METHOD ARTICLE

Liquid chromatography–tandem mass spectrometry for the simultaneous quantitation of ceftriaxone, metronidazole and hydroxymetronidazole in plasma from seriously ill, severely malnourished children [version 1; referees: 4 approved with reservations]

Martin Ongas¹,², Joseph Standing³,⁴, Bernhards Ogutu¹,², Joseph Waichungo⁵, James A. Berkley⁶⁻⁷, Karin Kipper⁴,⁸,⁹

¹Center for Research in Therapeutic Sciences, Strathmore University, Ole Sangale Road, Nairobi, Kenya
²KEMRI-Centre for Clinical Research, Nairobi, Kenya
³Inflammation, Infection and Rheumatology Section, UCL Great Ormond Street Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, UK
⁴Paediatric Infectious Diseases Research Group, Institute for Infection and Immunity, St. George’s, University of London, Cranmer Terrace, London, SW17 0RE, UK
⁵KEMRI-Wellcome Trust Research Programme, Kilifi, Kenya
⁶Centre for Tropical Medicine & Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, UK
⁷The Childhood Acute Illness & Nutrition (CHAIN) Network, Lenana Place, Nairobi, Kenya
⁸Analytical Services International, St George’s University of London, Cranmer Terrace, London, SW17 0RE, UK
⁹Institute of Chemistry, University of Tartu, Tartu, Estonia

Abstract

We have developed and validated a novel, sensitive, selective and reproducible reversed-phase high-performance liquid chromatography method coupled with electrospray ionization mass spectrometry (HPLC–ESI-MS/MS) for the simultaneous quantitation of ceftriaxone (CEF), metronidazole (MET) and hydroxymetronidazole (MET-OH) from only 50 µL of human plasma, and unbound CEF from 25 µL plasma ultra-filtrate to evaluate the effect of protein binding. Cefuroxime axetil (CEFU) was used as an internal standard (IS). The analytes were extracted by a protein precipitation procedure with acetonitrile and separated on a reversed-phase Polaris 5 C18-Analytical column using a mobile phase composed of acetonitrile containing 0.1% (v/v) formic acid and 10 mM aqueous ammonium formate pH 2.5, delivered at a flow-rate of 300 µL/min. Multiple reaction monitoring was performed in the positive ion mode using the transitions m/z 555.1→m/z 396.0 (CEF), m/z 172.2→m/z 128.2 (MET), m/z 188.0→m/z 125.9 (MET-OH) and m/z 528.1→m/z 364.0 (CEFU) to quantify the drugs. Calibration curves in spiked plasma and ultra-filtrate were linear (r² ≥ 0.9948) from 0.4–300 µg/mL for CEF, 0.05–50 µg/mL for MET and 0.02 – 30 µg/mL for MET-OH. The intra- and inter- assay precisions were less than 9% and the mean extraction recoveries were 94.0% (CEF), 98.2% (MET), 99.6% (MET-OH) and 104.6% (CEF in ultra-filtrate); the recoveries for the IS were 93.8% (in plasma) and 97.6% (in ultra-filtrate). The validated method was successfully applied to a pharmacokinetic study of CEF, MET and MET-OH in...
successfully applied to a pharmacokinetic study of CEF, MET and MET-OH in hospitalized children with complicated severe acute malnutrition following an oral administration of MET and intravenous administration of CEF over the course of 72 hours.

This article is included in the KEMRI | Wellcome Trust gateway.

**Corresponding author:** Karin Kipper (kkipper@sgul.ac.uk)

**Author roles:** Ongas M: Supervision, Writing – Review & Editing; Standing J: Validation, Writing – Original Draft Preparation; Ogutu B: Supervision, Writing – Original Draft Preparation; Waichungo J: Resources, Supervision; Berkley JA: Project Administration; Kipper K: Resources, Supervision, Writing – Review & Editing

**Competing interests:** No competing interests were disclosed. No writing assistance was utilized in the production of this manuscript.

**How to cite this article:** Ongas M, Standing J, Ogutu B et al. Liquid chromatography–tandem mass spectrometry for the simultaneous quantitation of ceftriaxone, metronidazole and hydroxymetronidazole in plasma from seriously ill, severely malnourished children [version 1; referees: 4 approved with reservations] Wellcome Open Research 2017, 2:43 (doi: 10.12688/wellcomeopenres.11728.1)

**Copyright:** © 2017 Ongas M et al. This is an open access article distributed under the terms of the Creative Commons Attribution Licence, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. The author(s) is/are employees of the US Government and therefore domestic copyright protection in USA does not apply to this work. The work may be protected under the copyright laws of other jurisdictions when used in those jurisdictions.

**Grant information:** JS has received funding from United Kingdom Medical Research Council Fellowships (grants G1002305 and M008665), and been supported by the National Institute for Health. KK has received funding from the People Programme (Marie Curie Actions) of the European Union’s Seventh Framework Programme (FP7/2007-2013) under REA grant agreement no. 608765 and from the Estonian Research Council grant agreement PUTJD 22. JB received funding for this work within the First-Line Antimicrobials in Children with Complicated Severe Acute Malnutrition (FLACSAM) trial from the MRC/DFID/Wellcome Trust Joint Global Health Trials Scheme (MR/M007367/1), and is funded by the Bill & Melinda Gates Foundation for the Childhood Acute Illness & Nutrition (CHAIN) Network (OPP1131320).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**First published:** 19 Jun 2017, 2:43 (doi: 10.12688/wellcomeopenres.11728.1)
Introduction
Serious infections are common in children, especially those with severe acute malnutrition (SAM) admitted sick to hospitals, with over 50% of patients estimated to be infected at any one time. Mortality remains high in this patient group, despite implementation of current treatment guidelines. Although empiric antibiotics are routinely given, it is not clear whether the currently recommended regimen is the most effective in the context of increasing antimicrobial resistance (AMR), and moreover whether expected therapeutic levels are achieved in this group of patients.

