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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.

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

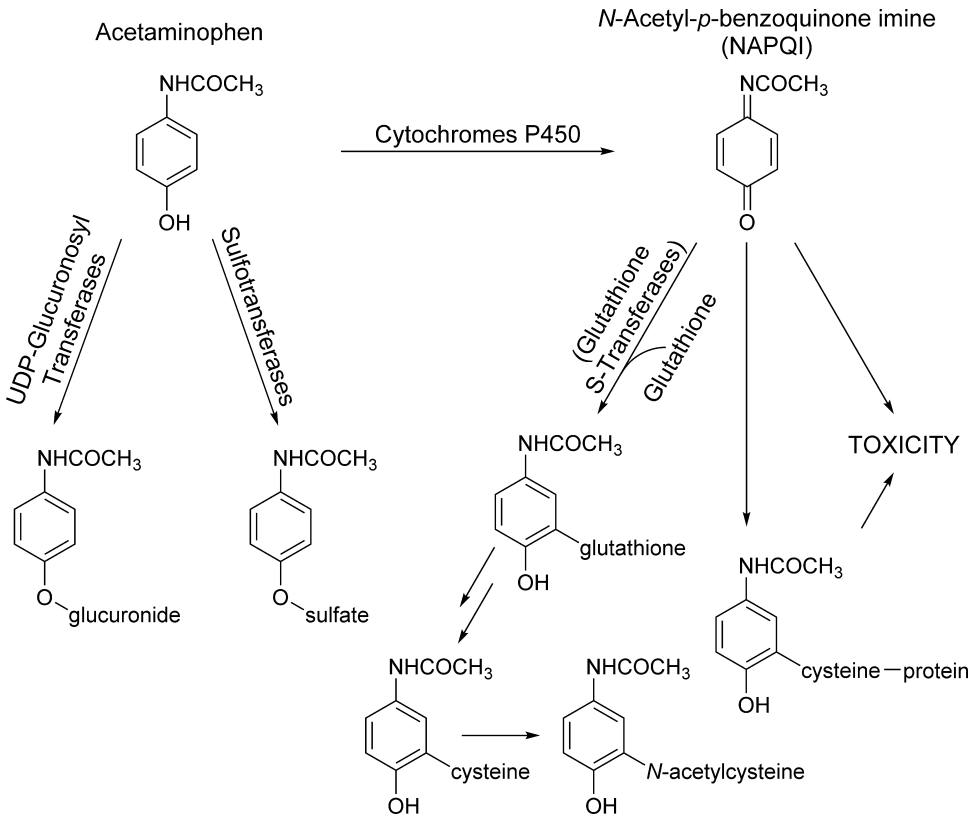

Fig. 1. Major acetaminophen metabolic pathways.

at the University of Utah and immediately stored at −80 °C until the time of preparation for analysis.

2.3. Calibrator, quality control (QC), and internal standard (IS) solutions

Individual stock solutions of analyte reference standards and IS were prepared at concentrations ranging from 0.1 to 1 mg/mL in methanol/water (1/1, v/v) using a Mettler Toledo XS3DU microbalance (Columbus, OH) and silanized class-A volumetric flasks. For reference standards obtained in salt form, all specified concentrations reflect the concentration of free analyte. Individual stock solutions were then pooled and diluted to prepare a combined working solution with 100 μg/mL acetaminophen, acetaminophen-gluc, and acetaminophen-sulf and 10.0 μg/mL acetaminophen-glut, acetaminophen-cys, and acetaminophen-NAC in methanol/water (1/1, v/v). Lower concentration working solutions were subsequently prepared by five ten-fold serial dilutions in methanol/water (1/1, v/v). Separate sets of analyte stock and working solutions were prepared for calibrator and QC applications. Acetaminophen reference material used for each set was obtained from two different chemical manufacturers. Metabolite reference standards of sufficient purity were generally not available from different manufacturers or lot numbers; however, calibrator and QC solution sets were prepared by different analysts. Individual IS stock solutions were pooled and diluted with water to prepare IS working solutions with the concentrations indicated in Sections 2.4.2 and 2.4.3. All stock and working solutions were stored at −20 °C in silanized glass tubes.

2.4. Sample preparation

2.4.1. Calibrator and QC samples

Prior to use for preparation of calibrator or QC samples, individual lots of biological matrix were prepared without IS, analyzed, and confirmed to be negative for all analytes and IS. Calibration standards and triplicate sets of QC samples were freshly prepared in silanized glass tubes for concurrent analysis with each validation or study sample batch. Analyte- and IS-free matrix (10 μL plasma or urine) was fortified with analyte working solutions to yield the nominal matrix concentrations provided in Table 1. Two additional analyte-free matrix samples accompanied each batch for preparation with and without IS.

2.4.2. Plasma sample preparation

Study samples were thawed at ambient temperature and gently mixed by vortexing before transfer of 10-μL aliquots to silanized glass tubes. To maintain equivalence in preparation and control for the addition of solvent that occurred during fortification of calibrator and QC samples with analyte working solution, methanol/water (1/1, v/v) was used to bring all samples to a total volume of 110 μL. Samples were then fortified with 10 μL of IS working solution containing 0.20 and 25 μg/mL of acetaminophen-d4 and acetaminophen-d3-sulf, respectively, in water. Acetonitrile (600 μL) was added, and each sample was vortex mixed for 30 s before 15 min of centrifugation at 1100 × *g*, ambient temperature in an IEC FL40 swing-out rotor centrifuge (Thermo Fisher Scientific Inc., Waltham, MA). Sample supernatants were transferred to a clean set of silanized glass tubes, placed in a 35 °C water bath, and evaporated to dryness under a 10–15 psi air stream in a TurboVap LV Evaporator (Zymark). Sample residues were reconstituted in 400 μL of 0.1% aqueous formic acid, vortex mixed for 30 s, and centrifuged for 5 min at 1100 × *g*, ambient temperature in the swing-out rotor centrifuge. From the top of each centrifuged

Table 1
Nominal calibrator and quality control concentrations.

Analyte	Matrix	Calibrator concentrations (μg/mL)	Quality control concentrations (μg/mL)			
			Level 1	Level 2	Level 3	Level 4
Acetaminophen, acetaminophen-gluc, acetaminophen-sulf	Plasma	0.050, 0.10, 0.25, 0.75, 1.0, 2.5, 7.5, 10, 50	0.15	0.80	8.0	40
Acetaminophen-glut	Plasma	0.0050[a], 0.010[a], 0.025, 0.075, 0.10, 0.25, 0.75, 1.0, 5.0	0.015[c]	0.080	0.80	4.0
Acetaminophen-cys	Plasma	0.0050[a], 0.010, 0.025, 0.075, 0.10, 0.25, 0.75, 1.0, 5.0	0.015	0.080	0.80	4.0
Acetaminophen-NAC	Plasma	0.0050[a], 0.010, 0.025, 0.075, 0.10, 0.25, 0.75, 1.0, 5.0[b]	0.015	0.080	0.80	4.0[c]
Acetaminophen	Urine	0.20, 1.0, 5.0, 10, 40, 70, 100, 400, 700, 1000	7.5	50	500	n/a
Acetaminophen-gluc, acetaminophen-sulf	Urine	0.20[a], 1.0, 5.0, 10, 40, 70, 100, 400, 700, 1000	7.5	50	500	n/a
Acetaminophen-glut, acetaminophen-cys, acetaminophen-NAC	Urine	0.020[a], 0.10, 0.50, 1.0, 4.0, 7.0, 10, 40, 70, 100	0.75	5.0	50	n/a

Note: n/a: Not applicable.
[a] Concentration present but excluded from analysis due to failure to meet LLOQ acceptance criteria.
[b] Concentration present but excluded from analysis due to non-linearity.
[c] Concentration present but excluded from analysis; concentration falls outside calibration range.

sample, 200 μL was carefully removed and transferred to a conical polypropylene autosampler vial.

