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Simultaneous determination of seven β-lactam antibiotics in human plasma for therapeutic drug monitoring and pharmacokinetic studies

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Abstract

There is strong evidence in literature supporting the benefit of monitoring plasma concentrations of β-lactam antibiotics in the critically ill to ensure appropriateness of dosing. The objective of this work was to develop a method for the simultaneous determination of total concentrations piperacillin, benzylpenicillin, flucloxacillin, meropenem, ertapenem, cephazolin and ceftazidime in human plasma. Sample preparation involved protein precipitation with acetonitrile containing 0.1% formic acid and subsequent dilution of supernatant with 0.1% formic acid in water. Chromatographic separation was achieved on a reversed phase column (C18, 2.6μm, 2.1* 50 mm) via gradient elution using water and acetonitrile, each containing 0.1% formic acid, as mobile phase. Tandem mass spectrometry (MSMS) analysis was performed, after electrospray ionization in the positive mode, with multiple reaction monitoring (MRM). The method is accurate with the inter-day and intra-day accuracies of quality control samples (QCs) ranging from 95%-107% and 95%-108%, respectively. It is also precise with intra-day and inter-day coefficient of variations ranging from 4 to 12 % and 5-14% respectively. The lower limit of quantification was 0.1μg/mL for each antibiotic except flucloxacillin (0.25μg/mL). Recovery was greater than 96% for all analytes except for ertapenem (78%). Coefficients of variation for the matrix effect were less than 10% over the six batches of plasma. Analytes were stable over three freeze-thaw cycles, and for reasonable hours on the bench top as well as post-preparation. This novel liquid chromatography tandem mass spectrometry method proved accurate, precise and applicable for therapeutic drug monitoring and pharmacokinetic studies of the selected β-lactam antibiotics.

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Keywords: β-lactam; antibiotics; LC-MS/MS; therapeutic drug monitoring; pharmacokinetics

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Highlights

- We present a method for simultaneous determination of seven beta-lactams in plasma
- The selected antibiotics are those commonly used in critically ill patients
- The method is accurate, precise and meets validation requirements by guidelines
- It proved applicable for therapeutic drug monitoring and pharmacokinetic studies

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1. Introduction

Severe infection and associated sepsis remain the most common causes of morbidity and mortality in critically ill patients worldwide [1]. Despite tremendous investments in new treatments, arresting poor clinical outcomes has been a global challenge [2]. For β-lactam antibiotics, which are the mainstay of therapy in severely ill patents, emerging evidence suggest that poor antibiotic exposure is a potential cause of poor clinical outcomes [3-5]. Therefore, optimising antibiotic dosing may be a key intervention to improve clinical outcomes. This is further supported by studies that correlate optimised antibiotic exposure with improved patient outcome [6-12].

However, optimization of antibiotic dosing is not an easy exercise. The pharmacokinetics of β-lactam antibiotics is difficult to predict in the critically ill patient population due to the unpredictable effects of pathophysiologic processes particularly during sever sepsis and malignancy [13,14]. The drug's apparent volume of distribution and clearance may be elevated leading to sub-therapeutic plasma concentrations [4,15-24]. On the other hand clearance may be unchanged [17] or decreased [4,18] and in the presence of organ dysfunction such acute kidney injury, diminished clearance may lead to massive accumulation and toxicity [25-27]. In patients with renal dysfunction, optimization antibiotic dosing is further complicated by the use of renal replacement therapy which provides significant and variable extracorporeal clearance for several β-lactams [28-34]. These pharmacokinetic challenges would mean that empiric fixed dose strategy is unlikely to ensure sufficient antibiotic exposure and as well empiric dose optimization is unrealistic due the little data available to guide clinicians.

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Confirmation of dosing appropriateness through monitoring of plasma concentrations is therefore essential for β-lactam antibiotics in the critically ill patient population. Routine therapeutic drug monitoring (TDM), enables a rational, patient specific dose adjustment thereby maximizing treatment efficacy while minimizing drug toxicity [35]. Even though it is has not yet been a standard practice across the world, studies have demonstrated the clinical utility of this methodology in critically ill patients [12,36-40]. The level of evidence supporting TDM program for these antibiotics is strong with increasing interest in further demonstrating its impact on clinical outcomes [41].

Associated with this increasing interest in β-lactam TDM, there has been an increasing effort to develop a rapid and efficient assay method to enable quick decisions on dose adjustment. A convenient assay method for routine use should have a short turnover time while using less sophisticated, cheap and easy to use instrumentation. For aminoglycoside antibiotics, immunochemical assays provide such an advantage. However, no such techniques are available for β-lactam antibiotics and previous attempts to develop such methods have been difficult [42,43]. Most of the studies that have described TDM for these antibiotics utilized high performance liquid chromatography (HPLC) assay methods [41]. For a comprehensive review of advances in the determination of β-lactam antibiotics by liquid chromatography using different detector systems, the reader is referred to the paper by Lara *et al*[44]. In general, a number of HPLC methods with ultraviolet detection are described for β-lactams including simultaneous determination of several β-lactams [45-49].

Liquid chromatography tandem mass spectrometry (LCMSMS) methods have also been described [50-53].The use of mass spectroscopy detectors allows a more definitive identification and quantitative determination of compounds, even with low resolution chromatography; a future not possible with other methods of HPLC detection [54]. LCMSMS

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also offers short time of analysis and is generally regarded as superior to HPLC methods because risk of false positive results is minimal. However, there are few LCMSMS methods for β-lactams that can analyse more than three analytes simultaneously in human plasma with many of the methods focusing on a single or few (two or three) analyte combinations. Further to this, the existing simultaneous methods do not encompass all relevant antibiotics as far as TDM in critical care is concerned. For example the method by Ohmori *et al* [50] can analyse eight β-lactams simultaneously. However this method does not include all commonly used drugs such as benzylpenicillin, ertapenem, ceftazidime and flucloxacillin which are of interest in β-lactam TDM and included in the present method. A method by Ahsman *et al* [51] described analysis of six β-lactams. Two of these, cefotaxime and deacetylecefotaxime, are not commonly used in intensive care units and thus are less relevant as far as application to TDM is concerned. The method by Carlier *et al* [52]can analyse 7 beta-lactams but does not include bezylpenicillin and ertapenem which were of interest in our TDM program.

The aim of this paper is to describe a newly developed, precise, accurate and reproducible LCMSMS method for simultaneous analysis of seven β-lactam antibiotics; three penicillins (benzylpenicillin, piperacillin, flucloxacillin), two carbapenems (meropenem, ertapenem) and two cephalosporins (cephazolin, ceftazidime). The combination of antibiotics selected in this study is different from previously described simultaneous LCMSMS methods and is aimed at those common antibiotics used in severely ill patients such as those in the intensive care unit (ICU) or with malignancy so as to enable the method applicable for TDM or pharmacokinetic studies.

2. Materials And Methods

2.1 Chemicals and reagents

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The drug materials were obtained as formulations for injections: piperacillin sodium (Tazocin, Pfizer Australia Pty Ltd); benzylpenicillin sodium (BenPen, CSL Limited); flucloxacillin sodium (Flucil, Aspen Pharmacare Australia); meropenem trihydrate (DBL Meropenem for injection, Hospira Pty Ltd Australia); ertapenem sodium (Invanz, Merck Sharp and Dohme); cephazolin sodium (Kefzol, Aspen Pharmacare Australia); ceftazidime pentahydrate (DBL ceftazidime for injections, Hospira Pty Ltd Australia); fluconazole, sodium chloride solution for injection (Aspen Pharmacare, Australia); acetonitrile HPLC grade (Optigen Scientific, Australia), methanol especially purified for HPLC (Ajax Finechem, Australia); formic acid for HPLC (Sigma-Aldrich, USA). Blank plasma was obtained from patients undergoing plasmapheresis at the Queen Elizabeth Hospital, Adelaide, South Australia. All water used was purified by reverse osmosis (Cascada™ RO-Water Purification System, Pall Life Sciences).

2.2 Instrumentation

The LCMSMS system used for analysis included a Shimadzu LC system combined with a triple quadrupole mass spectrometer API 3000 (Applied Biosystems–ABSciex, Foster City, CA, USA) equipped with an electrospray ionization source. The LC system was composed of two pumps for gradient flow (Model: LC-20AD) with a binary flow pumping mode at a rate of 0.3 mL/min and was equipped with a temperature controlled auto-sampler (Model: SIL-20AC), column oven (Model: CTO-20A), system controller (Model: CBM-20A) and on-line solvent degasser (Model: DGU 20A5). A reversed phase kinetex® C18 column with trimethylsilyl endcaping (Phenomenex, Part No. 00B-4462-AN, 2.6µm, 2.1* 50 mm) was used for chromatographic separation in combination with a pre-fitted in-line filter for column protection (Phenomenex, AFO-8497 KrudKatcher). Analyst software version 1.5 run on

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Windows XP Professional was used for instrument control, data acquisition and data processing.

