Research Article

LC–MS/MS method for simultaneous quantification of ten antibiotics in human plasma for routine therapeutic drug monitoring

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ABSTRACT

Background: Optimizing antimicrobial therapy to attain drug exposure that limits the emergence of resistance, effectively treats the infection, and reduces the risk of side effects is of a particular importance in critically ill patients, in whom normal functions are augmented or/and are infected with pathogens less sensitive to treatment. Achievement of these goals can be enhanced by therapeutic drug monitoring (TDM) for many antibiotics. A liquid chromatography tandem mass spectrometry (LC–MS/MS) method is presented here for simultaneous quantification of ten antimicrobials: cefazolin (CZO), cefepime (CEP), cefotaxime (CTA), ceftazidime (CTZ), ciprofloxacin (CIP), flucloxacillin (FLU), linezolid (LIN), meropenem (MER), piperacillin (PIP) and tazobactam (TAZ) in human plasma.

Methods: Plasma samples were precipitated with acetonitrile and injected into the LC–MS/MS. Chromatographic separation was on a Waters Acquity BEH C18 column. Compounds were eluted with water and acetonitrile containing 0.1 % formic acid, using a gradient (0.5–65 % B), in 3.8 min. The flow rate was 0.4 mL/min, and the run time was 5.8 min.

Results: The calibration curves were linear across the tested concentration ranges (0.5–250, CZO, CEP, CTA, CTZ and FLU; 0.2–100, MER and TAZ; 0.1–50, CIP and LIN and 1–500 mg/L, PIP). The intra and inter-day imprecision was < 11 %. Accuracy ranged from 95 to 114 %. CTZ and MER showed ionization suppression while CIP showed ionization enhancement, which was normalized with the use of the internal standard.

Conclusion: An LC–MS/MS method for simultaneous quantification of ten antimicrobials in human plasma was developed for routine TDM.

Introduction

Since the introduction of the first synthetic antibiotic, salvarsan, [1] in clinical use in 1910, and the subsequent discovery of penicillin in 1928, [2] antimicrobials have been used for the treatment of various infections, drastically improving patient survival. Their discovery has changed modern medicine, enabling the performance of many procedures, previously unthinkable, such as organ transplantation, cardiac surgery, as well as treatment of immunosuppressive cancer and autoimmune disorders. However, widespread overuse of antimicrobials,

Abbreviations: CZO, cefazolin; CEP, cefepime; CTA, cefotaxime; CTZ, ceftazidime; CIP, ciprofloxacin; CV, coefficient of variation; ESI, electrospray ionization; FLU, flucloxacillin; HPLC, high performance liquid chromatography; ICU, intensive care unit; LIN, linezolid; LLOQ, lower limit of quantification; LC–MS/MS, liquid chromatography tandem mass spectrometry; MER, meropenem; MIC, minimum inhibitory concentration; MRM, multiple reaction monitoring; NOR, norfloxacin; PK, pharmacokinetics; PIP, piperacillin; QC, quality control; R, resistant organism; Rt, retention time; RT, room temperature; r², coefficient of determination; S, susceptible, wild type organism; SIL-IS, stable isotope labelled internal standard; TAZ, tazobactam; TDM, therapeutic drug monitoring; ULOQ, upper limit of quantitation.

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https://doi.org/10.1016/j.jmsacl.2022.11.001
Received 30 March 2022; Received in revised form 13 October 2022; Accepted 15 November 2022
Available online 18 November 2022

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due to easy access and treatment effectiveness, has resulted in the emergence of multi-drug-resistant bacteria. The continuing rise of antimicrobial resistance in conjunction with the lack of new antimicrobials is an increasing and significant global concern [3].

Resistance to antibiotics can be acquired quickly since bacteria replicate rapidly. Long-term use of antibiotic at inadequate concentrations (sub-optimal exposure) is likely a contributing factor to resistance [4]. Another concern associated with inappropriate antimicrobial use, although perceived to be uncommon, is an increase in neurological toxicity [5] and seizures [6] associated with high β-lactam concentrations. Optimizing antibiotic dosing can minimize resistance emergence bacteria and reduce the side effects. This can be achieved by therapeutic drug monitoring (TDM), tailoring the drug dose to an individual. It is particularly important to individualize antimicrobial therapy in critically ill patients, in whom normal functions are augmented and standard treatment is less effective. These patients can be infected with pathogens that are less sensitive to treatment, resulting in poor clinical outcomes. Large variations in antimicrobial concentrations in plasma have been reported for patients in intensive care units (ICU) receiving guideline-directed, standard treatment. For example, sub-optimal exposure has been reported for fluoxacinill (FLU) [7], and increased neurological toxicity [5] and seizures [6] have been correlated with high meropenem (MER) and cefepime (CEP) concentrations, respectively, while both toxic and sub-therapeutic concentrations have been documented with linezolid (LIN) [8]. The literature indicates a relationship between serum concentrations of β-lactams and clinical outcomes in the critically ill [9], making TDM a potentially useful tool for dose optimization of this class of drugs.