2.4.3. Urine sample preparation

Study samples were thawed at ambient temperature and gently mixed by vortexing before transfer of 10-μL aliquots to silanized glass tubes. To maintain equivalence in preparation and control for the addition of solvent that occurred during fortification of calibrator and QC samples with analyte working solution, methanol/water (1/1, v/v) was used to bring all samples to a total volume of 110 μL. Samples were then fortified with 10 μL of IS working solution containing 10 and 100 μg/mL of acetaminophen-d4 and acetaminophen-d3-sulf, respectively, in water. Samples were diluted by addition of 380 μL of 0.1% aqueous formic acid followed by 20 s vortex mixing. To remove any solid particles that might be present, samples were centrifuged for 5 min at 1100 × *g*, ambient temperature in the swing-out rotor centrifuge. Sample supernatants (100 μL) were carefully transferred to a clean set of silanized glass tubes and diluted in an additional 300 μL of 0.1% aqueous formic acid. Following 20 s vortex mixing, 200 μL of each sample was transferred to a conical polypropylene autosampler vial.

2.5. HPLC–ESI–MS/MS analysis

HPLC–ESI–MS/MS was conducted on an Agilent 1260 Infinity HPLC system (inline solvent micro-degasser, binary LC pump, high-performance thermostatted autosampler, and 1290 Infinity thermostatted column compartment) interfaced with an Agilent 6460 triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). MassHunter Workstation software (Agilent Technologies, Santa Clara, CA) was used for instrument control, data acquisition, and ESI–MS/MS parameter optimization (version B.03.01) and for data analysis (version B.04.00).

Prepared samples were stored in the autosampler tray at 5 °C. Sample injection volumes ranged from 10 to 100 μL. Injection volumes within each batch did not vary, but adjustments were made over time as needed based on changes in instrument response (magnitude of chromatographic peak areas). Samples were injected in the following order: calibration standards (ascending concentrations), analyte-free samples with and without IS, QC set 1, approximately half of the validation/study samples, QC set 2, remaining validation/study samples, QC set 3. Between injections, the autosampler needle was washed with methanol/water (1/1, v/v). For the plasma assay only, the analytical system was equilibrated by injecting seven extra prepared matrix samples at the beginning of each batch. Chromatographic separation was achieved with an Agilent Poroshell 120 EC-C18 column (2.1 × 100 mm, 2.7 μm particle size, Agilent Technologies, Santa Clara, CA) maintained at 40 °C and using a gradient mobile phase consisting of 10 mM aqueous ammonium acetate, pH 3.5 (A) and methanol (B) at a flow rate of 0.25 mL/min. Mobile phase was maintained at 3% B for the first 6 min, increased linearly to 35% B over 3 min, maintained at 95% B for 3 min, decreased linearly to 3% B over 0.5 min, and then re-equilibrated at 3% B for 7.5 min, yielding a total run time of 20 min/injection. The MS diverter valve was only directed to the ion source during the anticipated retention time range for analytes and IS.

The mass spectrometer was operated in ESI + Agilent Jet Stream mode with multiple reaction monitoring (MRM). Acetaminophen-sulf and acetaminophen-d3-sulf were monitored in negative ionization mode; all other analytes and acetaminophen-d4 were monitored in positive ionization mode. Ultra-high-purity nitrogen was used for source and collision cell gas. The following settings were applied: 350 °C gas temperature, 10 L/min gas flow, 30 psi nebulizer pressure, 350 °C sheath gas temperature, 9 L/min sheath gas flow, 3500 V capillary voltage, 500 V nozzle voltage, and 250 V delta EMV. Analyte- and IS-specific MRM transitions, fragmentor voltages, collision energies, and dwell times are provided in Table 2. Wide resolution (FWHM approximately 1.2 amu) was applied in both mass analyzers.

2.6. Quantitation calculations and acceptance criteria

Throughout method validation and study sample analysis, calibration curves were constructed by plotting the analyte/IS chromatographic peak area ratio against the nominal analyte concentration in each calibration standard. Acetaminophen-d4 was used as the IS for acetaminophen, and acetaminophen-d3-sulf was used as the IS for all other analytes. Calibration curves were fit by linear regression for all analytes except acetaminophen-gluc, for which a quadratic regression was used. Weighting of $1/x^2$ was applied to all calibration curves. Back-calculated calibrator and QC concentrations determined by interpolation were required to be within ±20% of nominal concentration. Calibration standards that failed to meet this criterion were excluded from regression, and at least three-fourths of the calibrators were required to be included in regression. The lower limit of quantification (LLOQ) was defined as the lowest concentration of analyte with acceptable imprecision (≤20%) and mean accuracy (±20% of nominal concentration). At each QC concentration, at least two of the three replicates were required to meet the ±20% accuracy criterion in order for a sample batch to meet acceptance criteria for reporting of quantitative results.

2.7. Method validation

The methods were validated by assessment of LLOQ, intra- and inter-day accuracy and imprecision, specificity, recovery and ion-

Table 2
Analyte- and IS-specific ESI–MS/MS parameters.

Compound	MS segment	ESI mode	MRM transition (precursor → product *m/z*)	Fragmentor voltage (V)	Collision energy (V)	Dwell time (ms)	Transition type
Acetaminophen-gluc	2	+	328.1 → 152.1	100	5	200	Quantifier
Acetaminophen-d3-sulf	3	–	233.1 → 153.1	110	14	200	Quantifier
Acetaminophen-d3-sulf	3	–	233.1 → 107.1	110	30	200	Qualifier
Acetaminophen-sulf	3	–	230.1 → 150.1	110	14	200	Quantifier
Acetaminophen-sulf	3	–	230.1 → 107.1	110	30	200	Qualifier
Acetaminophen-cys	4	+	271.1 → 140.0	80	22	100	Quantifier
Acetaminophen-cys	4	+	271.1 → 182.0	80	10	100	Qualifier
Acetaminophen-d4	4	+	156.1 → 114.1	80	14	100	Quantifier
Acetaminophen	4	+	152.1 → 110.0	80	14	100	Quantifier
Acetaminophen-glut	5	+	457.1 → 140.0	110	42	100	Quantifier
Acetaminophen-NAC	6	+	313.1 → 208.0	80	14	100	Quantifier
Acetaminophen-NAC	6	+	313.1 → 140.0	80	34	100	Qualifier

ization efficiency, matrix effect, stock solution stability, and sample stability.