2.3 Chromatographic and mass spectroscopic conditions

20 μL of sample was injected into the LC system. Auto-sampler and column temperature were maintained at 4 °C and 35 °C, respectively. Chromatographic separation was performed by gradient elution using mobile phase A (water containing 0.1 % formic acid) and mobile phase B (acetonitrile containing 0.1% formic acid) with a total flow rate of 0.3 mL/min (Figure 1). Mobile phase B was kept at 0% from 0.0 min to 0.1 min, then increased linearly to 85% from 0.1 to 2.0 min. It was held at 85% from 2.0 to 5.5 min and then decreased linearly back to 0% from 5.5 to 6.0 min and finally held at 0% from 6.0 to 7.0 min. Flow was diverted to waste for the first 2.7 and after 4.9 minutes. The total run time was 7.0 minutes.

MSMS detector analysis was performed in positive mode with electrospray ionization. Direct infusion of 100 μg/mL of each analyte in mobile phase A was made to optimise parameters to detect the most intense signals of transitions from parent to product ions; which were subsequently monitored using multiple reactions monitoring (MRM) mode. Compound specific parameters for each transition are given in Table 1. Other working parameters maintained during analysis include curtain gas (CUR) 12 psi, collision activated dissociation (CAD) 10 psi, focusing potential (FP) 360 V, ion spray voltage (IS) 4200 V and the temperature of the turbo gas was set at 350 °C. Nitrogen was used as the nebulizer, auxiliary, collision and curtain gases. Dwell time was set at 100 ms for each mass transition.

2.4 Preparation of solutions, standards, and quality control samples

Vials of each antibiotic containing powder for injection were initially reconstituted with 5 mL of water and then were subsequently diluted to 10 mL with water to give seven primary stock

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solutions ranging from 50 mg/mL to 400 mg/mL. The primary stocks were then diluted with water to give 5 mg/mL standard solutions for each antibiotic and were stored in 1 mL aliquots at -80 °C under which condition β-lactams are known to be stable at least for few months [45,46,50,51]. These standard solutions were used to prepare fresh standards and QCs in plasma for every analytical run.

To prepare standards and QCs in plasma firstly, an intermediate 500 µg/mL solution containing all the seven β-lactam antibiotics was prepared by combining appropriate volumes of each 5 mg/mL standard solution (e.g. 250µl of each 5mg/mL solutions were combined and diluted with 750µl of water to give 500µg/ml combined solution). Secondly, this combined solution was diluted with water to give drug concentrations of 400, 250, 200, 100, 75, 50, 25, 10, 7.5, 5, 2.5 and 1 µg/mL. Finally, each of these combined β-lactam solutions were then diluted with drug-free blank plasma in a 1 : 9 ratio to give working standards and quality control samples. Plasma working standards (300µl each) were prepared at drug concentrations of 50, 25, 10, 5, 2.5, 1, 0.5, 0.25 (flucloxacillin only) and 0.1 µg/mL. Plasma quality control samples were prepared independently at a concentration of 40, 7.5, 0.75 and 0.25 µg/mL (for meropenem, ertapenem, cefazolin and ceftazidime) and at a concentration of 20, 7.5, 0.75, and 0.25 µg/mL (for benzylpenicillin and piperacillin). Flucloxacillin concentrations for quality control samples were 20, 7.5 and 0.75 µg/mL.

The internal standard (fluconazole) stock solution was prepared by diluting the 2 mg/mL solution for injections (available in vials) with water to give a 5 µg/mL solution. Aliquots of 1 mL containing 5 µg/mL solution were stored at -80 °C until used.

2.5 Sample Preparation

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Protein precipitation by acetonitrile containing 0.1% formic acid was used for sample preparation after testing various protein precipitants at different plasma to precipitant ratios as recommended by Polson et al. for human plasma [55].

During sample preparation, 15 μL of 5 μg/mL internal standard (Fluconazole) was added to each 300 μl standard calibrators and quality control samples put in 1.5 mL microcentrifuge tubes, and the samples were subsequently vortex mixed for 20 seconds. Plasma proteins were then precipitated by adding 600 μL of acetonitrile containing 0.1% formic acid and vortex mixed for about 30 seconds. The precipitant was separated by centrifugation at 12000 × g for six minutes at 4 °C (Sigma, 1-15K; Germany). 400 μL of the upper clear supernatant was then transferred into another 1.5 mL tube and diluted in 1 : 1 ratio with 400 μL of water containing 0.1% formic acid . After vortex mixing for 20 seconds, 300 μL was transferred into a flat bottom autosampler insert. The autosampler vials were then sealed with caps (SUN-Sri, Thermo Fisher Scientific Inc.) fitted with white PTFE septa (8-425 closure, Grace Davison Discovery Science).

2.6 Validation of the method.

Validation of the method was conducted in accordance with guidelines of the National Association of Testing Authorities, Australia (NATA) [56] and the Food and Drug Administration (FDA)[57].

2.6.1 Calibration curve and limit of quantification

Calibration curve was examined by running five sets of standard calibrators on five different days. For this validation each standard calibrator was analysed in five replicates. Calibration curves were generated by plotting the ratio of nominal concentration of the standard to that of

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internal standard versus the ratio of the standard peak area to the internal standard peak area. Different regression modes in combination with different weighting approaches were tested to select the best fit to the data. The acceptance criterion for calibration curve was that at least 75 % of standards should have acceptable accuracy and precision [58]. Accuracy was considered acceptable when deviations of the mean values of back calculated concentration from the nominal concentrations were within 15% for all standards except at the lower limit of quantification (LLOQ) where 20% was considered acceptable. Similarly, acceptable precisions were those with the coefficient of variation (CV) less than 15% except at the LLOQ, where it was less than 20%. The limit of quantification was validated by analysis of five replicates prepared independently of the standards with less than 20% relative standard deviation as well as less than 20% deviation from the nominal concentration as acceptance criteria [57].

2.6.2 Accuracy and precision.

The intra-day and inter-day accuracy and precision were assessed by using five concentration of QCs including the LLOQ run in five replicates. For flucloxacillin four concentrations of QCs were used. The intra-day data was collected by running two sets of calibrators and QCs within a day and the inter-day data was collected by running five sets of QCs with calibrators on five different days.

2.6.3 Recovery, matrix effect and specificity

Recovery (extraction efficiency) was determined at four QC levels run in five replicates each by comparing the peak areas of each analyte in spiked plasma samples with those of samples to which the same amounts of analyte was added after protein precipitation. Matrix effect was examined in two ways. Firstly, QC samples at four different concentrations (three for

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flucloxacillin) were prepared in plasma from six different donors and quantified using freshly spiked calibration curve. The accuracy and precision of calculated concentrations in reference to the nominal concentrations were then determined for each QC[59]. Secondly, the matrix effect was quantified according to the procedures demonstrated by Matuszewski et al.[60]. Analytes were spiked in post extraction matrix from six different plasma donors at three concentrations in duplicate; and in water as a 'neat solution'. Matrix effect was then calculated as the ratio of peak area obtained from post-extraction matrix samples to that of water samples expressed as percentage. Internal standard normalized matrix effect was also calculated from the six different lots of plasma by dividing the matrix effect of each analyte by the matrix effect of the internal standard. Specificity of the method was ascertained using six different sources of plasma and comparing chromatograms of blank plasma with the corresponding spiked plasma samples.

2.6.4 Stability

Bench top stability of samples in plasma was tested by preparing QCs at four different concentrations (three for flucloxacillin) in five replicates and analysing them after four hours of stay on the bench at room temperature prior to extraction. Accuracy and precision were determined using back calculated concentrations from an original standard curve plotted with freshly prepared and extracted standards.

Post preparative stability (auto-sampler stability) was examined using four QCs prepared and extracted in five replicates and stored in sealed auto-sampler vials in a cold room at 4 °C (auto-sampler temperature) for twelve hours before analysis. Samples were spiked after twelve hrs and the accuracy and precision were determined using back calculated concentrations from an original standard curve plotted with freshly prepared and extracted standards.

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Freeze and thaw stability was also assessed using QCs at four concentrations prepared in five aliquots. Analyte stability was determined after three freeze-thaw cycles: Aliquots of the QCs were initially frozen at -80 °C for twenty four hours and then allowed to thaw unassisted at room temperature. When completely thawed, samples were refrozen again at the same temperature for about sixteen hrs. Samples were then thawed similarly and refrozen for another cycle for about sixteen hrs after which they were thawed, extracted and analysed together with freshly prepared and extracted standards. Accuracy and precision were determined using back calculated concentrations.

2.6.5 Incurred samples analysis

Incurred samples analysis was performed in accordance with the European Medicine Agency (EMEA) guideline [58]. Twenty five previously analysed patient samples were reanalysed on different days in separate runs. Samples were randomly selected for re-analysis and were around C_{max} and in the elimination phase. Percent deviation of concentrations obtained for the initial analysis and the concentrations obtained by reanalysis from their respective means were determined.

