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METHOD ARTICLE

REVISED Determination of ceftriaxone in human plasma using liquid chromatography–tandem mass spectrometry [version 3; peer review: 1 approved, 2 approved with reservations, 1 not approved]

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Abstract

Ceftriaxone is a cephalosporin antibiotic drug used as first-line treatment for a number of bacterial diseases. Ceftriaxone belongs to the third generation of cephalosporin and is available as an intramuscular or intravenous injection. Previously published pharmacokinetic studies have used high-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV) for the quantification of ceftriaxone. This study aimed to develop and validate a bioanalytical method for the quantification of ceftriaxone in human plasma using liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). Sample preparation was performed by protein precipitation of 100 µl plasma sample in combination with phospholipid-removal techniques to minimize matrix interferences. The chromatographic separation was performed on an Agilent Zorbax Eclipse Plus C18 column with 10 mM ammonium formate containing 2% formic acid: acetonitrile as mobile phase at a flow rate of 0.4 ml/min with a total run time of 10 minutes. Both the analyte and cefotaxime (internal standard) were detected using the positive electrospray ionization (ESI) mode and selected reaction monitoring (SRM) for the precursor-product ion transitions m/z 555.0→396.1 for ceftriaxone and 456.0→324.0 for cefotaxime. The method was validated over the concentration range of 1.01-200 µg/ml. Calibration response showed good linearity (correlation coefficient > 0.99) and matrix effects were within the ±15% limit in 6 different lots of sodium

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heparin plasma tested. However, citrate phosphate dextrose plasma resulted in a clear matrix enhancement of 24% at the low concentration level, which was not compensated for by the internal standard. Different anticoagulants (EDTA, heparin and citrate phosphate dextrose) also showed differences in recovery. Thus, it is important to use the same anticoagulant in calibration curves and clinical samples for analysis. The intra-assay and inter-assay precision were less than 5% and 10%, respectively, and therefore well within standard regulatory acceptance criterion of ±15%.

Keywords

Ceftriaxone, bioanalytical method, human plasma, liquid chromatography tandem mass spectrometry

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Introduction

Antibiotic resistance development is a serious global health concern. The number of deaths from drug-resistant infections is predicted to increase from 700,000 to 10 million deaths annually by 2050 with an estimated cost of up to US$ 100 trillion[1,2]. The impact of resistance will increase patient mortality, morbidity, length of hospitalization, and health-care costs[3,4]. Furthermore, development of widespread antibiotics resistance decreases the number of effective antibiotics rapidly, and new drug discovery of novel drugs are not delivering new agents in sufficient rate to combat this rapidly increasing issue[5]. Therefore, all strategies to preserve efficacy of available drugs should be considered. Only with an in-depth understanding of the pharmacokinetic and pharmacodynamic (PK/PD) properties of a drug, can we achieve an evidence-based dosing (i.e. right drug, at the right dose and time). However, accurate and reliable bioanalytical methods for drug determination is a fundamental element to obtain reliable pharmacokinetic data.

Ceftriaxone is an important antibiotic drug that has been used as a first-line treatment for a number of bacterial infectious diseases for more than 30 years. Although the drug was discovered in the 1980s by Hoffmann-La Roche, some PK/PD properties, particularly in neonates, have not been well defined. Published pharmacokinetic studies were mostly performed in adults, excluding populations such as neonates with severe infections, infants, and malnourished young children[6-10]. To be able to perform PK/PD studies on these groups, a sensitive and selective bioanalytical method is needed.

Most of the previously published methods for ceftriaxone determination were performed by high performance liquid chromatography coupled with ultraviolet detection (HPLC-UV)[6-8,11,12], which is less sensitive and requires larger sample volume compared to LC-MS/MS assays. The large sample volumes required for the HPLC-UV detection render these assays inappropriate for measuring drug levels in neonates, infants and young children. Another drawback of the HPLC-UV techniques are long analysis times, often 10 to 20 minutes per sample.

The objective of this study was to develop and validate an accurate and sensitive bioanalytical method for ceftriaxone determination in low volume human plasma using LC-MS/MS. Only a few research publications have reported using LC-MS/MS for ceftriaxone determination in human biological samples[13-16]. Thus, this will be one of the first methods for ceftriaxone determination by LC-MS/MS and an alternative option to the already published methods.

Methods
Materials and reagents
Ceftriaxone disodium salt was supplied by Sigma-Aldrich Chemicals (St Louis, MO, USA). The internal standard, cefotaxime sodium salt, was from Santa Cruz Biotechnology (Dallas, TX, USA). Ceftriaxone-D$_3$ disodium salt hydrate was supplied by Medical Isotopes, Inc. (Pelham, NH, USA). Figure 1 shows
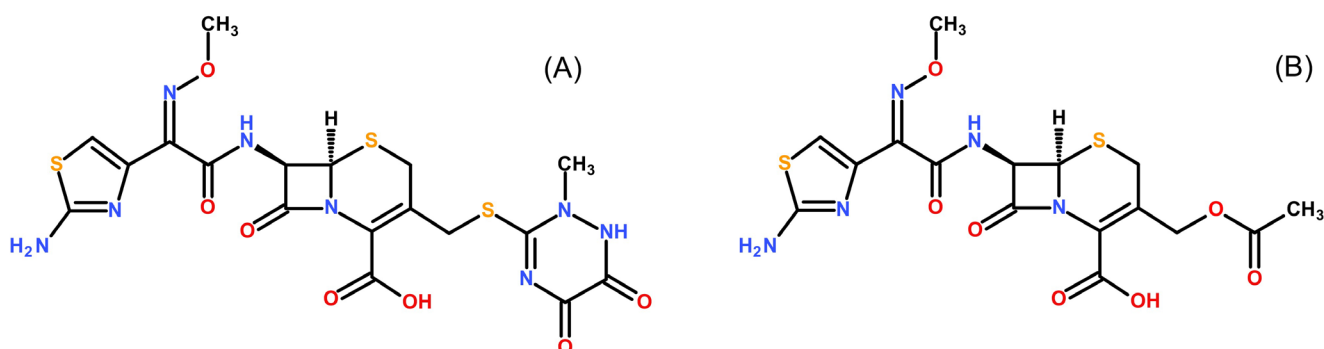

Figure 1. Molecular structures. Structures of ceftriaxone (**A**) and the internal standard cefotaxime (**B**) are shown.

the molecular structures of ceftriaxone and cefotaxime. Formic acid (LC-MS grade), ammonium formate (LC-MS grade) and ammonium bicarbonate (LC-MS grade) were supplied by Honeywell Fluka (Seelze, Germany). Acetonitrile, methanol and water (LC-MS grade) were obtained from J.T Baker (Phillipsburg, NJ, USA). Citrate phosphate dextrose (CPD) human plasma was provided by Thai Red Cross Society (Bangkok, Thailand). Ethylenediaminetetraacetic acid (EDTA), Li-heparin and Na-heparin human plasma were acquired from six different healthy donors at Faculty of Tropical Medicine, Mahidol University (Bangkok, Thailand). Ethical approval for the method development and validation was given by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (approval certificate no. MUTM 2018-028-01). All healthy volunteers provided a written informed consent before blood donation.

Sample preparation

Preparation of standard and working solutions. Stock solutions of ceftriaxone (10 mg/ml in its base form) and cefotaxime (10 mg/ml in its base form) were prepared in water and methanol, respectively. The solutions were stored in cryo vials at -80°C. Working solutions of ceftriaxone were prepared by serial dilution of the stock solution in water and used for spiking of plasma samples. All solutions were allowed to equilibrate to room temperature before use. Haemolysed plasma was made by adding frozen and subsequently thawed whole blood to spiked plasma samples in an amount of 1.5% of total volume, which equals 2-2.5 g/l haemoglobin, resulting in moderately haemolysed plasma.

Preparation of calibration standards and quality control samples. Calibration standards and quality control samples (QC) were prepared from two separate stock solutions to confirm the accuracy of the preparation. CPD human plasma was used to prepare calibration standards at concentrations of 1.01, 2.88, 8.21, 23.4, 66.7, and 200 µg/ml, including the lower limit of quantification (LLOQ: 1.01 µg/ml) and upper limit of quantification (ULOQ: 200 µg/ml), as well as over-curve dilution samples at 400 µg/ml. Quality control samples at 2.97, 24.1 and 155 µg/ml were prepared from a second stock solution. The final volume of working solution in plasma was less than 4% in all samples. Additional quality control samples were prepared with EDTA and heparin as anticoagulants.

Extraction procedure. Sample preparation was performed by protein precipitation followed by phospholipid removal using Phree phospholipid removal cartridge (Phenomenex, CA, USA) on an automated liquid handler, Freedom Evo 200 platform (TECAN, Mannedorf, Switzerland). Plasma samples (100 µl) were manually aliquoted into a 96-well plate followed by protein precipitation using 400 µl internal standard solution (acetonitrile containing cefotaxime at 2 µg/ml) except for the double blank which used 400 µl acetonitrile. The plate was mixed at 1,000 rpm for 10 minutes on a Mixmate (Eppendorf, Hamburg, Germany) and centrifuged at 1,100 × g at 20°C for 5 minutes. The supernatant (300 µl) was loaded on the Phree phospholipid removal plate and vacuum was applied until the whole

sample passed through the column. Finally, the extracted and cleaned sample was diluted with 500 µl water and mixed for 2 minutes at 1,000 rpm on a Mixmate and centrifuged at 1,100 × g for 2 minutes before injection.

Instrument and chromatographic conditions

Chromatography. The chromatographic separation was performed using a Dionex ultimate 3000 UHPLC (Thermo Scientific, CA, USA) consisting of a quaternary LC pump, a vacuumdegasser, a temperature-controlled micro-well plate autosampler set at 10°C and a temperature-controlled column compartment set at 40°C. The LC systems were controlled by Chromeleon Chromatography Data System (CDS) 6.80 software (Thermo Scientific, CA, USA). The analytical column was an Agilent Zorbax Eclipse Plus C18 (100 × 2.1 mm; I.D. 3.5 µm (Agilent technologies, CA, USA) connected with pre-column C18 AJ0-7596, 4 × 2.0 mm (Phenomenex, CA, USA). The mobile phases consisted of (A) acetonitrile-ammonium formate (10 mM with 2% formic acid) (12.5:87.5 v/v), (B) acetonitrile-methanol (25:75 v/v) and (C) 20 mM ammonium bicarbonate. The mobile phase gradient was A: 0-2.0 min (0.4 ml/min), B:C (5:95 v/v): 2.1-4.1 min (0.6 ml/min), B:C (90:10 v/v): 4.2-6.2 min (0.6 ml/min), and A: 6.3-10.0 min (0.4 ml/min), resulting in a total run time of 10 min. A sample volume of 2 µl was injected into the LC system.

Mass spectrometry. An API 5000 triple quadrupole mass spectrometer (SCIEX, MA, USA) was used for the detection and quantification. Data acquisition and analysis were performed using the Analyst® 1.7 software (SCIEX, MA, USA). The TurboV ionisation source (TIS) interface was operated in the positive ion mode with a drying temperature of 500°C. The interface voltage was set to 5.5 kV. The curtain, nebulizer, TIS gas pressure and declustering potential were set at 35, 50, 55 psi and 90 V, respectively. The selected reaction monitoring (SRM) was used to detect and quantify the precursor-product ion transitions m/z 555.0→396.1 for ceftriaxone and 456.0→324.0 for cefotaxime with a collision energy of 20 and 39 V, respectively.