To identify an appropriate LLOQ for each analyte in each matrix, analyte-free human plasma and urine were fortified with analyte working solutions at several concentrations near the target LLOQ ($n = 6$ for each concentration). Fortified samples were prepared and analyzed as described in Sections 2.4 through 2.6, and each appropriate LLOQ was selected based on the criteria described in Section 2.6. A LLOQ calibrator was included in each calibration curve, but the LLOQ test replicates were excluded from regression for determinations of accuracy and imprecision.

Accuracy and imprecision were determined from replicate samples of analyte-free human plasma and urine fortified with analytes at the QC concentrations indicated in Table 1. Intra-assay accuracy and imprecision at each concentration were determined from five replicate samples assayed within the same analytical batch. Inter-assay accuracy and imprecision at each concentration were calculated from a total of 20 replicates assayed over seven separate analytical batches. Accuracy is expressed as a percent of the nominal concentration and imprecision as percent coefficient of variation (% CV).

Specificity of the methods was assessed by analysis of human plasma and urine from six acetaminophen-abstinent individuals. To allow for identification of potential interfering peaks co-eluting with either analytes or IS, plasma and urine samples were prepared in triplicate according to the usual procedures (Section 2.4), and one plasma and urine sample from each individual was prepared similarly but without IS fortification. For samples prepared with IS, the specificity acceptance criterion for each lot was that the mean analyte/IS peak area ratio at the analyte retention time must be <20% of the corresponding peak area ratio from a concurrently assayed LLOQ sample. Additionally, qualification transitions were incorporated for some analytes (acetaminophen-sulf, acetaminophen-cys, and acetaminophen-NAC), and the presence of a qualifier ion peak with a signal-to-noise ratio greater than 3 was required for declaration of a positive result for these analytes. For samples prepared without IS, any peak area at the IS retention time was required to be <5% of the IS peak area in the LLOQ sample in order to meet acceptance criterion.

Recovery and ionization efficiency (suppression/enhancement) were determined at the QC concentrations indicated in Table 1. For each biological matrix, one QC set (set A, $n = 5$) was prepared according to the usual procedures detailed in Section 2.4. Another QC set (set B, $n = 5$) was prepared similarly but IS was added during the final step of sample preparation. A third QC set (set C, $n = 5$) was prepared with water in place of analyte-free matrix and with IS added during the final step of sample preparation. In the HPLC–ESI–MS/MS injection sequence, each QC set was interspersed so that comparable samples were injected consecutively (i.e., low QC A replicate 1, low QC B replicate 1, low QC C replicate 1, etc.). IS recovery was calculated by dividing each IS peak area from set A by the IS peak area from the corresponding set B sample. Ionization efficiency was calculated by dividing each analyte or IS peak area from set B by the analyte or IS peak area from the corresponding set C sample.

The influence of inter-individual variability in matrix effects on analyte accuracy and imprecision was assessed using analyte-free plasma or urine obtained from six individuals. Each lot was fortified with analytes at low and high QC concentrations (QC levels 2 and 4 for plasma, and levels 1 and 3 for urine). The fortified samples were then prepared for analysis according to the described procedures (Section 2.4)

Stability of analyte stock solutions was assessed by comparison of freshly prepared solutions to solutions that had been stored at −20 °C for approximately six or twelve months. Solutions were diluted in 0.1% aqueous formic acid to concentrations appropriate for injection on the HPLC–ESI–MS/MS ($n = 3$ for each comparison), and the test solutions were analyzed as described in Section 2.5. Stability was calculated by dividing the mean analyte/IS peak area ratios from test solutions prepared from stored stocks by the mean analyte/IS peak area ratios from test solutions prepared from the fresh stocks.

For plasma stability experiments, analyte concentrations were equivalent to the lowest and highest QC concentrations. Plasma fortification was performed in triplicate in silanized glass tubes by first evaporating appropriate amounts of analyte working solutions to dryness in a TurboVap LV Evaporator (35 °C water bath, 10–15 psi air stream) and then reconstituting each sample in 1 mL analyte-free plasma. Because reference standard material is costly and the urine stability samples required relatively high analyte concentrations, a urine sample for stability studies was obtained from a volunteer at the Center for Human Toxicology who was known to have ingested 1 g of acetaminophen approximately 3 h prior. This specimen was serially diluted in analyte-free urine from the same donor to obtain analyte concentrations in the low-to-mid and mid-to-high regions of the calibration curves. Stability samples were prepared for initial analysis immediately after fortification/collection, and aliquots of matrix (200 μL for plasma; 1 mL for urine) were also subjected to the following conditions: up to 24 h of storage at room temperature, up to 6 months (plasma) or 1 month (urine) of storage at −80 °C, and three cycles of freezing (at least 12 h storage at −80 °C) and thawing (60 min at room temperature). Analyte concentrations determined from stored aliquots were then compared to the concentrations determined from initial analysis. Additionally, stability of prepared samples was assessed following storage in the autosampler (5 °C) for 72 h.

2.8. Statistical software

All calculations for descriptive statistics were performed in Excel (version 14.0, Microsoft Corp., Redmond, WA).

3. Results and discussion

3.1. Method development

Table 1 provides a summary of the calibrator and QC concentrations used for both the plasma and urine assays. Based on previous knowledge about acetaminophen pharmacokinetics [27], calibration curve ranges were carefully selected to be most appropriate for each biological matrix and analyte. Because cytochrome P450-mediated oxidation accounts for a small portion of acetaminophen metabolism, concentrations of NAPQI-derived metabolites (acetaminophen-glut, acetaminophen-cys, and acetaminophen-NAC) can be anticipated to fall roughly one order of magnitude lower than the other analytes. Additionally, urinary analyte concentrations are generally expected to be significantly higher than the respective plasma concentrations. Calibrator concentrations for plasma and urine were also designed to be compatible with a single set of stock and working solutions so that costly metabolite reference standards could be used as economically as possible. As a result, the calibration range for acetaminophen in urine extends to much higher concentrations than typically necessary.

A variety of LC conditions were tested during method development. In addition to the Poroshell 120 EC-C18 column, which was ultimately employed for the assay, a Polaris C18-A column (2.0 × 150 mm, 5 μm particle size, Agilent Technologies, Santa Clara, CA) was considered. Potential aqueous mobile phase solutions included various ammonium acetate buffers, formate buffers, dilute formic acid, dilute acetic acid, and dilute trifluoroacetic acid. Acetonitrile was also explored as a potential organic mobile phase solvent. Additionally, numerous variations in pump flow rate, pump timetable program, and column temperature were tested. Ultimately, the conditions reported in Section 2.5 were found to provide optimal chromatographic separation within a reasonable run time. Typical retention times for analytes and IS can be observed in Fig. 2 and 3 (plasma) and in electronic supplementary data Fig. S1 and S2 (urine).