Measuring drug concentration for various antimicrobials in clinical laboratories is generally performed by high performance liquid chromatography (HPLC), immunoassay or liquid chromatography tandem mass spectrometry (LC–MS/MS). HPLC instruments, although reliable, are relatively insensitive. The extraction procedure for HPLC analysis is generally lengthy, as the separation of the analytes is essential for quantitation, and the overall run time per sample is long, with results generally not available on the same day of sample collection. Immunoassays, on the other hand, have the advantage of a short turn-around time; however, these assays tend to lack specificity and commercial kits are not always available, particularly for newer classes of antibiotics. LC–MS/MS technology, which is becoming more readily available in clinical labs, can overcome these drawbacks with results being available much sooner than possible with HPLC. Simultaneous methods for measurement of many analytes, for example 10–15 antibiotics, allows for a broad range of compounds to be measured daily. This in turn reduces not only the turn-around time, but also pre-analytical and operator time, as well as the reagent and storage costs. Additionally, sicker patients tend to be prescribed more than one antibiotic at a time, but changes in their prescribed regimens are also frequent. It is, therefore, useful to have a multiplexing method that allows for the results to be available before the next dosing occasion. Given that quantification of antimicrobials, using either HPLC or LC–MS/MS technology, is generally limited to in-house developed methods, the selection of the analytes to be included in the method, generally, is tailored to the clinical need, the lab, or the research group. Consequently, the current published LC–MS/MS methods are quite diverse in the classes of antimicrobials chosen for measurement. Simultaneous quantification of five or more antimicrobials [10–29] has been reported in the literature, with only few methods enabling quantification of ten or more analytes [14,16,18,19,25,25]. Among these, protein precipitation is the predominant sample extraction procedure with one method reporting the use of a solid phase extraction [15]. Analytical run time for the methods ranged from 4 [18] to 12 [13] minutes. Some of the current methods have narrow analytical ranges with either the lower limit of quantitation (LLOQ) being too high, above the minimum inhibitory concentration (MIC), or the upper limit of quantitation (ULOQ) being too low [16,18,19], thus requiring re-analysis of the samples after dilution, and thereby delaying the availability of results.

Among the current published methods that enable quantification of ≥ 10 antimicrobials, one used analyte analogue as the internal standard [13] rather than stable isoote labelled internal standard (SIL-IS), as recommended in LC–MS/MS [30] analysis, with the remaining using a SIL-IS for some but not all. Although most of the published methods state that the methods are fit for TDM purposes, none have reported on whether the methods have been implemented in the clinic for routine antimicrobial monitoring.

To date, no method has been published for measuring the set of antimicrobials presented here. The assay developed includes quantification of different classes of antibiotics (i.e., penicillins, carbapenems, cephalosporins, oxazolidinones and quinolones), commonly prescribed in ICUs. The aim of this work was to improve the current LC–MS/MS method used for routine monitoring of antimicrobials in our lab, particularly focusing on resolving the signal suppression (i.e., changes in signal intensity affected by the co-eluting interferences) and cross-signal contribution (i.e., interferences between the analyte and SIL-IS from the naturally occurring isotopes). The initial LC–MS/MS method included simultaneous quantitation of five antimicrobials using norfloxacin (NOR) as the internal standard. The LC–MS/MS method presented herein, simultaneously quantifies ten antimicrobials: cefazolin (CZO), CEP, cefotaxime (CTA), cefazidime (CTZ), ciprofloxacin (CIP), FLU, LIN, MER, piperacillin (PIP) and tazobactam (TAZ) in human plasma and utilizes SIL-IS for each analyte.

**Materials and methods**

**Chemicals and reagents**

Cefazolin sodium (98 % purity), cefazolin 13C2-N (CZO-IS; 98.6 % isotopic purity), cefepime dihydrochloride monohydrate (98 % purity), cefepime 13H2 sulfate (CEP-IS; 98.6 % isotopic purity), cefotaxime 12H2 (CTA-IS; 99.8 % isotopic purity), cefazidime pentahydrate (97 % purity), cefazidime 12H2 (CTZ-IS; 99.4 % isotopic purity), ciprofloxacin (98 % purity), ciprofloxacin 13H2 (CIP-IS; 98.3 % isotopic purity), fluoxacillin sodium (97 % purity), fluoxacillin 13C4 (FLU-IS; 99.5 % isotopic purity), linezolid (98 % purity), linezolid 12H2 (LIN-IS; 98.3 % isotopic purity), meropenem trihydrate (97 % purity), meropenem 13H2 (MER-IS; 99.6 % isotopic purity), piperacillin 14H2 (PIP-IS; 98.9 % isotopic purity), tazobactam sodium (96 % purity), tazobactam sodium 13C2-N (TAZ-IS; 97.9 % isotopic purity) were purchased from Toronto Research Chemicals (PM Separations, Australia). Cefotaxime sodium (96.4 % purity) and formic acid, LC–MS grade, were obtained from Sigma-Aldrich (St Louis, MO, USA). LC–MS grade acetonitrile, methanol and water were from Thermo Fisher Scientific (Scoresby, VIC, Australia). Water was further filtered using a Simplicity UV purification system from Millipore Australia (North Ryde, NSW, Australia). Disodium hydrogen phosphate was obtained from Chem-Supply (Gillman, SA, Australia).Expired, drug free, human plasma was obtained from blood bank (SydPath, St Vincent’s Hospital, Sydney).

