were incubated with 5, 10, 20, 30, and 40 μL of β-glucuronidase/sulfatase, respectively, and the hydrolyzed samples were subjected to LC-MS/MS analysis as previously stated. Fig.2 showed the optimization of enzymatic time and enzyme amount. The results (Fig.2 a) indicated that 20 μL of β-glucuronidase/sulfatase was sufficient to deconjugate paraben conjugates. To determine the optimal hydrolysis time, 4 mL pooled urine samples were incubated with 20 μL of β-glucuronidase/sulfatase for 1, 2, 4, 8, 12, and 16 h, respectively, and hydrolyzed samples were subjected to LC-MS/MS analysis as previously stated. The results (Fig.2 b) indicated that maximal deconjugation was achieved within 4 h of enzyme incubation for all parabens, and paraben levels detected remained stable for up to 16 h of enzyme incubation. Considering the variations in individual urine samples, we chose to perform sample deconjugation by incubating with 20 μL of β-glucuronidase/sulfatase for 16 h (overnight) to ensure complete hydrolysis of parabens in all samples.

SPE cleanup

Sorbents in SPE cartridges may affect the recoveries of target analytes. Thus, it is crucial to use suitable SPE cartridges for effective extraction of target analytes with good recoveries. In previous studies, different SPE cartridges were used for the

cleanup of urinary parabens.[18, 27, 30] Therefore, we tested four types of SPE cartridges including HLB, MCX, MAX, and C18 cartridges for the preliminary experiment. The C18 cartridge (ENVI, 500 mg, 3 mL) was finally selected for subsequent experiments due to its relatively higher recoveries of parabens and lower commercial price.

Any given SPE cartridge may retain non-target matrix substances from urine samples, which may potentially interfere with LC-MS/MS analysis of target analytes. Water or aqueous methanol solutions are often used to remove such matrix substances in a SPE cleanup procedure. Usually, solutions containing lower concentrations of methanol produce better recoveries of analytes, but are less effective in removing matrix substances. Solutions containing higher concentrations of methanol are more effective in removing matrix substances, but may also elute some target analytes. In the present study, we performed the SPE cleanup based on our developed SPE procedure for the urinary hydrroxylated polyaromatic hydrocarbons.[31] We found that cleanup with 4 mL of deionized water followed by 4 mL of 30% methanol effectively removed interfering substances without compromising the recoveries of target parabens. Cleanup with 40% methanol resulted in decreased recoveries of methyl and ethyl parabens. Therefore, we chose to use water followed by 30% methanol for SPE cleanup in subsequent experiments.

Optimization of chromatographic resolution and ESI-MS/MS parameters

Optimization of chromatographic separation and sensitivity

To our knowledge, *n*-butyl and benzyl parabens have never been chromatographically separated with adequate resolution. *n*-Butyl and benzyl parabens were coeluted in the

chromatogram in a previous study,[15] resulting in inaccurate quantification of each analyte. To achieve better chromatographic separation of *n*-butyl and benzyl parabens, we tested analytical columns with different packing (C8, C18, and NH_2) and different length (15 and 25 cm). Our results indicated that the five parabens were separated with highest resolutions on a ZORBAX Eclipse Plus C8 column (150 × 4.6 mm, 5.0 μm, Agilent Technologies).

Chromatographic separation of analytes may be affected by mobile phase characteristics such as solvent polarity and buffer constituents, and elution conditions such as flow rate and gradient program. Methanol and water were used in a binary gradient program for chromatographic separation of parabens in a previous study.[15] In the present study, we tested various mobile phases composed of methanol, acetonitrile, water, and buffers and found that the best analyte separation was achieved using a mobile phase composed of methanol, acetonitrile, and 0.5‰ formic acid in water (Table 1). Compared with methanol, acetonitrile enhanced the sensitivity of detection by making the analyte peaks narrower. A mobile phase of 70% organic solvents was used at the initial phase of elution to shorten retention times of parabens. Fig.3 shows a chromatogram of the five paraben standards at a concentration of 5.0 μg/L. Retention times of methyl, ethyl, *n*-propyl, *n*-butyl, and benzyl parabens were 5.95, 7.09, 9.50, 12.30, and 13.12 min, respectively. In particular, *n*-butyl and benzyl parabens were well separated.