2.7 Application: Analysis of patient samples. The current method is being used for ongoing study of TDM of β-lactam antibiotics in critically ill patients receiving antibiotic therapy in intensive care unit at The Queen Elizabeth Hospital, Adelaide, Australia. Ethics approval for the study was granted by the Human Research Ethics Committees (HREC) of the Queen Elizabeth Hospital (TQEH) (Ref. No. HREC/12/TQEHLMH/14) and the University of South Australia (UniSA) (Application ID: 0000031080). For assessment of pharmacodynamic target attainment, corrections for protein binding were made based on analyses of protein binding performed in critically ill patients (data submitted) or published protein binding data. Accordingly for piperacillin unbound concentrations were determined from the

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corresponding total concentrations by the equation y= 0.885x -4.813, where y is unbound (free) plasma concentration and x is the total plasma concentration. This method has also been applied to a pharmacokinetic study of piperacillin in patients with haematological malignancies who succumb to febrile neutropenia. Ethics approval for this study was granted by the HREC of TQEH (Ref. No. HREC/12/TQEHLMH/157) and UniSA (Application ID: 0000031077).

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3. **Results**

3.1 Sample preparation, chromatography and mass spectrometry

Upon screening for protein precipitation efficiency and effect on the stability of β-lactams, 10% (w/v) trichloroacetic acid (TCA) in 0.5 : 1 ratio, acetonitrile in 2 : 1 ratio and methanol in 2 : 1 ratio showed comparable pellet size (data not shown). This is in accordance with previous reports of comparable percent protein precipitation, 91%, 92% and 90%, respectively [55]. However, 10% TCA appears to affect stability, particularly for meropenem. Both acetonitrile and methanol proved to be favourable as a protein precipitant. However acetonitrile was chosen because of the better peak shapes that resulted with the mobile phase combination used in this study.

The mobile phase combination and gradient for the HPLC system were optimised by alternating the organic mobile phase B between acetonitrile and methanol (each containing 0.1 % formic acid) and comparing the different precipitation methods and the symmetry of chromatogram peak shapes. With methanol as mobile phase B, while using supernatant as injectate after acetonitrile protein precipitation, peak shapes were inconsistent with multiple peaks appearing for some analytes. Acetonitrile was finally chosen as mobile phase B due to very good peak shapes consistent across all analytes as well as a significant reduction in the instrument operational pressure in comparison with methanol.

A representative chromatogram with simultaneous analysis showing typical peak shapes for each analyte is depicted in Figure 2. All of the seven analytes including the internal standard eluted after 3.4 minutes within a one minute interval. Further chromatographic separation was not required as the mass to charge ratios were distinct with no cross-talk for each analyte and

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therefore allow monitoring of unique transitions for MSMS analysis in MRM mode. The internal standard fluconazole is eluted in the middle, 40 seconds apart from flucloxacillin and within 30 seconds of the rest of the analytes. It is therefore, a suitable common internal standard for all of the analytes. For analysis of samples from patients receiving fluconazole treatment, one of the analytes can potentially serve as internal standard for the others. For example we have validated and used (data not shown here) benzyl penicillin as internal standard with this method.

3.2 Calibration curve and limit of quantification

The calibration curves generated by the ratio of peak areas of standards to that of internal standard at eight standard concentration (seven for benzyl penicillin, piperacillin and flucloxacillin) showed that quadratic regression with a weighting scheme of 1 / (x * x) best described the data set generated for all the seven analytes. The calibration range was from 0.1 to 50 μg/mL for meropenem, ertapenem, ceftazidime and cephazolin; 0.1 to 25 μg/mL for benzylpenicillin and piperacillin; and 0.25 to 25 μg/mL for flucloxacillin. Table 2 shows the data for five calibration curves. The mean regression coefficient (r^2) for all standard curves was greater than 0.99 and this high correlation is consistent with very low standard deviation (0.1% to 0.4%). There is variation, however, in the coefficients of quadratic equations from run to run. It is, therefore, necessary to include standards together with QCs in every run of data analysis [57]. Mean coefficients and standard deviations from the five inter-day runs are given in Table 2.

The LLOQ for all analytes was 100 ng/mL except for flucloxacillin for which it was 250 ng/mL. The LLOQs are sufficiently lower than expected trough concentration or real plasma levels [61-66] and therefore the method can accurately determination concentrations in the expected low ranges. Concentrations above the upper limit of quantification can be diluted

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with blank drug free plasma as needed to put the concentration in the range of the calibration curve. Figure 3 depicts typical chromatograms of each analyte at the LLOQ. The signal to noise ratio at the LLOQ for all analytes was greater than 5 : 1.

3.3 Accuracy, precision and recovery

Table 3 shows a summary of accuracies and precisions for intra-day and inter-day runs. For all analytes, mean accuracies of the intra-day QC samples ranged from 95% to 107% while that of LLOQ samples range from 88% to 108%. The CV for all intra-day QC samples ranged from 4 to 12 %. The CVs for LLOQ were also less than 12% for all analytes except flucloxacillin for which it was 15%. The mean inter-day accuracies ranged between 95% to 108 for all QCs including the LLOQs with the CVs ranging from 5% to 14%. In general the method is accurate and precise for each antibiotic as per the requirements of the FDA and NATA guidelines [56,57]. The mean recovery at all QC concentrations was greater than 96% for all analytes except for ertapenem for which 78% recovery was noted (Table 2). Though relatively low, the recovery of ertapenem is sufficiently high and the analyte demonstrated good sensitivity, precision and accuracy. Generally, the extent of recovery is not considered as an issue in bioanalytical method development given adequate sensitivity, precision and accuracy[67].

3.4 Stability

Table 4 summarizes the percent accuracy and coefficient of variation for bench-top, post-preparative and freeze-thaw stability evaluation at the various QC concentrations. In general, the antibiotics were stable under the conditions tested with the percent accuracy and coefficient of variations falling in the acceptable ranges, 85%-115% and within 15%, respectively.

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3.5 Matrix effect

The mean accuracies of all QC samples run in plasma from six different donors were all within 10% of the nominal concentration and ranged 95-100% for benzylpenicillin, 93-100% for ceftazidime, 98-101% for cephazolin, 96-105% for ertapenem, 94-100% for flucloxacillin, 93-103% for meropenem and 90-104% for piperacillin. The mean coefficient of variation was also within the acceptable limit (less than 15%) for each analyte at each QC concentration indicating no significant variability in analyte signal due to difference in the source of plasma. Quantitative analysis showed notable matrix effect (Figure 4), particularly for meropenem and ceftazidime which also have the smallest retention times of all the analytes. The highest matrix effect observed for meropenem is comparable to that reported by Ahsman et al.[51] using a procedure that involves acetonitrile protein precipitation. The matrix effect for the internal standard (fluconazole) was negligible. The internal standard normalized matrix effect was comparable to the matrix effect observed for each analyte (Figure 4). CV of normalized matrix effect was less than 15% for each analyte. Despite variable among analytes, the matrix effect was consistent for each analyte. There was no variation among the six different batches of plasma with less than 10% CV of the mean matrix effect for each analyte. This finding supports the above observation of good precision and accuracy of QC samples spiked in six different batches of plasma against a standard curve.

3.6 Selectivity

Representative chromatograms of the blank and double blank plasma samples are given in Figure 5. Analyte signals of spiked blank plasma samples can be seen at their respective

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retention times in Figure 3. The absence of any interfering signal from endogenous compounds in the drug free human plasma at the retention times of the analytes and the internal standard (in Figure 5) indicates the selectivity or specificity of the method.

3.7 Incurred sample analysis

For each of the twenty-five samples subjected to incurred sample reanalysis, the concentration obtained for the initial analysis and the concentration obtained by reanalysis were within 20% of their mean. I.e. 100% of the repeats resulted in concentration within the limits of EMEA guideline.

3.8 Analysis of patient samples

The developed method has proved successful in the analysis of patient samples for TDM. Daily TDM was performed using this method for patients admitted to ICU. Figure 6 A and B illustrate the steady state plasma concentration profile (corrected for protein binding) after daily TDM of piperacillin-tazobactam (4.5 g via intravenous intermittent infusion every eight hours) in a patient for four consecutive days and in four different patients after first TDM respectively. Peak concentrations were determined by sampling immediately after the end of bolus infusion (ranged from 30 min-63 min) and varied 121 to 254 mg/L for these patients. Variable peak concentrations have been observed in critically ill patients (not relevant to efficacy and also depends on variable bolus infusion and sampling time); e.g. 178-316 mg/L at steady state by Roberts et al.[5], 72 to 179 mg/L in the first 24hr of dosing by Taccone et al.[4]. As the time free concentrations remains above MIC is more important for efficacy of β-lactams, intermediate samples taken at specific times in the dosing interval can allow determination of the specific pharmacodynamic target considered, e.g. free concentration greater than MIC for 50% of the dose interval (50% $fT_{>MIC}$), and trough concentration just

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before the next dose enable assessment of the attainment of more robust pharmacodynamic targets (i.e. 100% $fT_{>MIC}$ or 100% $fT_{>4\text{-}5xMIC}$). The trough concentrations that were observed (Figure 6) ranged from 8 to 20 mg/L and are in agreement with previous reports [12]. Similarly, figure 7 shows unbound concentration profile of meropenem in a critically ill patient with renal dysfunction undergoing continuous venovenous haemodiafiltration after TDM for two consecutive days.