**UHPLC–MS/MS equipment and conditions**

The LC–MS/MS system included a Shimadzu Ultra-Fast Liquid Chromatography system coupled to a Shimadzu-8050 triple quadrupole mass spectrometer (Shimadzu Oceania, Rydalmere, NSW, Australia). The LC system consisted of a solvent delivery system (Nexera X2 LC-30AD), an autosampler (Nexera X2 SIL-30AC) maintained at 8 °C, a vacuum degasser (DGU-20A5R), a column oven (Prominance CTO-20A) set to 40 °C and a system controller (CBM-20A). Compounds were chromatographically separated on a Waters Acquity BEH C18 (2.1 x 50 mm, 1.7 μm) column with gradient elution using water and acetonitrile, each containing 0.1 % formic acid, as mobile phases A and B, respectively. The initial gradient starting at 0.5 % B was held for 0.2 min and then linearly increased to 65 % B over 3.8 min. The gradient
was ramped up to 100 % B where it was held for one minute before returned to initial conditions and equilibration for a further minute. The flow rate was 0.4 mL/min, and the total run time was 5.8 min.

A Shimadzu 8050 tandem mass spectrometer equipped with an electrospray ionization (ESI) source interface, and operated in positive ion mode, was used for multiple reaction monitoring (MRM) analysis. Two product ions were selected for each analyte and one for each of the SIL-IS. Optimization parameters are shown in Table 1 and the mass spectra data are illustrated in Fig. 1. Mass resolution for first and third quadrupoles were set to unit with a full-width-half-mass of 0.51–0.80 Daltons. Data acquisition and processing used Shimadzu LabSolution software version 5.96. Optimized ESI parameters were as follows: 1) nitrogen was used as the nebulizing gas, set to 2.8 L/min, and also used as the heating and drying gas at flow rates of 9 L/min, 2) capillary voltage was set to 4 kV, and 3) interface, heating block and de-solvation line temperatures were set to 250 °C, 400 °C and 150 °C, respectively.

Preparation of calibrators and quality control (QC) samples

Two independently weighed out stock powders of each analyte at concentrations of 2 g/L (CEP, CTA, CTZ, CIP, LIN and TAZ), 4 g/L (MER, PIP) and 10 g/L (CZO, FLU) were used for preparation of the working calibrators and QC. A minimum of 10 mg of each was accurately weighed-out and dissolved in either methanol (CEP, CTA, CTZ, LIN and PIP), acidified methanol (0.1 M hydrochloric acid in methanol (0.05/99.95 v/v)) (CIP), water (CZO, MER, and TAZ) or buffer (0.1 M di-sodium hydrogen phosphate, pH = 7.4, (adjusted with concentrated ortho-phosphoric acid) containing 0.3 % sodium chloride) (FLU). Stock solutions were sonicated for 10 min, except TAZ (20 min), and stored in glass vials at −80 °C. Working calibrators at concentrations of 0.1, 0.4, 1.0, 10, 20 and 50 mg/L (CIP, LIN); 0.2, 0.8, 2.0, 20, 40 and 100 mg/L (MER, TAZ); 0.5, 2.0, 5.0, 50, 100 and 250 mg/L (CZO, CEP, CTA, CTZ and FLU) and 1.0, 4.0, 10, 100, 200 and 500 mg/L (PIP) were prepared in drug free plasma and stored as aliquots (50 μL) in microfuge plastic tubes at −80 °C. Working QC solutions at concentrations of 0.5, 4.0 and 40 mg/L (CIP, LIN); 1.0, 8.0 and 80 mg/L (MER, TAZ); 2.5, 20 and 200 mg/L (CZO, CEP, CTA, CTZ and FLU) and 5.0, 40 and 400 mg/L (PIP) were also prepared in drug free plasma and stored as aliquots (50 μL) in microfuge plastic tubes at −80 °C. Working QC calibrators/QC were prepared first and used to prepare the subsequent calibrators/QC by dilution, with drug-free plasma. To ensure the integrity of the plasma, the final volume of water and methanol content in drug free plasma and stored as aliquots (50 μL) in microfuge plastic tubes at −80 °C until analysis. The highest calibrator and QC were prepared first and used to prepare the working calibrators/QC, by dilution, with drug-free plasma. To ensure the integrity of the plasma, the final volume of water and methanol content in drug free plasma and stored as aliquots (50 μL) in microfuge plastic tubes at −80 °C until analysis.

