



# Accurate analysis of parabens in human urine using isotope-dilution ultrahigh-performance liquid chromatography-high resolution mass spectrometry

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## ARTICLE INFO

### Article history:

Received 27 November 2017

Received in revised form

18 December 2017

Accepted 19 December 2017

Available online 24 December 2017

### Keywords:

Parabens

Isotope-dilution UHPLC-HRMS

Urine sample analysis

## ABSTRACT

An analytical method that utilizes isotope-dilution ultrahigh-performance liquid chromatography coupled with hybrid quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS or called UHPLC-HRMS) was developed, and validated to be highly precise and accurate for the detection of nine parabens (methyl-, ethyl-, propyl-, isopropyl-, butyl-, isobutyl-, pentyl-, hexyl-, and benzyl-parabens) in human urine samples. After sample preparation by ultrasound-assisted emulsification microextraction (USAEME), the extract was directly injected into UHPLC-HRMS. By using negative electrospray ionization in the multiple reaction monitoring (MRM) mode and measuring the peak area ratios of both the natural and the labeled-analogues in the samples and calibration standards, the target analytes could be accurately identified and quantified. Another use for the labeled-analogues was to correct for systematic errors associated with the analysis, such as the matrix effect and other variations. The limits of quantitation (LOQs) were ranging from 0.3 to 0.6 ng/mL. High precisions for both repeatability and reproducibility were obtained ranging from 1 to 8%. High trueness (mean extraction recovery, or called accuracy) ranged from 93 to 107% on two concentration levels. According to preliminary results, the total concentrations of four most detected parabens (methyl-, ethyl-, propyl- and butyl-) ranged from 0.5 to 79.1 ng/mL in male urine samples, and from 17 to 237 ng/mL in female urine samples. Interestingly, two infrequently detected pentyl- and hexyl-parabens were found in one of the male samples in this study.

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## 1. Introduction

On a daily basis, consumers in modern society are exposed to a variety of chemicals through dermal application of cosmetic or personal care products, and ingestion of processed foods or pharmaceutical products. One such class of chemicals is parabens, esters of *p*-hydroxybenzoic acid, which are commonly applied as antimicrobial agents and preservatives in above mentioned products due to their low toxicities and capabilities against different populations of microorganisms by various mechanisms [1]. Although parabens have been approved and commonly employed as preservatives in various industries, there is emerging observation that they interfere with endocrine system processes – specifically, some of parabens might increase women's risk of developing breast cancer [2–5]. Recently, a spectrum of parabens have been detected in human

body fluid, including blood, serum, breast milk, and human urine, which indicate that humans have been exposed to paraben residues via dermal contact of cosmetic and personal care products, or ingestion of processed food [6–15]. Although paraben residues had previously been detected in various human specimens, few analytical methods for the simultaneous determination of various and differently sized ester side chain (from methyl- to hexyl-, including two pairs of isomers, and benzyl-) have, to date, been reported [13].

The method developed in this study is a variation of isotope dilution mass spectrometry (IDMS), which is valuable for its high precision, excellent accuracy, and definable values. As of now, IDMS has been applied to determine various organic chemicals in food, human urine, infant formula, plasma, and red blood cells [16–21]. As a continuation of our work to analyze the presence of parabens residues in human specimen, and what their presences might mean for health, nine parabens were selected in the development of the method based on ultrasound-assisted emulsification microextraction (USAEME) coupled with isotope-dilution UHPLC-HRMS. Nine parabens were used in the process of developing and validating

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this method, and their specific characteristics (names, structures, and log octanol-water partition coefficients ( $\log K_{ow}$ )) are shown in Table S1. Moreover, the precision and trueness of the developed method were examined, and its applicability in the detection of low to trace-levels of target parabens in the human urine samples was also demonstrated.

## 2. Experimental

### 2.1. Chemicals and reagents

All chemicals and solvents used in this study were purchased in the highest available purity from Merck (Darmstadt, Germany), Mallinckrodt Baker (Phillipsburg, NJ, USA), and Sigma-Aldrich (St. Louis, MO, USA). The analytical standards: methyl-paraben (MeP), ethyl-paraben (EtP), propyl-paraben (PrP), isopropyl-paraben (iPrP), butyl-paraben (BuP), isobutyl-paraben (iBuP), benzyl-paraben (BzP), pentyl-paraben (PeP) were obtained from Alfa Aesar (Lancashire, UK), and hexyl-paraben (HxP) was from TCI Ltd. (Tokyo, Japan), purities of all compounds were >98%. Deuterated standards:  $d_4$ -methyl-,  $d_5$ -ethyl-,  $d_7$ -propyl-,  $d_7$ -isopropyl-,  $d_9$ -butyl-,  $d_9$ -isobutyl-,  $d_4$ -benzyl-,  $d_4$ -pentyl- and  $d_4$ -hexyl-paraben were purchased from Toronto Research Chemicals (Ontario, Canada). Stock solutions of individual analytes (1.0 mg/mL) were prepared in methanol. The mixture of analytes for working standards and sample fortification were prepared daily from the stock solution by appropriate dilution. All stock solutions and mixtures were stored in a refrigerator at 4 °C.  $\beta$ -Glucuronidase (986400 units/g-solid) and *H. pomatia* sulfatase (20230 units/g-solid) were obtained from Sigma-Aldrich.