The method has also been applied to a pharmacokinetic study of piperacillin. Figure 8 depicts the total plasma concentration profile of piperacillin after a single dose administration of 4.5g Tazocin® via intravenous bolus infusion (over 30 min) in twelve patients with haematological malignancy who succumbed to febrile neutropenia following high dose chemotherapy.

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4. Discussion

We present a method for the simultaneous determination of seven β-lactam antibiotics commonly used in severely ill patients such as those in the ICU or with malignancy. The ability to simultaneously assay several antibiotics in a single analytical run is of great advantage to ensure the practicality of a method for routine use as it enables samples from different patients receiving different antibiotics to be assayed together at the same time. Time is saved by combining multiple analytes with the same standard curve analysis. For the purposes of routine TDM, given the need of short turn-over time, it would be impractical to individually assay samples from different patients receiving different β-lactam antibiotics. Even though TDM of β-lactam antibiotics is not yet part of standard patient care, evidence is increasingly suggesting its benefits in ICU patients and hence the need of for an efficient and rapid assay method is growing [41].

A simple sample preparation procedure is important to minimize sample turnover time. In the present method, acetonitrile containing 0.1% formic acid has been added to plasma in a 2 : 1 ratio for protein precipitation. It allowed a simple and efficient protein extraction process with excellent analyte recoveries. Protein precipitation using acetonitrile alone [68], acetonitrile followed by formic acid [51] or organic extraction [69] has been described earlier for β-lactam antibiotics. Acetonitrile is one of the most efficient protein precipitants particularly at precipitant to plasma volume ratios of 2 : 1 or greater and has excellent reproducibility. It is also the precipitant of choice among organic solvents due to its lowest ionization suppression effect [55]. To ensure good peak shape of all analytes and maintain the retention time, it was found necessary to add water containing 0.1 % formic acid in 1 : 1 ratio to the supernatant after precipitation. The addition of 0.1% formic acid in the water for

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dilution as well as the precipitant improved peak shapes and may enhance ionization efficiency of analytes[70]. In addition, the auto-sampler stability may be better for some β-lactams with 0.1% formic acid, particularly meropenem which appears to be the least stable of all analytes. Ahsman et al.[51] reported that twenty four hour degradation was better for meropenem when it was reconstituted in 0.1% formic acid as compared to pure water (after acetonitrile protein precipitation).

Analyte stability is a significant challenge in the development of assay methodology for β-lactams and may restrict the choices of mobile phase and various efficient protein precipitants that could be used in LCMSMS. For example, there has been a concern on the use of methanol as a mobile phase amid reports of relative instability of β-lactams in methanol as compared to acetonitrile[71] , while Kantiani et al. [72] showed that this may be unlikely as the chromatographic run time is often less than 10 minutes, a time period not sufficiently long for the compounds to undergo any degradation . Despite such concerns, methanol has been used even as the protein precipitant in LCMSMS methods for some β-lactams with prolonged exposure [73,74]. While further study on the effect of methanol on individual β- lactams (possibly differential effect) is warranted, we avoided it both as a mobile phase and as a precipitant. Among other precipitants tested, 10% TCA led to noticeable degradation possibly because, in the presence of an acid in high concentration, degradation of β-lactams is very likely due to instability of the four member β-lactam ring [44,75]. β-lactams are also unstable when zinc sulphate is used as a precipitant despite the latter being very efficient protein precipitant [55]. Zinc has been shown to catalyse degradation of penicillins [76]. Potentially it could also affect analyte ionization and MS interface integrity requiring solvent diversion to waste to reduce involatile salt build up[55].

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β-lactams are known to achieve efficient ionization via the electrospray technique and therefore would undergo easy ionization from the TurboIonSpray® source used in this method. Positive ionization mode was used because, generally most of the β-lactams have higher sensitivity in positive mode [44]. However they are amenable to negative ionization given the fact that all contain a carboxylic acid moiety and for some, negative mode has been reported [77] to produce a high signal to noise ratio. In positive ion mode, both β-lactam class specific fragments of m/z 160 and compound specific fragments [M + H – 159]+ are produced together with other fragments including adducts of sodium and methanol [M+CH3OH + H]+ [44] providing a wide choice for monitoring of unique ion-transitions using tandem-mass spectroscopic technique coupled to the HPLC system. The transitions from Q1 to Q3 were distinct for each anayltes in the current method with similar Q3 used only for benzylpenicillin and flucloxacillin for which there was no 'cross-talk' with clear baseline resolution.

Across the concentration ranges of the established calibration curves, the precision and accuracy of this method meet the requirements guidelines for bioanalytical method development [56,57]. The accuracy is not affected by the storage condition as the three freeze-thaw cycles met stability criteria. Additional confirmatory tests are possible if the need arises or if reanalysis is necessary for any reason. As sample preparation is simple and does not take a long time, concerns of bench-top stability may not be an issue with this method. In our method, twelve hour post-preparative stability was acceptable for all analytes. However, when instability is identified, large sample runs must be validated particularly for those β-lactams with known limited stability, specifically meropenem and ertapenem.

Despite the notable matrix effect observed, particularly for meropenem and ceftazidime, signal intensity was importantly consistent across the six batches of plasma tested and did not

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affect the desired LOQ levels to detect clinically relevant concentrations. Practically, since all the calibration samples are always run in plasma, much of the matrix effect noted (by comparing peak area against neat solution) is duly accounted for as long as there are no variations across different batches. In the current method, there was no such variation with the accuracy and CV of QC samples run in six different batches of plasma falling within the limits of guidelines. This indicates that no further sources of variation arise due to the use of different plasma sources. Therefore, estimates of plasma concentration quantified using this method from clinical samples of different patients can be considered reliable and comparable.

5. Conclusion

The method presented here is accurate, precise and reliable for the determination of piperacillin, benzylpenicillin, flucloxacillin, meropenem, ertapenem, cephazolin and ceftazidime in human plasma. It has been successfully applied to a pharmacokinetic study and therapeutic drug monitoring proving reproducibility. Complementary to the few simultaneous LCMSMS methods available for β-lactams, it offers an advantage by combining the most common antibiotics for which there is an increasing TDM interest in the critically ill. It, thus, provides an important alternative for research and clinical analysis of the unique combination of β-lactams presented.

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Table 1. Compound specific instrument parameters.

	Antibiotic	Molecular Weight (g)	Precursor Ion (Q1)	Product Ion (Q3)	Declustering Potential (DP) V	Entrance Potential (EP) V	Collision Energy (CE) eV	Collision Cell Exit Potential (CXP) V
t1.2	Meropenem	383.5	384.3	114.1	15	4	37	2
t1.3	Ertapenem	475.5	476.9	432.9	8	6	14	33
t1.4	Ceftazidime	546.5	547.2	167.1	10	5	31	4
t1.5	Cephazolin	454.5	455.4	156.1	5	6	21	3
t1.6	Benzylpenicillin	334.4	335.6	160.2	15	7	19	11
t1.7	Piperacillin	517.5	519	143.1	15	7	23	11
t1.8	Flucloxacillin	453.9	454.6	160.1	15	7	23	11
t1.9	Fluconazole (IS[a])	306.3	307.3	127.1	54	10	40	19

[a] Internal standard

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Table 2. Summary of standard curves, retention times and extraction efficiency.

Antibiotics	Quadratic coefficient		Linear coefficient		Constant		r^2		Range (µg/ml)	Retention time (min)	Extraction Efficiency	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD (%)			% Recovery	% Deviation
Meropenem	1.35E-09	5.74E-10	3.07E-04	5.42E-05	-5.05E-03	0.006234	0.992	0.3	50-0.1	3.4	101	6.5
Ertapenem	8.65E-10	6.48E-10	2.34E-04	2.26E-05	-1.13E-03	0.004336	0.992	0.3	50-0.1	3.5	78	6.6
Ceftazidime	7.44E-10	4.23E-10	1.53E-04	1.08E-05	-5.66E-04	0.002473	0.992	0.2	50-0.1	3.4	96	5.6
Cephazolin	-1.47E-09	6.75E-10	3.36E-04	4.04E-05	-1.15E-03	0.003106	0.996	0.1	50-0.1	3.8	107	5.6
Benzylpenicillin	-9.11E-09	2.27E-09	6.73E-04	8.28E-05	-2.93E-03	0.007398	0.993	0.3	25-0.1	4.2	96	4.8
Piperacillin	-2.96E-09	1.37E-09	2.38E-04	3.08E-05	8.36E-04	0.002629	0.992	0.4	25-0.1	4.1	98	4.1
Flucloxacillin	-1.91E-09	3.83E-10	1.29E-04	1.21E-05	1.64E-03	0.001536	0.992	0.4	25-0.25	4.4	98	4.7
Fluconazole (Internal Standard)										3.7	106	5.5

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1 Table 3. Summary of accuracy and precision for intra-day and inter-day runs.

