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Bioanalytical development and validation of liquid chromatographic–tandem mass spectrometric methods for the quantification of total and free cefazolin in human plasma and cord blood

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ABSTRACT

Objectives: Cefazolin is a commonly prescribed β-lactam antibiotic for prophylaxis against skin infections following surgery, including caesarean sections. Assessment of maternal and neonatal exposure is important for correlating drug concentrations to clinical outcomes. Thus, bioanalytical methods for the quantification of both total and free cefazolin in maternal plasma and cord blood can assist in the comprehensive evaluation of cefazolin exposure.

Design and methods: Specimen preparation for the measurement of total cefazolin was performed via protein precipitation with acetonitrile containing the internal standard cloxacillin. Ultrafiltration was used to isolate free cefazolin. Processed samples were analyzed on a Prelude SPLC system coupled to a TSQ triple quadrupole Vantage mass spectrometer. Methods were validated following FDA bioanalytical guidelines.

Results: The analytical measuring ranges of these methods were 0.48–480 μg/mL and 0.048–48 μg/mL for total and free drug, respectively. Calibration curves were generated using $1/x^2$ weighted linear regression analysis. Total cefazolin demonstrated inter- and intra-assay precision of ≤20% at the LLOQ and ≤11.2% at other levels. Free cefazolin demonstrated inter- and intra-assay precision of ≤18.5% at the LLOQ and ≤12.6% at other levels, respectively. Accuracy (%DEV), carryover, matrix effects, recovery and stability studies were also acceptable based on FDA recommendations. Furthermore, it was demonstrated that samples prepared in cord blood can be accurately quantified from an adult plasma calibration curve, with recoveries ≤9.1% DIF and ≤11.9% DIF for total and free cefazolin, respectively.

Conclusions: The described LC–MS/MS methods allow for the measurement of total and free cefazolin in both plasma and cord blood.

1. Introduction

Cephalosporin antibiotics have become one of the most commonly prescribed classes of β-lactams due to their broad spectrum of anti-bacterial activity, low toxicity profile, and ease of administration [1,2]. Consequently, cephalosporins have been utilized for the treatment of a number of soft tissue and skin infections in both perioperative and post-surgical settings, particularly as prophylactic agents [1,3–5]. Cephalosporins are semi-synthetic compounds initially derived from the fungus *Cephalosporium acremonium* [6]. Structurally, these drugs are comprised of a β-lactam moiety fused to a 3,6-dihydro-2H-1,3-thiazine ring [1]. There are several generations of cephalosporin antibiotics, and family members are stratified based on their anti-bacterial activity and route of administration.

Cefazolin, a first-generation cephalosporin, is widely used to manage skin infections, and has also shown therapeutic efficacy in the treatment of pulmonary infections and methicillin-susceptible *Staphylococcus aureus* [7,8]. Like other first-generation cephalosporins, cefazolin is effective in treating Gram-positive bacteria, but does not elicit equally effective bactericidal effects against Gram-negative bacteria [6,9]. Moreover, cefazolin has been administered prophylactically to prevent post-surgical skin infections, including those

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incurred during cesarean deliveries [10,11]. Cesarean delivery is the primary risk factor for postpartum maternal infections caused by Gram-positive bacteria, and studies have demonstrated that the infection rate in a post-cesarean setting can be as high as 38% [12]. Notably, the prophylactic administration of cefazolin prior to skin incision reduced post-cesarean morbidity as well as endometritis, and has been recommended by the American College of Obstetrics and Gynecology as a perioperative prophylactic agent during cesarean deliveries [11–13]. While the majority of the drug is predominantly protein bound by albumin (~90% bound), cefazolin elicits its antimicrobial effects in the unbound, or free, form [14–16]. Thus, determination of both total and free cefazolin concentrations may be helpful in better characterizing the pharmacokinetics of the cephalosporin in a variety of clinical scenarios, including in a post-cesarean setting. Cefazolin quantification in the mother, as well as the neonate, can provide clarity on neonatal drug exposure, as well as its potential impact on post-delivery outcomes, including the prevention of neonatal sepsis [17]. Neonatal drug exposure measurement has been assessed in many matrices including urine, blood, meconium, hair, and umbilical cord blood. Umbilical cord blood is a convenient matrix to assess neonatal exposure because it should reflect recent changes in neonatal exposure at the time of delivery without having to perform a venipuncture.

Cefazolin quantification has been previously reported using high performance liquid chromatographic (HPLC) [18–20] or liquid chromatographic–tandem mass spectrometric (LC–MS/MS) [21–25] approaches. However, none of the aforementioned methods have extensively evaluated the acceptability of cord blood as a matrix for cefazolin determination. While several methods provide strategies for directly quantifying free drug concentration, only the methods published by Zhang et al. [21] and Douglas et al. [23] utilized tandem mass spectrometry as an analytical detector [18–20,22]. Here we present a comprehensive method describing the development and validation of LC–MS/MS methods for the quantification of total and free cefazolin via ultrafiltration in plasma and cord blood. The method uses a low volume of specimen for determination, requiring less than 250 μL for determination of both total cefazolin and free drug within a sample.

2. Materials and methods

2.1. Chemicals

Cefazolin (97% purity) as its sodium salt, its structural analogue cloxacillin (94% purity) as its hydrated sodium salt (Fig. 1), and ethylenediaminetetraacetic acid (EDTA) were acquired in powder form from Sigma Aldrich (St. Louis, MO). Drug-free human K_2EDTA plasma was purchased from Biological Specialty Corporation (Colmar, PA). Drug-free human K_2EDTA cord blood plasma was purchased from Golden West Biologicals (Temecula, CA). HPLC-grade water, acetonitrile, and formic acid were acquired from Fisher Scientific (Pittsburgh, PA).