Method validation

Method development and validation was performed in 2017 and 2018. The method was validated according to the US Food and Drug Administration (FDA, 2001) and European Medicines Agency (EMA, 2012) guidelines on bioanalytical method validation[17,18]. The EMA 2012 and the new FDA 2018 guidelines are very similar as described by Kaza *et al.*[19]. Accuracy and precision were determined by analysing five replicates of five concentrations (1.01, 2.97, 24.1, 155, 200 µg/ml) from four separate runs. The over-curve samples of 400 µg/ml were diluted with blank plasma (1:10) to evaluate dilution integrity. Accuracy was calculated by comparing the mean measured concentration to the nominal concentration at each QC level. Precision of the assay was evaluated by using analysis of variance (ANOVA) via the Analysis ToolPak add-in to Microsoft Excel 2016 (Microsoft, Redmond, WA, USA) and reported as the relative standard deviation (%RSD). Acceptance criteria for precision and accuracy are ±15%, except for LLOQ where ±20% is acceptable.

Linearity, selectivity and recovery. Linearity was evaluated by individually analysing the calibration standards from four separate runs. The regression model that resulted in the best accuracy of back-calculated concentrations of the calibration curves and QC samples was selected as the most appropriate regression model. Linear regression models, non-weighted and with weighting ($1/x$ and $1/x^2$), as well as quadratic model with $1/x$ weighting, were evaluated. Acceptance criteria for linearity are that 75% of non-zero calibrators should be within ±15%, except for LLOQ where ±20% is acceptable.

Selectivity was evaluated by injecting blank extracted samples and potentially interfering drugs during a regular analysis run. Six blank heparin plasma samples from six different blood donors and samples containing different anticoagulants (EDTA, CPD, Li-heparin and haemolysed Na-heparin) were used for the analysis. Potentially interfering drugs (i.e. acetaminophen, doxycycline and azithromycin, at a concentration of 100 ng/ml in methanol-water 20:80 v/v equivalent to a pre-extraction sample concentration of 1.5 µg/ml) were also evaluated. The occurrence of a peak response at the retention time of the analyte or internal standard indicates an interference and would require further investigation. Acceptance criteria for selectivity are that interference should be less than 20% of LLOQ and less than 5% of the internal standard response.

Recovery was determined by comparing two sets of samples. One set was spiked with ceftriaxone and internal standard before extraction (i.e. pre-spiked) and extracted as described in the method, including internal standard. However, to minimize variations as the Phree plate will retain some extraction liquid, a fixed volume of 150 µl extracted Phree eluate was taken and mixed with 500 µl water. The second set was extracted blank plasma with post-extract addition of ceftriaxone and internal standard, where 150 µl extracted blank plasma Phree eluate were taken and mixed with 350 µl water and 150 µl spiked water solution containing the same nominal concentration of ceftriaxone and internal standard as set 1. Thus, both sets contained the same volume ratio of extracted biological sample, acetonitrile and water. Recovery was determined by comparing the peak response of individual pre-spiked samples of set 1 to the average peak response of post-extract addition samples in set 2. Five replicates of each concentration at 2.97, 24.1 and 155 µg/ml were evaluated.

carry-over testing. The carry-over effect was investigated by injecting three replicates of blank samples after five injections of samples at ULOQ concentrations. To verify that this carry-over would not accumulate over time, carry-over was therefore tested in all 4 precision and accuracy batches and the carry-over set was positioned to run after approximately 50 sample injections had passed from the precision and accuracy batch. The presence of a signal greater than 20% of the LLOQ or 5% of the internal standard indicates carry-over.

Matrix effects. Six blank heparin plasma samples from six different blood donors, single plasma samples from different donors containing different anticoagulants (EDTA, CPD, Li-heparin and haemolysed Na-heparin) as well as neat solutions containing acetaminophen, azithromycin and doxycycline were evaluated.

Matrix effect was first assessed by post-column infusion (qualitative visualization)[20,21] infusing 10 µl/min of 1 µg/ml ceftriaxone and 1 µg/ml cefotaxime (internal standard) in water, to confirm that there was no signal that could potentially interfere at or around the retention times of ceftriaxone and the internal standard.

The quantitative evaluation of matrix effects was done by using a simplified approach described by Matuszewski *et al.*[22]. The matrix factor was calculated by comparing the peak response of extracted blank plasma using post-extract addition of ceftriaxone and internal standard to the average peak response of the analytes in neat matrix free reference solution at the same nominal concentrations. As in the recovery test, the same volume ratio of acetonitrile and extracted sample/water was maintained. Two concentrations (low and high) at 2.97 and 155 µg/ml were evaluated. Heparin plasma from six different donors was used for the analysis. The acceptance criteria for matrix effects was achieved when the RSD of the internal standard normalised matrix factor calculated from the 6 lots of donor matrix was below ±15%.

Stability. Stock solution 10 mg/ml was evaluated at -80°C and at +4°C for 5 days, and in room temperature (+24°C) for 8 hrs. The lowest concentration of working solution 50 µg/ml was also evaluated at -80°C for 5 days and at room temperature (24°C) for 5 hrs. Spiked plasma stored at ambient temperature and at 4°C for 48 h was used to evaluate short-term stability. Long-term stability of spiked samples at -80°C was evaluated after 7 months. Freeze-thaw stability was evaluated for plasma samples and haemolysed plasma samples for five cycles. The samples were stored at -80°C for 24 h followed by unassisted thawing at room temperature for 2–3 h and subsequent re-freezing at -80°C. The stability of precipitated samples stored at ambient temperature (about 23°C) for 4 h was also evaluated. The stability of extracted samples in the LC autosampler kept at 10°C was evaluated by re-injecting the calibrators and QC samples 65 h after initial injection. The acceptance criteria for stability was achieved when the RSD of stability samples was below ±15%, and the accuracy of mean concentrations was within ±15% of nominal concentration.

Results and discussion

The calibration range of 1.01-200 µg/ml was based on pharmacokinetic data from previously published studies[6,8,23], taking into account the sensitivity and linearity of the MS instrument. Reported population mean peak levels of ceftriaxone was reported to be below 200 µg/ml after a standard 2-g daily dose in critically ill patients with sepsis[8]. There is a possibility that some clinical samples have higher concentrations of ceftriaxone than covered by the calibration range. However, to maintain the ability to quantify these high-concentration samples, sample dilution integrity needs to been shown. An over-curve sample concentration of 400 µg/ml was evaluated for dilution integrity and demonstrated that such samples can be diluted and quantified using the developed method. Mean plasma concentrations, 24 h

after administration of ceftriaxone, were reported to be 5.3, 9.3 and 15.1 µg/ml after 0.5-g, 1-g, and 2-g of intravenous dose, suggesting adequate sensitivity to quantify the drug in patients to evaluate the pharmacokinetic properties[6].

Sample preparation and extraction

Various extraction solvents were evaluated for protein precipitation. Adding an acid, such as acetic acid or formic acid, often improves the precipitation of proteins and can improve recovery. However, acidic storage conditions affected the stability of ceftriaxone and degradation was observed. Sample dilutions with mobile phase buffer containing ammonium formate 10 mM with 2% formic acid (pH of about 2) resulted in substantial degradation of ceftriaxone leaving only 5% of its initial concentration after 2 days when stored at +4°C and less than 1% when stored for 3 days. However, dilutions in solutions with neutral pH resulted in 95–100% of initial concentration at same storage conditions and time durations described above. Neat acetonitrile and methanol both worked well as protein precipitation solvents. The results indicated that acetonitrile yielded lower ceftriaxone extraction recovery than methanol. However, methanol likely extracted more interfering components from plasma samples which gave more matrix effects compared to acetonitrile. To improve the sample clean-up further, three different phospholipid removal filtration plates were evaluated; HybridSPE (Supelco, PA, USA), Ostro (Water, MA, USA) and the Phree plate. The HybridSPE plate retained ceftriaxone, giving very low recovery yield. Both Phree and Ostro phospholipid removal plates showed similar performance with a recovery difference of less than 10% compared to only protein precipitation. The Phree plate was selected based on price and performance.

Instrumentation and chromatographic condition

Peak tailing of ceftriaxone has been observed and reported in the literature previously[14,15,24]. Various chromatographic columns (i.e. C18, C6-phenyl, CN and amide stationary phases) and mobile phases were screened in this study, but peak tailing of ceftriaxone could not be eliminated completely. Best peak shape was obtained with the C18 end capped column from Agilent Zorbax Eclipse Plus and used throughout validation experiments.

To evaluate the effectiveness of the sample clean-up and how much phospholipids passes through the LC column, fragment ions m/z 104 and 184 was monitored as described by Ismaiel *et al*,[25]. Protein precipitation resulted in a significant amount of phospholipids left in the sample while phospholipid removal plates resulted in a clean sample with very low amount of phospholipids left in the sample. No phospholipid interference was seen at the retention time of analyte or IS. Residues of strongly retained phospholipids could be eluted by utilising a LC-washout gradient of acetonitrile-methanol (25:75 v/v) preventing accumulation on the LC-column or interference of late eluting phospholipids in subsequent injections. Phospholipid removal plates also filtrated proteins and particles, and are particular useful for clinical studies with a large number of samples to process (i.e. less problems and downtime).

The ESI MS was operated in the positive ion mode and generated several abundant ceftriaxone fragment ions; m/z 396.3, 324.1, 167.3, 125.4 and 112.0 (Figure 2). Three of these fragment ions (*m/z* 396.3, 167.3 and 125.4) were evaluated for signal intensity and selectivity, and for any signs of interference. The precursor-product ion transition *m/z* 555.0→396.1 was
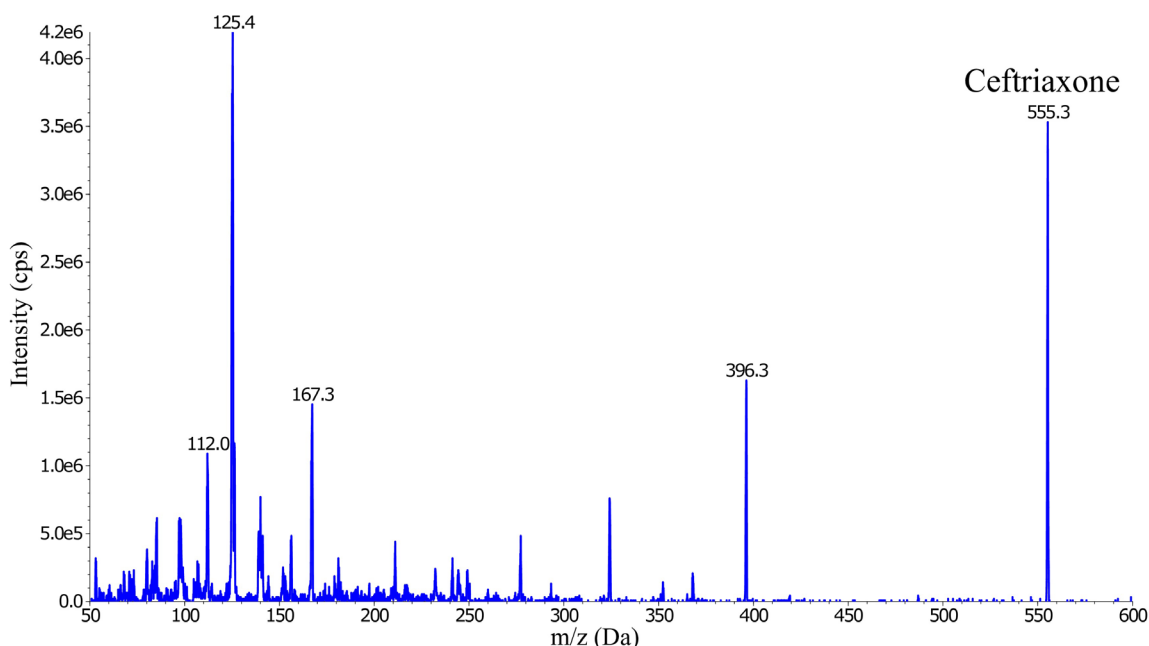
Figure 2. Collision energy scan and fragmentation product ions of ceftriaxone (555.3 *m/z*).

selected as the quantification trace because it showed approximately twice the intensity compared to the other two fragments.