### 2.2. Sample collection

The collection of human urine used as samples came from 18 healthy volunteers (8 males and 10 females) within our department, their ages ranging from 22 to 28 years old. To avoid contamination, the volunteers were asked to wash their hands with fresh water (not using soaps or personal care products) before sampling. The samples were collected in sterilized polyethylene bottles. In addition, deionized water (5 mL) in separated sterilized polyethylene bottle was used as the field blank during each sample collection. Prior to their use, all samples were stored at -20 °C. Since paraben preservatives are ubiquitous, to eliminate contamination, all glassware used were submerged in a solution of 5% (w/w) sodium hydroxide in ethanol for at least 12 h. They were then cleaned and further washed using deionized water, ethanol and acetone before left out to dryness, and followed by heating at 250 °C overnight. After performing these procedures, chemical background of the target compounds was detected neither in glassware nor in the field blank samples by the UHPLC-HRMS analysis, as described below. To further control for potential sources of contamination, the use of personal care products, such as moisturizers (hand cream, body oils, etc.) and make-up by laboratory personnel was restricted.

### 2.3. USAEME procedure

The procedure used for USAEME is detailed in our previous report [22], and was done for the development of this method with minor modifications. An aliquot of urine sample 1.0-mL was transferred into a 2.0 mL conical tube, and deuterated standards were added in the sample (final concentration: 20 ng/mL each). The sample was gently mixed with 100  $\mu$ L of 1.0 M ammonium acetate buffer (pH 6.8) and 10  $\mu$ L of enzyme solution (10000 units/mL of  $\beta$ -glucuronidase and 2500 units/mL of sulfatase), and then incubated 2 h at 37 °C. After enzymatic de-conjugation, the USAEME was

employed as follows: the sample solution was first mixed with 0.1-g of sodium chloride, and immersed into an ultrasonic bath at 40 °C. Then, ethyl acetate 200- $\mu$ L (used as an extractant) was quickly injected into the urine sample. During ultrasonication of 1.0 min, a cloudy suspension formed, and contained miniscule droplets of the extractant. Phase separation was then accomplished using a centrifuge at 7000 rpm for 5 min. Afterward, the supernatant was collected, and set underneath a mild flow of nitrogen until evaporated to dryness. The residue was re-dissolved in a 38% (v/v) of acetonitrile solution, and UHPLC-HRMS analysis was done to the extract for final determination.

### 2.4. UHPLC-HRMS analysis

Chromatographic separation was performed on a Dionex Ulti-Mate 3000 UHPLC system consisting of a quaternary rapid separation pump with TCC-3000RS column thermostat and WPS-3000TRS autosampler (Thermo Fisher, Waltham MA, USA). The UHPLC column used in this study was an Acquity UPLC HSS T3 column (1.8- $\mu$ m, 2.1  $\times$  100 mm, Waters Corp., Milford MA, USA). The injection volume was 2.0- $\mu$ L, and the flow rate of the mobile phase was set at 0.6 mL/min. The mobile phase was a compilation of water (A) and acetonitrile (B), and at the gradients: 0.0 min 38% B, 3.0 min 41% B, 3.4 min 99% B, a 0.6-min hold, and then a reversion to initial conditions. The total running time was 4.0 min, plus a 3-min stabilization time.

A hybrid quadrupole time-of-flight mass spectrometry (Compact, Bruker Daltonik GmbH, Bremen, Germany) equipped Apollo II ion funnel electrospray ionization (ESI) source was used for detection. In negative ion mode, the quantitation of parabens and deuterated-analogue were put under the multiple reaction monitoring (MRM) mode. Each target analyte (including deuterated-analogue) was detected by one parent ion and one product ion, and the mass transitions for each analyte, and their corresponding optimized MS/MS conditions, are listed in Table 1. The cluster of sodium formate ions  $[(\text{NaHCOO})_n\text{HCOO}]^-$ ,  $n = 1$  to 6, corresponding to the ions of  $m/z$  112.9856, 180.9731, 248.9605, 316.9479, 384.9353 and 452.9227 were used as in-run mass correction ions for high resolution, which infused via a syringe pump. Capillary voltage was set to 3500 V. Nebulizer pressure was 3.5 bar, and dry gas flow was delivered at 10 L/min. Nitrogen was used as the collision gas.

### 2.5. Method evaluation

The above mentioned method, after development, was evaluated using the spiked urine of male #1 as the quality control (QC) sample, and the standard solutions at two concentration levels were spiked at the final concentrations of 5 ng/mL (QC low) and 50 ng/mL (QC high) for each analyte. Calibration standards were prepared in deionized water with deuterated standards at a concentration of 20 ng/mL [13]. For the real samples, quadratic calibration curves with  $1/x$  weighting were performed. The limits of detection (LODs) and limits of quantitation (LOQs), defined as signal/noise (S/N) ratios of 3 and 10, respectively, were determined through USAEME coupled with UHPLC-HRMS analytical procedures of the spiked real samples, as described in previous reports [23]. Using intra-day and inter-day analyses of the two concentration levels of spiked urine samples, precision and trueness were calculated. Repeatability (or called intra-day precision) came from the same day analysis of five spiked urine samples ( $n = 5$ ), whereas reproducibility (or called inter-day precision) was found on five successive days ( $n = 5$ ). The mean recovery of these spiked samples was calculated as the trueness, which was assumed that 100% of

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