2.2. Preparation of reagents and standards

Concentrations of cefazolin and cloxacillin are presented as their free base forms. Cefazolin working solutions for preparation of calibration standards were prepared for determination of total cefazolin by initially preparing a solution containing 23,800 μg/mL cefazolin in 1:1 acetonitrile:water by weighing standard material and diluting volumetrically. Working solutions were prepared at final concentrations of 48 μg/mL, 480 μg/mL, and 4,800 μg/mL via serial dilution of the initial stock solution. Calibration standards were prepared at final concentrations of 0.48, 2.4, 4.8, 12, 24, 48, 120, 240, and 480 μg/mL by spiking K_2EDTA plasma with the appropriate stock solution. The concentrations reflect the quantity of the cefazolin sodium salt in solution. Total organic volume added to plasma was $<2\%$. Quality control (QC) materials were prepared using working solutions prepared from an independent weighing from the solutions used to prepare calibration standards. Working solutions for preparation of QC materials were prepared at concentrations of 190, 2400, and 48,000 μg/mL in 1:1 acetonitrile:water. Working solutions for the preparation of QC materials were diluted into K_2EDTA plasma at final concentrations of 0.48 (lower limit of quantification, LLOQ), 1.4 (low QC), 18.0 (mid QC), and 410 (high QC) μg/mL.

Cefazolin working solutions for preparation of calibration standards were prepared for the determination of free cefazolin by initially preparing a solution of 1900 μg/mL cefazolin in 1:1 acetonitrile:water by weighing standard material and diluting volumetrically. Working

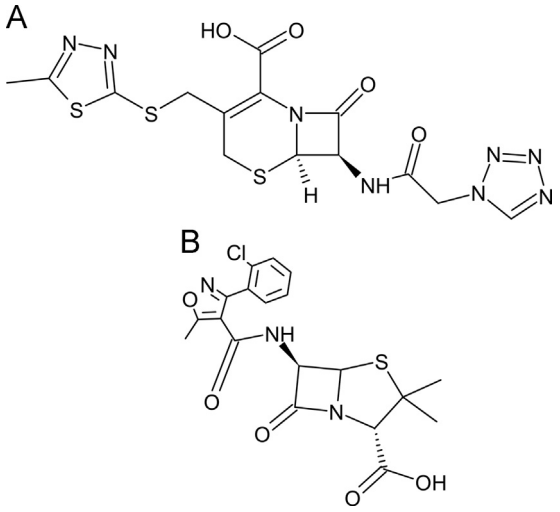

Fig. 1. Structures of (A) the beta-lactam antibiotic cefazolin and its (B) structural analog, cloxacillin.

solutions were prepared at final concentrations of 0.19 μg/mL, 19 μg/mL, and 190 μg/mL via serial dilution. Calibration standards were prepared at final concentrations of 0.048, 0.095, 0.24, 0.48, 0.95, 2.4, 4.8, 9.5, 24, and 48 μg/mL by spiking K_2EDTA plasma ultrafiltrate with the appropriate stock solution. QC materials were prepared from working solutions prepared independently from solutions for preparation of calibration standards. A working stock of 1900 μg/mL was prepared by weighing standard material and diluting volumetrically, and subsequent working stock solutions were prepared as previously described. These working solutions were used to prepare QC materials in plasma ultrafiltrate at final concentrations of 0.048 (LLOQ), 0.14 (low QC), 1.8 (mid QC), and 41 (high QC) μg/mL.

Internal standard solutions were prepared in HPLC grade acetonitrile for total cefazolin determination and HPLC grade water for free cefazolin determination at final concentrations of 9.2 μg/mL and 1.8 μg/mL, respectively, by diluting a 1.8 mg/mL stock solution that was prepared by weighing cloxacillin and diluting it volumetrically using 50% acetonitrile.

2.3. Sample preparation

2.3.1. Total cefazolin

Total cefazolin concentrations in plasma and cord blood were determined after samples had been subjected to protein precipitation. Twenty microliters of calibrator or QC was diluted 1:50 with acetonitrile at ambient temperature containing 9.2 μg/mL cloxacillin in a Fisherbrand 1.5 mL polypropylene microcentrifuge tube (Waltham, MA; final cloxacillin concentration of 9.0 μg/mL). This mixture was vortexed for 5 min using a Scientific Industries Vortex Genie 2 (Bohemia, New York). After mixing, samples were centrifuged at 17,921 x g for 5 min using an Eppendorf 5415D microcentrifuge (Hamburg, Germany). One-hundred microliters of supernatant was diluted four-fold with HPLC grade H_2O in a Nunc 96 DeepWell 2 mL polypropylene plate (Rochester, NY) and loaded onto the instrument for analysis. During the development of this assay, we compared drug recovery from samples prepared in borosilicate tubes and Nunc deepwell plates. We noticed comparable recovery ($>$ 95%) between the borosilicate tubes and deepwell plates (data not shown). The injection volume was 10 μL for downstream LC–MS/MS analysis.

2.4. Free cefazolin

Free cefazolin concentrations in plasma and in cord blood were determined after samples had been treated using an ultrafiltration technique. Two-hundred microliters of plasma was loaded onto a Corning SPIN-X UF 500 μL 10 k MW cutoff concentrator device (Corning, NY) and centrifuged at 1646 x g for 30 min to produce an ultrafiltrate. Twenty microliters of ultrafiltrate, or 20 μL of calibrators or QC materials previously prepared in ultrafiltrate from blank plasma, were diluted 1:50 with HPLC H_2O at ambient temperature containing 1.9 μg/mL cloxacillin in a Fisherbrand 1.5 mL polypropylene microcentrifuge tube (Waltham, MA; final cloxacillin concentration of 1.8 μg/mL). This mixture was briefly vortexed using a Scientific Industries Vortex Genie 2. Four-hundred microliters of this mixture was loaded into Nunc 96 DeepWell 2 mL polypropylene plate and loaded onto the instrument for analysis. The injection volume was 10 μL for downstream LC–MS/MS analysis.