Antibiotic	Concentration	Intra-day		Inter-day	
		Mean Accuracy (%)	Mean CV (%)	Mean Accuracy (%)	Mean CV (%)
Meropenem	100ng/ml (LLOQ)	103	10	105	10
	250ng/ml	102	10	101	7
	750ng/ml	103	6	100	7
	7500ng/ml	103	7	100	6
	40000ng/ml	100	8	96	7
Ertapenem	100ng/ml (LLOQ)	88	11	97	14
	250ng/ml	96	7	101	10
	750ng/ml	98	12	102	7
	7500gn/ml	99	7	103	6
	40000ng/ml	103	4	99	6
Ceftazidime	100ng/ml (LLOQ)	97	12	102	14
	250ng/ml	107	8	105	10
	750ng/ml	105	5	99	6
	7500gn/ml	98	4	99	5
	40000ng/ml	100	6	97	8
Cephazolin	100ng/ml (LLOQ)	92	11	99	7
	250ng/ml	104	8	105	9
	750ng/ml	103	6	102	7
	7500gn/ml	102	4	99	5
	40000ng/ml	99	7	95	7
Benzylpenicillin	100ng/ml (LLOQ)	93	9	105	9
	250ng/ml	106	6	102	8
	750ng/ml	105	5	100	7
	7500gn/ml	96	7	99	5
	20000ng/ml	101	6	103	11
Piperacillin	100ng/ml (LLOQ)	99	11	102	14
	250ng/ml	102	11	108	11
	750ng/ml	103	7	98	14
	7500gn/ml	95	8	98	7
	20000ng/ml	103	8	99	9
Flucloxacillin	250ng/ml (LLOQ)	108	15	100	13
	750ng/ml	104	11	101	11
	7500gn/ml	96	7	96	5
	20000ng/ml	98	7	102	11

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Table 4. Summary of accuracy and precision of QCs for bench-top, freeze-thaw and post-preparative stability studies. (Values represent mean % accuracy / % CV)

	Bench-top stability (4hrs)				**Freeze-Thaw stability (-80 °C)**				**Post-Preparative stability (12hrs)**			
	40000/ 20000[a] (ng/ml)	7500 (ng/ml)	750 (ng/ml)	250 (ng/ml)	40000/ 20000[a] (ng/ml)	7500 (ng/ml)	750 (ng/ml)	250 (ng/ml)	40000/ 20000[a] (ng/ml)	7500 (ng/ml)	750 (ng/ml)	250 (ng/ml)
Meropenem	99/4	94/4	93/7	96/8	90/7	110/2	109/13	114/4	92/5	98/4	90/7	86/10
Ertapenem	98/4	96/6	106/6	111/12	108/4	107/7	112/7	110/8	91/4	97/4	96/3	95/8
Ceftazidime	91/4	89/5	105/8	105/7	102/5	109/11	102/4	98/7	105/4	111/7	105/5	103/5
Cephazolin	102/3	94/2	108/4	105/8	109/6	108/4	107/5	110/5	105/4	103/5	100/5	109/8
Benzylpenicillin	100/11	90/8	100/8	100/6	113/13	105/5	108/5	108/2	99/13	94/7	90/3	97/3
Piperacillin	90/7	90/4	110/7	102/12	105/10	104/7	107/4	113/12	104/9	100/5	95/4	104/11
Flucloxacillin	100/7	91/7	104/12	-	105/8	106/5	113/12	-	110/9	98/9	97/12	-

[a] 20000ng/ml for benzylpenicillin, piperacillin and flucloxacillin

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1 Figure 1. Gradient Curve

2 Figure 2. Representative chromatogram of the seven β-lactams and the internal standard
3 fluconazole run simultaneously.

4 Figure 3. Typical chromatogram of each analyte at their respective lower limit of
5 quantification.

6 Figure 4. Matrix effect of analytes. Values represent averages with the corresponding
7 95% confidence intervals.

8 Figure 5. Representative chromatograms of blank (A) and double blank (B) plasma
9 samples.

10 Figure 6. Steady state free plasma concentration profile of piperacillin after daily TDM in
11 a patient receiving 4.5 g Tazocin® every eight hours (A), and after first TDM in four
12 different patients (B). MIC marked is for *Pseudomonas aeruginosa*.

13 Figure 7. Free plasma concentration profile of meropenem in a patient undergoing
14 continuous venovenous haemodiafiltration (TDM-1 after 1g IV every 12 hr dosing;
15 TDM-2 after 1g IV every 8hr dosing).

16 Figure 8. Total plasma concentration profile of piperacillin after single dose
17 administration of 4.5g Tazocin® IV over 30 minutes in twelve patient with
18 haematological malignancy and febrile neutropenia.

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Highlights

- We present a method for simultaneous determination of seven beta-lactams in plasma
- The selected antibiotics are those commonly used in critically ill patients
- The method is accurate, precise and meets validation requirements by guidelines
- It proved applicable for therapeutic drug monitoring and pharmacokinetic studies