Analyte- and IS-specific ESI–MS/MS parameters are summarized in Table 2. Ion source and MS/MS conditions were optimized during direct infusion of individual analyte solutions (10 μg/mL in methanol/water (1/1, v/v)) at 10 μL/min into 0.25 mL/min mobile phase flow with a composition approximately equivalent to that at the time of analyte elution from the HPLC column. MRM transitions for analyte quantification were selected based on the ability to provide acceptable specificity and optimal product ion abundance for maximum sensitivity. For acetaminophen-sulf, acetaminophen-d3-sulf, acetaminophen-cys, and acetaminophen-NAC, additional qualification transitions were monitored to enhance assay specificity. Samples were required to have a qualifier ion peak with a signal-to-noise ratio greater than 3 in order for a quantifiable analyte concentration to be reported, and this was particularly helpful for samples with concentrations near the LLOQ. Many of these quantifier and qualifier mass transitions have previously been utilized in LC–MS/MS methods for these analytes [21–23,25,28,29].

At the time of initial method development, deuterated analogues of sufficient purity for use as IS (≥95%) were unavailable for some analytes. During preliminary testing, acetaminophen-d4 performed well as an IS for acetaminophen. However, the instrument response was much greater for acetaminophen and acetaminophen-d4 than for the other analytes. This is readily apparent by examination of the peak areas in Fig. 2b and electronic supplementary data Fig. S1b; even when concentration and molecular weight differences are taken into account, peak areas for acetaminophen are significantly larger than for the other analytes. As a result, acetaminophen-d4 did not perform well as an IS for the other analytes. Both acetaminophen and acetaminophen-d4 showed evidence of ion suppression at the high end of the calibration curve. This self-induced ion suppression was not problematic for quantification of acetaminophen, but it did limit the utility of acetaminophen-d4 as an IS for other analytes. Preliminary testing showed that acetaminophen-d3-sulf performed well as an IS for acetaminophen-sulf and for the remaining analytes. It provided consistent peak areas across the calibration curve ranges and allowed for linear curve fits for all analytes except acetaminophen-gluc, for which a quadratic curve fit was applied. IS working solution concentrations of acetaminophen-d4 and acetaminophen-d3-sulf were selected based on the relative instrument response for each.

Due to the wide variability in the chemical nature of the analytes (i.e., relatively non-polar parent drug and several highly polar metabolites), non-specific sample preparation procedures were expected to be most suitable. Thus, plasma samples were prepared for analysis by protein precipitation, and the relatively high urinary analyte concentrations inspired the testing and eventual implementation of a dilution-only urine sample preparation. As evidenced by the validation results in Section 3.2, these simple and efficient preparation procedures performed well.

3.2. Method validation

Appropriate LLOQ concentrations were determined based on the criteria described in Section 2.6. LLOQ values for the plasma and urine assays are presented in Tables 3 and 4, respectively, along with intra-assay accuracy and imprecision data for each LLOQ. Representative MRM chromatograms are provided for analyte- and IS-free plasma (Fig. 2a), plasma LLOQ calibrators fortified with IS (Fig. 2b), analyte- and IS-free urine (electronic supplementary data Fig. S1a), and urine LLOQ calibrators fortified with IS (electronic supplementary data Fig. S1b).

Most calibration curves were fit well by linear regression with $1/x^2$ weighting. Acetaminophen-gluc, however, required quadratic regression. Also, in plasma, the acetaminophen-NAC curve was linear only up to 1.0 μg/mL. Back-calculation of calibrator and QC concentrations by interpolation consistently yielded values within ±20% of nominal concentration, and coefficients of determination (R^2) for calibration curves were typically ≥0.99. No evidence of chromatographic carryover was observed when analyte-free matrix samples were injected immediately after the highest calibration standards.

Intra- and inter-assay accuracy and imprecision data for each QC concentration are summarized in Tables 3 and 4 for plasma and urine, respectively. The methods were found to be highly accurate and precise. Mean values for intra- and inter-assay accuracy in plasma ranged from 85 to 112% and 90 to 108%, respectively. In plasma, intra- and inter-assay imprecision did not exceed 14% and 15%, respectively. Mean values for intra- and inter-assay accuracy in urine ranged from 87 to 107% and 92 to 111%, respectively. In urine, intra- and inter-assay imprecision did not exceed 8% and 13%, respectively.

Analysis of human plasma and urine from six acetaminophen-abstinent individuals demonstrated that the assays provide requisite specificity. In plasma samples fortified with IS, mean peak area ratios (analyte/IS) for each lot were <5% of the LLOQ peak area ratio for all analytes. Similarly, in plasma samples that were not fortified with IS, peak areas in the IS MRM transitions were <0.1% of the IS peak areas in the LLOQ samples. In urine samples fortified with IS, mean peak area ratios for acetaminophen-cys/IS were

Table 3
LLOQ and intra- and inter-assay accuracy and imprecision for determination of acetaminophen and metabolites in human plasma.

Analyte	Nominal concentration (μg/mL)	Intra-assay accuracy and imprecision[a]			Inter-assay accuracy and imprecision (n = 20)		
		Observed concentration (μg/mL)[b]	Mean accuracy (%)	Imprecision (% CV)	Observed concentration (μg/mL)[b]	Mean accuracy (%)	Imprecision (% CV)
Acetaminophen	0.050[c]	0.046 ± 0.002	92	5.3	n/a	n/a	n/a
	0.15	0.15 ± 0.01	99	7.1	0.15 ± 0.01	101	7.2
	0.80	0.83 ± 0.01	104	1.4	0.82 ± 0.03	102	4.2
	8.0	7.9 ± 0.5	98	6.3	7.9 ± 0.3	99	4.2
	40	39 ± 4	98	9.1	39 ± 3	98	6.8
Acetaminophen-gluc	0.050[c]	0.040 ± 0.006	80	13.9	n/a	n/a	n/a
	0.15	0.14 ± 0.01	92	6.1	0.15 ± 0.02	101	11.5
	0.80	0.81 ± 0.04	102	5.2	0.83 ± 0.08	104	9.2
	8.0	7.2 ± 0.4	91	5.5	7.5 ± 0.5	94	6.5
	40	40 ± 5	100	13.7	40 ± 4	101	8.8
Acetaminophen-sulf	0.050[c]	0.050 ± 0.003	100	6.5	n/a	n/a	n/a
	0.15	0.15 ± 0.01	100	6.8	0.15 ± 0.02	101	12.4
	0.80	0.80 ± 0.02	100	2.3	0.79 ± 0.06	99	7.4
	8.0	8.2 ± 0.5	102	5.9	8.0 ± 0.4	100	4.4
	40	43 ± 2	107	5.8	42 ± 2	105	5.2
Acetaminophen-glut	0.025[c]	0.028 ± 0.004	111	13.6	n/a	n/a	n/a
	0.080	0.089 ± 0.004	112	4.3	0.086 ± 0.007	107	8.0
	0.80	0.89 ± 0.03	111	3.4	0.87 ± 0.06	108	6.7
	4.0	4.4 ± 0.2	111	5.4	4.3 ± 0.3	107	7.3
Acetaminophen-cys	0.010[c]	0.0097 ± 0.0004	97	4.3	n/a	n/a	n/a
	0.015	0.013 ± 0.001	85	8.2	0.013 ± 0.002	90	14.5
	0.080	0.070 ± 0.005	87	6.7	0.074 ± 0.005	93	6.8
	0.80	0.71 ± 0.03	89	4.4	0.74 ± 0.05	92	6.9
	4.0	3.6 ± 0.2	89	6.9	3.7 ± 0.3	93	7.2
Acetaminophen-NAC	0.010[c]	0.0082 ± 0.0008	82	9.4	n/a	n/a	n/a
	0.015	0.014 ± 0.002	93	12.3	0.015 ± 0.002	97	11.9
	0.080	0.084 ± 0.004	105	4.9	0.083 ± 0.005	104	6.4
	0.80	0.75 ± 0.05	94	6.1	0.77 ± 0.05	97	6.3

Note: n/a: not applicable.
[a] LLOQ n = 6; QC n = 5.
[b] Reported values are mean concentration ± standard deviation.
[c] LLOQ.