Bulk plasma ultrafiltrate for preparation of standards and QC materials was prepared using a single lot of human plasma using large capacity filtration devices identical in composition to those prepared for individual specimen preparation (Corning SPIN-X UF 20 mL capacity 10 k MW cutoff concentration devices). Plasma was added to spin devices and centrifuged at 1645 x g for 30 min using a Thermo Scientific Jouan C4i swinging bucket centrifuge (San Jose, CA). Ultrafiltrate was pooled from collections and used in the preparation of the calibrators and QC materials.

2.5. Instrument and acquisition parameters

Chromatographic separation was performed on the Thermo Scientific Prelude SPLC system (San Jose, CA) operating in laminar only mode (LX). Specimens were maintained at 4 °C in a CTC PAL autosampler (Carrboro, NC). Cefazolin and its structural analog were chromatographically separated using a Phenomenex Kinetex C8 (50 × 2.1 mm^2, 1.7 μm particle size) column (Torrance, CA) maintained at 40 °C. The mobile phase system consisted of water containing 2% acetonitrile and 0.1% formic acid (mobile phase A, MPA) and acetonitrile containing 0.1% formic acid (mobile phase B, MPB). A third solvent consisting of 45:45:10 acetonitrile:isopropanol:acetone was introduced to the analytical column during the wash step. To reduce carryover, 10 μL of 1 mM EDTA was injected into the injection port and flowed through the column between analyses. Full chromatographic conditions are listed in Table 1. The total method time was 5 min.

Detection was performed on a Thermo TSQ Vantage triple quadrupole mass spectrometer (San Jose, CA) using a heated electrospray ionization source and operated in positive ionization mode with nitrogen sheath gas supplied by a Parker-Balston N2-80 nitrogen generator (Haverhill, MA). The detection window was from 0.5 to 2.5 min of the chromatographic run. Collision cell gas was ultra-high purity argon supplied by Airgas (Radnor, PA). The electrospray source was operated under the following conditions, which was optimized for cefazolin product ion generation: spray voltage 4500 V, vaporizer temperature 200 °C, sheath gas pressure 30, aux gas pressure 35, and capillary temperature 200 °C. Quantifier transitions for cefazolin and cloxacillin were 455 → 156 m/z (collision energy (CE): 15 eV) and 437 → 278 m/z (CE: 14 eV), respectively. Qualifier transitions for cefazolin and cloxacillin were 455 → 323 m/z (CE: 9 eV) and 437 → 160 m/z (CE: 15 eV), respectively.

2.6. Data evaluation

XCalibur 2.2 software (Thermo Scientific) was used for data acquisition and processing. Calibration curves were generated using linear regression with $1/x^2$ weighting and peak areas generated after Gaussian smoothing using the Genesis integration algorithm. Microsoft Office Excel 2010 was used to determine coefficients of variation. Outliers were identified using the Grubb's test for outliers. Carryover effects were evaluated using EP Evaluator Release 8 (Data Innovations, South Burlington, VT). Figures were prepared using ACD/ChemSketch, R, and Inkscape.

Table 1
Chromatographic conditions for the determination of cefazolin and free cefazolin.

Step	Start time (min)	Flow rate (mL/min)	%A	%B	Gradient
1	0	0.5	95	5	Step
2	0.5	0.5	5	95	Linear ramp
3	2.5	0.5	5	95	Step
4	3.0	0.05+0.75 Wash	5	95	Step
5	3.5	0.5	95	5	Linear ramp
6	3.75	0.5	95	5	Step

Coefficient of variation (%CV) is defined below, where σ is the standard deviation and $\bar{x}$ is the average concentration observed.

$$\%\mathrm{CV} = \frac{\sigma}{\bar{x}} \times 100 \tag{1}$$

%DEV is defined below, where $x_{Expected}$ is the theoretical concentration of the QC material, and $\bar{x}_{Observed}$ is the average concentration determined for a QC material by solving for its concentration using the aforementioned regression equation relating peak area ratio to calibrator concentration.

$$\%\mathrm{DEV} = \frac{\left(\bar{x}_{Observed} - x_{Expected}\right)}{x_{Expected}} \times 100 \tag{2}$$

%DIF is defined below, where $x_{Reference}$ is the average measured concentration of the QC material in a reference condition, and $\bar{x}_{Treated}$ is the average concentration measured for the same level QC material treated differently.

$$\%\mathrm{DIF} = \frac{\left(\bar{x}_{Treated} - x_{Reference}\right)}{x_{Reference}} \times 100 \tag{3}$$

%ME, %PE, and %RE are presented as relative recoveries, where $\bar{x}_1$ is the average determined peak area intensity for a reference treatment for a component (cefazolin or cloxacillin) determined from a QC material and $\bar{x}_2$ is the average area intensity test treatment for a QC material. For example, in determining cefazolin matrix effects, $\bar{x}_1$ is the average area intensity for cefazolin in unextracted preparation and $\bar{x}_2$ is the average area intensity for cefazolin in post-extracted material.

$$\%\mathrm{ME},\ \ \%\mathrm{PE},\ \%\mathrm{RE} = \frac{\bar{x}_2}{\bar{x}_1} \times 100 \tag{4}$$

3. Method validation

The LC–MS/MS methods were validated based on FDA Guidance for Industry: Bioanalytical Method Validation recommendations [26]. Validation metrics included intra- and inter-assay precision and accuracy, linearity, stability, carryover, and matrix effects. These parameters were fully characterized for total cefazolin in human plasma. Free cefazolin was validated in human plasma ultrafiltrate by addressing precision, accuracy, and carryover. Cord blood was evaluated by measuring recovery of cefazolin in cord blood and cord blood ultrafiltrate spiked with cefazolin from a calibration curve prepared in non-cord blood plasma.

