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Simultaneous quantification of acetaminophen and five acetaminophen metabolites in human plasma and urine by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry: Method validation and application to a neonatal pharmacokinetic study

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ABSTRACT

Drug metabolism plays a key role in acetaminophen (paracetamol)-induced hepatotoxicity, and quantification of acetaminophen metabolites provides critical information about factors influencing susceptibility to acetaminophen-induced hepatotoxicity in clinical and experimental settings. The aims of this study were to develop, validate, and apply high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC–ESI–MS/MS) methods for simultaneous quantification of acetaminophen, acetaminophen-glucuronide, acetaminophen-sulfate, acetaminophen-glutathione, acetaminophen-cysteine, and acetaminophen-*N*-acetylcysteine in small volumes of human plasma and urine. In the reported procedures, acetaminophen-d4 and acetaminophen-d3-sulfate were utilized as internal standards (IS). Analytes and IS were recovered from human plasma (10 μL) by protein precipitation with acetonitrile. Human urine (10 μL) was prepared by fortification with IS followed only by sample dilution. Calibration concentration ranges were tailored to literature values for each analyte in each biological matrix. Prepared samples from plasma and urine were analyzed under the same HPLC–ESI–MS/MS conditions, and chromatographic separation was achieved through use of an Agilent Poroshell 120 EC-C18 column with a 20-min run time per injected sample. The analytes could be accurately and precisely quantified over 2.0–3.5 orders of magnitude. Across both matrices, mean intra- and inter-assay accuracies ranged from 85% to 112%, and intra- and inter-assay imprecision did not exceed 15%. Validation experiments included tests for specificity, recovery and ionization efficiency, inter-individual variability in matrix effects, stock solution stability, and sample stability under a variety of storage and handling conditions (room temperature, freezer, freeze-thaw, and post-preparative). The utility and suitability of the reported procedures were illustrated by analysis of pharmacokinetic samples collected from neonates receiving intravenous acetaminophen.

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Abbreviations: gluc, glucuronide; sulf, sulfate; NAPQI, *N*-acetyl-*p*-benzoquinone imine; glut, glutathione; cys, cysteine; NAC, *N*-acetylcysteine; LC–MS/MS, liquid chromatography–tandem mass spectrometry; HPLC–ESI–MS/MS, high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry; QC, quality control; IS, internal standard(s); MRM, multiple reaction monitoring; LLOQ, lower limit of quantification; ULOQ, upper limit of quantification.

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1. Introduction

Acetaminophen (paracetamol) has been widely used for nearly a century and is currently one of the most commonly used medications in the United States [1–4]. Acetaminophen is an effective and well-tolerated analgesic and antipyretic agent when used as indicated [5–7]. At supratherapeutic doses, however, the drug has long been known to produce liver injury [8–11], and acetaminophen overdose is currently the leading cause of acute liver failure in the United States [12]. Consequently, acetaminophen is frequently utilized as a model hepatotoxicant [13–15], and studies of the precise mechanistic pathways that ultimately result in acetaminophen-induced liver injury are still underway [16].

Drug metabolism plays a key role in acetaminophen-induced hepatotoxicity (Fig. 1) [17,18]. Acetaminophen metabolism occurs primarily in the liver, where the drug undergoes glucuronidation and sulfation by UDP-glucuronosyltransferases and sulfotransferases, respectively. The non-toxic glucuronide (acetaminophen-gluc) and sulfate (acetaminophen-sulf) metabolites are efficiently excreted in the urine. Acetaminophen can also be oxidized by hepatic cytochrome P450 enzymes to form the reactive intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI). At therapeutic doses, only a small portion (5–15%) of acetaminophen is bioactivated to yield NAPQI. This electrophilic species can be detoxified by conjugation with glutathione, either non-enzymatically or with the aid of glutathione *S*-transferase enzymes. The acetaminophen-glutathione conjugate (acetaminophen-glut) undergoes rapid hydrolysis by gamma-glutamyl transpeptidase and dipeptidases to form acetaminophen-cysteine (acetaminophen-cys), and acetaminophen-cys is subsequently acetylated by *N*-acetyltransferases, thus producing acetaminophen-*N*-acetylcysteine (acetaminophen-NAC) [19,20]. Given a sufficiently high dose of acetaminophen, the glutathione detoxification pathway can be saturated by NAPQI, and excess electrophile will instead bind covalently to hepatic proteins [17,18]. Toxicity is thought to result from a combination of inactivation of critical hepatic proteins via NAPQI binding and oxidative stress [13,16].