Table 4
LLOQ and intra- and inter-assay accuracy and imprecision for determination of acetaminophen and metabolites in human urine.

Analyte	Nominal concentration (μg/mL)	Intra-assay accuracy and imprecision[a]			Inter-assay accuracy and imprecision (n = 20)		
		Observed concentration (μg/mL)[b]	Mean accuracy (%)	Imprecision (% CV)	Observed concentration (μg/mL)[b]	Mean accuracy (%)	Imprecision (% CV)
Acetaminophen	0.20[c]	0.20 ± 0.01	98	4.2	n/a	n/a	n/a
	7.5	7.1 ± 0.4	94	5.0	7.5 ± 0.4	100	5.7
	50	53 ± 1	106	1.3	52 ± 2	105	4.1
	500	494 ± 13	99	2.6	496 ± 25	99	5.0
Acetaminophen-gluc	1.0[c]	1.02 ± 0.03	102	2.6	n/a	n/a	n/a
	7.5	6.5 ± 0.4	87	5.6	7.4 ± 0.9	99	11.9
	50	49 ± 2	98	3.1	51 ± 3	102	6.5
	500	488 ± 20	98	4.1	517 ± 53	103	10.2
Acetaminophen-sulf	1.0[c]	1.0 ± 0.1	103	4.9	n/a	n/a	n/a
	7.5	6.9 ± 0.4	92	5.9	7.9 ± 0.7	106	8.7
	50	50 ± 1	101	1.7	54 ± 3	108	6.4
	500	491 ± 25	98	5.0	554 ± 47	111	8.4
Acetaminophen-glut	0.10[c]	0.111 ± 0.004	111	3.2	n/a	n/a	n/a
	0.75	0.69 ± 0.04	92	5.6	0.72 ± 0.07	96	9.3
	5.0	5.1 ± 0.1	101	1.6	4.8 ± 0.3	96	6.7
	50	54 ± 2	107	4.2	53 ± 3	105	6.6
Acetaminophen-cys	0.10[c]	0.10 ± 0.01	96	7.4	n/a	n/a	n/a
	0.75	0.67 ± 0.04	89	6.4	0.76 ± 0.09	101	12.2
	5.0	5.0 ± 0.1	100	2.5	5.1 ± 0.4	102	7.9
	50	51 ± 2	102	3.1	53 ± 4	106	8.4
Acetaminophen-NAC	0.10[c]	0.114 ± 0.004	114	3.4	n/a	n/a	n/a
	0.75	0.70 ± 0.05	93	7.3	0.69 ± 0.05	92	7.9
	5.0	5.2 ± 0.1	104	2.8	4.8 ± 0.3	96	7.3
	50	54 ± 2	107	3.7	50 ± 4	100	7.9

Note: n/a: not applicable.
[a] LLOQ n = 6; QC n = 5.
[b] Reported values are mean concentration ± standard deviation.
[c] LLOQ.

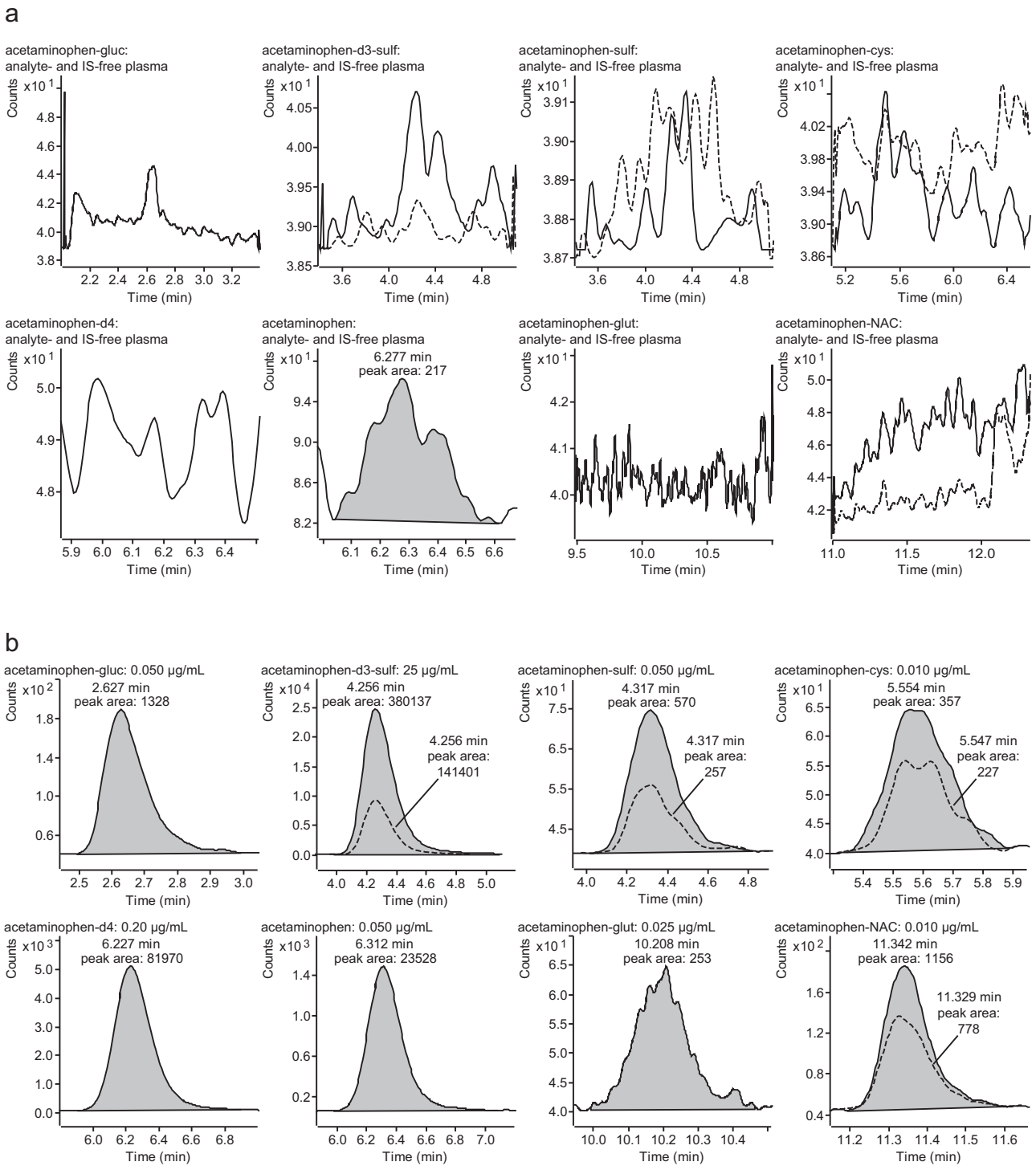

Fig. 2. Representative MRM chromatograms for determination of acetaminophen and metabolites in human plasma. Specific precursor → product ion transitions for each analyte and IS are provided in Table 2. Solid lines depict MRM traces for quantifier transitions and dashed lines depict MRM traces for qualifier transitions. (a) Analyte- and IS-free plasma. (b) Plasma fortified with IS and with acetaminophen and metabolites at each LLOQ; MRM chromatograms are derived from three sample injections (the three lowest calibrators). Nominal concentrations for analytes and IS are provided in the heading of each MRM trace.