3.1. Precision and accuracy

Intra-assay precision was determined by replicate injection ($n=6$) of total or free cefazolin QC materials at four levels. Observed means, SDs and %CVs were assessed at each level. The LLOQ was defined as the lowest calibrator on the calibration curve, and in accordance with FDA recommendations, yielded accuracy and precision criteria of $\leq 20\%$DEV from theoretical concentrations and imprecision of $\leq 20\%$CV. Targets for levels above the LLOQ were $\leq 15\%$DEV from theoretical concentrations and imprecision of $\leq 15\%$CV. Total cefazolin QC materials were prepared at the lower limit of quantification (LLOQ; 0.48 μg/mL), low (1.4 μg/mL), mid (18 μg/mL) and high (410 μg/mL) concentrations in plasma by spiking stock solutions into plasma. Free cefazolin QC materials were set at LLOQ (0.048 μg/mL), low (0.14 μg/mL), mid (1.8 μg/mL) and high (41 μg/mL) concentrations in plasma ultrafiltrate. Inter-assay precision was determined by evaluating the observed concentrations for each level from three separate analytical runs and sample preparations, which is consistent with FDA recommendations. Concentrations determined were those generated from the calibration curve performed on each run.

3.2. Carryover

Carryover was determined by alternating injections of low (L) concentration calibrator (either 0.48 or 0.048 μg/mL for cefazolin or free cefazolin, respectively) and high (H) concentration calibrator (either 410 or 41 μg/mL for cefazolin or free cefazolin, respectively) and comparing the mean concentration of the low calibrators injected prior to high calibrator to those injected after. Specifically, calibrators were injected in the order: L1, L2, L3, H1, H2, L4, H3, H4, L5, L6, L7, L8, H5, H6, L9, H7, H8, L10, H9, H10, and L11. Acceptability criteria were mean post-high injection levels <3SD greater than pre-high injection levels. These acceptability criteria are consistent with CLSI protocol EP10-A2 [27]. Moreover, during routine analysis, injections of solvent A (0.1% formic acid) were injected following injection of the highest concentration calibrator. Acceptability criterion was post-injection blank signal, measured as chromatographic peak area $<20\%$ of the signal observed for the lowest concentration calibrator.

3.3. Stability

Stability studies were conducted for total cefazolin analysis. Low, mid, and high QC levels were subjected to injection matrix stability, sample matrix stability, and freeze–thaw stability. Injection matrix stability experiments were conducted by re-analyzing QC extracts maintained at 4 °C four days after preparation by re-analyzing calibrators and QC materials, quantifying on the basis of the new calibration curve, and comparing to the original values. Sample matrix stability experiments were conducted by analyzing quality control materials after incubating for 24 h at room temperature. Freeze–thaw stability experiments were conducted by comparing freshly compared quality control material to material that had undergone $n=4$ freeze–thaw cycles using a freezer maintained at −20 °C. Stock stability experiments were conducted where stock solution was incubated at room temperature, 4 °C, and −20 °C for 3 weeks. Acceptability was determined as ≤ ±15%DIF from the signal acquired from the material that had been stored at −20 °C.

3.4. Matrix effects

Matrix effects studies were conducted for total cefazolin analysis. Matrix effects on ionization were assessed using methods previously described by Matuszewski and colleagues [28] using sets of QC materials prepared at low, mid, and high concentrations. These materials were prepared for analysis in three independent sets: unextracted, pre-extracted, and post-extracted. Briefly, unextracted samples were prepared using the described protocol but substituting MPA (0.1% formic acid) in place of plasma, pre-extracted samples containing QC levels spiked into plasma from six individual lots, and a post-extracted set, which involved the addition of a spiking solution into post-extracted plasma matrix from the aforementioned six independent lots. The final concentrations at low, mid and high QCs were maintained across all three sets. The raw peak area values generated by these analyses were compared to calculate matrix effects, recovery efficiency, and processing efficiency. Individual set %CVs were also determined. Matrix effects were evaluated by the recovery ratio of post-extracted:unextracted; recovery efficiency was evaluated by the recovery ratio of pre-extracted:post-extracted; processing efficiency was evaluated by the recovery ratio of pre-extracted:unextracted.

3.5. Cord blood comparison

The described LC–MS/MS method was also used to assess drug quantification of total cefazolin in cord blood and free cefazolin in cord blood ultrafiltrate, respectively. Acceptability of quantifying drug concentrations in cord blood from an adult human plasma (non-cord blood) calibration curve was determined by calculating %DIF of low, mid, and high QC levels as compared to adult plasma QC material analyzed on the same calibration curve. Cefazolin working solutions for the preparation of QC materials for total and free cefazolin were used to generate equivalent concentration materials in both cord blood plasma and plasma ultrafiltrate. Cord blood plasma ultrafiltrate was prepared using the same protocol for determination of free cefazolin: briefly, Corning ultrafiltration devices were implemented for the filtration of 200 μL sample. A pool of ultrafiltrate was generated for the preparation of QC materials. Cord blood materials were prepared at the same low, mid, and high QC concentrations as prepared for total and free cefazolin determination. Acceptability limits were defined as ≤ ±15%DIF from material prepared in human plasma or human plasma ultrafiltrate; this acceptability threshold has been implemented for stability-challenged samples, and was thus applied when comparing plasma and cord blood results.

