Determination of ceftriaxone in human plasma using liquid chromatography–tandem mass spectrometry [version 1; peer review: 2 approved with reservations]

Thamrong Wongchang1,2, Markus Winterberg1,3, Joel Tarning1,3, Natthida Sriboonvorakul2, Sant Muangnoicharoen2, Daniel Blessborn1,3

1Mahidol-Oxford Tropical Medicine Research Unit (MORU), Faculty of Tropical Medicine, Mahidol University, Bangkok, 10400, Thailand
2Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok, 10400, Thailand
3Centre for Tropical Medicine & Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, OX3 7FZ, UK

Abstract
Ceftriaxone is a cephalosporin antibiotic drug used as first-line treatment for several bacterial diseases. Ceftriaxone belongs to the third generation of antibiotics and is available as an intramuscular or intravenous injection. Previously published pharmacokinetic studies have mainly 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 in combination with phospholipid-removal techniques for cleaning up 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. Both the analyte and cefotaxime (internal standard) were quantified 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 no significant matrix effects were observed. The intra-assay and inter-assay precision were less than 5% and 10%, respectively, and therefore well within standard regulatory acceptance criterion of ±15%.
**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\(^{12}\). The impact of resistance will increase patient mortality, morbidity, length of hospitalization, and health-care costs\(^{14}\). Furthermore, development of widespread antibiotics resistance decreases the number of effective antibiotics rapidly, and new drug discovery does not demonstrate a healthy pipeline of novel drug to combat this rapidly increasing issue\(^5\). Therefore, all strategies to preserve efficacy of available drugs should be considered. Only through 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 several 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\(^{10-12}\). 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)\(^{6-10,11}\), 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\(^{13-15}\). Thus, this will be among 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). Figure 1 shows 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 sought from the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (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) and cefotaxime (10 mg/ml) 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

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**Figure 1. Molecular structures.** Structures of ceftriaxone (A) and the internal standard cefotaxime (B) are shown.
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 extraction 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, Männedorf, Switzerland). Pipette tips, 96-well plates and seal mats were all methanol-washed before use. Plasma samples (100 μl) were manually aliquoted into a 96-well plate. To the first well which is the double blank was 400 μl acetonitrile added and to all remaining wells was 400 μl internal standard solution added (acetonitrile containing cefotaxime 2 μg/ml). 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 binary LC pump, a vacuum degasser, 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 Chromelene 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 ceftoxime with a collision energy of 20 and 39 V, respectively.

**Method validation**

The method was validated according to the US Food and Drug Administration (FDA) guidelines on bioanalytical method validation16. 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).

**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/x²), as well as quadratic model with 1/x weighting, were evaluated.

Selectivity was evaluated by injecting blank extracted samples and potentially interfering drugs during post-column infusion. 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) were also evaluated. The occurrence of a peak response at the retention time of the analyte or internal standard indicates matrix interference.

Recovery was determined by comparing the peak response of individual QC samples to the average peak response of extracted blank plasma post-spiked at the same nominal concentration. Five replicates of each concentration were evaluated.

**Matrix and carry-over effects.** Matrix effect was assessed by both post-column infusion (qualitative visualization) and post-extraction spiking (quantitative evaluation). Heparin plasma from six different donors was used for the analysis as well as haemolysed plasma. Different anticoagulants EDTA, CPD, Li-heparin were evaluated. The matrix factor was calculated by comparing the peak response of post-spiked blank plasma samples to the average peak response of analyte in neat reference solution at the same nominal concentrations. Two concentrations (low and high) at 2.97 and 155 μg/ml were evaluated.

The carry-over effect was investigated by injecting three replicates of blank samples after five injections of samples at ULOQ concentrations. The presence of a signal greater than 20% of the LLOQ indicates carry-over.
Stability. 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.

Results and discussion

The calibration range of 1.01-200 μg/ml was based on pharmacokinetic data from previously published studies,[4-17] 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 sepsis9. 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 be 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. 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 properties8.

Deuterium-labelled ceftriaxone (d3) was evaluated as an internal standard. It was substituted by cefotaxime because ceftriaxone interfered with the ceftriaxone-d3 signal in the LC-MS/MS instrument. This could be explained by the isotopic distribution of ceftriaxone, were some isotopes have the same mass as ceftriaxone-d3 and hence cause interference.10,19. 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.

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. Neat acetonitrile and methanol both worked well as protein precipitation solvents. The results indicated that acetonitrile yielded lower ceftriaxone extraction recovery than methanol. However, higher reproducibility was achieved with acetonitrile. To improve the sample purity 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. 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.14,15,20. 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.

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

Validation

The US FDA (2001) guideline on bioanalytical method validation was followed for assay validation.16 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.21

Linearity, selectivity and recovery. The calibration curve was evaluated for linearity by different calibration models. The model that described the best concentration-response relationship was a linear regression with 1/x2 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 use of Phree and Ostro plates to remove phospholipids showed similar recovery of ceftriaxone but the Phree plate was selected based on price and performance. In contrast, almost 100% of ceftriaxone was absorbed by the HybridSPE, and therefore this extraction approach was excluded. The Phree plate and heparin plasma was used for determining ceftriaxone recovery. The results showed a recovery of 30–35%. There was a clear recovery difference using different anticoagulants, where CPD plasma generally achieved 10–15% higher recovery compared to heparin and EDTA about 5–10% higher compared to heparin.
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%.