slightly greater than 20% in most of the test lots (range: 20–24%); however, these samples did not produce detectable peaks in the acetaminophen-cys qualifier MRM transition. For all other analytes, mean peak area ratios (analyte/IS) for each urine lot were <19% of the LLOQ peak area ratio. In urine samples that were not fortified with IS, peak areas in the IS MRM transitions were <1% of the IS peak areas in the LLOQ samples.

Acetaminophen-d4 and acetaminophen-d3-sulf were recovered from plasma at 86 ± 6% and 81 ± 7%, respectively (mean% recovery ± SD, $n = 20$). These values suggest that small but consistent amounts of IS and, presumably, analytes were lost during the supernatant transfer step that occurred prior to sample evaporation. Results for ionization efficiency and matrix effect experiments for the plasma assay are summarized in Table 5. For acetaminophen and acetaminophen-sulf, mean ionization efficiencies ranged from

Table 5
Ionization efficiency and matrix effect for determination of acetaminophen and metabolites in human plasma.

	Ionization efficiency (%)[a] ($n=5$)				Accuracy and imprecision of matrix effect samples (%)[a] ($n=6$)	
Compound	QC level 1	QC level 2	QC level 3	QC level 4	QC level 2	QC level 4
Acetaminophen	96 ± 6	93 ± 3	97 ± 5	97 ± 2	101 ± 3	108 ± 1
Acetaminophen-d4	95 ± 10	97 ± 3	96 ± 5	98 ± 6	n/a	n/a
Acetaminophen-gluc	32 ± 4	32 ± 1	42 ± 1	51 ± 2	111 ± 6	109 ± 5
Acetaminophen-sulf	90 ± 5	87 ± 5	91 ± 7	91 ± 4	107 ± 3	113 ± 3
Acetaminophen-d3-sulf	91 ± 16	90 ± 4	84 ± 4	91 ± 5	n/a	n/a
Acetaminophen-glut	n/a	61 ± 4	57 ± 2	56 ± 9	132 ± 16	117 ± 15
Acetaminophen-cys	381 ± 200	168 ± 29	109 ± 7	101 ± 4	114 ± 4	114 ± 3
Acetaminophen-NAC	75 ± 3	73 ± 5	74 ± 3	n/a	104 ± 3	n/a

Note: n/a: not applicable.
[a] Reported values are mean% ± standard deviation.

87 to 97%, suggesting insignificant influence of plasma matrix components on analyte ionization. Results for acetaminophen-gluc, acetaminophen-glut, and acetaminophen-NAC were indicative of ion suppression, with mean ionization efficiencies ranging from 32 to 75%. In contrast, results for acetaminophen-cys suggest that ion enhancement occurred at lower analyte concentrations, with mean ionization efficiencies of 381% and 168% at QC levels 1 and 2, respectively. In spite of these variable ionization efficiencies, results from the matrix effect experiment demonstrate that the influence of plasma matrix components on analyte ionization was relatively consistent across six different individuals. With the exception of acetaminophen-glut, mean accuracies ranged from 101 to 114% and standard deviations did not exceed 6%. Matrix effect results for acetaminophen-glut were less accurate and less precise (see Table 5); however, acetaminophen-glut is arguably the least important of the analytes in this matrix (see further discussion in Section 3.3), and these results were therefore considered acceptable for this particular analyte.

Acetaminophen-d4 and acetaminophen-d3-sulf were recovered from urine at 104 ± 8% and 104 ± 7%, respectively (mean% recovery ± SD, $n=15$). As expected for a dilution-only sample preparation, these values are close to 100%. Results for ionization efficiency and matrix effect experiments for the urine assay are summarized in Table 6. Across all analytes, mean ionization efficiencies ranged from 94 to 104%, which suggests that urine matrix components neither suppressed nor enhanced analyte ionization. Results from the matrix effect experiment demonstrate that the analytes could be quantified with acceptable accuracy and imprecision in urine from six different individuals. Across all analytes, mean accuracies ranged from 87 to 115% and standard deviations did not exceed 12%.

Analyte stock solutions appeared stable (within ±20% of freshly prepared solution) following six months of storage at −20 °C. After twelve months of storage at −20 °C, acetaminophen-glut stock solution had deteriorated to less than 40% of the values obtained from fresh stock solution; however, significant deterioration was not observed for any of the other analyte stock solutions. Based on these results, it was determined that acetaminophen, acetaminophen-gluc, acetaminophen-sulf, acetaminophen-cys, and acetaminophen-NAC stock solutions could be used up to one year after the preparation date, and acetaminophen-glut stock solution could be used up to six months after the preparation date.

Results of plasma stability experiments are summarized in Table 7. Under all tested storage and handling conditions, acetaminophen-glut quickly deteriorated in human plasma, presumably as a result of hydrolysis by gamma-glutamyl transpeptidase and dipeptidases. Acetaminophen-cys is a major hydrolysis product of acetaminophen-glut, and the decline in acetaminophen-glut was accompanied by a concomitant increase in acetaminophen-cys concentrations. For many analytes, such instability would be cause for concern. However, in this case, investigators are often primarily interested in using the sum of acetaminophen-glut, acetaminophen-cys, and acetaminophen-NAC concentrations as a surrogate for the amount of NAPQI that formed. Thus, acetaminophen-glut instability is not a major concern here because the critical information is essentially retained through measurement of acetaminophen-cys. If a particular research question required differentiation between relative amounts of acetaminophen-glut, acetaminophen-cys, and acetaminophen-NAC, it seems likely that addition of peptidase inhibitors during sample collection would prevent or at least minimize acetaminophen-glut degradation, but such experiments were beyond the scope of this study.

Acetaminophen, acetaminophen-gluc, and acetaminophen-sulf were adequately stable in human plasma under all tested storage and handling conditions (Table 7). For these analytes, mean concentrations were within ±20% of initial values following storage of fortified human plasma at room temperature for 24 h, at −80 °C for six months, and after three freeze-thaw cycles. These results are in agreement with previous studies where acetaminophen [25,30–33] and acetaminophen-gluc [25] have been found to be quite stable in human plasma under typical sample handling conditions. The results for stability of acetaminophen-cys in human plasma were obscured due to the degradation of acetaminophen-glut to acetaminophen-cys. However, previous work has shown that acetaminophen-cys was adequately stable in human plasma/serum for up to 24 h at room temperature and through three freeze-thaw cycles [25,28,29]. Acetaminophen-NAC appeared adequately stable in human plasma following 24 h of storage at room temperature and after three freeze-thaw cycles, but significant deterioration was evident at QC level 1 after six months of storage at −80 °C (mean stability: 74% of initial concentration). After only one month of storage at −80 °C, stability for acetaminophen-NAC at QC levels 1 and 3 was acceptable at 88 ± 8% and 93 ± 11%, respectively (mean% of initial observed concentration ± SD). All analytes were adequately stable in prepared plasma samples that were stored in the autosampler at 5 °C for up to 72 h. Across all analytes and QC concentrations, mean concentrations of post-preparative stability samples were within ±11% of freshly prepared QC samples (Table 7). Taken together, the stability experiment results suggest that plasma samples can be handled at room temperature during routine sample preparation and subjected to several freeze-thaw cycles without concern for analyte degradation. Furthermore, plasma samples should ideally be assayed within the first few months after sample collection.