To resolve this question, a large clinical trial of metronidazole (MET) and ceftriaxone (CEF) versus standard care (penicillin or ampicillin plus gentamicin) is planned. However, first, a study of the pharmacokinetics (PK) of MET and CEF is needed in order to optimize the dosing strategy in severely malnourished children, since they may have altered absorption, body composition, volume of distribution, available plasma proteins for binding, or metabolism and elimination through hepatic and renal pathways. A quantitative determination of MET and CEF in plasma is essential in order to evaluate the pharmacokinetics of these co-administered antibiotics (Figure 1).

Previous studies have indicated the activity of MET and its two principle metabolites, 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (the “alcohol” metabolite, MET-OH) and 2-methyl-5-nitroimidazole-1-acetic acid (the “acid” metabolite) against a broad range of anaerobic bacteria. Several methods have been reported for quantification of either MET or MET and its metabolites in human plasma or serum. O’Keefe et al. evaluated the activity of the metronidazole metabolites against anaerobic bacteria; however, the LC-UV method was limited in quantifying lower levels of the metabolites in a biological matrix due to its low sensitivity and poor selectivity. Silva et al. developed an HPLC-MS-MS method for the quantitation of metronidazole in plasma. The method required large sample volumes and complex sample preparation steps, with large volumes of extraction solvents.

CEF, like other β-lactam antibiotics, is highly protein bound. Wong et al. reported average protein binding of 89.5%. It has also been noted that ceftriaxone protein binding is nonlinear, becoming saturated at higher concentrations and linked with serum albumin concentrations in critically ill patients.

Given the significant effects of protein binding on clinical exposure to highly bound drugs, and given that the free drug is important for antimicrobial effect, it was necessary to develop a method to measure the unbound ceftriaxone appropriate for use in seriously ill malnourished children. Some of the methods reported previously give approaches to measurement of unbound fractions of compounds using equilibrium dialysis, which are more prone to environmental interference and much more laborious in sample preparations. Other methods involved the use of HPLC with UV detection, but did not consider the protein binding of CEF.

Figure 1. Chemical structures of ceftriaxone (A), metronidazole (B), hydroxymetronidazole (D) and cefuroxime axetil, IS (C).
We aimed to develop the first simultaneous HPLC-ESI-MS/MS method for rapid, simple, reliable, sensitive and selective quantification of MET, CEF and MET-OH in a small volume (50 µL) of human plasma, and unbound CEF from (25 µL) plasma ultrafiltrate.

Materials and methods
Chemicals
Ceftriaxone sodium (CEF; batch no. 3.2, purity 90.4%; MW=554.58 g/mol), metronidazole (MET; batch no. 2.1, purity 100%, MW=171.15 g/mol) and cefuroxime axetil (CEFU, batch no. 4.0, purity 97.3%, MW=510.47 g/mol) were purchased from European Directorate for the Quality of Medicines and Healthcare (Strasbourg, France). Hydroxymetronidazole (MET-OH; Lot no. 4276, purity 98.2%, MW=187.15 g/mol) was purchased from LGC (Teddington, UK). Acetonitrile and methanol (both LC-MS grade), formic acid (85%; AnalaR® grade) and ammonium formate (AnalaR® grade) were purchased from Sigma Aldrich (St. Louis, MO, USA). Deionized water was prepared using a Smart2 Pure® water purification system (Thermo-scientific, Niederelbert, Germany). Blank human plasma with Li-heparin for the preparation of calibrators and quality controls was obtained from Kenya Medical Research Institute, Centre for Clinical Research (Nairobi, Kenya).

Sample preparation
Total drug. To a 50 µL aliquot of plasma (blank, standard, quality control, or patient sample) 200 µL of internal standard (CEFU; of a 1.25 µg/mL solution in acetonitrile) was added. The 1.5 mL polystyrene tubes were vortex-mixed for 3 minutes to precipitate the plasma proteins, followed by centrifugation (4000 x g; 10 min, 4°C). The supernatant (100 µL) was transferred into another clean 1.5 mL polystyrene tube and diluted with 400 µL of 20% methanol in water. The samples were vortex-mixed for 3 minutes and submitted for analysis by LC-MS/MS.

Unbound ceftriaxone. About a 300 µL aliquot of patient plasma was taken into a clean 1.5mL polystyrene tube and incubated on a Grant JB Series incubation bath (Grant Instruments, Cambridge, UK) at 37°C for 1 h, then transferred into Centrifree® Ultrafiltration Device (Merck Millipore Ltd, Darmstadt, Germany) and centrifuged on a Thermo Fisher Scientific SL 40R centrifuge (2000 x g; 30min, 37°C). 25 µL sample ultra-filtrate was taken into another clean 1.5 mL polystyrene tube and diluted with 200 µL of 20% methanol in water. The samples were vortex-mixed for 3 min and submitted for analysis by LC-MS/MS.

Preparation of analytical standards
Stock solutions of CEF (5 mg of the base/mL), MET and MET-OH (both 1 mg/mL) were prepared by dissolving an appropriate amount of each compound in acetonitrile. The stock solutions were further serially diluted with 20% methanol to make working standard solutions used to spike the blank plasma. Stock solution of CEFU (IS) was prepared by dissolving appropriate amount of the compound in acetonitrile, the stock solution was serially diluted with acetonitrile to make working standard solutions of 1.25 µg/mL and 1 µg/mL. All the stock solutions were stored at -20°C, protected from light (in amber sample vials) and used within three months.