4. Results and discussion

4.1. Liquid chromatographic–tandem mass spectrometric (LC–MS/MS) parameters

Cefazolin was chromatographically separated from matrix components under a gradient elution profile with a Phenomenex Kinetex C8 column (Table 1 and Fig. 2). Using the described method, cefazolin and cloxacillin eluted at 1.5 and 2.0 min, respectively. The Phenomenex Kinetex C8 column is a sub-2 μm porous particle column that lends itself to clinical applications because of fast, sharp peaks and moderate back pressure [29]. Due to the lack of availability of isotopically labeled cefazolin, a β-lactam structural analogue, cloxacillin, was used as an internal standard. Other methods described for cefazolin analysis also used structural analogues as internal standards [23–25]. Acetonitrile was chosen as mobile phase B as it demonstrated higher ionization efficiency than methanol (data not shown). The addition of EDTA into the injection port in line with the column, as well as introduction of a strong wash solvent during the wash step, was used in this method to mitigate carryover; during initial stages of development, carryover was observed to be ~1–2% of the signal intensity area of the high concentration material. This carryover was eliminated after replicate injections of blank mobile phase A (Fig. 3). This observation, combined with the necessity to achieve an appropriately dynamic analytical measuring range (AMR), resulted in the subsequent modifications to the LC method. The strong wash solvent was introduced using a second binary pump with a switching valve and a tee into the analytical column.

Optimized ionization source and mass spectrometry parameters for cefazolin and cloxacillin were determined by manually adjusting conditions during direct infusion. Both compounds were analyzed in their protonated $[M+H]^+$ forms. Selected reaction monitoring (SRM) mode ion transitions were selected on the basis of signal intensity and imprecision during suitability experiments. Moreover, though the molecular formula of these fragment ions was not confirmed with labeling studies, their masses indicate they are not products of common losses (condensation, decarboxylation, etc.) and may contain the tetrazole ring, which is the characteristic R^2 moiety for cefazolin. Chromatograms generated from qualifier ion transitions were evaluated on the basis of their peak shape. Application of qualifier transitions is especially important when utilizing a structural analogue as an internal standard for quantitative mass spectrometry. One major consequence of a structural analogue as an internal standard is that it often does not co-elute with the analyte. Potential interferences may cause ion suppression and significantly affect the accuracy of results.

The method published by Douglas et al. used *in vivo* microdialysis to determine free cefazolin concentrations, which is impractical for routine clinical testing [23]. The method published by Zhang et al. presented a relatively comprehensive validation for determining total and free cefazolin in human plasma, but did not provide details on comparative studies in cord blood, nor did it provide details on

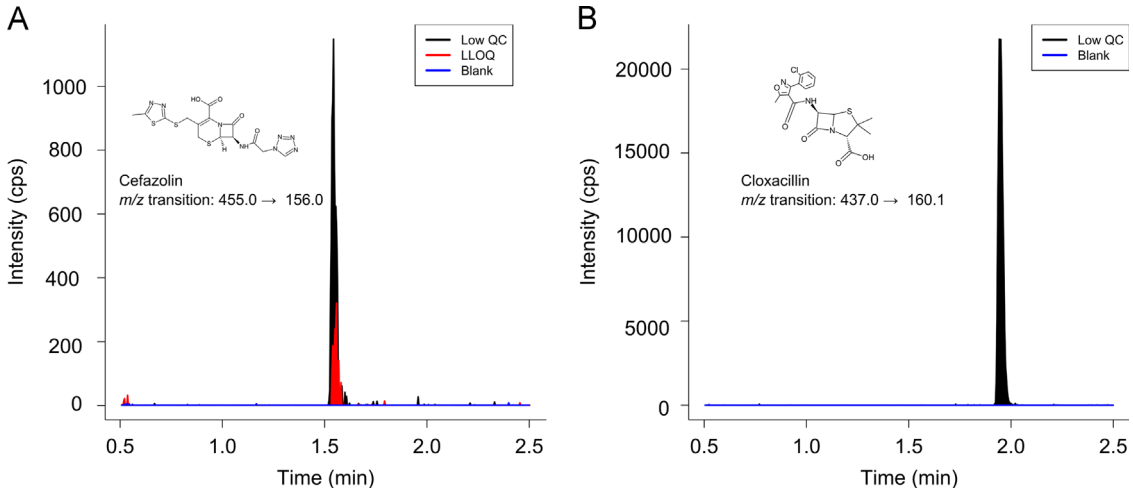

Fig. 2. Representative chromatograms for (A) cefazolin LLOQ, low QC and blank plasma and (B) cloxacillin internal standard and blank plasma.