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Figure 1

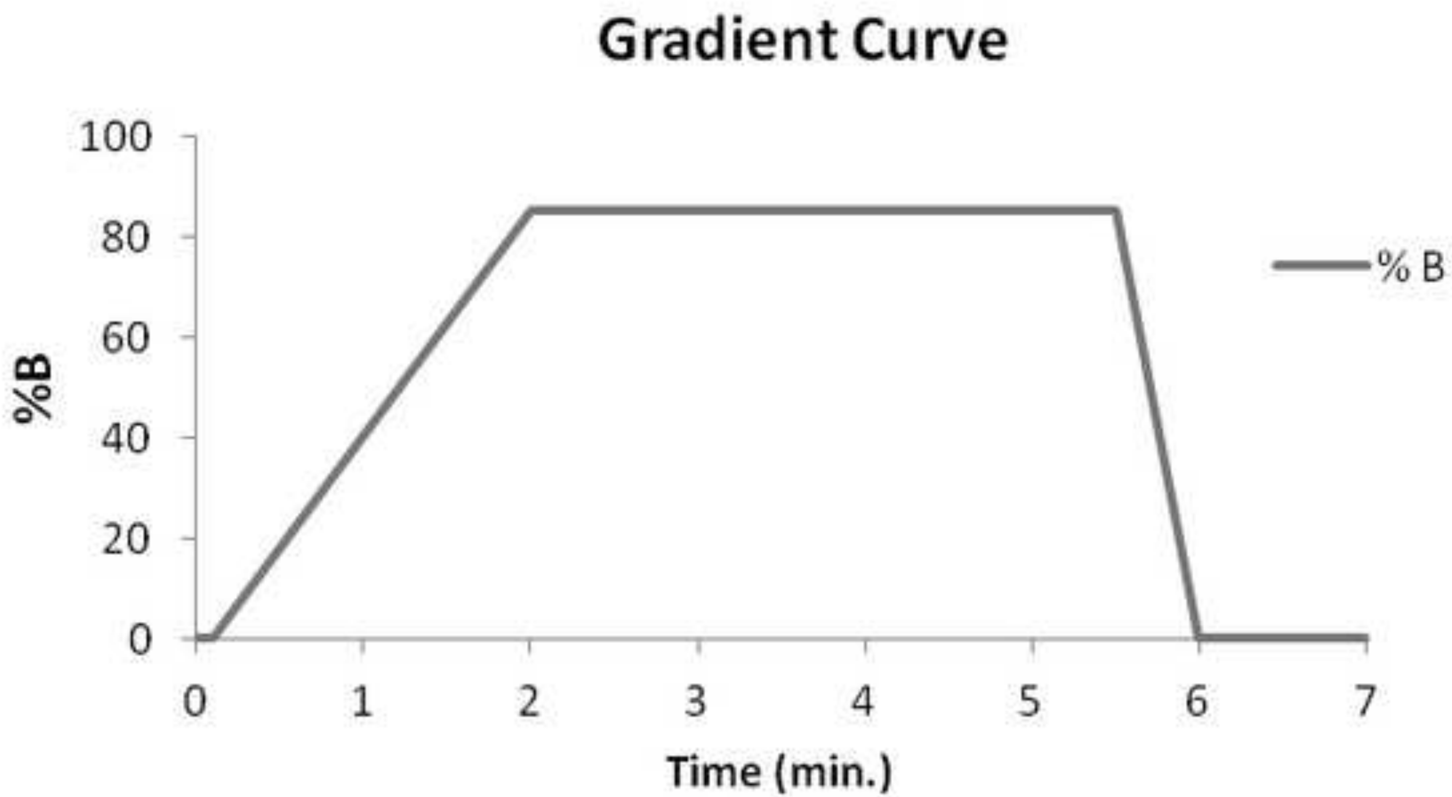

Figure 2

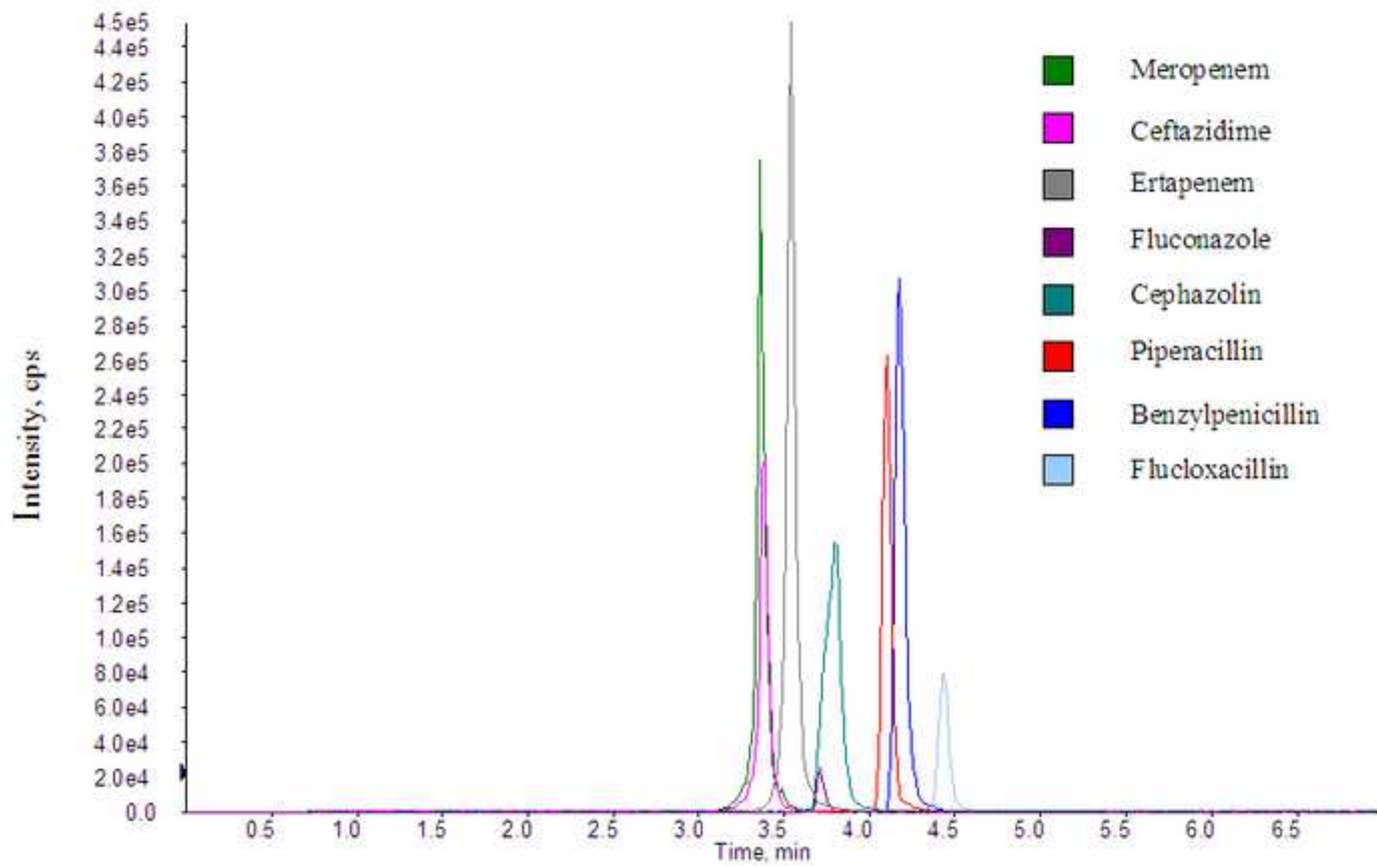

Figure 3

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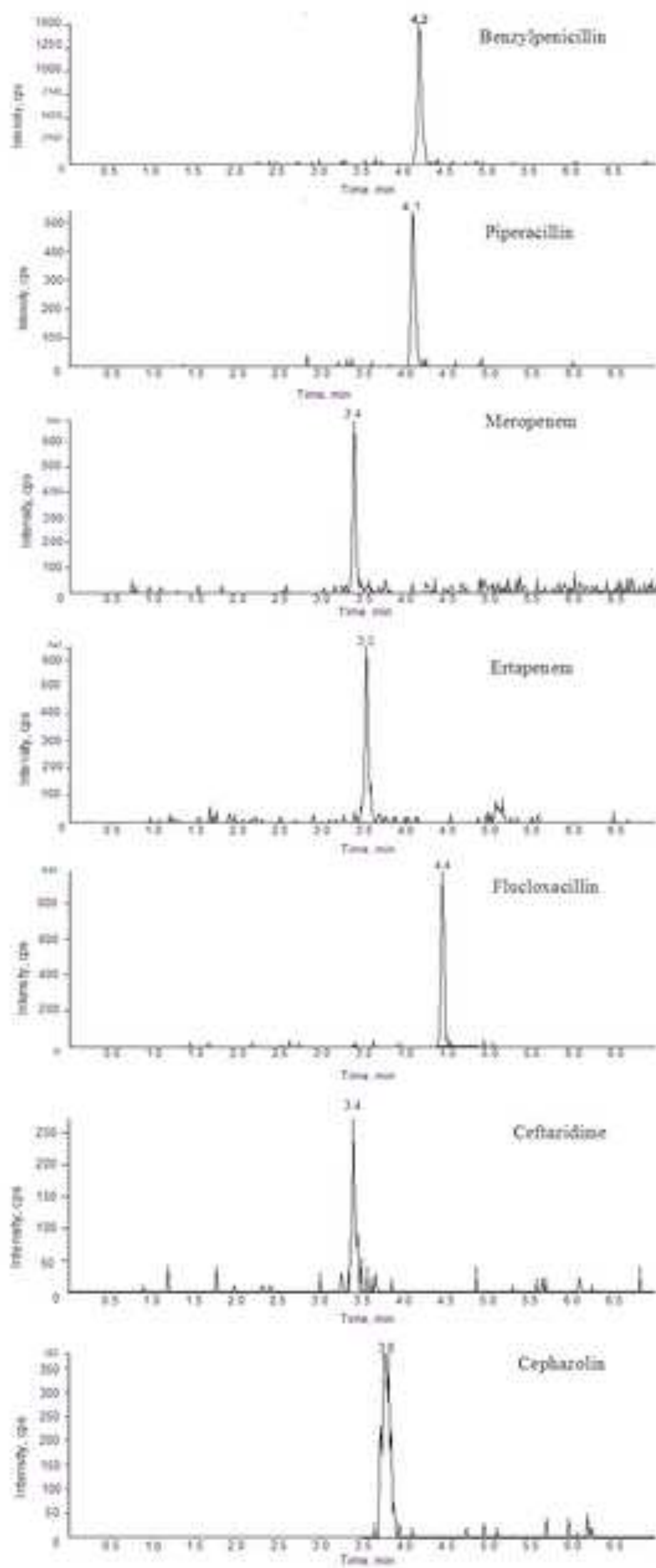
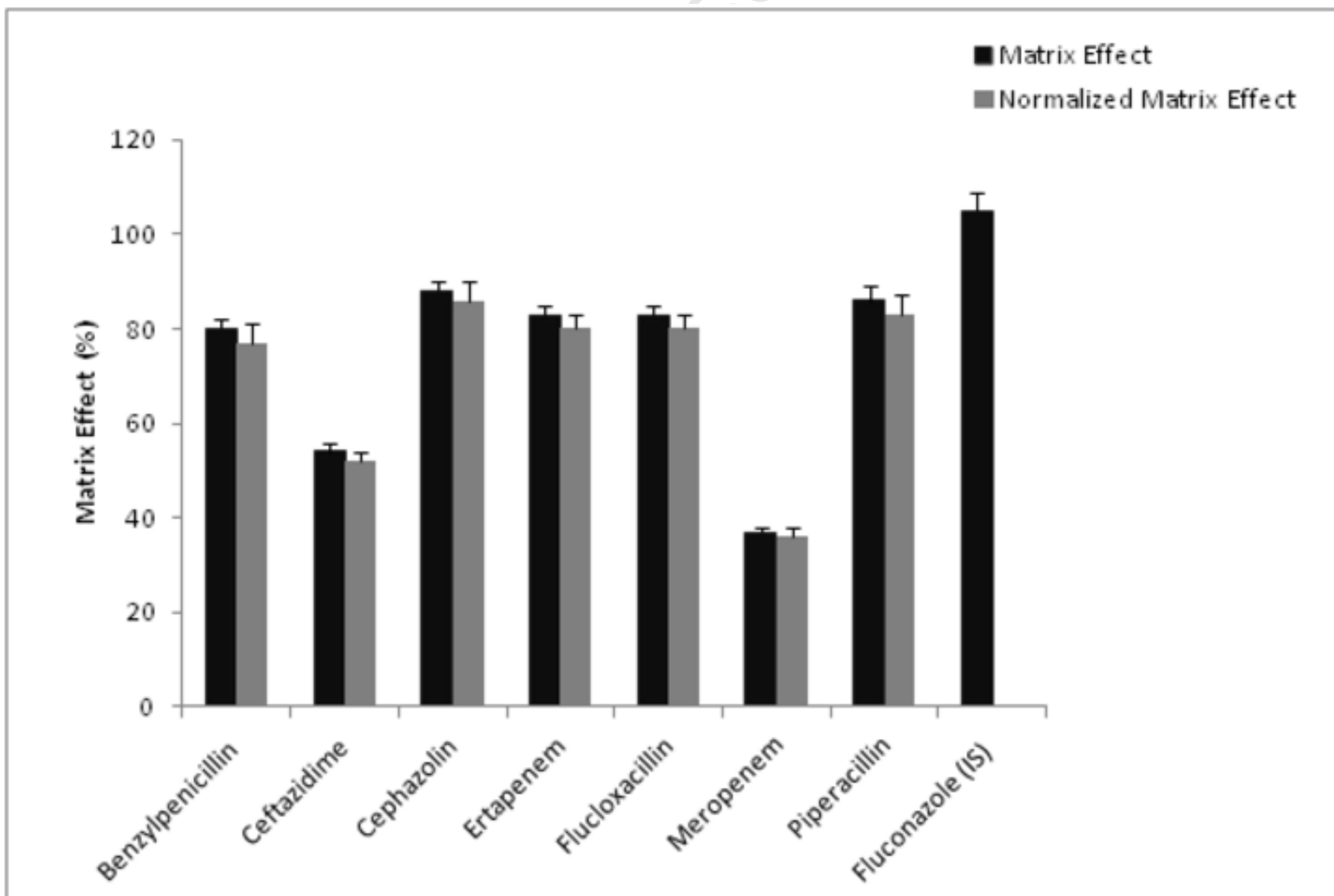