Results of urine stability experiments are summarized in Table 8. Acetaminophen-glut was not detected in the initial urine stability samples (see further discussion in Section 3.3), but all other analytes were adequately stable in human urine under the tested storage and handling conditions. For these analytes, mean con-

Table 6
Ionization efficiency and matrix effect for determination of acetaminophen and metabolites in human urine.

	Ionization efficiency (%)[a] ($n = 5$)			Accuracy and imprecision of matrix effect samples (%)[a] ($n = 6$)	
Compound	QC level 1	QC level 2	QC level 3	QC level 1	QC level 3
Acetaminophen	100 ± 5	99 ± 5	99 ± 2	101 ± 4	97 ± 1
Acetaminophen-d4	100 ± 6	103 ± 8	103 ± 9	n/a	n/a
Acetaminophen-gluc	96 ± 3	94 ± 3	94 ± 3	107 ± 12	99 ± 8
Acetaminophen-sulf	101 ± 4	99 ± 4	97 ± 3	112 ± 4	115 ± 2
Acetaminophen-d3-sulf	100 ± 7	103 ± 7	102 ± 6	n/a	n/a
Acetaminophen-glut	99 ± 4	99 ± 3	99 ± 4	101 ± 5	112 ± 8
Acetaminophen-cys	104 ± 5	99 ± 5	99 ± 4	108 ± 5	107 ± 1
Acetaminophen-NAC	99 ± 4	98 ± 2	98 ± 4	87 ± 4	91 ± 1

Note: n/a: not applicable.
[a] Reported values are mean% ± standard deviation.

Table 7
Stability of acetaminophen and metabolites in human plasma.

		Stability in plasma (%)[a] ($n = 3$)			Stability in prepared samples (%)[b] ($n = 3$)
Analyte	Nominal concentration (μg/mL)	24 h at room temperature	6 months at −80 °C	3 freeze-thaw cycles	72 h in 5 °C autosampler
Acetaminophen	0.15	99 ± 8	103 ± 10	102 ± 6	95 ± 5
	40	109 ± 9	102 ± 8	105 ± 9	98 ± 8
Acetaminophen-gluc	0.15	105 ± 6	99 ± 13	96 ± 3	101 ± 7
	40	120 ± 11	96 ± 10	97 ± 9	111 ± 4
Acetaminophen-sulf	0.15	97 ± 12	103 ± 9	82 ± 8	102 ± 9
	40	112 ± 8	101 ± 8	102 ± 7	98 ± 6
Acetaminophen-glut	0.080	0 ± n/a	35 ± 3	15 ± 1	94 ± 4
	4.0	0.16 ± 0.03	46 ± 4	23 ± 2	107 ± 7
Acetaminophen-cys	0.015	365 ± 29	127 ± 11	197 ± 31	90 ± 10
	4.0	307 ± 24	123 ± 13	160 ± 17	97 ± 6
Acetaminophen-NAC	0.015	94 ± 10	74 ± 16	90 ± 11	102 ± 14
	0.80	106 ± 8	87 ± 10	100 ± 7	98 ± 7

Note: n/a: not applicable.
[a] Reported values are mean% of initial observed concentration ± standard deviation.
[b] Reported values are mean% of observed concentration from freshly prepared samples ± standard deviation.

centrations were within ±19% of initial concentrations following storage of human urine at room temperature for 24 h, at −80 °C for one month, and after three freeze-thaw cycles. Additionally, all analytes were adequately stable in prepared urine samples that were stored in the autosampler at 5 °C for up to 72 h. Mean concentrations of post-preparative stability samples were within ±10% of freshly prepared QC samples.

3.3. Application to neonatal pharmacokinetic samples

The methods presented in this paper have been successfully applied for determination of acetaminophen and metabolites in the plasma and urine of neonatal clinical study participants receiving therapeutic doses of intravenous acetaminophen. The analysis of samples from this special patient population was greatly facilitated by minimizing the required sample volume

Table 8
Stability of acetaminophen and metabolites in human urine.

	Stability in urine (%)[a] ($n = 3$)				Stability in prepared samples (%)[b] ($n = 3$–5)	
Analyte	Initial concentration (μg/mL)	24 h at room temperature	1 month at −80 °C	3 freeze-thaw cycles	Nominal concentration (μg/mL)	72 h in 5 °C autosampler
Acetaminophen	1.5	103 ± 3	96 ± 7	98 ± 4	7.5	98 ± 1
	15	108 ± 4	81 ± 3	99 ± 1	500	97 ± 8
Acetaminophen-gluc	54	100 ± 7	92 ± 3	94 ± 5	7.5	101 ± 9
	489	99 ± 6	82 ± 5	91 ± 7	500	98 ± 11
Acetaminophen-sulf	43	98 ± 2	99 ± 4	98 ± 3	7.5	102 ± 3
	416	108 ± 5	86 ± 6	102 ± 1	500	102 ± 8
Acetaminophen-glut	<0.10	n/a	n/a	n/a	0.75	106 ± 3
	<0.10	n/a	n/a	n/a	50	103 ± 9
Acetaminophen-cys	2.1	98 ± 5	106 ± 14	96 ± 4	0.75	104 ± 7
	21	104 ± 6	90 ± 5	96 ± 4	50	105 ± 12
Acetaminophen-NAC	1.4	103 ± 8	99 ± 9	91 ± 5	0.75	110 ± 9
	14	111 ± 6	89 ± 5	96 ± 4	50	106 ± 12

Note: n/a: not applicable.
[a] Reported values are mean% of initial observed concentration ± standard deviation.
[b] Reported values are mean% of observed concentration from freshly prepared samples ± standard deviation.