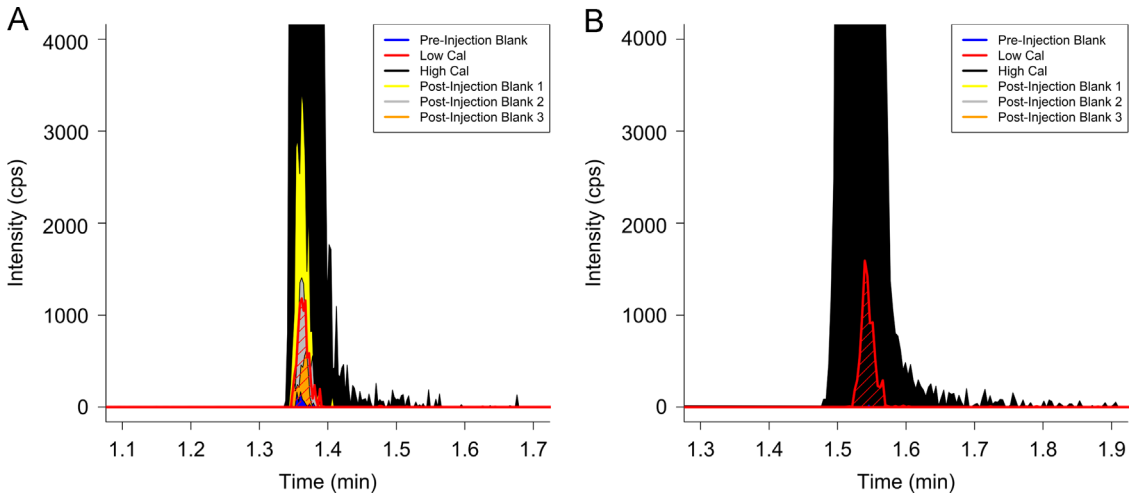

Fig. 3. Representative chromatograms of carryover optimization sequences. Chromatograms from (A) are prior to optimization. Chromatograms from (B) are post-optimization.

improving analytical selectivity through the introduction of a qualifier ion transition [21]. Evaluation of qualifier ion transitions adds additional analytical specificity to a mass spectrometry method because interferences may have differential efficiency at producing signals for different ion transitions. Cord blood, while not an independent matrix, was validated in terms of precision and accuracy studies to assess any potential interferents on drug quantification and to better characterize the overall ruggedness of the analytical method. This method was validated using FDA Bioanalytical Method Validation guidelines because it is a well-established set of guidelines suitable for pharmacokinetic studies, which will be the primary application for the assay.

Ultrafiltration was chosen to isolate the free fraction of cefazolin because it is rapid, relatively inexpensive to implement on a limited study scale, and only requires a microcentrifuge, which is commonly available in a clinical research laboratory. The internal standard was added to the ultrafiltrate of processed material because the addition of material prior to filtration would disrupt the equilibrium of the system. As a result, we used standard filtration conditions, particularly those designed to filter albumin, to sample the free fraction of cefazolin using ultrafiltration. The topic in general has been evaluated in the literature, particularly in the application of free phenytoin determination by immunoassay following ultrafiltration [30].

4.2. *Precision, accuracy and calibration curve analysis*

Intra- and inter-assay precision and accuracy for total cefazolin and free cefazolin were determined following the recommendations of the FDA guidelines as previously described (Table 2). Total cefazolin intra-assay precision ranged from 6.2% to 20.0%CV at the LLOQ. Intra-assay accuracy as %DEV ranged from −4.3% to 14.8%. Total cefazolin inter-assay precision ranged from 6.2% to 19.1% at the LLOQ. Inter-assay accuracy as %DEV ranged from −7.7% to 11.9%. Free cefazolin intra-assay precision ranged from 7.2% to 17.8% at the LLOQ. Free cefazolin intra-assay accuracy ranged from −6.7% to 3.4%. Free cefazolin inter-assay precision ranged from 5.7% to 18.5% and inter-assay deviation ranged from −1.9% to 2.5%. All observed results were acceptable for all QC levels.

The AMRs of the assays were 0.48–480 μg/mL for total cefazolin and 0.048–48 μg/mL for free cefazolin (Fig. 4 and data not shown). The AMRs are sufficiently dynamic and encompass typical cefazolin concentrations observed following standard dosing, as total cefazolin plasma concentrations typically achieve levels ~1.4–141 μg/mL [22]. The AMR was expanded ~3-fold beyond this limits to account for unexpected over-dosing, under-dosing, as well as studies that adjust the timing of dosing. Standard dosing protocols include 1–2 g

Table 2
Inter-assay and intra-assay precision and accuracy.

QC level	Concentration (μg/mL)	Intra-assay precision and accuracy				Inter-assay precision and accuracy			
		Mean (μg/mL)	SD (μg/mL)	%CV	%DEV	Mean (μg/mL)	SD (μg/mL)	%CV	%DEV
Total cefazolin									
LLOQ	0.48	0.547	0.110	20.0	14.8	0.534	0.102	19.1	11.9
Low	1.4	1.52	0.171	11.2	6.5	1.49	0.151	10.1	4.4
Mid	18	18.7	1.93	10.3	4.7	18.7	1.49	8.0	4.6
High	410	388	23.9	6.2	−4.3	374	23.3	6.2	−7.7
Free cefazolin									
LLOQ	0.048	0.0445	0.00790	17.8	−6.7	0.0467	0.00650	18.5	1.1
Low	0.14	0.145	0.0147	10.1	1.1	0.140	0.0176	12.6	−1.9
Mid	1.8	1.79	0.186	10.4	0.0	1.83	0.153	8.4	2.5
High	41	41.9	3.01	7.2	3.4	41.3	2.33	5.7	1.9

