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Bioanalysis of propylparaben and *p*-hydroxybenzoic acid, and their sulfate conjugates in rat plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

Two rugged liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods for the determination of propylparaben, its major metabolite, *p*-hydroxybenzoic acid (*p*HBA), and their sulfate conjugates have been developed and validated in citric acid-treated rat plasma. To prevent propylparaben being hydrolyzed to *p*HBA *ex vivo*, rat plasma was first treated with citric acid; then collected and processed at a reduced temperature (ice bath). Stable isotope labeled internal standards, d_4-propylparaben, $^{13}C_6$-*p*HBA, and the d_4-labeled internal standards of their sulfate conjugates were used in the methods. The analytes were extracted from the matrix using protein precipitation, followed by chromatographic separation on a Waters ACQUITY UPLC HSS T3 column. Quantification using negative ion electrospray was performed on a Sciex API 4000 mass spectrometer. The analytical ranges were established from 2.00 to 200 ng/mL for propylparaben, 50.0–5000 ng/mL for *p*HBA, 50.0–10,000 ng/mL for the sulfate conjugate of propylparaben (SPP) and 200–40,000 ng/mL for the sulfate conjugate of *p*HBA (SHBA). Inter- and intra-run precision for the quality control samples were less than 5.3% and 4.4% for all analytes; and the overall accuracy was within ±5.7% of the nominal values. The validated bioanalytical methods demonstrated excellent sensitivity, specificity, accuracy and precision and were successfully applied to a rat toxicology study under the regulations of Good Laboratory Practices (GLP). Strategies have been developed and applied toward overcoming the challenges related to analyte stability, and environmental and endogenous background.

1. Introduction

Parabens, esters of *p*-hydroxybenzoic acid (*p*HBA), are very effective antimicrobial agents and are widely used as preservatives in foods, cosmetics, toiletries and pharmaceuticals including pediatric formulations [1,2]. Their antimicrobial activity increases as the chain length of the ester group increases. Methyl- and propylparabens are the most extensively used for the above purposes [1]. After being absorbed, parabens are mainly hydrolyzed to *p*HBA, which is further metabolized to glycine, glucuronide and sulfate conjugates, and excreted in urine [3].

Humans are exposed to environmental parabens through food consumption, personal care, cosmetic application, and the use of medicines. The reported total amount of parabens consumed daily is estimated to be a total of 76 mg, which includes approximately 1 mg from food; 50 mg from personal care products/cosmetics; and 25 mg from drugs [2]. Parabens and their main metabolite, *p*HBA, have been reported to have estrogenic activity due to their structure similarity to alkylphenols. As suggested in published *in vitro* studies [4–6], the level of estrogenic response increased with the alkyl group size, with butyl ester showing the greatest activity, followed by propyl, ethyl and methyl. It was also reported that parabens' estrogenic activity could induce the growth of human breast cancer cells and influence the expression of estrogen-dependent genes [7–9]. A research study in rats from Oishi [10] also suggested that propylparaben might adversely affect the secretion of testosterone and the function of the male reproductive system. It was further reported that the main metabolite, *p*HBA, had estrogenic activity, including effects on vaginal cornification and uterotrophic activity in both immature and adult ovariectomized mice [4]. For these reasons, exposure of parabens in children has arisen as a matter of concern by regulatory bodies. A recent reflection paper from

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EMA [11] highlighted the importance of understanding the exposure of propylparaben in immature metabolic enzyme systems and necessity of determining maximal oral intake of propylparaben for children under 2 years of age. For the purpose of understanding the disposition of propylparabens *in vivo*, Bristol-Myers Squibb Co. has carried out a toxicological study, in accordance with the GLP regulations, to investigate the potential toxicity of propylparaben in juvenile rats when given orally once daily from postnatal days (PNDs) 4 through 90. To provide accurate information for total propylparaben exposure, validated bioanalytical assays to quantify propylparaben and its major metabolites (*p*HBA, SPP and SHBA) were needed.

Several methods have been developed to analyze parabens in different matrices and media. Gas chromatography–mass spectrometry has been used to measure parabens in formulations [12]. And more recently, high performance liquid chromatography (HPLC) coupled with chemiluminescence [13], UV [14] and mass spectrometry (MS) [15,16] were reported for paraben measurements in cosmetic products, food and biological samples. However, no LC–MS/MS methods are available for the simultaneously measurement of propylparaben and its major metabolites, *p*HBA and sulfate conjugates of propylparaben and *p*HBA, in rat plasma/blood.

Herein, we present the method development and validation of two LC–MS/MS methods to support the quantitation of propylparaben, *p*HBA; SPP and SHBA in rat plasma. Rat plasma is known to have significant *ex vivo* esterase activity [17] and propylparaben is known to be hydrolyzed by carboxyl esterase to *p*HBA [3]. Therefore, it was necessary to stabilize the analytes during sample collection, storage and processing by inhibiting the enzymatic hydrolysis process. A number of strategies, including addition of various esterase inhibitors and pH adjustment, have been reported [18–20] to inhibit the enzymatic hydrolysis process. In our methods, stability was controlled by adjusting plasma pH with citric acid, and performing sample collection and processing at a low temperature (on wet ice bath). We overcame other challenges including the environmental background for propylparaben and high endogenous levels of *p*HBA and its sulfate conjugate in the blank rat plasma. The established LLOQ was 2.00 ng/mL for propylparaben and 50.0 ng/mL for *p*HBA based on an extraction volume of 25 μL; the LLOQ was 50.0 ng/mL for sulfate of propylparaben and 200 ng/mL for sulfate of pHBA based on an extraction volume of 10 μL. These two assays demonstrated good linearity, selectivity, accuracy and precision for analytes under quantitation. The validated methods were successfully applied to support the toxicity study in juvenile rats and helped in developing an understanding of the potential toxicity of propylparaben when given orally in pediatric formulations.