Chromatographic conditions
The equipment consisted of an Agilent Technologies HPLC-ESI-MS/MS system (Santa Clara, CA, USA), composed of a 1260 µ Quaternary Pumps, 1260 Autosampler and 1260 Thermosetting Column Compartment (TCC). Chromatographic separation was performed on a Polaris 5 C18-A (150 mm x 3.0 mm I.D; 3.0 µm particle size) analytical column from Agilent Technologies(Santa Clara, CA, USA) with a C18 guard cartridge (4 mm x 3.0 mm, 3.0 µm) (Phenomenex, Torrance, CA, USA) maintained at 30°C. The mobile phase consisted of (A) 10mM aqueous ammonium formate pH 2.5 and (B) 0.1% formic acid in acetonitrile. A linear gradient elution was used to deliver the mobile phase, 40% solvent B at time 0 min, and 100% from 1.8 min, to 5.5 min, and back to 40% from 6 min to 12 min, (re-equilibration step). The flow rate was set at 300 µL/min, an injection volume of 5 µL was used to optimize the drug signals and for analysis.

Mass spectrometry
Mass spectrometric detection of analytes was performed on a 6410 Triple Quadrupole Mass Spectrometer with an Electrospray Ionization (ESI) source from Agilent Technologies (Santa Clara, CA, USA) in positive ionization mode. Nitrogen was used as the nebulizing, desolvation and collision gas, the optimized ion source parameters were: ion spray voltage 4.0 kV, exit potential 7V, RF lens 0.5V.

Source temperature was 100°C and desolvation temperature 300°C. High purity nitrogen from Genius NM32LA generator (Peak Scientific, Scotland, UK) was used as both sheath and auxiliary gas set at 20 l/min and 12 l/min, respectively.

Selected reaction monitoring (SRM) was employed for the data acquisition, the analytical parameters optimized for the compounds were declustering potentials (DP) and collision energies (CE) (Table 1), and the scan dwell time was set at 500 ms. for each channel. Data acquisition and analysis were accomplished with Mass Hunter software (version A.02.00; Agilent Technologies).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion</th>
<th>MRM Transition (m/z)</th>
<th>DP (V)</th>
<th>CE (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEF</td>
<td>[M+H]+</td>
<td>555.1→396.0</td>
<td>60</td>
<td>18</td>
</tr>
<tr>
<td>MET</td>
<td>[M+H]+</td>
<td>172.2→128.2</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>MET-OH</td>
<td>[M+H]+</td>
<td>188.0→125.9</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>CEFU</td>
<td>[M+NH4]+</td>
<td>528.1→364.0</td>
<td>60</td>
<td>18</td>
</tr>
</tbody>
</table>
Validation
Method validation was performed as per the US Food and Drug Administration Guidance for Industry Bioanalytical Method Validation\(^4\). The method was validated for selectivity and sensitivity, inter-day and intra-day accuracy and precision, extraction recovery, matrix effect and stability. Method’s linear range was evaluated and lower limit of quantification was set to fit for purpose for the actual clinical trial samples. Carry-over was assessed in accordance with the European Medicines Agency guideline\(^1\).

Selectivity of the method was assessed and assured by analysis of six blank plasma samples from different sources, each blank sample was tested for interference using the proposed extraction procedure and chromatographic/mass spectrometric conditions and compared with those obtained with an aqueous solution of the analyte at a concentration near to the lower limit of quantification (LLOQ).

Extraction recovery was determined by comparing the response of extracted quality control plasma samples with the response of post extracted plasma samples spiked at similar concentrations to the quality control samples.

Stability (ST%) studies were evaluated via sample and solution concentrations, where:

\[
ST\% = \frac{c_t}{c_0} \times 100\%.
\]

(i)

ST% is the stability of the chemical compound in the sample over the period of time. \(c_0\) is the initial concentration, determined without introducing any extra pauses in the analysis process. \(c_t\) is the concentration obtained after the storage period with time \(t\).

Sub-stock solution stability was evaluated for CEF, MET and MET-OH, by comparing the response generated from the same solution at preparation and after being stored at -20°C for a period of 28 days. All the analytes were found to be stable within the period investigated and fresh stock solutions were prepared thereafter, fresh IS solution was prepared daily from weighing the compound in acetone. The curves were constructed by plotting peak area ratios of analytes against the nominal concentrations of CEF, MET and MET-OH from only 50 µL of human plasma, and unbound CEF from 25 µL plasma ultra-filtrate based on the physicochemical properties of these compounds and the area of method application. Moreover, the concerns raised by Berezhkovskiy et al.\(^2\) on temperature dependency of protein binding and the need to maintain the physiological temperature (37°C) through the sample processing time were considered in sample pretreatment.

The method took into account the therapeutic and overdose concentration ranges. The method has been validated and proved to be reliable for the determination of the drugs in human plasma. During the method development, several chromatographic conditions were optimized for all analytes such as the mobile phase composition, \(pH\) and various flow rates. Various ratios (80:20, 70:30, 60:40 v/v) of acetonitrile and 10mM ammonium formate were tested as starting eluent for chromatographic separation. The variation in the mobile phase led to considerable changes in the chromatographic parameters, like peak symmetry and retention time. The pH effect showed that optimized conditions are reached when the pH value of the buffer is adjusted to 2.5 with formic acid, producing well resolved and sharp peaks for all analytes assayed. Henceforth, in the present method the pH adjusted to 2.5 and the chosen LC gradient ensured sharp chromatographic peaks with the best possible baseline-resolved separations of CEF, MET, MET-OH and CEFU (IS) within 4 minutes with a total runtime of 12 minutes. With the optimized SRM transitions, the stable and most intense product ions of CEF (m/z 396.0), MET (m/z 128.2), MET-OH (m/z 125.9) and CEFU (m/z 364.0) were detected (Figure S1).

Method validation
Selectivity. All the lots of blank plasma used for selectivity studies met the acceptance criteria, no significant interferences at the retention times of the analytes or internal standard were found. Figure 2 shows the typical chromatograms of extracted blank plasma, blank plasma spiked with IS (Zero sample), a spiked plasma sample with the analytes at LLOQ and ULOQ level. It can be seen that there were no interfering peaks from endogenous compounds observed at the retention times of the analytes and the IS. Moreover, no interference was observed from plasma samples fortified with commonly used \(\beta\)-lactam antibiotics (cefadroxil and cefaclor), processed and analyzed as described under the proposed sample preparation procedure.