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

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.

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

QC, Quality Control; RSD, Relative Standard Deviation.
Using the same anticoagulant in both calibrators and study samples is therefore important to avoid a bias in the result.

**Matrix effect and carry-over.** Matrix effect evaluation during post-column infusion did not show any increase or drop in concomitant drugs or plasma with different anticoagulants, including haemolysis-plasma, did not show any increase or decrease in the signal. To determine matrix effects quantitatively, extracted blank plasma post-spiked was compared with neat solution at the same nominal concentration, revealing no significant matrix suppression or enhancement. Some of the QC1 samples experienced some degree of enhancement, of which CPD as anticoagulant at QC1 level exhibited a 27% signal enhancement, while the QC3 sample had no effect at all but with a tendency to suppress the signal (Table 3). Matrix effects have also been reported by other authors, affecting only the lowest concentrations. The internal standard did not show any matrix effects. A stable isotope internal standard would have been desirable in this case and would most likely have experienced the same matrix effect and compensated for any potential differences in the signal. Carry-over was prevented by adding a washout step using ammonium bicarbonate and an increase in run-time (Figure 3).

**Stability.** 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 haemolisys. 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 Threo phospholipid removal plate (Table 4). Extracted samples in the LC autosampler, 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 for sample preparation could reduce some matrix interferences and preserve the MS instrument and column, 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.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>EDTA</th>
<th>CPD</th>
<th>Li-Hep</th>
<th>Na-Hep</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC 1, 2.97 μg/ml</td>
<td>1.12</td>
<td>1.18</td>
<td>1.10</td>
<td>1.03</td>
<td>1.07</td>
<td>1.04</td>
<td>1.10</td>
<td>1.27</td>
<td>1.10</td>
<td>1.17</td>
</tr>
<tr>
<td>QC 3, 155 μg/ml</td>
<td>0.93</td>
<td>0.87</td>
<td>0.93</td>
<td>0.91</td>
<td>0.93</td>
<td>0.88</td>
<td>0.93</td>
<td>0.96</td>
<td>0.90</td>
<td>0.91</td>
</tr>
<tr>
<td>IS for QC 1, 2 μg/ml</td>
<td>0.99</td>
<td>1.03</td>
<td>1.00</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
<td>1.03</td>
<td>1.02</td>
<td>1.03</td>
<td>1.05</td>
</tr>
<tr>
<td>IS for QC 3, 2 μg/ml</td>
<td>1.01</td>
<td>1.05</td>
<td>1.03</td>
<td>1.05</td>
<td>1.01</td>
<td>1.03</td>
<td>1.04</td>
<td>1.04</td>
<td>1.03</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Hep, Heparin; QC, Quality Control; IS, internal standard.

Table 3. Matrix effects from different donors in heparin plasma and different anticoagulants. Donors A to F are individual donors collected using sodium heparin as anticoagulant. Other anticoagulants were collected from individual donors and are not from the same source.
Figure 3. Overlay of ceftriaxone at LLOQ concentration and the first blank injection after injecting five ULOQ samples, presenting no significant carry-over.

Table 4. 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.

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>QC1, 2.97 μg/ml</th>
<th>RT 24 hrs</th>
<th>RT 48 hrs</th>
<th>4°C 24 hrs</th>
<th>4°C 48 hrs</th>
<th>F/T cycle 3</th>
<th>F/T cycle 5</th>
<th>Precipitated 4hrs in RT</th>
<th>-80°C 224 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPD</td>
<td></td>
<td>106</td>
<td>100</td>
<td>102</td>
<td>103</td>
<td>97.7</td>
<td>94.0</td>
<td>94.2</td>
<td>103</td>
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<tr>
<td>CPD haemolysis</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>105</td>
<td>-</td>
<td>113</td>
<td>-</td>
<td>-</td>
<td>88.4</td>
<td>99.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na-Hep</td>
<td>103</td>
<td>-</td>
<td>109</td>
<td>-</td>
<td>-</td>
<td>91.3</td>
<td>97.6</td>
<td>91.9</td>
<td>93.7</td>
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<tr>
<td>Na-Hep haemolysis</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>98.3</td>
<td>-</td>
<td>-</td>
<td>95.7</td>
<td>99.5</td>
<td>103</td>
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<table>
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<th>RT 48 hrs</th>
<th>4°C 24 hrs</th>
<th>4°C 48 hrs</th>
<th>F/T cycle 3</th>
<th>F/T cycle 5</th>
<th>Precipitated 4hrs in RT</th>
<th>-80°C 224 days</th>
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<td>Na-Hep haemolysis</td>
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<td>94.2</td>
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<td></td>
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</table>

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

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).

Grant information
This work was supported by the Wellcome Trust (104926) and the Bill & Melinda Gates Foundation (OPP1133769).

Acknowledgements
We would like to thank Karnrawee Kaewhao at Mahidol-Oxford Tropical Medicine Research Unit for valuable assistance in the development and validation of the method.

References


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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). 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

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). 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-13CD₃ 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-13CD₃ signal as it did in our evaluation of ceftriaxone-D₃. 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-D₃ 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:
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 al2) 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.

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

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-D₃), 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-D₃ was excluded (i.e. the level of interference between ceftriaxone and ceftriaxone-D₃). A SIL-IS of D₅ 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) 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.