Figure 4



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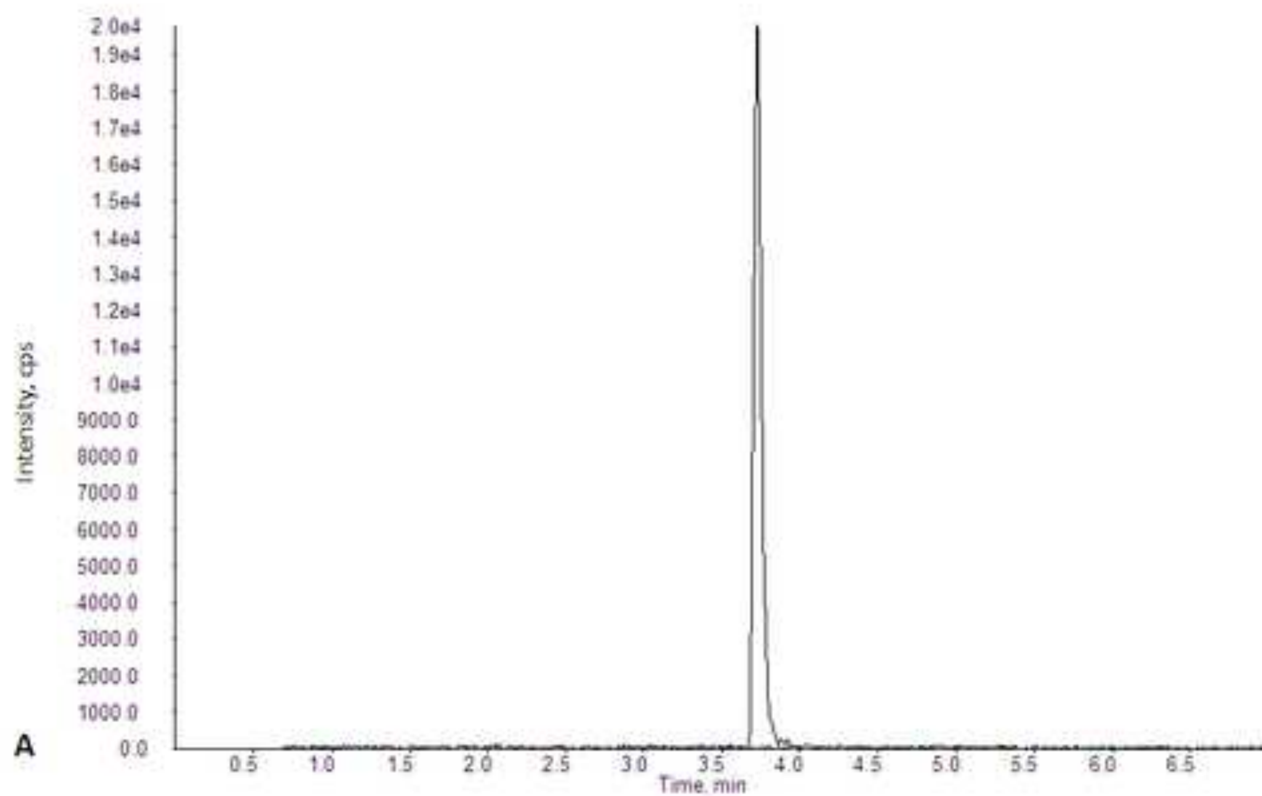