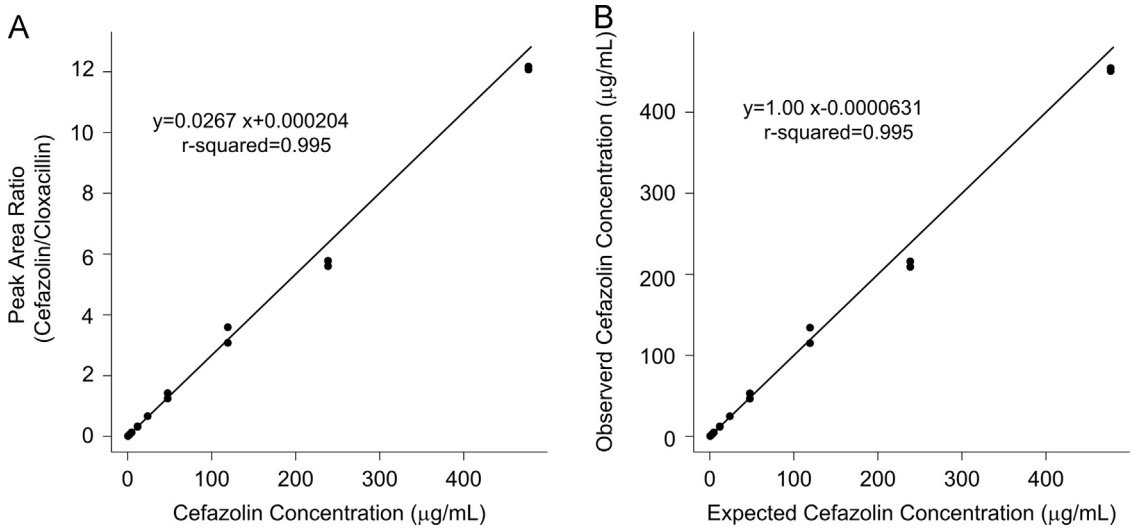

Fig. 4. Representative calibration curves for total cefazolin analysis. The *y*-axis of (A) is the peak area ratio observed between cefazolin and cloxacillin quantifier transitions. The *y*-axis of (B) is the calculated concentration using linear regression.

cefazolin t.i.d. for treatment of infections or 1–2 g single dose for prophylaxis during surgery [2,31,32]. The analytical measuring range of unbound cefazolin spans 1 order of magnitude less than the analytical measuring range for total cefazolin; this is consistent with plasma protein binding resulting in free concentrations ~10% of total concentrations [33]. Both assays utilized standard curves constructed using $1/x^2$ weighted linear regressions of the peak area ratio of cefazolin and cloxacillin relative to the cefazolin concentration of calibrators. The standard curve intercepts and linear coefficients were used to determine total cefazolin and free cefazolin concentrations in quality control materials and unknown specimens. The average correlation coefficient R^2 for regression fittings was ≥ 0.99 (Fig. 4). The upper limit of quantification was determined by observing the highest concentration that provided a linear calibration curve, in this method that concentration (480 or 48 μg/mL for total cefazolin and free cefazolin) provided an analytical measuring range of three orders of magnitude. The upper end of the calibration curve does appear to slightly under-recover, as reflected by the high QC bias of −7.7%, but this small value was still within the acceptability criterion defined by FDA Bioanalytical Method Validation guidelines. Adoption of an isotopically labeled internal standard of cefazolin may mitigate this bias.

4.3. Carryover

Carryover analysis was conducted according to CLSI guidelines by evaluating the determined average concentration of low concentration standards injected in alternating series with high concentration standards [27]. Overcoming issues with carryover was critical during method development because of the broad dynamic range of the method. Carryover was not noticed when observing analogous signal of the cloxacillin internal standard, suggesting potential accumulation of cefazolin in the SPLC system at high concentrations. Based on these observations, a stringent wash of 45:45:10 acetonitrile:2-propanol:acetone was introduced during the wash step as well as introducing EDTA into the injection port post-sample injections. These additional steps were relatively facile to implement as the EDTA could be added to a well in the 96-well plate used for specimen preparation and the stringent wash step was easy to introduce by using a union instead of a turboflow column in the TX position of the SPLC. The addition of these steps greatly reduced carryover (Fig. 3). In order to continuously monitor carryover, injection sequences were programmed to inject blank MPA (0.1% formic acid) after the highest concentration calibrator (480 or 48 μg/mL cefazolin for total cefazolin and free cefazolin, respectively). After introduction of the stringent wash and post-injection EDTA bolus we did not experience any issues with carryover during assay validation.

Table 3
Cefazolin stability studies.

QC Level	Freeze–thaw cycle, $n=6$			Sample matrix stability, $n=6$			Injection matrix stability, $n=6$		
	Control mean	Treated mean	%DIF	Control mean	Treated mean	%DIF	Control mean	Treated mean	%DIF
Low (1.4 μg/mL)	1.54	1.39	−9.7	1.53	1.34	−15.0	1.54	1.47	−0.8
Mid (18 μg/mL)	17.9	16.6	−7.3	18.1	18.1	2.7	17.9	18.8	2.7
High (410 μg/mL)	388	345	−11.2	388	350	−9.8	379	362	−4.4

The observed signal of a post-injection blank following a high QC sample was $<2\%$ of the signal observed at the LLOQ, indicating the successful abrogation of carryover.

4.4. Stability

Stability studies were conducted to evaluate cefazolin stability in injection matrix, sample matrix, and after serial freeze–thaw cycles (Table 3). Injection matrix stability was evaluated after allowing extracts to incubate at 4 °C for four days in the autosampler prior to injection and comparison to freshly prepared quality control material. Percent differences from this experiment ranged from −4.4% to 2.7%. Sample matrix stability was evaluated by allowing a control material to thaw and sit at room temperature for 24 h prior to processing and analysis. Comparison of these samples to freshly thawed and analyzed samples yielded percent differences ranging from −15.0% to 2.7%. Freeze–thaw stability was evaluated by comparing recovery of QC material that had undergone $n=4$ freeze–thaw cycles prior to analysis relative to quality control material prepared fresh. Comparison of these samples to freshly thawed and analyzed samples yielded percent differences ranging from −11.2% to −7.3%. The recovery determined in these experiments was within acceptable criteria ($\leq \pm 15\%$ DIF). However, the freeze–thaw cycle data all indicate negative differences, which suggest some instability of the analyte to sustain free–thaw cycles, leading to degradation.