Deuterium-labelled ceftriaxone (D_3) was evaluated in the method development phase as an internal standard. In positive ion mode it generated two fragment ions containing deuterium. The two fragment ions (m/z 399.0 and 327.0) were evaluated for signal intensity and selectivity, and for any signs of interference. Unfortunately, ceftriaxone interfered with the ceftriaxone-D_3 signal in the LC-MS/MS instrument. This could be explained by the naturally occurring isotope distribution of atoms in the structure, were the naturally abundant isotope in ceftriaxone (M+3) have the same mass as ceftriaxone-D_3 and hence cause interference[26,27]. The signal contribution from a ULOQ sample was about 40% of the ceftriaxone-D_3 internal standard signal (concentration of 1 µg/ml). Lowering the calibration range (ULOQ) and increasing the D_3-internal standard concentration would still produce a signal contribution to D_3-internal standard with more than 5%, thus over the acceptance limit for signal

interference to internal standard. There are other stable isotope internal standards, but these could not be evaluated due to time and funding restrictions. Thus, a substitute internal standard (cefotaxime) was chosen, which belongs to the same class of antibiotic as ceftriaxone but the two drugs are not administered together.

Validation
Accuracy and precision were evaluated by an ANOVA approach and all concentration levels were within the acceptance criteria, including the over-curve dilution integrity samples (Table 1). Alternative anticoagulants (EDTA, Na-heparin, Li-heparin) were evaluated at low and high QC levels and were within the acceptance criteria (Table 2). Raw data are available on Figshare[28].

Linearity, selectivity and recovery. The calibration curve was evaluated for linearity by different calibration models. The model that described the best concentration-response relationship

Table 1. Accuracy and precision of ceftriaxone determination. The method was validated by analysing five replicate samples of each concentration and repeated over four days. Accuracy and precision must not exceed 15% for each concentration, except for the LLOQ that should not deviate by more than 20%.

Value	Nominal conc. (µg/ml)	Intra-assay precision (%RSD)	Inter-assay precision (%RSD)	Total-assay precision (%RSD)	Accuracy (%)
LLOQ	1.01	4.31	4.18	4.29	0.50
QC 1	2.97	4.22	3.95	4.18	-13.6
QC 2	24.1	3.94	5.57	4.24	-8.90
QC 3	155	2.21	8.68	4.00	-13.0
ULOQ	200	3.29	8.71	4.59	2.80
Over-curve	400	3.59	9.29	4.95	-3.50

LLOQ, lower limit of quantification; QC, quality control; ULOQ, upper limit of quantification; Over-curve, i.e. sample dilution 10 times; RSD, relative standard deviation.

Table 2. Accuracy and precision of ceftriaxone in different anticoagulants. The method was validated by analysing five replicate samples of each concentration and repeated over four days. Accuracy and precision must not exceed 15% for each concentration. However, accuracy is not reported since the QC samples were compared against a calibration curve using CPD plasma and the recovery difference would bias the accuracy result.

Anticoagulant	Nominal conc. (µg/ml)	Intra-assay precision (%RSD)	Inter-assay precision (%RSD)	Total-assay precision (%RSD)
EDTA, QC 1	2.97	5.52	5.56	5.54
Na-Heparin, QC 1	2.97	7.53	13.5	8.75
Li-Heparin, QC 1	2.97	7.35	9.00	7.64
EDTA, QC 3	155	3.81	4.76	3.98
Na-Heparin, QC 3	155	4.10	10.5	5.62
Li-Heparin, QC 3	155	3.77	5.40	4.07

QC, Quality Control; RSD, Relative Standard Deviation.

was a linear regression with $1/x^2$ weighting, resulting in an accuracy of back-calculated concentration ranging from 92.1–104%. For selectivity, no interfering peaks were present in the blank plasma injections from the six different donors. Moreover, injection of possible concomitant drugs (i.e. acetaminophen, azithromycin and doxycycline) did not produce any interference. Blank plasma samples with CPD, EDTA, sodium heparin, lithium heparin and a sodium heparin sample with haemolysis were also evaluated. None of the anticoagulants or the haemolysis sample produced any interference.

The Phree plate and heparin plasma was used for determining ceftriaxone and internal standard recovery. Recovery was calculated as:

$$Recovery = \frac{(Response\ of\ pre-spiked\ plasma\ sample)}{(Response\ of\ blank\ plasma\ post\ extract\ addition\ (post-spiked))}$$

The recovery results for heparin plasma is presented in Table 3. There was a recovery difference for ceftriaxone using different anticoagulants, where CPD plasma generally achieved 10–15% higher recovery compared to heparin, and EDTA about 5–10% higher compared to heparin. Using the same anticoagulant in both calibrators and study samples is therefore important to avoid a bias in the result.

carry-over testing. Carry-over was a problem and difficult to eliminate. Initially an Agilent 1260 infinity system (Agilent technologies, CA, USA) was used and extensive testing with advanced needle wash programming and rotor changes was performed without being able to eliminate the carry-over. Later, a Dionex ultimate 3000 UHPLC was used, switching stainless steel to biocompatible tubing and introducing injection rotor switching during run did not prevent the carry-over issue. However, the carry-over did reduce over-time as the mobile phase flowed through the system and was eliminated given enough time (> 20 min) between injections. This is similar to a few other publications where ceftriaxone had a short retention time but a long total run time[12,14,15,29]. To reduce the time between injections, different washout solvents and solution mixes were tested. Carry-over was minimized by using a LC-washout gradient of acetonitrile-methanol (25:75 v/v), which reduced the elimination time to less than 20 min. Total run time could be shortened to 10 minutes by adding a second short washout step, using 20 mM ammonium bicarbonate (pH 8), and increasing the flow rate from 0.4 to 0.6 ml/min during both washouts (Figure 3).

Matrix effect. Matrix effect evaluation by post-column infusion can visualise the suppression zones caused by co-eluting

Table 3. Absolute recovery in heparin plasma.

Concentration/ Sample:	No: 1	No: 2	No: 3	No: 4	No: 5	Average	SD	RSD
QC 1, 2.97 µg/ml	33%	33%	28%	28%	34%	30%	3.00	9.88%
QC 3, 155 µg/ml	34%	38%	35%	34%	35%	35%	1.87	5.27%
IS for QC 1, 2 µg/ml	100%	101%	100%	98%	102%	100%	1.05	1.05%
IS for QC 3, 2 µg/ml	97%	99%	97%	99%	98%	98%	0.96	0.98%

QC, Quality Control; IS, internal standard.
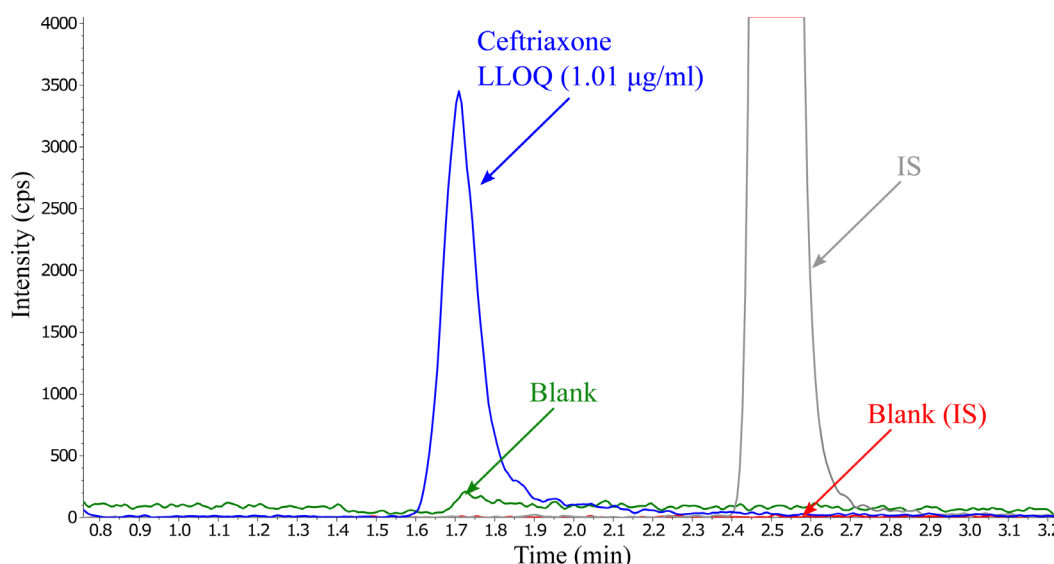
Figure 3. Overlay of ceftriaxone at LLOQ concentration containing internal standard (2 µg/ml) and the first blank injection after injecting five ULOQ samples, presenting no significant carry-over.

compounds, e.g. phospholipids are one such group of compounds known to cause suppression effects. If suppression happens at or around the retention time of the analyte or internal standard, adjustment to the mobile phase and sample preparation is required to create separation or to eliminate the co-eluting compounds. The developed method did not show any increase or drop at the retention time of ceftriaxone or internal standard signals from any of the six-donor blank sodium heparin plasma samples. Similarly, injection of extracted blank plasma with different anticoagulants, including haemolysis-plasma, and injection of possible concomitant drugs did not show any visual increase or decrease in the signal. However, the suppression/enhancement may not have been large enough to show up in the post-column visualised matrix evaluation (Figure 4).