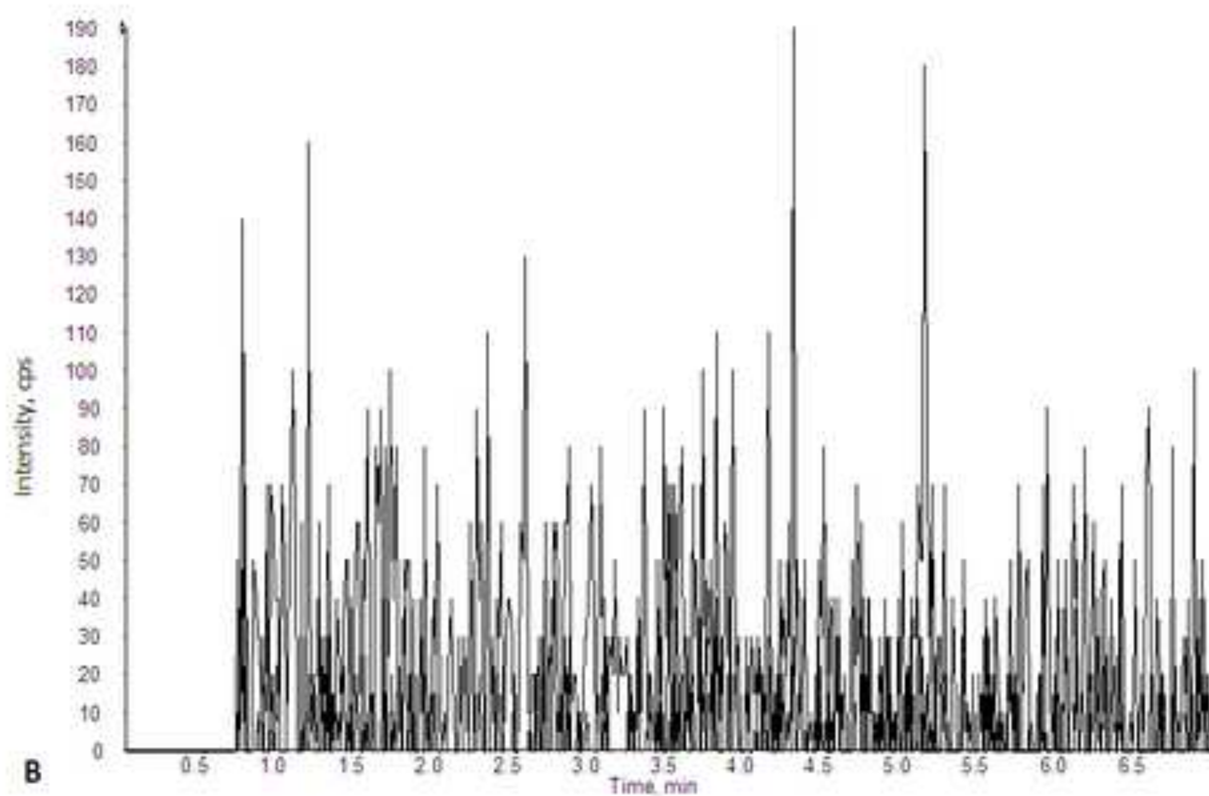

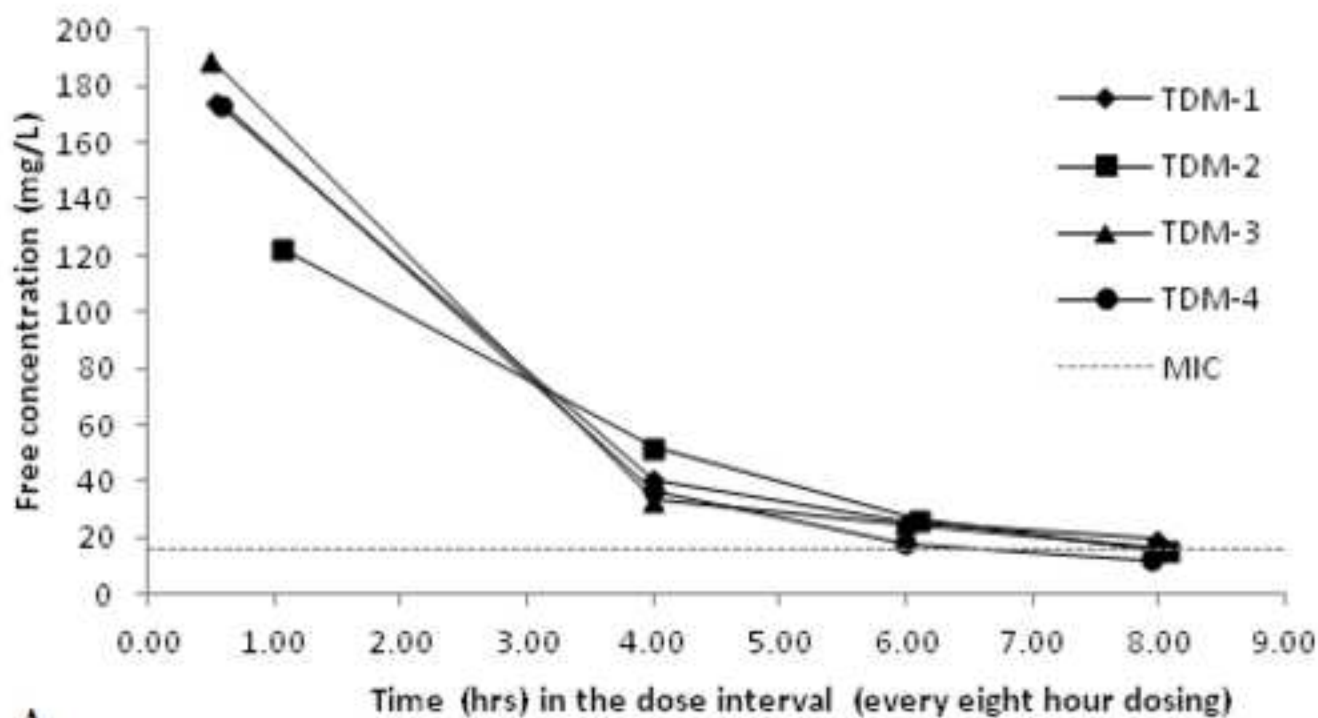
Free concentration (mg/L)
200
180
160
140
120
100
80
60
40
20
0
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
Time (hrs) in the dose interval (every eight hour dosing)
TDM-1
TDM-2
TDM-3
TDM-4
MIC
A

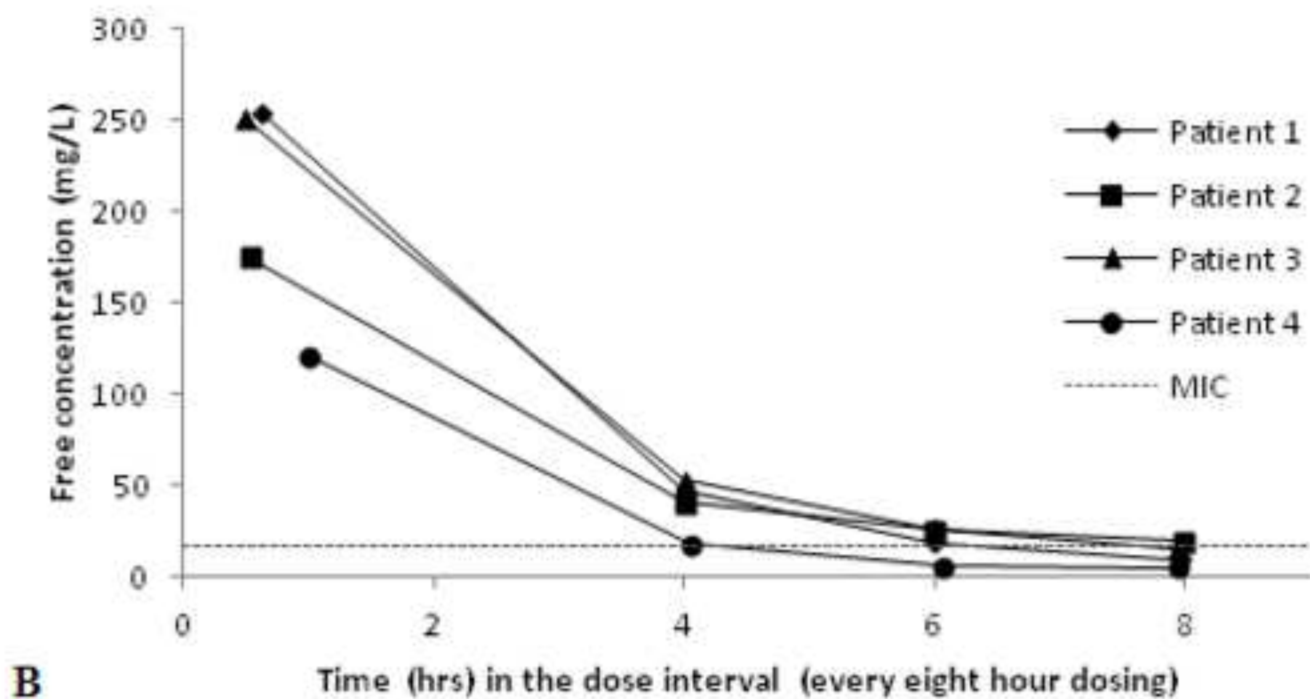
Free concentration (mg/L)
300
250
200
150
100
50
0
0
2
4
6
8
Time (hrs) in the dose interval (every eight hour dosing)
Patient 1
Patient 2
Patient 3
Patient 4
MIC
B

Figure 7

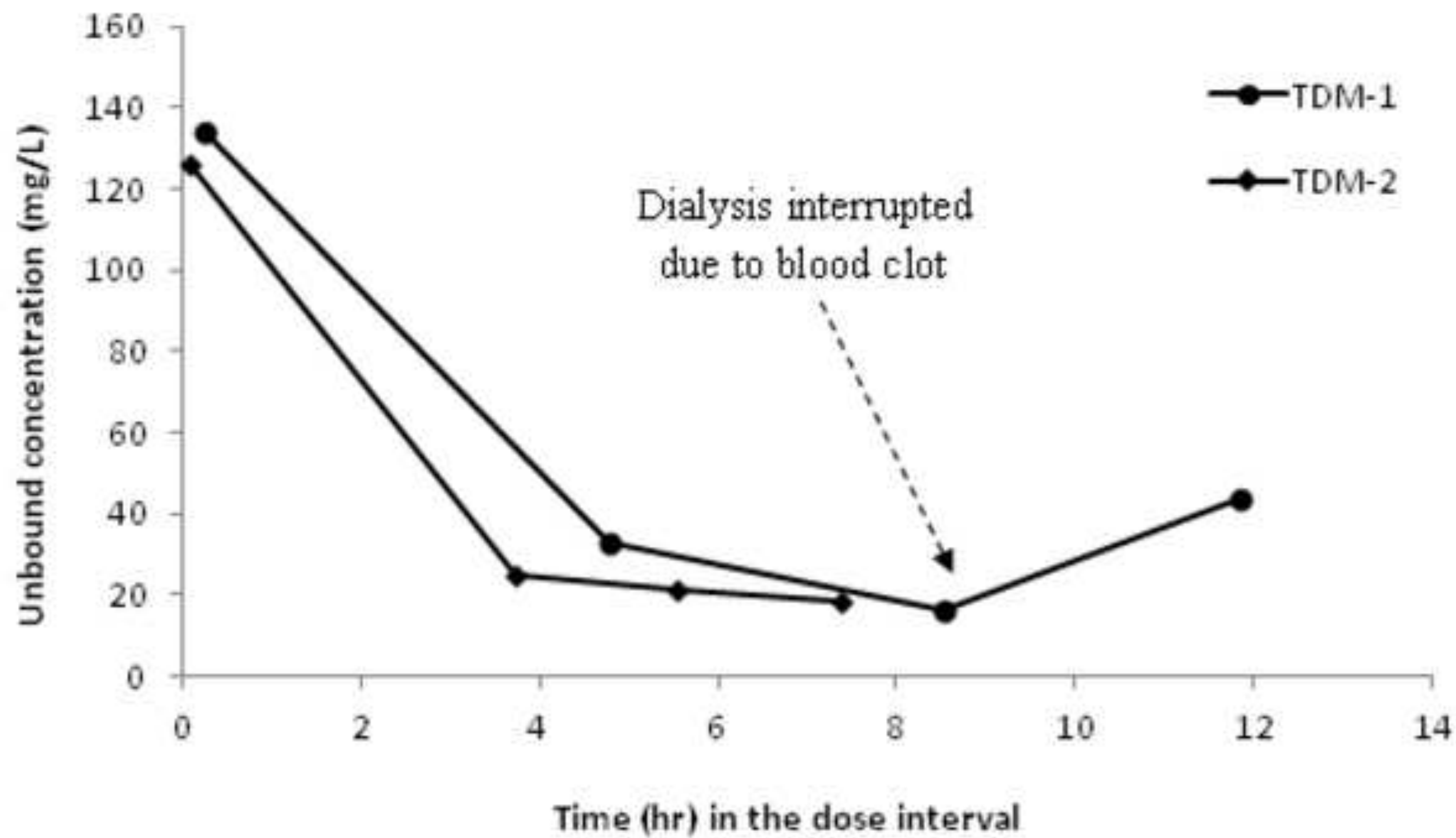

Figure 8