4.5. Selectivity and matrix effects

No peaks were observed near the retention times for cefazolin or cloxacillin in blank plasma (Fig. 2). Moreover, six independent lots of human plasma were evaluated in the process and showed no interfering peaks (data not shown). Matrix effects were evaluated by comparing peak area signals generated from cefazolin spiked into water (unextracted) and processed according to the protocol, cefazolin spiked into plasma and processed according to protocol (pre-extracted), or spiked into blank plasma extract (post-extracted). Matrix effects are determined by comparing the apparent signal from post-extracted material to unextracted material. The recovery efficiency for matrix effects at low, mid, and high QC levels ranged 88.5–106.6% for cefazolin and 91.7–98.9% for cloxacillin (Table 4). At each QC level, there is less than 10% difference in overall matrix effects between cefazolin and cloxacillin, further supporting the use of cloxacillin as an internal standard. Recovery efficiency was determined by comparing the peak area intensities of pre-extracted material to post-extracted material. These recoveries ranged from 74.6% to 87.9% for cefazolin and 101.4% to 107.6% for cloxacillin. It is expected for recovery of cefazolin to be less than that of cloxacillin as cefazolin must be isolated from the plasma matrix, whereas the internal standard is in solvent. Processing efficiency was evaluated by comparing pre-extracted material to un-extracted material. The recoveries ranged from 68.7% to 93.7% for cefazolin and 93% to 106% for cloxacillin. The overall processing efficiency for cefazolin reflects the minimal matrix effects seen in addition to the decreased recovery from plasma. However, as there are no relative matrix effects and cefazolin concentrations are determined by the peak area ratio of drug to internal standard, the method may be used for cefazolin quantification.

4.6. Cord blood comparison

In lieu of full validation by preparing calibration and quality control material sets in cord blood, we validated its acceptability as a specimen matrix by evaluating recovery of cefazolin spiked directly into cord blood or cord blood ultrafiltrate. These concentrations were determined using calibration curves generated using total cefazolin calibrators in human plasma or free cefazolin calibrators in human plasma ultrafiltrate for total cefazolin and free cefazolin, respectively. The values determined from the cord blood plasma quality control materials were compared to the values determined from plasma and plasma ultrafiltrate. The difference from the two values was calculated and a %DIF was determined, with an acceptability criteria of $\leq \pm 15\%$. The relative recovery for cefazolin in cord blood and cord blood ultrafiltrate ranged −4.9% to 9.1% for total cefazolin and −1.4% to −11.9% for free cefazolin (Table 5). These values were within our limit of acceptability of $\leq \pm 15\%$DIF. These results suggest that cord blood plasma and cord blood plasma ultrafiltrate may be analyzed alongside plasma and plasma ultrafiltrate specimens using the aforementioned conditions described in the protocol. This is the first demonstration verifying acceptability for analysis of both total and free cefazolin in plasma and plasma ultrafiltrate as well as cord blood plasma and cord blood plasma ultrafiltrate.

The described LC–MS/MS methods facilitate the quantification of total and free cefazolin in both adult and cord blood plasma. These methods take advantage of convenient ultrafiltration techniques for separating the free drug from plasma proteins. The methods are fast at 5 min per analysis and only require 20 μl of plasma for determination of total cefazolin and 200 μl of plasma for determination of free cefazolin. However, there are some limitations to the described methods. The steps required to achieve satisfactory carryover performance with additional wash steps that could be prohibitive if the HPLC does not easily facilitate introduction of strong wash solvent during an analysis. Another limitation is the necessity of consuming an ultrafiltration device for assessing free cefazolin concentrations. The free cefazolin method uses standard ultrafiltration conditions to remove plasma proteins, but the calibrators and quality control materials were prepared directly in plasma ultrafiltrate. Ideally, a method would utilize well-characterized materials of known free drug concentration as calibrators and quality controls. Additionally, alternative methods to ultrafiltration, such as rapid equilibrium dialysis, may reduce the amount of technician time required for analysis. Moreover, these methods have not been compared to a reference laboratory due to a lack

Table 4
Total cefazolin matrix effects studies describing recoveries for the analyte cefazolin (CFZ) and cloxacillin (CLX).

QC level	Analyte peak area			Internal standard peak area			Matrix effects (%)		Recovery efficiency (%)		Processing efficiency (%)	
	Un-extracted	Post-extracted	Pre-extracted	Un-extracted	Post-extracted	Pre-extracted	CFZ	CLX	CFZ	CLX	CFZ	CLX
Low	720	768	675	37,976	37,576	40,159	106.6	98.9	87.9	106.9	93.7	106
Mid	10,697	9858	7353	32,609	29,911	30,325	92.2	91.7	74.6	101.4	68.7	93.0
High	223,649	197,838	163,387	30,618	28,727	30,921	88.5	93.8	82.6	107.6	73.1	101.0

Table 5
Comparison of total cefazolin recovery from plasma and cord blood plasma and comparison of free cefazolin recovery from plasma ultrafiltrate and cord blood plasma ultrafiltrate.

QC level	Plasma (μg/mL)	Cord blood plasma (μg/mL)	%DIF
Total cefazolin			
Low	1.05	1.14	9.1
Medium	13.6	13.0	−4.9
High	281	294	4.7
Free cefazolin			
Low	0.144	0.142	−1.4
Medium	1.92	1.86	−3.4
High	43.3	38.1	−11.9

of testing availability. Lastly, we have not evaluated the described methods in a patient cohort; this will be performed in forthcoming studies.

5. Conclusions

A rugged LC–MS/MS method for the quantification of total and free cefazolin in both adult and cord blood plasma has been developed and validated.

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