Table 1

| Analyte | R (min) | MRM (m/z) | CE (eV) | Dwell time (ms) | SIL-IS | R (min) | MRM (m/z) | CE (eV) | Dwell time (ms) |
|---------|---------|-----------|---------|-----------------|--------|---------|-----------|---------|-----------------|
| CEP     | 1.71    | 241.0 → 227.0 | 11      | 25              | CEP-IS | 1.70    | 242.4 → 227.0 | 11      | 5               |
| TAZ     | 1.77    | 300.9 → 207.1 | 16      | 30              | TAZ-IS | 1.77    | 304.0 → 168.0 | 14      | 15              |
| CTZ     | 1.82    | 273.9 → 80.1  | 25      | 20              | CTZ-IS | 1.81    | 276.5 → 85.1  | 22      | 5               |
| MER     | 1.83    | 384.0 → 68.0  | 41      | 15              | MER-IS | 1.82    | 390.0 → 147.1 | 19      | 5               |
| CIP     | 2.04    | 331.9 → 288.1 | 19      | 30              | CIP-IS | 2.03    | 340.1 → 296.1 | 20      | 20              |
| CTA     | 2.11    | 456.1 → 396.2 | 12      | 15              | CTA-IS | 2.12    | 461.0 → 401.2 | 12      | 10              |
| CZO     | 2.25    | 454.9 → 323.0 | 13      | 15              | CZO-IS | 2.25    | 460.1 → 326.1 | 11      | 5               |
| LIN     | 2.50    | 338.0 → 235.2 | 20      | 15              | LIN-IS | 2.50    | 341.1 → 297.3 | 20      | 5               |
| PIP     | 2.87    | 518.1 → 143.0 | 8       | 15              | PIP-IS | 2.87    | 523.2 → 148.1 | 24      | 5               |
| FLU     | 3.52    | 454.0 → 160.0 | 17      | 15              | FLU-IS | 3.52    | 460.0 → 160.0 | 17      | 10              |

R, retention time; MRM, multiple reaction monitoring; CE, collision energy; SIL-IS, stable isotope labelled internal standard; CEP, cefepime; TAZ, tazobactam; CTZ, ceftazidime; MER, meropenem; CIP, ciprofloxacin; CTA, cefotaxime; CZO, cefazolin; LIN, linezolid; PIP, piperacillin; FLU, flucloxacillin.
Fig. 1. Mass spectrometry fragmentation pattern for cefazolin (CZO), cefepime (CEP), ceftaxime (CTA), ceftazidime (CTZ), ciprofloxacin (CIP), flucloxacillin (FLU), linezolid (LIN), meropenem (MER), piperacillin (PIP) and tazobactam (TAZ).
Specificity and selectivity

Cross-signal contribution. Cross-signal contribution between the analytes and SIL-IS from naturally occurring isotopes and isotopically impure SIL-IS was assessed. Individual analytes, prepared in pure solution at the ULOQ, and the SIL-IS at concentrations of 1 mg/L, were individually injected into the LC-MS/MS, while response of all analytes were monitored. Any peak observed at the retention time (R_t) and MRM, except for the analyte being injected, was considered a cross-signal contribution. Acceptance criteria were set to the response being ≤ 20 % of the analyte LLOQ and ≤ 5 % of the SIL-IS response.

Suppression and enhancement of ionization. To evaluate the effect on ionization efficiency of the endogenous and/or exogenous compounds present in the matrix, calibrators were prepared identically in pure solution (water) and in plasma. Extracted samples were analyzed and their slopes compared. Slope ratios (plasma /water) between 0.85 and 1.15 indicate absence of suppression or enhancement of ionization by the matrix. Additionally, patient samples (n = 35) requested for routine antimicrobial monitoring were spiked with a pure solution mixture containing all the analytes. The concentrations of the solution used for spiking were 2 mg/L (CIP, LIN), 5 mg/L (MER, TAZ), 25 mg/L (CEP, CTA, CTZ, CZO, and FLU) and 50 mg/L (PIP). Each sample was analyzed in duplicate; neat and spiked. The recovered concentrations were calculated by subtracting the concentrations of the neat samples from the spiked. The acceptance criteria were set to 85–115 % recovery from the spiked concentration with 67 % of the samples falling within the predefined criteria.

Carryover

Carryover was assessed by injecting the highest calibrator followed by two double blank plasma extracts. Acceptance criteria of the carry-over, according to the guidelines, states that the response in the blank sample should not exceed 20 % of the response of analyte LLOQ.

Stability of the stock solutions and samples

Stability of the analytes in plasma and whole blood was assessed at three concentrations in triplicate at room temperature (RT) and at 4 °C for up to 24 and 48 h, respectively. Short- and long-term stability in plasma was also tested at −20 and −80 °C for two weeks and 8 months, respectively. The freeze–thaw stability over three cycles was evaluated (−80 °C to RT). Stability of the extracted samples in the autosampler at 8 °C was assessed for up to 24 h. The stability of the stock solutions was assessed at −80 °C for up to 11 months for all analytes and up to 20 and 24 months for linezolid and ceftazidime, respectively. Stability of the analyte at the defined condition was accepted, if the mean concentration was ± 15 % from the freshly prepared samples at the same concentration.

Ethical considerations

Samples described in the study were collected for measurement of antimicrobial concentrations for clinical purposes and retained by an accredited clinical pathology service and the identities of the donors were not necessary for the research described herein. The use of these samples for research purposes is consistent with the Australian National Statement on Research Ethics (section 3.2.6, https://www.nhmrc.gov.au/about-us/publications/national-statement-ethical-conduct-human-research-2007-updated-2018#toc_725).