In the ESI-MS/MS analysis, as ionization efficiency is affected by ionic strength of the mobile phase during the ESI process, we tested mobile phases containing certain

additives speculated to enhance signal response.[29] The mobile phases tested included 5 mM ammonium acetate in water, 0.5‰ formic acid in water, and 0.5‰ acetic acid in water. Our results indicated that 0.5‰ formic acid in water provided the most stable response and was thereby chosen as the mobile phase for ESI-MS/MS analysis.

Optimization of MS/MS parameters

Table 2 shows the optimized parameters for MS/MS analysis of the five parabens. Parameters including spray voltage, source temperature, collision gas (CAD), curtain gas (CUR), ion source gas 1 (GS_1), ion source gas 2 (GS_2), declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were the same for all five parabens in the present study. Compared with previously reported optimal parameters for MS/MS analysis,[15, 22] we found that optimal conditions for MS or MS/MS analysis may be different on different instruments.

Identification of parent ions and fragment ions was critical for analyte quantification. By Q1 scan in the range of m/z 50−300, m/z 151.1, m/z 165.1, m/z 179.1, m/z 193.1, and m/z 227.1 were identified as parent ions $[M\text{-}H]^{-1}$ for methyl, ethyl, *n*-propyl, *n*-butyl, and benzyl parabens, respectively. m/z 135.9 and m/z 91.8 were identified as fragment ions for all five parabens (Fig.4). According to the molecular structures of parabens, fragment ion m/z 135.9 was formed by neutral loss of the alkyl group from the parent ion $[M\text{-}H]^{-1}$ and fragment ion m/z 91.8 was formed by neutral loss of CO_2 (44) from fragment ion m/z 135.9. In the MS spectra of all five parabens, fragment ion m/z 91.8 showed higher intensities than fragment ion m/z 135.9. Therefore,

fragment ion m/z 91.8 was selected as the daughter ion for analyte quantification. These parameters were also consistent with those reported by González-Mariño et al. [30]

Method evaluation and application

The LC-ESI-MS/MS method for parabens quantification was evaluated under optimized conditions. Calibration curves were obtained using standard solutions of the five parabens over a concentration range of 1.0−500.0 μg/L. Correlation coefficients (R^2) of the five calibration curves ranged from 0.998 to 0.999, demonstrating excellent linearity. Recoveries were determined at three concentration levels (3.2, 32, and 80 ng) by spiking five parabens standards into urine samples. Recoveries of parabens at 3.2, 32.0, and 80.0 ng were 80.6−89.6%, 80.6−92.8%, and 88.3−95.6% (n = 5), respectively.

The precision of the method was investigated by repeated analysis of standard solution at different concentrations (5.0, 25.0, and 100.0 μg/L). Intra-day precision was assessed by the analysis of standard solution six times within a single day and inter-day precision was determined by analyzing standard solution once a day for five consecutive days. Relative standard deviations (RSD) determined were 1.2−4.5% for intra-day analysis and 2.2−7.1% for inter-day analysis.

Limits of detection (LODs), defined as signal levels with a signal-to-noise (S/N) ratio of 3:1, were calculated to be 3, 3, 3, 3, and 1 pg for methyl, ethyl, *n*-propyl, *n*-butyl, and benzyl parabens, respectively. Compared with previous studies, our method was more sensitive. Ye et al. reported LODs of 13, 10, 18, 10, and 10 pg for methyl, ethyl,

n-propyl, *n*-butyl, and benzyl parabens, respectively, in a method using atmospheric pressure chemical ionization (APCI) mode.[15]