Calibration curves and limit of quantification. Calibration curves were constructed by plotting peak area ratios of analytes and IS against the nominal concentrations of CEF, MET and MET-OH. The curves for drugs spiked in plasma were found to be linear over the concentration ranges of 0.4–300 µg/mL (CEF),
Figure 2A. Representative chromatograms of ceftriaxone (RT 2.59 min), metronidazole (RT 2.67 min), hydroxymetronidazole (RT 2.69 min), and cefuroxime (IS) (RT 3.71 min) from extracted spiked samples: zero sample LLOQ.
Figure 2B. Representative chromatograms of ceftriaxone (RT 2.59 min), metronidazole (RT 2.67 min), hydroxymetronidazole (RT 2.69 min), and cefuroxime (IS) (RT 3.71 min) from extracted spiked samples: zero sample ULOQ.
Figure 2C. Representative chromatograms of ceftriaxone (RT 2.59 min), metronidazole (RT 2.67 min), hydroxymetronidazole (RT 2.69 min), and cefuroxime (IS) (RT 3.71 min) from extracted spiked samples: zero sample in human plasma.
Protein precipitation with acetonitrile was used to extract the analytes and the IS from plasma samples. This method was found to be efficient given the small sample volume (50 µL) used that would otherwise be impossible to use with the liquid-liquid extraction techniques employed in previously reported publications.12,13,15 for MET and16–20 for CEF. This still yielded higher recoveries with better reproducibility (Table S2).

Accuracy and precision. To evaluate the inter-assay precision and accuracy, six replicates of quality control plasma samples were analyzed together with one independent calibration standard curve, this was done in three consecutive days; while intra-assay precision and accuracy were evaluated through analysis of quality control plasma samples in replicate of six in the same day. Inter-assay and intra-assay precision were expressed as coefficient of variation (CV%). The accuracy was expressed as the percent ratio between the experimental concentrations and the nominal concentration for each sample. A similar assessment was done for plasma ultra-filtrate to determine the accuracy and precision for the unbound ceftriaxone. Assay accuracy and precision data are shown in Table 2.

Stability (ST %)
The results of all the stability studies obtained were well within the acceptable limits of accuracy (±15%) and precision (CV ≤ 15%) (Table 3).

Sub-stock stability. All analytes indicated good stability at the storage temperature, 95.4–96.1% of the original concentration was found after storage period of 28 days.

Freeze and thaw stability. Spiked plasma samples were subjected to three freeze-thaw cycles and the analytes concentrations assessed after the third cycle. This was done also for plasma ultra-filtrate spiked with CEF to assess the stability of free ceftriaxone in calibrators and quality control samples. The freeze-thaw cycles were carried out in a -20°C freezer. The freeze and thaw stability data presented in Table 5 is consistent with previously reported data by Silva et al.12 and Ilomuanya et al.15 for MET stability. Ilomuanya et al.15 in his freeze/thaw cycle evaluations indicated that after the fourth freeze/thaw cycle the concentrations of MET was < 90%, suggesting that MET is not very stable after three freeze/thaw cycles. This is however the first published ultra-filtrate stability data for CEF.

Short term stability or bench-top stability. Plasma samples at low and high quality control levels were kept at room temperature for a minimum of eight hours, then processed and analyzed (Table 3). Some studies have reported stabilities of metronidazole over a longer duration than in this method.12,15. Our choice for the 8 h period was to report an analytically relevant study under which the three drugs can be analyzed. The results indicated that the drugs were stable and therefore the sample processing procedure outlined within this method can be used to process large number of samples without the risk of sample degradation due to room temperature exposure.

Silva et al.12 reported the stability of MET over a period of 48h, the mean stability ranging between 93.6% – 100.6%. This stability data was in agreement with what we have reported in this method, however we report the first stability study of MET-OH and CEF in plasma ultra-filtrate.

24 h stability in the autosampler. The results of post processing stability in Table 3 indicated that all the drugs were stable after 24 h in the autosampler and the integrity of data obtained after such re-assay would not be questionable. Ilomuanya et al.15 reported the autosampler stability of MET for 72h, however the data reported showed that MET was stable up to 24h and at 72h, the stability was greatly reduced to 40.6% – 58.7%.

Long term stability at -20°C. The stability data reported in this study show that all the analytes were stable (90.6%–99.1%) within the period investigated. Since the stability at -20°C was acceptable, there was no need to evaluate the stability at -80°C, as our aim was to report a method that is affordable to resource limited laboratories.

Carry-over. A processed blank sample was injected after a high concentration calibration standard at the upper limit of quantification (ULOQ). No significant peak indicating carry-over was detected.

Matrix effect (ME%). The protein precipitation method of sample preparation is known to be prone to matrix effect.16–29. Chromatography of analytes or IS, as well as accuracy of the method may be affected by matrix effect, ion suppression or enhancement, due to co-eluting endogenous components. Therefore, to ensure the selectivity of the method, matrix effect studies were carried out. Two different methods were used to access and determine matrix effect. In the first method, regions of ion suppression or enhancement were evaluated by direct post column infusion of a mixture of analytes and IS at high concentration at the rate of 10 µL/min, while injecting a blank extracted plasma. This was to determine...
Table 2. Intra-assay and inter-assay accuracy and precision of metronidazole (MET), ceftriaxone (CEF), and hydroxymetronidazole (MET-OH) in plasma, and CEF in ultra-filtrate (CEF\textsuperscript{uf}) at LLOQ, LOQ, MOQ and HOQ.