A more precise approach is the quantitative matrix effect test that was calculated as:

$$\text{Matrix factor} = \frac{(\textit{Response of blank plasma post extract addition}\,(\textit{pre}-\textit{spiked}))}{(\textit{Average peak response in neat matrix free reference solution})}$$

and showed a small matrix enhancement effect (18%) for donor B at QC1 level (Table 4). To evaluate if the signal enhancement could be compensated by the internal standard, the internal standard normalised matrix factor was calculated as:

$$\text{Normalised matrix factor} = \frac{(\textit{Matrix factor for analyte})}{(\textit{Matrix factor for internal standard})}$$

The six different sources of plasma from donors A-F collected in sodium heparin anticoagulant showed an average normalized
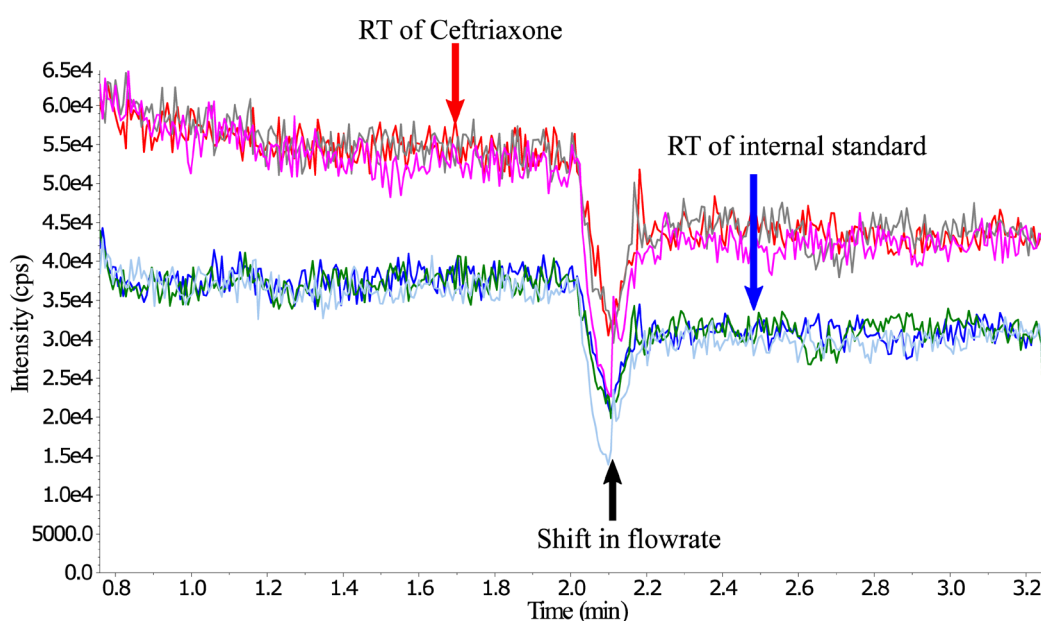
Figure 4. Post-column infusion (qualitative visualization of matrix effects) infusing 10 µl/min water solution of 1 µg/ml ceftriaxone (producing the higher signal intensity) and 1 µg/ml cefotaxime (internal standard, producing the lower signal intensity). Overlay of 3 injections of extracted blank plasma samples, Donor A & Donor B (both heparin) and one blank plasma with CPD as anticoagulant. *RT = Retention time.

Table 4. Matrix effects from different donors in heparin plasma. Donors A to F are individual donors collected using sodium heparin as anticoagulant.

Concentration/ Donor:	A	B	C	D	E	F	Average	SD	RSD
QC 1, 2.97 µg/ml	1.12	1.18	1.10	1.03	1.07	1.03	1.08	0.057	5.27%
QC 3, 155 µg/ml	0.93	0.87	0.93	0.91	0.93	0.88	0.90	0.027	2.96%
IS for QC 1, 2 µg/ml	0.99	1.03	1.00	1.01	1.01	1.01	1.01	0.013	1.32%
IS for QC 3, 2 µg/ml	1.01	1.05	1.03	1.05	1.01	1.03	1.03	0.016	1.59%
Normalised QC1/IS	1.13	1.15	1.10	1.02	1.06	1.02	1.07	0.057	5.30%
Normalised QC3/IS	0.93	0.84	0.90	0.87	0.92	0.86	0.88	0.036	4.12%

QC, Quality Control; IS, internal standard. Values less than 0.85 or higher than 1.15 would imply a matrix effect. However, if a matrix effect is present, then the RSD calculated from the different blank lots of matrices, should not be greater than ±15%.

matrix factor for QC1 of 1.07 ± 0.057 SD and an RSD of 5.3% (Table 4). This suggests that the precision of the method is not affected by different lots of plasma using the same anticoagulant when compared to the method precision in Table 2.

For the plasma samples containing different anticoagulants (i.e. EDTA, CPD, Li-heparin and haemolysed Na-heparin), the sample using CPD as anticoagulant resulted in a 27% signal enhancement at a concentration of 2.97 µg/ml concentration level. The CPD QC1 plasma normalised matrix factor was slightly lower but still showed a 24% signal enhancement (Table 5). Unfortunately, due to limited supply of volunteer donor blood, no further investigation could be done. However, matrix effects have also been reported by other authors, affecting mainly the lowest concentrations. Common features for all methods is the use of protein precipitation using either methanol or acetonitrile and all methods used C18 LC-columns for separation[14,15,30,31]. LC-MS separation and detection might not be able to avoid matrix effects. Ahsman et al.[30] reported a positive matrix effect for ceftriaxone and an accuracy of 117.2% for the QC1 level but the overall CV for the six lots stayed below 10%. Herrera-Hidalgo et al.[31] also reported a mean matrix effect of 119.2 ± 6.4% at the QC1 level and Lefeuvre et al.[32], reported matrix effects of about 130%, but in this case the stable isotope internal standard (ciprofloxacin-D8) did compensate for the effects. Ongas et al.[14] did not observe matrix effects in the post-column infusion evaluation and did not proceed with the quantitative matrix investigation and Meenks et al.[33] did not investigate matrix effects. Page-Sharp et al.[13] investigated matrix effects and reported no effects in their method, even though they use very similar extraction, LC and mobile phase settings as the other publications. One difference is that they used only 20 µl plasma volume whereas 100 µl plasma have been most commonly used in other publications. Decosterd et al.[34], reported that even with matrix effects at 165%, the use of ceftriaxone-13CD3 stable isotope internal standard compensated fully for this matrix effect. This resulted in a normalized matrix factor of 101.7%, demonstrating the importance of using a suitable internal standard. In our case, ceftriaxone interfered with the

ceftriaxone-D$_3$ signal in the LC-MS/MS instrument and could therefore not be used and the alternative internal standard in this work (cefotaxime) did not show any matrix effects and can therefore do little to compensate for matrix effects affecting ceftriaxone. The six lots of donor heparin plasma did not show matrix effects and only one plasma sample using CPD as anticoagulant showed matrix effects. However, a more suitable stable isotope internal standard (ceftriaxone-13CD$_3$) would have been desirable and should compensate for any potential differences in the signal[34].

Stability. Ceftriaxone stock solutions showed good stability above 98% at -80°C and at +4°C for 5 days and on the workbench at +24°C for 8 hrs. Also, the weakest working solution of 50 µg/ml demonstrated good stability of about 98% for storage at -80°C for 5 days and for 5 hrs on the workbench at +24°C. The stability samples were quantified using a calibration curve in CPD plasma. Stability samples in CPD plasma were compared to the average measured concentration of CPD QC samples added in the same run. The CPD calibration curve was also used to quantify heparin and EDTA stability samples due to limited supply of volunteer donor blood. However, since EDTA and heparin have different recovery from plasma compared to CPD, a direct comparison would be biased. Thus, stability samples were instead compared with the average measured concentration of the precision and accuracy of each anticoagulant. Short-term stability for up to 24 h at ambient temperature (about 23°C) and 4°C for ceftriaxone was confirmed in all anticoagulants and for CPD plasma up to 48 h. Long-term stability at -80°C was evaluated after 7 months (224 days) and showed good stability for all anticoagulants. QC samples in all anticoagulants presented good stability after freeze-thaw over five cycles, including plasma with moderate haemolysis. Protein precipitated samples also showed good stability when stored at ambient temperature (about 23°C) for 4 h prior to transferring the supernatant to the Phree phospholipid removal plate (Table 6).

Shrestha et al.[24] demonstrated rapid and substantial degradation of ceftriaxone in stress condition using acid hydrolysis with

Table 5. Matrix effects using different anticoagulants. Anticoagulants were collected from individual donors and are not from the same source.

Concentration/Anticoagulant	EDTA	CPD	Li-Hep	Na-Hep haemolysis
QC 1, 2.97 µg/ml	1.10	1.27	1.10	1.17
QC 3, 155 µg/ml	0.93	0.96	0.90	0.91
IS for QC 1, 2 µg/ml	1.03	1.02	1.03	1.05
IS for QC 3, 2 µg/ml	1.04	1.04	1.03	1.05
Normalised QC1/IS	1.06	1.24	1.07	1.12
Normalised QC3/IS	0.89	0.92	0.88	0.87

Hep, Heparin; QC, Quality Control; IS, internal standard. Values less than 0.85 or higher than 1.15 would imply a matrix effect.

Table 6. Stability of ceftriaxone in plasma under different conditions. Due to the recovery difference between anticoagulants, EDTA, Na-heparin and Li-heparin are compared to the average concentration of the four precision and accuracy batches for each anticoagulant and are presented as percentages.

QC1, 2.97 µg/ml	RT 24 hrs	RT 48 hrs	4°C 24 hrs	4°C 48 hrs	F/T cycle 3	F/T cycle 5	Precipitated 4hrs in RT	-80°C 224 days
CPD	106	100	102	103	97.7	94.0	94.2	103
CPD haemolysis	-	-	-	-	-	88.4	99.3	-
EDTA	105	-	113	-	103	103	98.0	95.5
Na-Hep	103	-	109	-	100	98.7	96.8	93.7
Na-Hep haemolysis	-	-	-	-	91.3	97.6	91.9	-
Li-Hep	105	-	98.3	-	95.7	99.5	103	96.0
QC3, 155 µg/ml	RT 24 hrs	RT 48 hrs	4°C 24 hrs	4°C 48 hrs	F/T cycle 3	F/T cycle 5	Precipitated 4hrs in RT	-80°C 224 days
CPD	99.8	99.6	101	103	99.1	95.0	101	109
CPD haemolysis	-	-	-	-	-	98.6	94.3	-
EDTA	105	-	104	-	97.9	95.5	90.9	92.8
Na-Hep	103	-	106	-	97.5	93.7	88.2	94.5
Na-Hep haemolysis	-	-	-	-	90.4	88.5	88.8	-
Li-Hep	101	-	108	-	96.1	94.2	91.4	98.8

Hep, heparin; RT, ambient room temperature (about 23°C), F/T, freeze and thaw, "-", not available.