Results

Analytes were eluted at R_t ranging from 1.7 to 3.5 min under the chromatographic conditions defined above. R_t for the analytes and their corresponding SIL-IS were as follow: 1.71, 1.77, 1.82, 1.83, 2.04, 2.11, 2.25, 2.50, 2.87 and 3.52 for CEP, TAZ, CTZ, MER, CIP, CTA, CZO, LIN, PIP and FLU, respectively (Table 1). A representative chromatogram for a LLOQ sample is shown in Fig. 2.

Linearity

The assays were linear across the tested concentration ranges (0.5–250, CZO, CEP, CTA, CTZ and FLU; 0.2–100, MER and TAZ; 0.1–50, CIP and LIN and 1–500 mg/L, PIP). An example of a calibration curve for each analyte is shown in Supplementary Figure S1. Mean r^2 of the calibration curves were 0.995 or greater. Summary of the slope, intercept and r^2 for each calibration curve is presented in the in supplementary Table S1.

Accuracy and precision

Precision and accuracy data are listed in Table 2. The maximum intra- and inter-day imprecision was < 7 % for all analytes at all concentrations, except CIP, which was 11.4 % at the low QC. Accuracy within batch and between the batches ranged from 95 to 110 %, except the inter-batch-accuracy of CTA and CIP at the low QC was 112 and 114 %, respectively. All accuracies were within the predefined acceptance criteria of ± 15 %. The LOD for CZO, CEP, CTA, CTZ, CIP, FLU, MER, LIZ, PIP and TAZ was 18, 35, 30, 104, 62, 8, 7, 18, 9 and 43 µg/L, respectively.

Specificity and selectivity

Cross-signal contribution

Cross-signal contribution from naturally occurring isotopes of the analytes at ULOQ for each analyte to the SIL-IS for the selected MRM transitions was < 5 % for all SIL-IS, except for CTA-IS and CZO-IS (5.4 and 8.6 %, respectively). The contribution effect at the second highest calibrator (concentration of 100 mg/L) was 2.3 and 4.5 % for CTA-IS and CZO-IS, respectively. No analyte cross-signal contribution was observed from the SIL-IS at concentrations of 1 mg/L.

Suppression and enhancement of ionization

CTZ and MER showed ionization suppression while CIP showed ionization enhancement. Slope area ratios (plasma/water) were 0.21, 0.69 and 1.23 for CTZ, MER and CIP, respectively. The ionization effect was normalized with the use of the SIL-IS. Slope ratios (absolute and normalized for the IS) of the remaining analytes were in the range of 0.87–1.08. The recovery data for the spiked patient samples shown in Fig. 3 were within the predefined acceptance criteria (85–115 %) for all the analytes, except for CEP (635; recovery ranged from 81 to 124 %).

Carryover

The response observed in the first double blank plasma extract injected after the highest calibrator was ≤ 20 % of the area of the LLOQ sample for five out of ten antibiotics and ≤ 5 % for all the SIL-IS. CEP, CTZ, CTA, CIP and FLU showed carryover of 21, 48, 27, 220, and 25 % of the area of the LLOQ, respectively, corresponding to concentrations of 0.11, 0.24, 0.14, 0.22 and 0.13 mg/L, respectively. This was reduced to < 8 % in the second double blank injection for all analytes, except CTZ (16 %) and CIP (100 %).

Stability

Stability data for each antibiotic is summarized in Table 3 and supplementary Table S2. CEP, TAZ, MER and PIP were each more stable in whole blood than in plasma. CZO, CTA and LIN were stable in both matrices, whereas CIP and FLU were more stable in plasma. Long term storage in plasma at −80 °C was acceptable for all analytes for at least eight months. Extracted samples stored in the autosampler at 8 °C were stored in plasma at −80 °C was acceptable for all analytes for at least eight months. Extracted samples stored in the autosampler at 8 °C were...
stabile for up to 24 h. The three freeze–thaw cycles did not affect the stability of the analytes. Individual stocks were stable for up to eleven months at −80 °C, except CTZ (3 months). LIN stock solution was stable for up 20 months.

Discussion

Method development: Singly-charged protonated ions were selected for all analytes, except for CEP and CTZ, including their SIL-IS, where doubly charged ions were used due to a higher response. The PIP peak intensity was the highest of all so, to prevent detector signal saturation, non-optimal collision energy was used and the response was reduced by 30 %.