Susceptibility to acetaminophen-induced hepatotoxicity is likely to be influenced by variability in the major acetaminophen metabolic pathways. Therefore, in both clinical and experimental settings, quantification of the major acetaminophen metabolites is essential to achieve a thorough understanding of factors affecting hepatotoxicity risk. In recent years, a number of liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods have been published for the sensitive and specific quantification of acetaminophen and metabolites in various human and rodent matrices [21–26]; however, most of these included only the parent drug and one or two metabolites as analytes [22–25]. One recently reported assay included acetaminophen, acetaminophen-gluc, acetaminophen-sulf, acetaminophen-glut, acetaminophen-cys, and acetaminophen-NAC, but the method required two 16-min analytical injections per sample, one for each ionization mode, in order to achieve adequate sensitivity. Additionally, the assay was validated for analysis of rat plasma, not human matrices [21].

We sought to develop and validate methods for simultaneous quantification of acetaminophen, acetaminophen-gluc, acetaminophen-sulf, acetaminophen-glut, acetaminophen-cys, and acetaminophen-NAC in human plasma and urine by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry (HPLC–ESI–MS/MS). Furthermore, we aimed to optimize the sensitivity of the assays so that the required sample volume could be minimized. This sample volume minimization was particularly important because the assays were intended for use in a neonatal pharmacokinetic study, an application where sample volumes are extremely limited.

Herein, we report novel procedures for simultaneous quantification of acetaminophen and five acetaminophen metabolites in human plasma and urine by HPLC–ESI–MS/MS. Details of the methods are provided along with comprehensive validation results. The utility and suitability of the assays are illustrated by a brief summary of the analysis of pharmacokinetic plasma and urine samples collected from neonates receiving intravenous acetaminophen.

2. Materials and methods

2.1. Materials

Analyte-free human plasma (sodium heparin; from individual donors) was obtained from BioChemed Services (Winchester, VA). Analyte-free human urine was obtained from acetaminophen-abstinent volunteers at the Center for Human Toxicology. Human urine for matrix stability experiments was obtained from a volunteer at the Center for Human Toxicology approximately 3 h after ingestion of 1 g acetaminophen (500-mg caplets, Kroger, Cincinnati, OH). The following reference standards and deuterated internal standards were obtained from Toronto Research Chemicals (Toronto, ON, Canada): acetaminophen (98%), 4-acetamidophenyl β-D-glucuronide sodium salt (98%), 4-acetaminophen sulfate potassium salt (98%), acetaminophen glutathione disodium salt (95%), 3-cysteinylacetaminophen trifluoroacetic acid salt (95%), 3-(*N*-acetyl-L-cystein-*S*-yl)-acetaminophen disodium salt (95%), acetaminophen-d4 (98% chemical purity, 99% isotopic purity), and 4-acetaminophen-d3 sulfate (acetaminophen-d3-sulf, 98% chemical purity, 99% isotopic purity). Acetaminophen (analytical standard) and ammonium acetate (≥98%) were obtained from Sigma–Aldrich (St. Louis, MO). Glacial acetic acid was obtained from Spectrum Chemicals (New Brunswick, NJ). Formic acid (88%) was obtained from Fisher Scientific (Pittsburgh, PA). LC–MS grade acetonitrile and methanol were obtained from Honeywell Burdick and Jackson (Morristown, NJ). Ultrapure water (18.2 MΩ) for preparation of aqueous solutions was obtained by passage of deionized water through a Milli-Q Gradient A10 filtration system equipped with a Q-Gard 2 purification pack (EMD Millipore, Billerica, MA). Silanized glassware was prepared by vapor-phase silanization with hexamethyldisilazane (Pierce, Rockford, IL) under vacuum in an oven at 250 °C for 2 h.

2.2. Authentic clinical samples for assay verification

Clinical samples were collected from subjects enrolled in an Institutional Review Board-approved study (Children's National Health System, Washington, DC) in which acetaminophen (Ofirmev, 10 mg/mL, Mallinckrodt Pharmaceuticals, Dublin, Ireland) was administered by 30-min intravenous infusions at 15 mg/kg/dose to neonates with a clinical indication for intravenous analgesia. Patients <28 weeks gestation received 5 doses at 12-h intervals; patients ≥28 weeks gestation received 7 doses at 8-h intervals. Pharmacokinetic samples were collected prior to the first acetaminophen dose and throughout the 3-day study period, up to 24 h after the final dose. Blood samples (0.2 mL) were obtained from indwelling arterial lines and collected in sodium heparin Vacutainer tubes (BD, Franklin Lakes, NJ). Blood samples were centrifuged at 4 °C for 10–15 min at 1500 × *g*. Plasma supernatants were transferred to cryovials and stored at −70 °C. Urine samples were collected from gel-free study diapers (Cuddle Buns Preemie diapers, Small Beginnings Inc., Hesperia, CA) and stored at −70 °C. Batches of de-identified study samples were shipped overnight on dry ice to the Center for Human Toxicology

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