The optimized method was used to determine parabens in ten urine samples collected from students in an elementary school in the Province of Guangdong in southern China. All samples showed detectable levels of methyl, ethyl, *n*-propyl, and *n*-butyl parabens. Benzyl paraben, however, was only detected in one sample. The test results are summarized in Table 3. Median concentrations determined were 0.9, 2.0, and 0.4 μg/L for methyl, ethyl, and *n*-propyl parabens, respectively. Levels of *n*-butyl and benzyl parabens were at least one order lower than those of methyl, ethyl, and *n*-propyl parabens, likely due to more frequent use of methyl, ethyl, and *n*-propyl parabens as preservatives. Interestingly, median urine levels of methyl and *n*-propyl parabens in Chinese students determined in the present study were much lower than those in a US population according to a report by the US Centers for Disease Control and Prevention (CDC). The US CDC (2006) reported that the median urine concentrations of methyl, ethyl, and *n*-propyl parabens in the US population were 43.9, 1.0, and 9.1 μg/L, respectively.[16] The composition of urinary parabens was also quite different between the Chinese students tested in this study and the general US population. Methyl and *n*-propyl parabens were the main parabens found in the US population, while ethyl paraben was the main paraben found in Chinese students in the present study.

Conclusion

A simple and sensitive method was developed for the determination of five parabens

in human urine by SPE-LC-MS/MS. Urine samples were enzymatically hydrolyzed and concentrated by SPE before being subjected to LC-MS/MS analysis. The experimental procedures including enzymatic hydrolysis, SPE, chromatographic separation, and MS/MS analysis were optimized for sensitive and accurate analyte determination. Five parabens were adequately separated under optimized conditions. To our knowledge, this is the first report on adequate chromatographic separation of *n*-butyl and benzyl parabens in human urine samples.

The method developed showed excellent linearity with good recovery of all paraben analytes. In addition, small intra-day and inter-day variations demonstrated the reproducibility of the method. The method was successfully used to determine parabens in urine samples from school students in southern China.

Acknowledgements

This study was supported by the National Instrumentation Program (No. 2011YQ170067) and the National Natural Science Foundation of China (No. 41303094). This is contribution no. IS-1900 from GIGCAS.

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Table 1 Gradient mobile phases program for the separation of five parabens

Time (min)	Methanol	Acetonitrile	Water (0.5‰ formic acid)
0	60%	10%	30%
10	60%	10%	30%
18	58%	10%	32%
20	60%	40%	0%
25	60%	40%	0%
28	60%	10%	30%
40	60%	10%	30%

Table 2 Optimized MS/MS parameters for the determination of five parabens

Parameter	Optimized value
Source temperature, TEM (^{o}C)	450
Ionization voltage (V)	4500
Ion source (GS1) settings	50
Ion source (GS2) settings	60
Curtain gas settings	30
CAD gas settings	10
Declustering potential (V)	-50
Entrance potential (V)	-6
Collision energy (V)	-50
Collision cell exit potential (V)	-5

Table 3 Method evaluation and its application in urine samples from students in Southern China

Compounds	Intra-day precision (RSD, %, n=6)			Inter-day precision (RSD, %, n=5)			Recovery (%, n=5)			Urinary parabens in students (μg/L, n=10)		
	Concentration level (ug/L)			Concentration level (ug/L)			Spiked amount (ng)			Mean	Median	Range
	5	25	100	5	25	100	3.2	32	80			
MeP	2.5	3.0	4.5	2.4	2.9	5.6	80.6	88.5	95.6	7.3	0.9	0.0-31.0
EthP	2.5	4.5	2.9	2.2	5.0	6.9	89.6	92.8	93.7	5.3	2.0	0.2-25.8
n-ProP	4.5	4.3	2.5	5.4	3.1	7.1	84.2	80.6	88.4	3.0	0.4	0.1-23.1
n-ButP	4.9	4.4	3.3	4.4	5.2	4.8	86.7	83.5	89.1	0.06	0.04	0.01-0.20
BeP	2.1	1.2	2.9	5.6	6.0	3.0	87.7	89.9	88.3	0.0003	0.00	0-0.0003

MeP: methyl paraben; EthP: ethyl paraben; *n*-ProP: *n*-propyl paraben; *n*-ButP: *n*-butyl paraben; BeP: benzyl paraben;

RSD: relative standard deviations; LODs: limits of detection.