The initial in-house LC–MS/MS method, developed and used for routine monitoring of antimicrobials, included simultaneous measurement of five analytes: CIP, MER, FLU, PIP and TAZ with NOR as the IS. When additional compounds were incorporated into the method and the analytical ranges for the current analytes were expanded, ionization suppression was observed for MER, CTZ and CEP, with the latter two also displaying a non-linear mode of regression. Meanwhile, FLU and PIP became non-linear after the ULOQ was increased from 100 to 250 mg/L and 300 to 500 mg/L, respectively, implying that NOR was not a suitable internal standard. Further, when SIL-IS were incorporated into the assay, the issue of cross-signal contribution between the analyte and the SIL-IS, arising from the presence of naturally occurring isotopes, needed to be addressed. Cross-signal contribution was observed for CTZ, CZO and FLU resulting in non-linear calibration curves. To mitigate the effect, the approach of utilizing a less abundant SIL-IS isotope with a mass that has the least contribution from the analyte isotopes was implemented [31]. For CTZ-IS, CZO-IS and FLU-IS, the isotopes m/z 461, 460 and 460, respectively, were used instead of the most abundant isotopes (m/z = 459, 459 and 458, respectively). However, this only mitigated the effect for FLU, while CTZ and CZO required a baseline separation, in addition to utilizing a less abundant SIL-IS isotope, to prevent the cross-signal contribution between the two. Changing the gradient from 5 to 95 % B in 3.5 min to 0.5–65 % B in 3.8 min resolved the issue. Despite the mitigating strategies undertaken to counteract the effect, cross-signal contribution for CTZ and CZO to the SIL-IS remained greater (5.4 and 8.6 %, respectively) than the pre-defined criteria (≤5 % of the IS response). While this could have been further reduced by increasing the amount of the SIL-IS used in the extraction, at the expense of increasing the cost, calibration curves remained linear, implying that the amount of the SIL-IS used was sufficient to counteract the effect. This was further reflected by the accuracy and precision data obtained for the two analytes (Table 2). A chromatogram for FLU, CTZ and CZO and their corresponding SIL-IS is presented in supplementary Figure S2, demonstrating the effect of the cross-signal contribution from the analyte isotopes to the SIL-IS.

Various mobile phases (0.1 % formic acid in water, ammonium formate and ammonium acetate buffers, pH 2.5–7) combined with the gradient (60–100 % B) were trialed for their suitability. Using ammonium formate buffer (25 mM, pH = 3) and acetonitrile as mobile phases A and B, respectively, allowed for quantitation of an additional compound, ceftriaxone. However, the overall response was greatly reduced for all compounds, while the LLOQ, for some, became too high. The gradient profile 0–90 and 0–100 % B resulted in sharper peaks with less tailing. However, compound resolution was poorer, while hydrophilic compounds had a lower column retention. With the aim of utilizing the
simplest extraction protocol, protein precipitation was the method of choice, because of simplicity and speed. Also, antimicrobial concentrations are relatively high (mg/L), allowing for direct sample dilution prior to injection into the LC-MS/MS, without the need for sample concentration.

Given that carryover for CEP, CTZ, CTA, CIP and FLU was > 20% of the LLOQ response, in attempt to mitigate the effect, various combinations of wash solutions and rinse protocols were investigated. Using a 70% isopropanol:water mix containing 0.1% formic acid to wash the needle (internally and externally), and the port and the pump, reduced

| Cefazolin (CZO) | Mean concentration (mg/L) ± SD | CV (%) | Accuracy % |
|----------------|---------------------------------|--------|------------|
| 0.5            | 0.49 ± 0.009                    | 1.8    | 99         |
| 3.0            | 3.01 ± 0.09                     | 3.1    | 106        |
| 20             | 21.7 ± 0.93                     | 4.3    | 101        |
| 200            | 203.6 ± 8.1                     | 4.0    | 102        |

| Cefepine (CEP) | Mean concentration (mg/L) ± SD | CV (%) | Accuracy % |
|----------------|---------------------------------|--------|------------|
| 0.5            | 0.51 ± 0.004                    | 0.8    | 101        |
| 2.5            | 2.59 ± 0.06                     | 2.5    | 104        |
| 20             | 20.3 ± 0.1                      | 0.5    | 102        |
| 200            | 200.2 ± 3.3                     | 1.6    | 100        |

| Cefotaxime (CTA) | Mean concentration (mg/L) ± SD | CV (%) | Accuracy % |
|------------------|---------------------------------|--------|------------|
| 0.5              | 0.50 ± 0.005                    | 1.0    | 100        |
| 2.5              | 2.49 ± 0.04                     | 1.7    | 99         |
| 20               | 19.7 ± 0.9                      | 4.5    | 99         |
| 200              | 202.3 ± 13.0                    | 6.4    | 101        |

| Cefazidime (CTZ) | Mean concentration (mg/L) ± SD | CV (%) | Accuracy % |
|------------------|---------------------------------|--------|------------|
| 0.5              | 0.51 ± 0.07                     | 1.5    | 101        |
| 2.5              | 2.49 ± 0.04                     | 1.7    | 99         |
| 20               | 19.7 ± 0.9                      | 4.5    | 99         |
| 200              | 202.3 ± 13.0                    | 6.4    | 101        |

| Ciprofloxacin (CIP) | Mean concentration (mg/L) ± SD | CV (%) | Accuracy % |
|---------------------|---------------------------------|--------|------------|
| 0.1                 | 0.10 ± 0.003                    | 2.6    | 99         |
| 0.5                 | 0.51 ± 0.05                     | 10.6   | 102        |
| 4                   | 4.20 ± 0.20                     | 4.8    | 105        |
| 40                  | 38.4 ± 2.2                      | 5.7    | 96         |

| Fluoroxacillin (FLU) | Mean concentration (mg/L) ± SD | CV (%) | Accuracy % |
|----------------------|---------------------------------|--------|------------|
| 0.5                  | 0.50 ± 0.005                    | 1.0    | 100        |
| 2.5                  | 2.66 ± 0.05                     | 1.7    | 107        |
| 20                   | 20.3 ± 0.11                     | 0.5    | 102        |
| 200                  | 203.6 ± 7.0                     | 3.4    | 102        |