0.1 M hydrochloric acid, resulting in 19.6% degradation of ceftriaxone in 30 minutes. Our experience, with ceftriaxone stored in the dark at +4°C in a solution of 10 mM ammonium formate containing 2% formic acid (pH about 2), was that ceftriaxone had experienced 95% degradation after 48 hrs with only 5% remaining. This could potentially be a problem if plasma samples are precipitated using acids or if acids are used in post-extraction dilutions where ceftriaxone will be in acidic conditions during the LC analysis. Ahsman et al.[30] reconstituted the samples using 0.1% aqueous formic acid before LC-analysis and Meenks et al.[33] precipitated plasma samples using 1% formic acid in methanol. However, none of them reported LC-stability data. Herrera-Hidalgo et al.[31] performed post-extraction dilution of samples using 0.5% formic acid in water and reported an LC-stability of 4 hrs. This would be a limiting factor in the number of samples that can be processed. A majority of the published quantification methods for ceftriaxone avoid using acids in extraction or dilutions. In our method, extracted samples in the LC autosampler, stored up to 65 h, showed less than 10% variation in QC concentrations if the full set of calibrators and QC was re-injected. However, comparing the original injection with the 65-h injection did show a loss of about 20%; however, the change is equal over the whole concentration range and will not be noticed if the full set of calibrators and QC are re-injected.

Conclusion

The use of LC-MS/MS resulted in higher sensitivity and selectivity than HPLC-UV. The developed method requires only a small volume of plasma (100 µl) and will allow for pharmacokinetic studies in children and other groups with limited sampling capabilities. However, there might still be a limitation for very small children, infants and neonates where only a very small amount of blood can be obtained from venepuncture or capillary sampling. Moreover, the incorporation of phospholipid removal techniques during sample preparation reduced particles and matrix interferences that could otherwise risk clogging the system and/or accumulate on the column. This sample preparation technique should preserve the MS instrument and column over time, enabling long-term usage without interruptions. Carry-over problems were solved by modifying the LC-gradient program by including an additional washout sequence. However, the spiked QC samples in EDTA and heparin plasma showed lower recovery than CPD. Thus, it is important to use the same anticoagulant in calibration curves and clinical samples for analysis. Spiked plasma samples showed good stability in various conditions over a short term and the extracted samples can be re-injected from the LC autosampler up to 65 h after extraction.

Data availability

Figshare: Supplementary files ceftriaxone plasma. https://doi.org/10.6084/m9.figshare.7775819.v1[28].

The following underlying data are available:

- Long-term stability 224 days.txt (Quantification data for long-term stability calculations of ceftriaxone in CPD, EDTA, Na-heparin and Li-heparin plasma)

- Precision and Accuracy run 1.txt (Quantification data for run 1 out of 4, for the accuracy and precision used in ANOVA calculations)

- Precision and Accuracy run 2.txt (Quantification data for run 2 out of 4, for the accuracy and precision used in ANOVA calculations)

- Precision and Accuracy run 3.txt (Quantification data for run 3 out of 4, for the accuracy and precision used in ANOVA calculations]

- Precision and Accuracy run 4.txt [Quantification data for run 4 out of 4, for the accuracy and precision used in ANOVA calculations)

- Recovery and matrix effects.txt (Peak areas of extracted QC samples, blank plasma post spiked and reference in neat solution for recovery and matrix effect calculations).

- Stability 4 hrs Haemolysis and Precipitation at RT.txt (Quantification data for the stability of precipitated samples in clear plasma and haemolysed plasma in different anticoagulants, stored 4 h in room temperature before transferring supernatant to Phree plate).

- Stability Freeze and Thaw.txt (Quantification data for testing repeated freeze and thaw stability of ceftriaxone in plasma using different anticoagulants including haemolysed plasma).

- Stability LC-stability over 65 hrs.txt (Quantification data testing ceftriaxone stability, comparing the difference in quantified concentration from original injected samples re-injection 65 h later).

- Stability RT and 4C 4hrs-48hrs.txt (Quantification data testing ceftriaxone stability in plasma with different anticoagulants stored in room temperature or in 4°C for 24 h (CPD tested up to 48 h)).

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

Acknowledgements

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Open Peer Review

Current Peer Review Status: ✖ ? ? ✔

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Version 3

Reviewer Report 15 August 2022

https://doi.org/10.21956/wellcomeopenres.19879.r51333

✔   **Daan Touw** (iD)
Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

The authors describe the development and validation of an LC-MSMS assay for ceftriaxone in human plasma. Analysis of beta-lactam antibiotics such as ceftriaxone is relevant from a patient perspective. The pharmacodynamic driver for ceftriaxone efficacy is the time the concentration of the free drug is above the minimal inhibitory concentration of the targeted microorganism. Especially in critically ill patients, pharmacokinetics of beta-lactam antibiotics may be altered and dosing guided by the measurement of plasma concentration may be of great help and has demonstrated clinically useful.

The authors follow the guidelines issued by the FDA and the EMA for bioanalytical method validation.

The research is well conducted and scientifically sound. Data are available and the method is well described to replicate the method in other laboratories.

Presently, only the total concentration of the drug is analysed. A next step would be the analysis of the free drug (not protein bound plasma fraction).

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Analytical method development, therapeutic drug monitoring, clinical toxicology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 07 February 2022

https://doi.org/10.21956/wellcomeopenres.18966.r47994

Stanislas Grassin-Delyle (iD)

[1] Department of Health Biotechnology, UFR Simone Veil - Santé, Université Versailles Saint Quentin, Versailles, France
[2] Department of Respiratory Diseases, Foch Hospital, Suresnes, France

Wongchang and colleagues describe a method for the quantification of ceftriaxone in human plasma by liquid chromatography-mass spectrometry.

The introduction clearly documents the state of the art and the need for specific and sensitive methods for the quantification of ceftriaxone in human plasma. It appears that other methods have been published since the initial submission of the article and would be worth mentioning in the introduction e.g. Meenks S *et al*., (2021)[1] and Herrera-Hidalgo L *et al*., (2021)[2]. In addition, the possible advantages of the described method over existing methods should be better highlighted in the Discussion.

Chromatographic separation:
As I understand it, the mobile phase consists of 2 binary mixtures plus a third phase of ammonium bicarbonate, with the application of a composition gradient and a flow rate gradient during the 10-minute run. This gradient appears complex and the simultaneous use of acetonitrile and

methanol as organic modifiers is unconventional, as is the use of both ammonium formate and ammonium bicarbonate. The pH values of the different phases should be clarified and the use of such a complex mixture justified, especially as it appears that a simpler phase can be used with a shorter run time (Herrera-Hidalgo L *et al.*, 2021[2]).

Method validation:
The authors refer to the 2001 FDA guideline while an updated version was published in 2018. The authors should clarify which parameters are assessed according to the guideline, e.g. stability of stock solutions or incurred sample reanalysis are not reported. Quantitative values could be presented for recovery for ceftriaxone and internal standard, comparison of different reagents used for sample preparation (acids, solvents, phospholipid precipitation plates), carry-over.

Matrix effect:
The results between the two methods used (post-column infusion and injection of plasma spiked before or after the sample preparation procedure) and the nature of the anticoagulants tested in each procedure should be better explained. Mean values and their dispersion are not shown in Table 3. Why do individual values appear for 6 donors with sodium heparin in Table 3, but only one value for the other anticoagulants? Which anticoagulants were tested with the post-column infusion method? With what concentration of ceftriaxone? The presentation of the plots obtained with the first method could help interpretation and the exact procedure used to calculate the matrix effect value detailed in full. The results presented as supplementary data in the file "Recovery and matrix effects.txt" do not help to understand how the value of +27% was observed, nor how this effect would be compensated by the internal standard.

What does the sentence "The high concentration level of 155 µg/ml showed suppression of the signal but stayed within acceptable limits (Table 3)." mean? If the value is within acceptable limits, then there is no suppression of the signal.

Results with clinical samples:
For publications reporting the development of analytical methods, demonstration that the method works correctly on real clinical samples is highly desirable.

Minor comments:
- Abstract: ceftriaxone is a third generation cephalosporin (not antibiotic).

- Internal standard is not "quantified.

- Preparation of stock solutions: it should be specified whether the 10 mg/ml of stock solutions refer to the sodium salts or base drug.

- "Sample preparation" may be preferred to "sample extraction" as the preparation procedure consists in protein precipitation and phospholipid removal.

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Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Partly

Are sufficient details provided to allow replication of the method development and its use by others?
Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mass spectrometry; metabolomics; pharmacokinetics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 27 May 2022

Daniel Blessborn, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Wongchang and colleagues describe a method for the quantification of ceftriaxone in human plasma by liquid chromatography-mass spectrometry.

The introduction clearly documents the state of the art and the need for specific and sensitive methods for the quantification of ceftriaxone in human plasma. It appears that other methods have been published since the initial submission of the article and would be worth mentioning in the introduction e.g. Meenks S *et al*., (2021)[1] and Herrera-Hidalgo L *et al* ., (2021)[2]. In addition, the possible advantages of the described method over existing methods should be better highlighted in the Discussion.

Response:
Thank you for these suggestions. We believe that the introduction should describe a short overview of what have been published prior to the validation of the assay. In our case, validation

was completed in mid-2018 and the method was published in early 2019, and we think it would be somewhat misleading if methods published after 2019 were to be added to the introduction. However, we have added the latest developments and compared these with our result. As suggested, we have added a section on matrix effects and stability in the discussion, as well as the publications of Meenks S et al., (2021) and Herrera-Hidalgo L et al., (2021).

Chromatographic separation:
As I understand it, the mobile phase consists of 2 binary mixtures plus a third phase of ammonium bicarbonate, with the application of a composition gradient and a flow rate gradient during the 10-minute run. This gradient appears complex and the simultaneous use of acetonitrile and methanol as organic modifiers is unconventional, as is the use of both ammonium formate and ammonium bicarbonate. The pH values of the different phases should be clarified and the use of such a complex mixture justified, especially as it appears that a simpler phase can be used with a shorter run time (Herrera-Hidalgo L *et al*., 2021[2]).

Response:
The acetonitrile/methanol mixture was initially explained in the result and discussion section "instrument and chromatographic conditions". In the revised manuscript, we clarified and explained how both acetonitrile/methanol and ammonium bicarbonate were used to reduce carry-over and that the increased washout flow rate reduced the run time to 10 minutes. The pH of ammonium bicarbonate has been added as suggested.

The article by Herrera-Hidalgo L et al., (2021) had indeed a simpler mobile phase and shorter run time, but their method also showed lower sensitivity compared to our assay (i.e. lower limit of quantification of 3.00 vs 1.01 µg/ml). They also reported matrix effect of QC1-124%, QC2-112% and QC3-123%, a carry-over but kept it below 20%, and a short LC-stability of only 4 hrs. In fact, most of the published methods have some sort of drawbacks, e.g. matrix effects, carry-over effects, long run times, limited calibration ranges, low sensitivity and/or bad peak symmetry etc. These aspects makes the method development of ceftriaxone more challenging and we have added this in the discussion section.

Method validation:
The authors refer to the 2001 FDA guideline while an updated version was published in 2018. The authors should clarify which parameters are assessed according to the guideline, e.g. stability of stock solutions or incurred sample reanalysis are not reported. Quantitative values could be presented for recovery for ceftriaxone and internal standard, comparison of different reagents used for sample preparation (acids, solvents, phospholipid precipitation plates), carry-over.

Response:
As the reviewer suggested, we have added the stability data of stock solutions and working solutions as well as a table showing the recovery of ceftriaxone and internal standard in heparin plasma.