<table>
<thead>
<tr>
<th>Intra-assay (n=6)</th>
<th>Compound</th>
<th>Nominal concentration (µg/mL)</th>
<th>Mean estimated concentration (µg/mL) ±SD</th>
<th>Precision (CV %)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MET</td>
<td>0.05</td>
<td>0.051 ± 2.0</td>
<td>3.9</td>
<td>101.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>0.148 ± 7.7</td>
<td>7.8</td>
<td>98.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20.44 ± 4.0</td>
<td>3.9</td>
<td>102.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>37.75 ± 5.6</td>
<td>5.9</td>
<td>94.4</td>
<td></td>
</tr>
<tr>
<td>CEF</td>
<td>0.4</td>
<td>0.39 ± 2.5</td>
<td>3.2</td>
<td>97.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>1.10 ± 1.5</td>
<td>1.7</td>
<td>91.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>112.02 ± 3.5</td>
<td>3.7</td>
<td>93.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>219.51 ± 6.8</td>
<td>7.5</td>
<td>91.5</td>
<td></td>
</tr>
<tr>
<td>MET-OH</td>
<td>0.02</td>
<td>0.018 ± 1.7</td>
<td>2.6</td>
<td>90.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.057 ± 4.7</td>
<td>4.9</td>
<td>95.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>11.43 ± 2.5</td>
<td>2.7</td>
<td>95.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>24.49 ± 8.6</td>
<td>8.4</td>
<td>102.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.41 ± 5.5</td>
<td>5.3</td>
<td>100.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>1.27 ± 5.5</td>
<td>5.2</td>
<td>105.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>112.32 ± 5.1</td>
<td>5.5</td>
<td>93.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>253.03 ± 8.2</td>
<td>7.8</td>
<td>105.4</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inter-assay (n=18)</th>
<th>MET</th>
<th>0.05</th>
<th>0.051 ± 1.4</th>
<th>2.7</th>
<th>101.1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.15</td>
<td>0.155 ± 5.6</td>
<td>5.4</td>
<td>103.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20.59 ± 3.3</td>
<td>3.2</td>
<td>103.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>38.79 ± 5.6</td>
<td>5.8</td>
<td>97.0</td>
<td></td>
</tr>
<tr>
<td>CEF</td>
<td>0.4</td>
<td>0.40 ± 2.7</td>
<td>2.9</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>1.15 ± 3.8</td>
<td>3.9</td>
<td>95.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>114.54 ± 5.1</td>
<td>5.4</td>
<td>95.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>226.75 ± 5.2</td>
<td>5.5</td>
<td>94.5</td>
<td></td>
</tr>
<tr>
<td>MET-OH</td>
<td>0.02</td>
<td>0.019 ± 1.2</td>
<td>2.3</td>
<td>95.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.059 ± 5.2</td>
<td>5.3</td>
<td>98.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>12.01 ± 4.8</td>
<td>4.8</td>
<td>100.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>24.51 ± 4.7</td>
<td>4.6</td>
<td>102.1</td>
<td></td>
</tr>
<tr>
<td>CEF\textsuperscript{uf}</td>
<td>0.4</td>
<td>0.43 ± 5.8</td>
<td>7.4</td>
<td>107.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>1.22 ± 8.3</td>
<td>8.1</td>
<td>101.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>115.32 ± 5.1</td>
<td>5.3</td>
<td>96.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>251.30 ± 6.2</td>
<td>5.9</td>
<td>104.6</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Stability (ST%) of metronidazole (MET), ceftriaxone (CEF), hydroxymetronidazole (MET-OH) with the coefficient of variation (CV%) in plasma and CEF in ultra-filtrate (CEF<sub>uf</sub>) (n=5).

<table>
<thead>
<tr>
<th>Stability parameters</th>
<th>Spiked conc.</th>
<th>MET</th>
<th>CEF</th>
<th>MET-OH</th>
<th>CEF&lt;sub&gt;uf&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(µg/mL)</td>
<td>0.15</td>
<td>40</td>
<td>1.2</td>
<td>240</td>
</tr>
<tr>
<td>Benchtop stability in matrix (room temperature, 8 h</td>
<td>Mean of stability of samples</td>
<td>0.16</td>
<td>39.32</td>
<td>1.18</td>
<td>230.4</td>
</tr>
<tr>
<td></td>
<td>CV %</td>
<td>3.8</td>
<td>1.4</td>
<td>4.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Freeze-thaw stability (3 freeze-thaw cycles at -20ºC</td>
<td>Mean of stability of samples</td>
<td>0.14</td>
<td>37.40</td>
<td>1.15</td>
<td>228.6</td>
</tr>
<tr>
<td></td>
<td>CV %</td>
<td>3.2</td>
<td>1.8</td>
<td>3.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Auto-sampler stability (24 h at 18ºC)</td>
<td>Mean of stability of samples</td>
<td>0.15</td>
<td>37.50</td>
<td>1.09</td>
<td>228.9</td>
</tr>
<tr>
<td></td>
<td>CV %</td>
<td>3.3</td>
<td>4.3</td>
<td>5.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Long-term stability (90 days at -20ºC)</td>
<td>Mean of stability of samples</td>
<td>0.14</td>
<td>37.52</td>
<td>1.11</td>
<td>217.4</td>
</tr>
<tr>
<td></td>
<td>CV %</td>
<td>6.0</td>
<td>4.7</td>
<td>4.2</td>
<td>7.3</td>
</tr>
<tr>
<td>Sub-stock solution stability (28 days at -20ºC)</td>
<td>Nominal Conc. (µg/mL)</td>
<td>50</td>
<td>300</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean of stability of samples</td>
<td>47.89</td>
<td>288.39</td>
<td></td>
<td>28.63</td>
</tr>
<tr>
<td></td>
<td>CV %</td>
<td>3.1</td>
<td>2.8</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ST %</td>
<td>95.8</td>
<td>96.1</td>
<td>95.4</td>
<td></td>
</tr>
</tbody>
</table>

which of the analytes experienced matrix effect; Figure S2A (iv) revealed that only MET showed interference (ion enhancement) at its retention time. In the second method, matrix effect (ion enhancement) was evaluated for MET in six different lots of plasma by comparing the response of post extracted plasma samples spiked with 0.15 µg/mL (LLOQ) and 40 µg/mL (ULOQ) of metronidazole with the response of neat standard solutions spiked at similar concentrations. The matrix effect encountered with this method (Table S3) was much lower than what had been previously reported<sup>15</sup>. This was due to the small sample volumes that were used in sample processing, where:

\[
ME\% = \frac{\text{Response}_{\text{POEM}}}{\text{Response}_{\text{NEAT}}} \times 100\%.
\]

Response<sub>POEM</sub> is the average concentration of post extraction spiked matrix and Response<sub>NEAT</sub> is the average concentration of the analyte in a neat solution.