| Linezolid (LIN)      | Mean concentration (mg/L) ± SD | CV (%) | Accuracy % |
|----------------------|---------------------------------|--------|------------|
| 0.06                 | 0.06 ± 0.01                     | 0.8    | 101        |
| 0.5                  | 0.52 ± 0.01                     | 1.0    | 103        |
| 4                    | 3.86 ± 0.07                     | 1.8    | 97         |
| 40                   | 38.4 ± 1.2                      | 3.2    | 96         |

| Meropenem (MER)      | Mean concentration (mg/L) ± SD | CV (%) | Accuracy % |
|----------------------|---------------------------------|--------|------------|
| 0.2                  | 0.20 ± 0.001                    | 0.5    | 101        |
| 1                    | 1.06 ± 0.03                     | 2.5    | 106        |
| 8                    | 8.13 ± 0.36                     | 4.4    | 102        |
| 80                   | 86.3 ± 3.7                      | 4.3    | 108        |

| Piperacillin (PIP)   | Mean concentration (mg/L) ± SD | CV (%) | Accuracy % |
|----------------------|---------------------------------|--------|------------|
| 0.85                 | 0.86 ± 0.005                    | 0.6    | 101        |
| 5                    | 4.89 ± 0.09                     | 1.8    | 97.9       |
| 40                   | 38.2 ± 0.5                      | 1.4    | 95.6       |
| 400                  | 402.4 ± 11.5                    | 2.9    | 100.6      |

| Tazobactam (TAZ)     | Mean concentration (mg/L) ± SD | CV (%) | Accuracy % |
|----------------------|---------------------------------|--------|------------|
| 0.2                  | 0.20 ± 0.001                    | 0.5    | 100        |
| 1                    | 1.09 ± 0.07                     | 6.6    | 109        |
| 8                    | 8.19 ± 0.30                     | 3.7    | 102        |
| 80                   | 78.5 ± 4.3                      | 5.4    | 98         |

CV, coefficient of variation; SD, standard deviation.
the carryover to < 20 %, except for CIP (64 %). This, however, resulted in an additional 2.7 min per injection extending the total run time to almost 9 min. Alternatively, re-injection of low concentration samples that happen to be analyzed after a high concentration sample, may be performed, to account for potential carryover issues. The actual increases in the LLOQ concentrations for the analytes are marginal and unlikely to be clinically significant, except for CIP for which the carryover was equivalent to 0.22 mg/L, increasing the LLOQ to 0.3 mg/L. It is
noteworthy the CIP ULOQ was excessively high (5 times higher than required) and given that CIP is measured as a peak rather than trough, the actual concentrations in patient samples are unlikely to exceed 10 mg/L, and, therefore, the percent of carryover will be significantly lower. The carryover for CIP was estimated to be approximately 0.4% of the analyte concentration, resulting in a concentration increase equivalent to 0.02, 0.032, 0.04, 0.1 and 0.2 mg/L at 5, 8, 10, 25 and 50 mg/L CIP concentrations, respectively. To maintain the potential carryover to <20%, patient samples with CIP concentrations of ≤0.1 (LLOQ), ≤0.25 and ≤0.5 mg/L analyzed after samples containing CIP ≥5, ≥10 and ≥20 mg/L, respectively, would need to be re-injected.

Stability of β-lactam antibiotics has been documented in the literature [32]. Generally, they are unstable in plasma and whole blood matrices at RT; hence proper sample handling is required to ensure result credibility. The stability data outlined in Table 3 and supplementary Table S2 shows that PIP, CEP and, particularly, CTZ are highly unstable at RT in a plasma matrix. However, if stored as whole blood, whether refrigerated or not, the analytes appear to be more stable. While plasma matrix is used for analysis, samples are collected as whole blood to allow to 0.02, 0.032, 0.04, 0.1 and 0.2 mg/L analyzed after samples containing CIP ≥5, ≥10 and ≥20 mg/L, respectively, would need to be re-injected.

Another advantage of this method, compared to the existing ones, is the use of plasma samples from patients treated with antimicrobials, for patient spike analysis. This is the first report in the literature for antimicrobial quantification. Generally, for method validation, 6–10 different plasma samples obtained from healthy volunteers are used to perform matrix effect and specificity and selectivity experiments. However, samples obtained from patients admitted to the ICU are unlikely to be similar in composition to the plasma of healthy volunteers. Using samples from this population group for validation purposes is more likely to identify potential interferences arising from drug co-administration.