2. Experimental

2.1. Chemicals and reagents

Reference standards for propylparaben and *p*HBA were obtained from U.S. Pharmacopeia Convention (Rockville, MD); n-propyl 4-hydroxybenzoate-2, 3, 5, 6-d_4 (d_4-propylparaben, stable labeled internal standard for propylparaben) was obtained from Medical Isotopes, Inc. (Pelham, NH); 4-hydroxybenzoic acid-(ring-$^{13}C_6$) ($^{13}C_6$-*p*HBA, stable labeled internal standard for *p*HBA) was obtained from Sigma–Aldrich (St. Louis, MO). Propylparaben sulfate ammonium salt, 4-benzoic acid sulfate dilithium salt, d_4-propylparaben sulfate ammonium salt (stable labeled internal standard for SPP) and d_4-4-benzoic acid sulfate dilthium salt (stable labeled internal standard for SHBA) were obtained from Chemtos (Austin, TX). The chemical structures for all four analytes are shown in Fig. 1. Citric acid (anhydrous) and HPLC grade

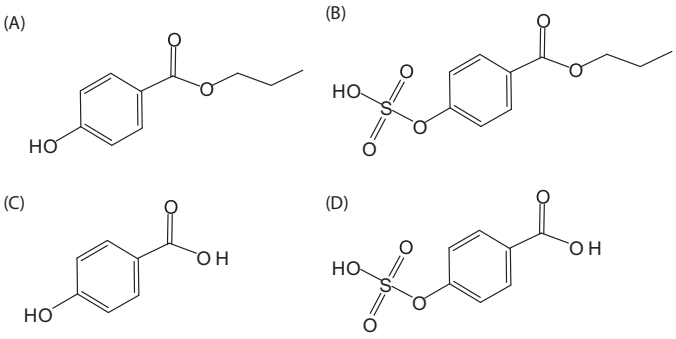

Fig. 1. Chemical structures of: (A) propylparaben; (B) sulfate of propylparaben; (C) *p*HBA; and (D) sulfate of *p*HBA.

acetonitrile were purchased from Sigma–Aldrich (St. Louis, MO). HPLC grade methanol and isopropanol (IPA) were purchased from J.T. Baker (Phillipsburg, NJ). Formic Acid (SupraPur grade) and acetic acid (Glacier, ACS grade) were purchased from EMD Chemicals (Gibbstown, NJ). Deionized water was generated in house using a NANOpure Diamond ultrapure water system from Barnstead International (Dubuque, IA). Rat plasma and rat whole blood (K_2EDTA) were obtained from Bioreclamation (Westbury, NY).

2.2. LC–MS/MS system

The LC system used was a Shimadzu Nexera system (Shimadzu, Kyoto, Japan). An ACQUITY UPLC HSS T3 column (2.1 × 50 mm, 1.8 μm) from Waters Corporation (Milford, MA) was used for chromatographic separation. The autosampler temperature was set at 5 °C and the LC column was maintained at 60 °C. The samples were analyzed on a triple quadrupole API 4000 mass spectrometer from AB Sciex (Foster City, CA) with a Turbo Ionspray interface. The system was controlled by Analyst v1.4.2 software.

2.3. LC–MS/MS conditions

The mobile phases were 0.5% acetic acid in water and 0.5% acetic acid in acetonitrile. A gradient elution with a total run time of 4.5 min was used for the separation of propylparaben and *p*HBA (referred to as Method 1 henceforth), the gradient started from 5% B and held for 0.2 min; increased to 20% B in 0.8 min; increased to 40% in 2.5 min; increased to 100% B in 0.01 min; held for 0.49 min; decreased to 5% B in 0.01 min; and held for 0.49 min. The total run time was 4.5 min for Method 1. For the separation of SPP and SHBA (referred to as Method 2 henceforth), the gradient started from 5% and held for 0.2 min; increased to 18% B in 1.3 min; increased to 30% in 4.5 min; increased to 100% B in 0.01 min; held for 0.49 min; decreased to 5% B in 0.01 min; and held for 0.49 min. The total run time was 7 min for Method 2. The flow rate was maintained at 0.8 mL/min for both methods. A volume of 40 μL was injected to the MS for the analysis for Method 1 and 10 μL for Method 2, respectively. The analytes were monitored using selective reaction monitoring (SRM) in negative ion electrospray mode. The SRM transitions and optimized mass spectrometer conditions are shown in Table 1.

2.4. Stock solution, calibration standards and quality control preparation

Separate stock solutions for standard and quality controls were prepared in 20:80 (v:v) DMSO and acetonitrile at the concentrations of 1.00 mg/mL for propylparaben, SPP and SHBA and