The samples were prepared at two concentrations and the matrix effect determined as 107.6% (0.15 µg/mL) and 102.1% (40 µg/mL), n=6 at both levels. The values obtained at both levels were above 100% indicating the plasma-induced ion enhancement on the analysis of MET and suggesting that the endogenous compounds increased the signal intensity of the analyte in positive ESI mode. The effect of signal enhancement was higher at low concentration level.

Application of the method to real patient samples

The Optimising Antibiotic Treatment for Sick Malnourished Children (FLACSAM-PK) study was registered (NCT02746276) at ClinicalTrials.gov<sup>38</sup>. The validated method was successfully applied to a pharmacokinetic study of CEF, MET, MET-OH and unbound ceftriaxone in hospitalized children with complicated severe acute malnutrition (SAM) following an oral administration of MET and intravenous administration of CEF over the course of 72 hours. 81 hospitalized children with SAM and requiring IV antibiotics according to WHO and national guidelines were recruited (after obtaining ethical approval from the Kenya Medical Research Institute Scientific and Ethics Review Unit, approval number: KEMRI/SERU/CGMR-C023-3161 and informed consent from the parents/guardians) and treated with an oral dose of 7.5mg/Kg MET (Flagyl<sup>®</sup> oral suspension, 200 mg/5 mL) three times daily and IV injection of 80 mg/kg CEF (Ceftriaxone Rocephin<sup>®</sup>, 250 mg) once
daily 15 min after metronidazole dose. Blood samples (3.0 mL) were collected into Li-heparinized tubes, a pre-dose sample was taken before administering the drugs. Further sampling at 5, 30, 60 min after ceftriaxone dose and 2, 4, and 8 h after metronidazole dose. The sampling plan was such that each patient had only three blood draws after the base-line sample. The blood was centrifuged (3000 rpm; 5 min), plasma separated and stored at -80°C until analysis time.

The patient samples were successfully analyzed using this method and no interference of endogenous compounds resulting from altered plasma protein compositions was encountered.

Figure 3, shows a concentration–time profile of a baseline and three post-dose samples from a patient who had previously taken at least one metronidazole dose prior to study enrolment, this was evident from the significant levels of metronidazole and hydroxymetronidazole detected from the baseline sample.

We also addressed the recommendations by Wong et al. [17], as this method allows for direct measurement of unbound ceftriaxone from only 25 µL plasma ultra-filtrate. Figure 4 shows representative chromatograms of processed plasma samples from one of the study participants.

Figure 3. Example concentration-time data of each of the four blood samples (baseline and 3 post first dose), where ceftriaxone, metronidazole and hydroxymetronidazole were quantified. In 2 samples, unbound ceftriaxone was also quantified. This example shows a patient who has clearly taken at least one previous dose of metronidazole prior to study enrolment.
Figure 4A. Representative chromatograms of processed plasma study samples at baseline before drug administration.
Figure 4B. Representative chromatograms of processed plasma study samples at 5 min after administering ceftriaxone IV.
Figure 4C. Representative chromatograms of processed plasma study samples at 30 min after administering ceftriaxone IV.
Conclusions
The validated HPLC–ESI–MS/MS method allowed the simultaneous quantitation of metronidazole, hydroximetronidazole, ceftriaxone from only 50 µL human plasma, and of unbound ceftriaxone from 25 µL plasma ultra-filtrate. It provided simple and rapid analyses, as well as sensitive and reliable results. Thus, this method is suitable for routine high-throughput analyses and may be successfully applied to pharmacokinetic and bioequivalence of multiple doses evaluated in the present work in human subjects. The small sample volumes used makes it applicable to pediatric pharmacokinetics and bioequivalence studies, in which large sample volumes may be unethical or impractical to obtain.

Competing interests
No competing interests were disclosed. No writing assistance was utilized in the production of this manuscript.

Grant information
JS has received funding from United Kingdom Medical Research Council Fellowships (grants G1002305 and M008665), and been supported by the National Institute for Health. KK has received funding from the People Programme (Marie Curie Actions) of the European Union’s Seventh Framework Programme (FP7/2007-2013) under REA grant agreement no. 608765 and from the Estonian Research Council grant agreement PUTJD 22. JB received funding for this work within the First-Line Antimicrobials in Children with Complicated Severe Acute Malnutrition (FLACSAM) trial from the MRC/DfID/Wellcome Trust Joint Global Health Trials Scheme (MR/M007367/1), and is funded by the Bill & Melinda Gates Foundation for the Childhood Acute Illness & Nutrition (CHAIN) Network (OPP1131320).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supplementary materials

Figure S1. MRM product ion spectra of protonated (i) CEF (m/z 555.1→m/z 396.0), (ii) MET (m/z 172.2→m/z 128.2), (iii) MET-OH (m/z 188.0→m/z 125.9), and ammonium adduct of (iv) CEFU (m/z 528.1→m/z 364.0).

Click here to access the data.

Figure S2. Representative chromatograms of a direct post column infusion of blank extracted plasma (A, i-iv), MET at ULOQ (A, v) and a blank extracted neat solution showing absence of matrix effect (B, i-iv).

Click here to access the data.

Table S1. Regression parameters for ceftriaxone (CEF), metronidazole (MET) and hydroxymetronidazole (MET-OH) in spiked plasma. CEFuf: ceftriaxone in ultra-filtrate.