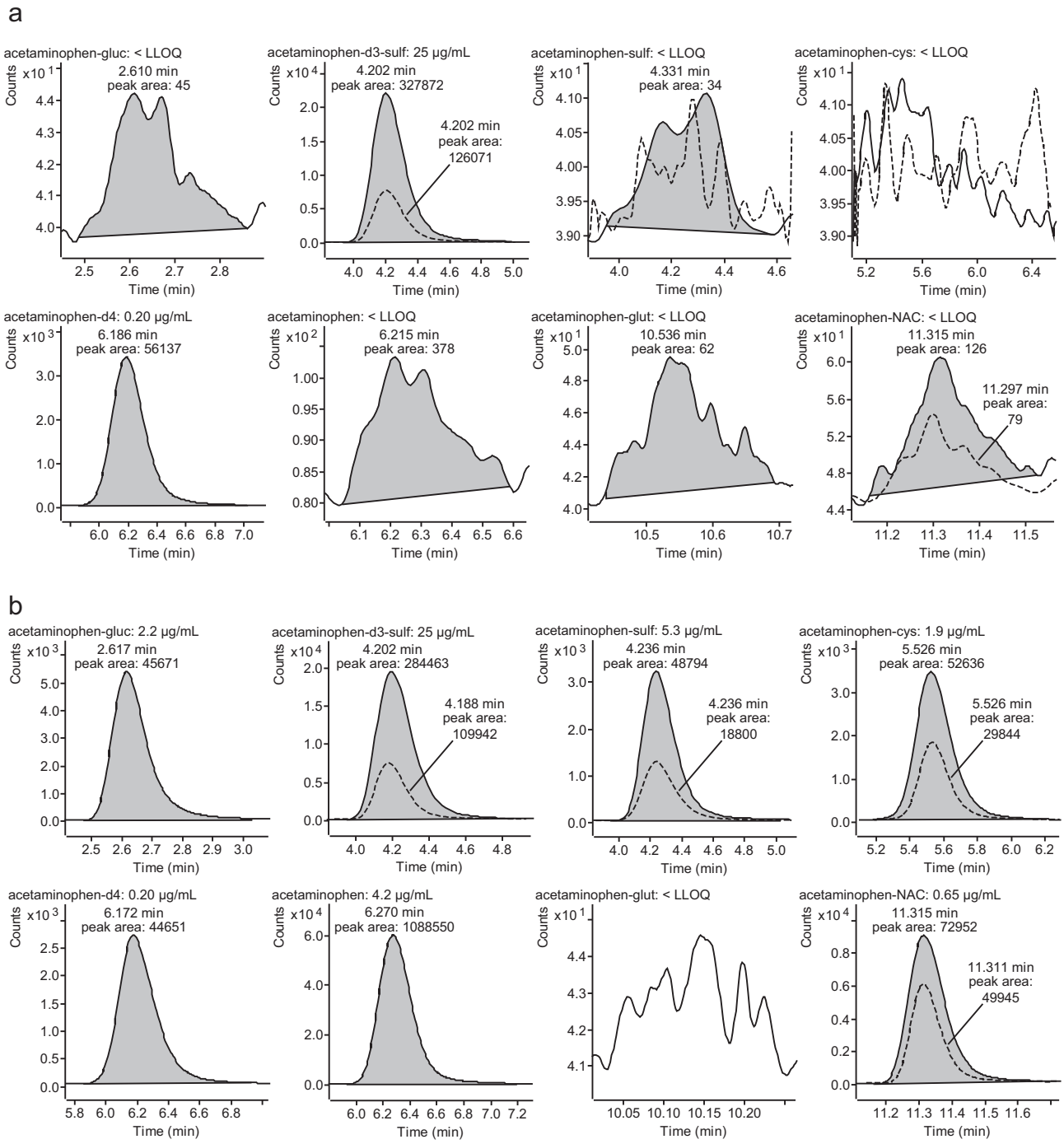

Fig. 3. Representative MRM chromatograms for determination of acetaminophen and metabolites in human plasma. Specific precursor → product ion transitions for each analyte and IS are provided in Table 2. Solid lines depict MRM traces for quantifier transitions and dashed lines depict MRM traces for qualifier transitions. (a) Pre-dose plasma sample from a clinical study participant. All analytes were determined to be <LLOQ. (b) A plasma sample collected from the same clinical study participant approximately 7 h after the first 15 mg/kg dose. Interpolated analyte concentrations are provided in the MRM trace headings.

(10 μL). Each pharmacokinetic sample consisted of only 200 μL of blood, so most plasma sample volumes were less than 100 μL. Representative MRM chromatograms from the plasma assay show all analytes to be <LLOQ in a pre-dose sample (Fig. 3a) and acetaminophen, acetaminophen-gluc, acetaminophen-sulf, acetaminophen-cys, and acetaminophen-NAC concentrations that are well above LLOQ in a sample collected from the same clinical study participant approximately 7 h after the first 15 mg/kg dose (Fig. 3b). Similar representative MRM chromatograms are provided for pre- and post-dose urine samples in electronic supplementary data Fig. S2. The percentage of post-dose plasma and urine samples that were <LLOQ did not exceed 1% for most analytes (Table 9). The exception was acetaminophen-glut, which was <LLOQ in nearly all plasma samples and in all of the urine samples. However, this was not unexpected because acetaminophen-glut is thought to be particularly short-lived in humans, perhaps due to relatively high expression of gamma-glutamyl transpeptidase compared to other species [20,34]. Additionally, results of stability experiments with fortified plasma showed that acetaminophen-glut was rapidly converted to acetaminophen-cys under routine

Table 9
Assay suitability for analysis of neonatal pharmacokinetic samples.

Analyte	Samples < LLOQ (%)		Undiluted samples > ULOQ (%)	
	Plasma[a]	Urine[b]	Plasma[a]	Urine[b]
Acetaminophen	0	1	0	0
Acetaminophen-gluc	0	1	0	0
Acetaminophen-sulf	0	1	9	3
Acetaminophen-glut	97	100	0	0
Acetaminophen-cys	0	1	8	5
Acetaminophen-NAC	1	1	34	11

[a] $n = 267$ post-dose samples.
[b] $n = 387$ post-dose samples.

storage and handling conditions (see Table 7 and discussion in Section 3.2). In spite of these expectations, acetaminophen-glut was included as an analyte in these assays because it is a critical intermediate in acetaminophen metabolism, and the ability to quantitate acetaminophen-glut makes the assays readily adaptable to other matrices where concentrations are anticipated to be quantifiable, such as rodent plasma, bile, and tissue [19,21,35]. The majority of the samples did not require dilution in order to fall within the upper limit of quantification (ULOQ) (Table 9). Acetaminophen-NAC in plasma had the highest percentage of samples requiring dilution (34%), which was largely a consequence of the fact that the upper range of the acetaminophen-NAC plasma curve was limited in order to maintain a linear fit. Those samples with analyte concentrations above the ULOQ required only minor dilution (no more than 10 fold), and they could easily be anticipated prior to being assayed based on patient dosing schedule and pharmacokinetic sample collection time.

4. Conclusion

A novel HPLC–ESI–MS/MS procedure for simultaneous quantification of acetaminophen and five acetaminophen metabolites in small volumes (10 μL) of human plasma and urine was developed and successfully validated. The utility, sensitivity, and suitability of the assays were demonstrated by analysis of samples from a pharmacokinetic study of intravenous acetaminophen in neonates. The reported methods were found to be sensitive, specific, accurate, precise, and efficient. These new methods will serve as powerful tools for researchers studying acetaminophen pharmacokinetics and acetaminophen-induced hepatotoxicity.

Authors' contributions

DGW and JNA secured funding. JNA designed and performed the clinical study. ADK, SFC, and DGW developed, validated, and applied the analytical methods. SFC and DGW drafted the manuscript, and all authors contributed to manuscript revisions and reviewed the final version

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2015.10.013.

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