Method applicability: The developed method has been implemented in the clinical lab for daily antimicrobial monitoring. Over the three-month period, (August –October 2016), 412 patient episodes were requested for monitoring of at least one of the antimicrobials. PIP with TAZ were the most frequently requested (24%), followed by FLU (23%), MER (20%), LIN (13%), CIP (7%), CZO (6%), CTA (5%), CEP and CTZ (1%). Antimicrobial concentrations for each patient episode are depicted in Fig. 4. Based on the target ranges set for each antibiotic, a substantial percentage of patients did not achieve the desired targets. This, however, may be misleading as the MIC, the infection type and the pathogen were not correlated with the individual concentration, while samples may not have been collected at trough concentrations either. Also, the very low antibiotic concentrations observed in some cases may not be a true representation of a sub-optimal dosing, but rather confirmatory checks to validate antibiotic flushig after changing to a different drug class. The MIC in Fig. 4 were set based on EUCAST PK/PD clinical breakpoints [35], which are not species related. The MIC breakpoints for the susceptible (S) and resistant (R) organisms were 4 and 8 mg/L (CEP, CTZ); 1 and 2 mg/L (CZO, CTA); 0.25 and 0.5 mg/L (CIP); 2 and 4 (FLU); 2 and 8 mg/L (MER); 2 and 2 mg/L (LIN) and 8 and 16 mg/L (PIP), respectively. For the optimal bactericidal activity, an individual MIC should be determined; otherwise, clinical breakpoints defined by EUCAST for various pathogens may be used. Studies [36] have shown that maximal bacterial efficacy for β-lactams is achieved when 40–70% of the time, within the dosing interval, the concentrations of the free fraction are 4–5 times above the MIC of the target pathogen. However, for clinically ill patients, the recommendation by the French Society of Pharmacology and Therapeutics and the French Society of Anesthesia and Intensive Care Medicine is plasma concentrations of the free fraction of 4–8 times the MIC of the causative pathogen for 100% of the dosing interval [37]. For antimicrobials exhibiting concentration dependent bactericidal effect, such as CIP, the target concentration is defined as a ratio of Cmax (maximum drug plasma concentration)/MIC > 10.

Limitations and future work: Measuring the unbound fraction of highly protein-bound drugs, such as FLU, may be of great importance. Small changes in the plasma protein concentration, often observed in the severely ill, will lead to a significant increase or decrease of the unbound...
Fig. 4. Antimicrobials concentrations of patients requested for therapeutic drug monitoring. MIC breakpoints for each agent were set based on the EUCAST PK/PD clinical breakpoints and are non-species related. The breakpoints for the susceptible (S) and resistant (R) organisms were 4 and 8 mg/L (CZO, CTA); 1 and 2 mg/L (CTZ, CEP); 0.25 and 0.5 mg/L (CIP); 2 and 4 (FLU); 2 and 8 mg/L (MER); 2 and 2 mg/L (LIN) and 8 and 16 mg/L (PIP), respectively. The target plasma concentration ranges set as 4 and 8 times the MIC breakpoint of the R organism were as per recommendation of the French Society of Pharmacology and Therapeutics (SFPT) and the French Society of Anesthesia and Intensive Care Medicine (SFAR) [37]. The targets of the total drug concentrations for CZO, CEP, CTA, CTZ, PIP and FLU, were based on the free drug fraction (%) and the MIC of the pathogen. For CZO, for example, the target free plasma concentration of 4 to 8 times the MIC of R (2 mg/L) is 8 and 16 mg/L, respectively. As the free fraction of CZO is approximately 20% of the total dose, the target total plasma concentration is 40–80 mg/L. The estimated free fraction (%) for CZO, CEP, CTA, CTZ, PIP and FLU were approximately 15–20, 80, 60–80, 90, 80 and 5–10 %, respectively. For CEP, the target plasma concentration (5–35 mg/L) were taken from the SEPT and SFAR guidelines where calculations were based on the MIC of 1 mg/L (Enterobacteriaceae) and not 8 mg/L (P. aeruginosa), since this would have resulted in a concentration above the defined toxic threshold. For CTA and MER, the target ranges were also from the SEPT and SFAR guidelines and were based on the MIC of 4 mg/L for S. aureus and 2 mg/L for P. aeruginosa, respectively. For CEP, the target plasma concentration (5–35 mg/L) were taken from the SEPT guidelines where calculations were based on the MIC of 1 mg/L (Enterobacteriaceae) and not 8 mg/L (P. aeruginosa), since this would have resulted in a concentration above the defined toxic threshold. For CTA and MER, the target ranges were also from the SEPT and SFAR guidelines and were based on the MIC of 4 mg/L for S. aureus and 2 mg/L for P. aeruginosa, respectively.
fraction. Similarly, measuring antimicrobial concentrations in other fluids and tissues, may better represent the drug concentration at the site of infection.

Conclusion

Overall simplicity of the method, in terms of extraction and a short run time, allowed for the method to be used daily for antimicrobial monitoring, providing results before the next dosing interval. Additional compounds could be incorporated into the method in the future, given the sample preparation and chromatography have already proved to be adequate for various classes of antimicrobials. Moreover, the wide assay analytical range allows for determination of free-drug concentrations, useful for some highly protein bound antimicrobials, and for pharmacokinetic peak levels to be measured simultaneously in the same method.

Funding

The authors received no financial support for the research, author-ship and/or publication of this article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The provision of a Shimadzu 8050 LC-MS/MS by Shimadzu Australia to undertake this work is greatly appreciated. The work in the manuscript was presented at the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) 2018 Congress, September 16–19, Brisbane, Australia in a poster format.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2022.11.001.

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