Click here to access the data.

Table S2. Extraction recoveries of ceftriaxone (CEF), metronidazole (MET), hydroxymetronidazole (MET-OH) and cefuroxime (CEFU) from spiked plasma samples and in ultra-filtrate. Standard deviation (SD); coefficient of variation (CV); internal standard (IS); n=6. CEFuf: ceftriaxone in ultra-filtrate; CEFUuf: cefuroxime in ultra-filtrate.

Click here to access the data.

Table S3. Matrix effects (ME %) for metronidazole (MET) in 6 plasmas. Standard deviation (SD); coefficient of variation (CV); internal standard (IS); n=6.

Click here to access the data.

References


Open Peer Review

Current Referee Status:  ?  ?  ?  ?

Version 1

Referee Report 17 November 2017

doi:10.21956/wellcomeopenres.12670.r26950

Pascal Houzé
CNRS (French National Center for Scientific Research) UMR8258 - U1022, Faculty of Pharmacy, Paris Descartes University, Paris, France

The authors report the development of the determination of ceftriazone and metronidazole in children by liquid chromatography coupled with mass spectrometry. Therapeutic adaptation in children is particularly important and especially in malnourished children. The theme developed by the authors is therefore totally up to date.

In general, the article is well constructed and the validation of method correctly performed in part on the study of stability performed under very varied conditions.

However, different points need to be clarified:

The introduction:
• Why did the authors not also measure the acid metabolite of metronidazole which is active as the parent molecule and the alcohol metabolite? To be explained by the authors

The material and methods
• Why the authors dilute the eluent to 1/5 in an aqueous solution of 20% methanol. Why such a large dilution? Why use an aqueous solution of methanol to dilute and not use mobile phase A?

• Would it not have been better to evaporate acetonitrile and take up the dry residue with mobile phase?

• For the quantification of the unbound fraction why do the authors start with 300 μl of serum to finally dilute in 1 ml of aqueous solution of methanol. Why not use less plasma and not dilute in such a large volume?

• The 300 μL used are a little high to qualify the method of micromethode as done by the authors

• For the quantification of the unbound fraction, what is the interest of incubating the plasma for 1 hour at 37 ° C before proceeding to ultrafiltration? Authors should explain this step

• the linearity domains for each molecule must be indicated in the paragraph corresponding to the preparation of the analytical standards
• how are the controls prepared for the study of the precision and accuracy of the method

The results and discussion

• In the section selectivity I do not understand the legend of Figure 2. In the text the authors speak of 4 chromatograms: Extracted blank, blank plasma spiked with IS, a spiked plasma with the analytes at LLOQ and ULOQ. Figure 2 shows only 3 chromatograms (A, B and C) and to my avis, the legends indicated do not correspond to the chromatograms presented. This point is major and must be clarified by the authors

• How did the authors determine the ULOQ values for all the measured compounds?

• For me the authors chose a bad example to illustrate their method of dosage. They should choose another child for whom there is no metronidazole at time T0. On the other hand, the chromatograms presented in FIG. 4 should correspond to those of the kinetics presented. Indeed, in FIG. 4A, chromatogram before injection, there is no peak of metronidazole nor of its metabolite, so this does not correspond to the chromatograms of the kinetics of FIG. 3

• Why was the determination of the unbound fraction of ceftriazone made only at 2 times and the determination of the total?

• How can the authors explain a very high concentration of free ceftriazone at T0 while the total form is undetectable at the same time? form at 4 times?

In conclusion, subject to making the changes mentioned above and especially to review the clinical illustration part of the end of article, this manuscript could be accepted for indexing.

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?
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?
Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Analytical Chemistry, Toxicology
I have read this submission. I 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.

Sandrine Lefeuvre
Laboratory of Biochemistry, CHR Orléans, Orléans, France

- Calibration and QCs preparation was not clearly explained. A paragraph detailing the preparation of calibrators and QCs in plasma and those used for unbound fraction is missing. How many points? Which matrix is used to quantify the free fraction?

- Target antibiotic concentrations should be determined for each patient, depending on the strain and the MIC. Has the MIC been taken into account to build the calibration range?

- Did the authors consider the impact of adding more methanol in preparing the high QC compared to the low QC? Furthermore, I am concerned that the different sample types (i.e. calibrators, QCs, and patient samples) were handled distinctly, especially with respect to the amount of methanol added to the sample prior to extraction.

- Why 6 min for the re-equilibration step of the analytical column? Could you explain?

- Dilution integrity was not experimented to validate the dilution test to be carried out on drug concentration beyond the calibration interval. Considering the wide range of concentrations expected at different stages of a treatment, the dilution process must be validated according to EMA guidelines.

- Incurred sample reanalysis is missing. Differences for instance in protein binding, sample inhomogeneity or concomitant medications, may affect the accuracy and precision of the analyte in such samples during processing and storage. It is therefore recommended to evaluate accuracy of incurred samples by reanalysis of study samples. In accordance with FDA and EMA guidelines.

- The 3 paragraphs (below) p9 should be in the Materials and Methods section. Not in the Results section

p9: “Accuracy and precision. To evaluate the inter-assay precision and accuracy, six replicates of quality control plasma samples were analyzed together with one independent calibration ..... A similar assessment was done for plasma ultra-filtrate to determine the accuracy and precision for the unbound ceftriaxone.”

p9: Carry-over. “A processed blank sample was injected after a high concentration calibration standard at the upper limit of quantification (ULOQ).”

p9 Matrix effect (ME%). …..”Two different methods were used to access and determine matrix effect. In the first method, regions of ion suppression or enhancement were evaluated by direct
post column infusion of a mixture of analytes and IS at high concentration at the rate of 10 μL/min, while injecting a blank extracted plasma.” … And ……” In the second method, matrix effect (ion enhancement) was evaluated for MET in six different lots of plasma by comparing the response of post extracted plasma samples spiked with 0.15 μg/mL (LLOQ) and 40 μg/mL (ULOQ) of metronidazole with the response of neat standard solutions spiked at similar concentrations.”