We referred to FDA2001 guideline as the validation took place at the same time the FDA2018 was being published. However, the validation presented here are also compliant with the EMA2012

guideline that includes carry-over testing and dilution integrity tests etc. In fact, the EMA-2012 and FDA-2018 are very similar. We have added EMA2012 in the reference as well and an open access article reference that explain the minor differences between these guidelines.

As for "comparison of different reagents used for sample preparation (acids, solvents, phospholipid precipitation plates), carry-over." These tests were performed during different stages of the method development using different concentrations and also in some cases a different model of the MS instrument. It would be difficult to present all the different parameters in one table without extensive explanations of differences and changes between each experiment. We believe that a simple explanation and overall result, as described in the article, would be most informative and hope that this is acceptable.

Matrix effect:
The results between the two methods used (post-column infusion and injection of plasma spiked before or after the sample preparation procedure) and the nature of the anticoagulants tested in each procedure should be better explained. Mean values and their dispersion are not shown in Table 3. Why do individual values appear for 6 donors with sodium heparin in Table 3, but only one value for the other anticoagulants? Which anticoagulants were tested with the post-column infusion method? With what concentration of ceftriaxone? The presentation of the plots obtained with the first method could help interpretation and the exact procedure used to calculate the matrix effect value detailed in full. The results presented as supplementary data in the file "Recovery and matrix effects.txt" do not help to understand how the value of +27% was observed, nor how this effect would be compensated by the internal standard.

Response:
We have revised this section to clarify what samples and anticoagulants were included in the post-column infusion evaluation as well as the concentration of the post-column infusion solution. A new figure was added in the revised manuscript, showing the post column infusion as overlay of Donor A and B in sodium heparin plasma and a CPD as anticoagulant to show that they did not differ visually.

Mean values and their dispersion have also been added to the matrix effect table as suggested. The matrix effect table have also been split in two separate tables as one table is for the 6 donors with sodium heparin and a separate table for the additional tests of different anticoagulants. FDA and EMA bioanalytical guidelines specify that 6 individual donors have to be screened for selectivity and that was done using sodium heparin as anticoagulant.
For the question "supplementary data in the file "Recovery and matrix effects.txt" and how the value of +27% was observed". To calculate the matrix effect, peak area of "Blank X no IS post addition QC 1 CPD" was divided with the average peak areas of "Neat Reference QC1 + IS", and that will result in 1.27 (+27%).

What does the sentence "The high concentration level of 155 µg/ml showed suppression of the signal but stayed within acceptable limits (Table 3)." mean? If the value is within acceptable limits, then there is no suppression of the signal.

Response:

The reviewer is correct, if within acceptable limits, there is no suppression of the signal. The sentence has been removed.

Results with clinical samples:
For publications reporting the development of analytical methods, demonstration that the method works correctly on real clinical samples is highly desirable.

Response:
We agree fully that a demonstration of the method performance with real clinical samples would be highly beneficial and a clinical study was also planned. However, due to several different circumstances, no clinical samples have been received as of this date.

Minor comments:
- Abstract: ceftriaxone is a third generation cephalosporin (not antibiotic).

  Response:
  Thank you for this suggestion. We have replaced, "antibiotic" with "cephalosporin", in the abstract.

- Internal standard is not "quantified.

  Response:
  Thank you for this suggestion. We have replaced, "quantified" with "detected".

- Preparation of stock solutions: it should be specified whether the 10 mg/ml of stock solutions refer to the sodium salts or base drug.

  Response:
  Thank you. Page 5, first paragraph: we have clarified that 10 mg/ml is the base form.

- "Sample preparation" may be preferred to "sample extraction" as the preparation procedure consists in protein precipitation and phospholipid removal.

  Response:
  Thank you for this suggestion. Page 5, third paragraph: we have changed "sample extraction" to "sample preparation", as suggested.

Competing Interests: No competing interests were disclosed.

Reviewer Report 19 November 2021

https://doi.org/10.21956/wellcomeopenres.18966.r45734

✖ **Sean O'Halloran**

School of Medicine, University of Western Australia, Perth, Australia

Thank you for revising the paper based on previous reviewer comments. I appreciate that some, but not all, of the previous comments have been addressed.

Previous comment regarding PKPD studies in neonates as an application but no case studies - not addressed.

Previous comment about checking Tazobactam as an interference - the authors have not provided a satisfactory response. The authors' expectation that it is the local laboratory's responsibility to check interferences that might be present in a local population group or that just because Tazobactam in not co-administered in their country is an insufficient or unsatisfactory response for an international peer-review publication platform.

Previous comment about recovery data being limited to Ceftriaxone - thank you for the authors' provision of data. The data is problematic. There is clearly a difference in recovery achieved for each compound which, again (corresponding to a previous comment) that the chosen internal standard is not satisfactory for this method.

Previous comment about lack of data on evaluating the isotopically-labelled version of Ceftriaxone - thank you for the authors' provision of data. The data is problematic. It is inappropriate practice to use a concentration of internal standard at an equivalent concentration to the LLOQ (1ug/mL). It is more appropriate to use an internal standard concentration, perhaps, midway, over the calibration range (in this case up to 155ug/mL).

Previous comment about inability of the chosen internal standard to compensate for matrix effects - thank you for the authors' provision of a literature reference to Decosterd *et al.* Clearly the referenced method using an isotopically labelled internal standard demonstrates superior performance for this crucial parameter in LCMSMS method validation which suggests that the offered method is less worthy of publication.

Previous comment about the differing methodologies for evaluating matrix effects (post-column infusion versus post extract addition etc) - thank you for the authors' inclusion of Matuszewski's methodology. However results from utilisation of both methodologies are inconsistent. This suggests that the post-column infusion adds contradictory data to the validation of the method.

Previous comment regarding spelling errors - thank you to the authors for correcting many, but some persist. Example "were its third isotope...."

Is the rationale for developing the new method (or application) clearly explained?

Partly

Is the description of the method technically sound?

Partly

Are sufficient details provided to allow replication of the method development and its use by others?

Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Chromatography, mass spectrometry, therapeutic drug monitoring, toxicology. pharmacology, drugs of abuse

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Version 1

Reviewer Report 19 July 2019

https://doi.org/10.21956/wellcomeopenres.16520.r35889

? **Lotte van Andel** 🆔

Department of Pharmacy and Pharmacology, Antoni van Leeuwenhoek Hospital, Division of Pharmacology, Netherlands Cancer Institute, Amsterdam, The Netherlands

The authors have described a method to quantify ceftriaxone using HPLC-MS. The introduction gives a strong argument on the importance of a more sensitive method to quantify ceftriaxone. However, this argument loses its strength throughout the article. HPLC-MS is generally more sensitive than HPLC-UV, but the expected concentrations are quite high. Moreover, an LLOQ of 1 µg/mL is not very low, hence I'd say that the method is not extremely sensitive. It would be interesting to know the LOD of the method.

I wonder if the need for a sensitive method was related to the last concentrations measured in order to enable the construction of a pharmacokinetic profile? If these concentrations were likely to fall below the LLOQ of a HPLC-UV method, the estimation of the terminal phase might not be possible. Hence, the need for a more sensitive method. From the presented data, the concentration after 24 h is 5.3 – 15.1 µg/mL. It seems likely that these concentrations could be measured using an HPLC-UV method. Moreover, it is stated that some samples might be above ULOQ and need dilution before analysis. Furthermore, samples are diluted ~8 times before analysis. If a similar extraction procedure was tested by drying the samples and reconstituting them again in 100 µL, perhaps the samples could be measured on an HPLC-UV system instead.

Finally, the authors claim that a disadvantage of HPLC-UV is the long run time of 10 – 20 min, whereas the current run time is 10 min. To me, this does not sound as a huge advantage over the other methods. The compound elutes at 1.6 min. Is the long run time required to stabilize the column? Has a shorter time been tested? Why are three mobile phases necessary? Elution occurs using mobile A only.

It is unclear to me what the authors have learned from previously published methods. Also, a recent publication from 2018 has not been cited (Mohamed, 2018[1]). It would be valuable if the authors could clarify how the current method has been improved in comparison to the previously published methods. This could be added to the introduction to clarify the huge advantage of the current method over the previously published ones. The conclusion merely states that "The use of LC-MS/MS resulted in higher sensitivity and selectivity than HPLC-UV." This could be explained further in the introduction or conclusion.

Has it been investigated if the lipids interfered in LC-MS analysis? Otherwise phospholipid removal might not have been necessary, saving time and cost. Would phospholipid removal be necessary for HPLC-UV analysis? It would be valuable to know the extraction yield without the use of the phospholipid removal plates. Moreover, this step in the sample pretreatment could induce problems, because the internal standard used is not a stable isotopically labelled one. Based on accuracy and precision results it seems to correct for variation sufficiently. But I'd be careful to introduce more steps during sample preparation when a structural analogue is used rather than a stable isotopically standard.

The authors have performed extensive interference analyses. However, to me it is not clear whether the interference between the analyte and IS was tested. This would have been useful. Moreover, the retention time of the internal standard is not specified.

During sample processing, the samples are diluted ~8 times. If a similar extraction procedure was tested by drying the samples and reconstituting them again in 100 µL, could the samples be measured on an HPLC-UV system?

The authors state "..revealing no significant suppression or enhancement", after which they proceed to explain that QC1 exhibited a 27% signal enhancement. To me, this sounds contradictory. I'd suggest to remove the statement.

The conclusion states that there might still be a limitation for infants if smaller amounts of blood are obtained. Therefore, it would be interesting to know the LOD of the method to assess whether the range could be widened.

Some remarks:
- ○ Acceptance criteria are not specifically stated.

- ○ I wonder why there was a specific interest in testing different coagulants.

- ○ It would be useful to know why the authors have methanol-washed pipette tips, well plates and seal mats before use.

- ○ Some rephrasing needs to be done such as "To the first well which is the double blank was 400 μL..".

References

1. Mohamed D, Kamal M: Enhanced HPLC-MS/MS method for the quantitative determination of the co-administered drugs ceftriaxone sodium and lidocaine hydrochloride in human plasma following an intramuscular injection and application to a pharmacokinetic study.*Biomed Chromatogr*. 2018; **32** (10): e4322 PubMed Abstract | Publisher Full Text

Is the rationale for developing the new method (or application) clearly explained?

Partly

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: LC-MS/MS, pharmacokinetics, mass balance, bioanalysis.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 20 Aug 2021

Daniel Blessborn, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

1. The authors have described a method to quantify ceftriaxone using HPLC-MS. The introduction gives a strong argument on the importance of a more sensitive method to quantify ceftriaxone. However, this argument loses its strength throughout the article. HPLC-MS is generally more sensitive than HPLC-UV, but the expected concentrations are quite high. Moreover, an LLOQ of 1 µg/mL is not very low, hence I'd say that the method is not extremely sensitive. It would be interesting to know the LOD of the method.