- CEF is highly bound to proteins; average protein binding of 89.5%. Could you explain Figure 3?

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?
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.

I have read this submission. I 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.

Referee Report 24 October 2017

doi:10.21956/wellcomeopenres.12670.r26783

I-Lin Tsai
Department of Biochemistry and Molecular Cell Biology, Taipei Medical University, Taipei, Taiwan

In the manuscript entitled “Liquid chromatography–tandem mass spectrometry for the simultaneous quantitation of ceftriaxone, metronidazole and hydroxymetronidazole in plasma from seriously ill, severely malnourished children”, the authors developed and validated a LC-MS/MS method to quantify drugs from plasma. The following are some comments for the manuscript:

1. Are the chromatograms in 2A and 2B generated from blank samples spiked with drugs at LLOQ and ULOQ respectively? Please indicate the peaks in 2A. What are “zero sample LLOQ” and “zero
sample ULOQ in the figure legend? What are the criteria of LLOQ for each analyte? How the authors defined the LLOQ?

2. Please indicate the spiked concentrations in figure 2C?

3. Please indicate the quantified concentrations in Figure 4.

4. Please use true scale (intensity) instead of 100% for all the figures.

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?
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.

I have read this submission. I 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.

Referee Report 24 July 2017
doi:10.21956/wellcomeopenres.12670.r24432

Laurens Manning
Department of Infectious Diseases, Fiona Stanley Hospital, Murdoch, WA, Australia

This is a straightforward methods paper for a simultaneous LCMS assay for ceftriaxone + metronidazole (+metabolite) from malnourished children. The necessity for good quality drug assays for use in vulnerable populations is a critical component for optimized PK, PK/PD and efficacy studies in the future. However, whilst I agree with the need for such an assay, I have a few concerns about how the data are reported and some of the analytical processes.

1. The assay is being reported in the conclusion as a low volume assay, but 300µL is required for the ultrafiltration component; I am not sure this can really be considered to be a microsampling technique. The total blood volume taken from very young, anaemic, malnourished children should
be a consideration in assay development, and it will almost certainly be an issue for ethics review boards.

2. Stability of the plasma assay has been reported. One of the challenges of working in tropical countries is ensuring that the assay is fit for purpose with respect to sample handling in the field. Often there are delays to plasma separation and the samples may have other delays before being placed into freezer conditions. Our group believes that in tropical and resource poor settings, the assay should account for stability at room temp (not just benchtop stability), tropical ambient temperatures and at 4 degrees.

3. As this assay has been reported as a simultaneous assay, it would be good to see the chromatograms overlaid with the 3 analytes and IS.

4. Could the authors please clarify throughout the manuscript whether MRM or SRM has been used?

5. My major critique is that of the example child used for the validation paper; the authors describe how the assay was used successfully for 81 children; but present a time concentration curve from a single child. The T0 samples in this child had >40000ng/mL. The explanation given is that the child must have had prior exposure to metronidazole. Whilst this is likely to be true, I don't believe this is appropriate for a methods paper that describes the utility of the assay. I would recommend reporting another ‘sample child' with a clear undetectable result at T0.

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.

I have read this submission. I 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 18 Aug 2017
Karin Kipper, St George’s, University of London, UK
Responses to the comments by Laurens Manning.

1. The assay is being reported in the conclusion as a low volume assay, but 300µL is required for the ultrafiltration component; I am not sure this can really be considered to be a microsampling technique. The total blood volume taken from very young, anaemic, malnourished children should be a consideration in assay development, and it will almost certainly be an issue for ethics review boards.

Response: This is a common misconception. For example, a 1ml sample taken at baseline then 3 time points for an 8kg child represents 0.6% of blood volume (80ml/kg) and would not have a measurable impact on haemoglobin concentration. No change to the manuscript.

2. Stability of the plasma assay has been reported. One of the challenges of working in tropical countries is ensuring that the assay is fit for purpose with respect to sample handling in the field. Often there are delays to plasma separation and the samples may have other delays before being placed into freezer conditions. Our group believes that in tropical and resource poor settings, the assay should account for stability at room temp (not just benchtop stability), tropical ambient temperatures and at 4 degrees.

Response: I don’t think anyone should be doing PK studies if they cannot do the separation soon after the blood was drawn. Our samples were immediately taken to labs with AC in Kilifi & Mombasa and Nairobi. We can confirm that is room temperature remained <28 degrees in all sites, therefore additional stability experiments are not needed to cover the sampling time and temperatures. Comment will be added to the manuscript.

3. As this assay has been reported as a simultaneous assay, it would be good to see the chromatograms overlaid with the 3 analytes and IS.

Response: Respectfully, we thought the chromatograms are best as represented for ease of understanding and interpretation by the reader. Overlaid chromatograms would be necessary with UV detection where overlapping peaks could be troublesome in integration, fortunately that is not the case with MS. No change to manuscript.

4. Could the authors please clarify throughout the manuscript whether MRM or SRM has been used?

Response: MRM was employed as depicted from mass transitions in Table 1 and Figures 2 and 4. SRM was investigated during the early stages of compound optimization. The manuscript will be edited to clarify.

5. My major critique is that of the example child used for the validation paper; the authors describe how the assay was used successfully for 81 children; but present a time concentration curve from a single child. The T0 samples in this child had >40000ng/mL. The explanation given is that the child must have had prior exposure to metronidazole. Whilst this is likely to be true, I don’t believe this is appropriate for a methods paper that describes the utility of the assay. I would recommend reporting another 'sample child' with a clear undetectable result at T0.
Response: Respectfully, we acknowledge this as a recommendation rather than a critique since the request is to have additional 'sample child' with a clear undetectable result at T0 for comparability purposes and ease of interpretation. Full results of this study are being published separately.

**Competing Interests:** No competing interests were disclosed.