   Response:
   Thank you for this insightful response. We did not evaluate LOD as it is not a validation parameter.
   As seen in figure 3, there was a small carryover effect. However, we verified that this carryover would not accumulate over time after repeated injections. Carryover was therefore tested in all 4 precision and accuracy batches, and was positioned to run after approximately 50 sample injections. As part of the validation, we tested an additional two concentration sets below the LLOQ; 0.5 µg/ml and 0.25 µg/ml. The results showed that 0.5 µg/ml would easily pass the carryover criteria as LLOQ. However, the 0.25 µg/ml would be too low to pass for LLOQ as the carryover was more than the 20% of the peak response. If there were no carryover, we would have pushed the sensitivity further. The chosen LOQ at 1.0 µg/ml produced robust and precise quantification, and should be sufficiently sensitive to evaluate the majority of pharmacokinetic studies.

2. I wonder if the need for a sensitive method was related to the last concentrations measured in order to enable the construction of a pharmacokinetic profile? If these concentrations were likely to fall below the LLOQ of a HPLC-UV method, the estimation of the terminal phase might not be possible. Hence, the need for a more sensitive method. From the presented data, the concentration after 24 h is 5.3 – 15.1 µg/mL. It seems likely that these concentrations could be measured using an HPLC-UV method. Moreover, it is stated that some samples might be above ULOQ and need dilution before analysis. Furthermore, samples are diluted ~8 times before analysis. If a similar extraction procedure was tested by drying the samples and reconstituting them again in 100 µL, perhaps the samples could be measured on an HPLC-UV system instead.

   Response:
   The concentrations (5.3 – 15.1 µg/ml) reported after 24 h is average values, which mean that 50% of all patients will have observed concentrations below and 50% above these reported values. Thus, you want to allow sufficient margin to enable quantification in the majority of patients that participate in the clinical trial.
   Using LC-UV should be possible, and a publication released one year after ours, by Cairoli et al 2020 did just that. They could measure down to 1 µg/ml using 100µl plasma and LC-DAD with evaporation and reconstitution to 100 µl. Though

matrix components would also be concentrated and could possibly pose an increased risk of interference.

3. Finally, the authors claim that a disadvantage of HPLC-UV is the long run time of 10 – 20 min, whereas the current run time is 10 min. To me, this does not sound as a huge advantage over the other methods. The compound elutes at 1.6 min. Is the long run time required to stabilize the column? Has a shorter time been tested? Why are three mobile phases necessary? Elution occurs using mobile A only.

Response:
The reviewer is correct, the elution occurs in isocratic mode using mobile phase A. Mobile phase B is used to flush out strongly retained components to avoid accumulation on the column, then Mobile phase C was added to help remove carryover and shorten the run time. Initially we had a 5-minute run time that included a washout gradient (A/B) to prevent more strongly retained components to accumulate on the column. However, there were still a carryover problem that was slowly reduced as the mobile phase flowed through the system and was eventually eliminated given enough time between injections (about 20 min). To reduce the waiting time between injections different washout solvents and solution mixes were tested, and by adding a washout step using ammonium bicarbonate, a final run-time of 10 minutes was achieved and the carry-over could be minimized but not completely eliminated.

4. It is unclear to me what the authors have learned from previously published methods. Also, a recent publication from 2018 has not been cited (Mohamed, 2018[1]). It would be valuable if the authors could clarify how the current method has been improved in comparison to the previously published methods. This could be added to the introduction to clarify the huge advantage of the current method over the previously published ones. The conclusion merely states that "The use of LC-MS/MS resulted in higher sensitivity and selectivity than HPLC-UV." This could be explained further in the introduction or conclusion.

Response:
The publication by Mohammed 2018 have now been cited in the introduction, and their method uses 450µl of sample and achieved a LOQ of 3 µg/ml. However, we do not claim that we have a superior method but merely an alternative option to already published methods. Also, many published ceftriaxone methods describe matrix effects, but not all of them have quantified these effects. Furthermore, methods report carryover result and many methods have short retention time but very long run time without explaining the reason. Some methods with short run times have included repeat injections of blank samples to reduce carryover, which makes the method unsuitable for routine analysis of clinical studies. We wanted to be clear and describe the problems we have encountered, as well as the different strengths and weaknesses and we hope that this information can help others setting up a method to quantify

ceftriaxone.
Also, after our publication, there were three new publications in 2020, two LC-MS and one DAD-UV using the same sample size (100µl) and similar calibration range. Both LC-MS methods experienced matrix effects, and one of them (Decosterd et.al. 2020) used ceftriaxone-13CD$_3$ that showed that it could compensate for the 150-175% matrix effects they otherwise would have experienced, and we have added this in the discussion section. Though they did not show any evaluation of interference between analyte and SIL-IS, or if the isotopes of ceftriaxone ads to the ceftriaxone-13CD$_3$ signal as it did in our evaluation of ceftriaxone-D$_3$. Theoretical predictions show that some interference could be present in small amounts, but their ULOQ go only to 100 µg/ml which would limit these effects.

5. Has it been investigated if the lipids interfered in LC-MS analysis? Otherwise phospholipid removal might not have been necessary, saving time and cost. Would phospholipid removal be necessary for HPLC-UV analysis? It would be valuable to know the extraction yield without the use of the phospholipid removal plates. Moreover, this step in the sample pretreatment could induce problems, because the internal standard used is not a stable isotopically labelled one. Based on accuracy and precision results it seems to correct for variation sufficiently. But I'd be careful to introduce more steps during sample preparation when a structural analogue is used rather than a stable isotopically standard.

   Response: Instrument and chromatographic conditions in the result section and conclusion section has been edited to clarify the reasons for phospholipid removal (also see the response of last question from reviewer 1). Briefly, phospholipid removal generally reduces matrix components due to removal of phospholipids and large particles. In early method development, we compared protein precipitation with different types of extraction plates. By using this type of sample cleanup, you can also reduce the risk of particles clogging the system causing analytical failures during the analytical run, and the risk of accumulation of strongly retaining components on the column causing deterioration of column performance. In our experience this will maintain the column performance and reduce the risk of analytical problems during the analysis and the need of re-analysis.

6. The authors have performed extensive interference analyses. However, to me it is not clear whether the interference between the analyte and IS was tested. This would have been useful. Moreover, the retention time of the internal standard is not specified.

   Response:
   More information on the interference testing between analyte and SIL-D$_3$ have been added to the manuscript. Retention time of the new internal standard is now shown in the updated figure 3.

7. During sample processing, the samples are diluted ~8 times. If a similar extraction procedure was tested by drying the samples and reconstituting them again in 100 µL, could the samples be measured on an HPLC-UV system?

   Response:
   Also see our response on comment 2 above. LC-UV should be possible, as seen in a publication released one year after ours (Cairoli et al 2020). They measured down to 1 µg/ml (LOQ) using 100 µl plasma and LC-DAD with evaporation and reconstitution to 100 µl. However, matrix components would also be concentrated and could possibly pose an increased risk of interference.

8. The authors state "..revealing no significant suppression or enhancement", after which they proceed to explain that QC1 exhibited a 27% signal enhancement. To me, this sounds contradictory. I'd suggest to remove the statement.

   Response:
   This statement have been removed and the matrix effect section have been re-written to clarify the validation findings.

9. The conclusion states that there might still be a limitation for infants if smaller amounts of blood are obtained. Therefore, it would be interesting to know the LOD of the method to assess whether the range could be widened.

   Response:
   Also see our response on comment 1 above. We did not evaluate LOD as it is not a validation parameter.
   The results showed that 0.5 µg/ml would easily pass the carryover criteria as LLOQ. However, the 0.25 µg/ml would be too low to pass for LLOQ as the carryover was more than the 20% of the peak response. The chosen LOQ at 1.0 µg/ml produced robust and precise quantification, and should be sufficiently sensitive to evaluate the majority of pharmacokinetic studies. The range could possibly be extended to 0.5 µg/ml, but this would require a re-validation.

Some remarks:
- Acceptance criteria are not specifically stated.

  Response:
  Acceptance criteria has been added to method section.

- I wonder why there was a specific interest in testing different coagulants.

Response:
From our experience we know that some studies want to streamline and make sample collection easy by using one anticoagulant for different measurements. The most commonly used anticoagulants are heparin and EDTA, so we test different anticoagulants in the validation to see if they are comparable and if a certain anticoagulant can be used. FDA guidelines also require validating the anticoagulants to be used.

○ It would be useful to know why the authors have methanol-washed pipette tips, well plates and seal mats before use.

Response:
The methanol washed tips and labware was part of the matrix effects investigation but it did not have a noticeable effect so the sentence has been removed in the updated manuscript.

Some rephrasing needs to be done such as "To the first well which is the double blank was 400 µL..".

Response:
The sentence has been re-written.

Competing Interests: No competing interests were disclosed.

Reviewer Report 04 July 2019

? **Sean O'Halloran**

School of Medicine, University of Western Australia, Perth, Australia

This manuscript outlines the determination of Ceftriaxone in human plasma by LCMSMS. The authors mention that a potential application of this method relates to performance of PKPD studies in neonates and malnourished young children however they do not include any case studies. The authors' stated objective is to offer this method to laboratories setting up this LCMSMS assay. Such literature can be important and useful but in this case the authors need to be very clear in their abstract about the particular details of the method, including any limitations, so that prospective laboratories can indeed assess the suitability of this method for their local application. As such, the abstract must include the finding that calibrators must match the anticoagulant used for specimen collection.

The authors claim, repetitively, that their validation is in accordance with FDA guidelines. Method validation protocols generally discourage the use of serial dilution of calibration material or stock. In the methodology description of selectivity by other compounds the author declare that the 'occurrence of a peak response at the retention time of the analyte or internal standard indicates matrix interference'. This is incorrect; such interference could be from a number of sources, not just matrix. In the same description there is lack of specific detail of the concentration of infused solution and concentration of potentially-interfering substances in the post-column infusion study. The authors must cite references that describe that this methodology is suitable for interference studies from specific compounds such as co-administered drugs. There are formulations of Ceftriaxone, albeit in specific countries only, that contain Tazobactam, so this is among other compounds that need to be specifically tested for interference.

The authors need to separate description and results of the matrix effects study from the carryover study into different sections. The presentation of the results of the matrix effects study is not clear. The authors use the phrase 'post-spiked' blank plasma however the literature tends to use the terminology 'post-extract addition'. The authors describe the use of neat reference solution however they do not describe in detail how that solution was prepared. A presentation of the matrix effects study results should consider methodology by Matuszewski et al.[1]. While their description of comparison between neat solution and post-extract spiked solution is correct for describing a matrix effect-study, the authors then go on to describe matrix effects in a QC sample. This needs clarification. The word 'tendency' is not useful. These descriptions of enhancement and suppression contradict the assertion in the abstract that no significant (poor choice of word) matrix effects were observed. The finding that Ceftriaxone is subject to matrix effects and the internal standard Cefotaxime is not, suggests that Cefotaxime is not suitable as an internal standard.

The standard and the internal standard were dissolved in two different solvents suggesting differing physicochemical properties; their relative and respective retention times in the method need to be made more explicit. In describing the evaluation of a labelled internal standard the authors do not make it clear whether or not they had purchased the labelled material, or that they had used the purchased labelled material (not mentioned in Materials and Reagents) to determine the MRM transitions by tuning for the D3 internal standard. Or did they rely only on the theoretical abundance of natural isotopes thereby dismissing the suitability of the compound? There is lack of information on the various product ions suitable for monitoring the D3 compound and whether or not the deuterium atoms are thought to be on those product ion fragments. CLSI tolerates 5% of the labelled internal standard signal at the ULOQ, and CLSI suggests that labelled compounds +3 mass units should suit analytes of molecular mass <1000. Also, under "Validation" the description of the recovery study is not clear, dealing with Ceftriaxone only. Evaluation of the internal standard recovery must also be included.

The authors declare that 'higher reproducibility was achieved with acetonitrile' to 'improve the sample purity', this is incorrect, and furthermore, reproducibility as an analytical method validation parameter is not presented anywhere in the paper, either in methodology or results. The authors admit some kind of problem with carryover. Detail must be provided. Increasing the run time to 10 minutes is presented as a way of dealing with carryover; this parameter must be presented in the abstract because the run-time is consequently within the range of HPLC assay run times – methodology and technology that is supposed to be surpassed by this LCMSMS method.

The abstract must also include the requirement for 100 µL of sample which does not compare favourably with the requirement of HPLC methods and where other authors (Page-Sharp et al[2]) require lower sampling volumes for paediatric patients.

This version of the manuscript must be edited further.
Ethical approval was sought; is not clear that the approval was given, please address.
Please clarify the use of a binary pump with three different mobile phases.
There is some repetition in the manuscript which must be removed.
In many parts of the manuscript there is a mixture of methodology and results, which needs to be corrected.

In the "Conclusion" section, the authors suggest that 'phospholipid removal techniques for sample preparation could reduce some matrix interferences'. This is unclear because the authors have already described such a phospholipid removal technique in the method they have presented.
In the description of the extraction procedure the authors detail 'To the first well which is the double blank was 400 µL of acetonitrile added'. This and other such phrases need to be edited and re-worded.
Other phrases or words that must be edited in their respective contexts include words like 'mainly', 'significant', 'a few' ,'only through', 'several', 'among', 'tendency' 'some degree', 'in the pipeline' etc. Typo example, 'were some isotopes'.

References
1. Matuszewski B, Constanzer M, Chavez-Eng C: Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC─MS/MS. *Analytical Chemistry*. 2003; **75** (13): 3019-3030 Publisher Full Text
2. Liquid Chromatography - Mass Spectrometry Methods;Approved Guideline. *Clinical Laboratory standards Institute*. 2014; **34** (16). Reference Source

Is the rationale for developing the new method (or application) clearly explained?

Partly

Is the description of the method technically sound?

Partly

Are sufficient details provided to allow replication of the method development and its use by others?

Partly

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Chromatography, mass spectrometry, therapeutic drug monitoring, toxicology. pharmacology, drugs of abuse

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 20 Aug 2021

Daniel Blessborn, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

1. This manuscript outlines the determination of Ceftriaxone in human plasma by LCMSMS. The authors mention that a potential application of this method relates to performance of PKPD studies in neonates and malnourished young children however they do not include any case studies. The authors' stated objective is to offer this method to laboratories setting up this LCMSMS assay. Such literature can be important and useful but in this case the authors need to be very clear in their abstract about the particular details of the method, including any limitations, so that prospective laboratories can indeed assess the suitability of this method for their local application. As such, the abstract must include the finding that calibrators must match the anticoagulant used for specimen collection.

    Response:
    Thank you for your suggestions. We have added additional information to the abstract, such as anticoagulant must match calibrators and collected specimen and that CPD plasma resulted in matrix enhancement in the low concentration level.

2. The authors claim, repetitively, that their validation is in accordance with FDA guidelines. Method validation protocols generally discourage the use of serial dilution of calibration material or stock. In the methodology description of selectivity by other compounds the author declare that the 'occurrence of a peak response at the retention time of the analyte or internal standard indicates matrix interference'. This is incorrect; such interference could be from a number of sources, not just matrix. In the same description there is lack of specific detail of the concentration of infused solution and concentration of potentially-interfering substances in the post-column infusion study. The authors must cite references that describe that this methodology is suitable for interference studies from specific compounds such as co-administered drugs. There are formulations of Ceftriaxone, albeit in specific countries only, that contain Tazobactam, so this is among other compounds that need to be specifically tested for interference.

    Response:
    Thank you for these insightful comments. To the best of our knowledge, regulatory guidelines (e.g. EMA 2012 and FDA 2018) does not mention exactly how to prepare different calibrators (i.e. serial or direct dilutions), but they do specify that QC samples should be prepared from a separate stock solution,

independently from calibration standards. We followed these guidelines, and used two independently prepared stock solutions for calibrators and QC samples. We believe that this is an acceptable approach.

The sentence 'occurrence of a peak response at the retention time of the analyte or internal standard indicates matrix interference' have been corrected and now state that "*The occurrence of a peak response at the retention time of the analyte or internal standard indicates an interference and would require further investigation.*"

We have also added the concentration of infused ceftriaxone and internal standard, and the concentration of potentially interfering drugs injected during post-column infusion (as suggested by reviewer). Tazobactam was not considered as it is not co-formulated in marketed products in the country we are located (Thailand). There are also other drug combinations, used in specific countries, and these needs to be evaluated by the analytical laboratory setting up the method depending on the specific requirements for the samples that will be analysed. We believe that it is beyond the scope of this paper to evaluate all possible combinations that might be of interest for different laboratories.

3. The authors need to separate description and results of the matrix effects study from the carryover study into different sections. The presentation of the results of the matrix effects study is not clear. The authors use the phrase 'post-spiked' blank plasma however the literature tends to use the terminology 'post-extract addition'. The authors describe the use of neat reference solution however they do not describe in detail how that solution was prepared. A presentation of the matrix effects study results should consider methodology by Matuszewski et al.[1]. While their description of comparison between neat solution and post-extract spiked solution is correct for describing a matrix effect-study, the authors then go on to describe matrix effects in a QC sample. This needs clarification. The word 'tendency' is not useful. These descriptions of enhancement and suppression contradict the assertion in the abstract that no significant (poor choice of word) matrix effects were observed. The finding that Ceftriaxone is subject to matrix effects and the internal standard Cefotaxime is not, suggests that Cefotaxime is not suitable as an internal standard.

Response: Thank you for your comments and suggestions to improve the manuscript. We have now rewritten that particular section, and separated carry-over effects from the text on matrix effects. The matrix effect investigation is in fact based on the article of Matuszewski et al. (2003), but follows the simplified approach presented in the same article. This approach uses two concentration levels of the analyte in 6 different sources of blank plasma. We have rephrased the terminology as suggested, and clarified the matrix effect evaluation was in accordance to Matuszewski et al. The sentence describing a matrix effect in a QC sample was indeed a mistake and we are grateful that the reviewer pointed out this typing error (this has been

corrected). Additional words and sentences have also been changed as suggested by the reviewer, and we hope that these edits now provide a clear and accurate method description.

4. The standard and the internal standard were dissolved in two different solvents suggesting differing physicochemical properties; their relative and respective retention times in the method need to be made more explicit. In describing the evaluation of a labelled internal standard the authors do not make it clear whether or not they had purchased the labelled material, or that they had used the purchased labelled material (not mentioned in Materials and Reagents) to determine the MRM transitions by tuning for the D3 internal standard. Or did they rely only on the theoretical abundance of natural isotopes thereby dismissing the suitability of the compound? There is lack of information on the various product ions suitable for monitoring the D3 compound and whether or not the deuterium atoms are thought to be on those product ion fragments. CLSI tolerates 5% of the labelled internal standard signal at the ULOQ, and CLSI suggests that labelled compounds +3 mass units should suit analytes of molecular mass <1000. Also, under "Validation" the description of the recovery study is not clear, dealing with Ceftriaxone only. Evaluation of the internal standard recovery must also be included.

   Response: The theoretical physicochemical properties of ceftriaxone (standard) and cefotaxime (internal standard) are similar, and both have very similar theoretical logD curves over the pH range. Both compounds can be dissolved in water, and the retention and separation are relatively close when using an isocratic mode (as can be seen in the revised figure 3). However, we decided to follow the CoA provided from the supplier when dissolving the standard and internal standard, thus explain why we used different solvents. Regarding the labelled internal standard (ceftriaxone-D$_3$), the source of this compound has been added in the material section, and we have restructured and re-written "Result and discussion – Instrument and chromatographic condition section" to explain better why ceftriaxone-D$_3$ was excluded (i.e. the level of interference between ceftriaxone and ceftriaxone-D$_3$). A SIL-IS of D$_5$ or higher would most likely work, but we have not been able to find such a labelled internal standard. Recovery of the new alternative internal standard (cefotaxime) have been added in the text as suggested, and the retention time and separation can now be seen in the updated figure 3.

5. The authors declare that 'higher reproducibility was achieved with acetonitrile' to 'improve the sample purity', this is incorrect, and furthermore, reproducibility as an analytical method validation parameter is not presented anywhere in the paper, either in methodology or results.
   The authors admit some kind of problem with carryover. Detail must be provided. Increasing the run time to 10 minutes is presented as a way of dealing with carryover; this parameter must be presented in the abstract because the run-time is consequently within the range of HPLC assay run times – methodology and technology that is supposed to be surpassed by this LCMSMS method.
   The abstract must also include the requirement for 100 µL of sample which does not

compare favourably with the requirement of HPLC methods and where other authors (Page-Sharp et al[2]) require lower sampling volumes for paediatric patients.

Response:
We fully agree that this sentence was unclear, and it has been edited for clarity in the revised manuscript. Details of the carry-over problem and how it was solved, although resulting in a longer run time, has now been added in the discussion. The analytical run time and sample volume required has been added in the abstract, as suggested.

6. This version of the manuscript must be edited further. Ethical approval was sought; is not clear that the approval was given, please address. Please clarify the use of a binary pump with three different mobile phases. There is some repetition in the manuscript which must be removed. In many parts of the manuscript there is a mixture of methodology and results, which needs to be corrected.

Response:
We thank the reviewer for highlighting these errors in the manuscript. Indeed, ethical approval was given and we have clarified this in the revised manuscript. The LC-pump was a quaternary pump (not a binary pump) and this has been corrected in the text. We have also re-arranged the text in the method and result sections as suggested by the reviewer.

7. In the "Conclusion" section, the authors suggest that 'phospholipid removal techniques for sample preparation could reduce some matrix interferences'. This is unclear because the authors have already described such a phospholipid removal technique in the method they have presented.
In the description of the extraction procedure the authors detail 'To the first well which is the double blank was 400 µL of acetonitrile added'. This and other such phrases need to be edited and re-worded.
Other phrases or words that must be edited in their respective contexts include words like 'mainly', 'significant', 'a few' ,'only through', 'several', 'among', 'tendency' 'some degree', 'in the pipeline' etc. Typo example, 'were some isotopes'.

Response:
The conclusion section has been edited and clarified to state that phospholipid plates commonly reduce matrix components due to removal of phospholipids, but also other particles. By using this type of sample cleanup, you can reduce the risk of particles clogging the system causing analytical failures during the run as well as reducing the amount of matrix components passing through the column. The description of the extraction procedure was also rewritten as suggested. Other phrases and words listed by the reviewer have been edited.

Competing Interests: No competing interests were disclosed.