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Authors: Saiyuri Naicker, Yarmarly C.Guerra Valero, Jenny L.Ordóñez Meija, Jeffrey Lipman, Jason A. Roberts, Steven C. Wallis, Suzanne L. Parker

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A UHPLC-MS/MS method for the simultaneous determination of piperacillin and tazobactam in plasma (total and unbound), urine and renal replacement therapy effluent.

Saiyuri Naicker[*,a,e], Yarmarly C. Guerra Valero[a], Jenny L. Ordóñez Meija[a], Jeffrey Lipman[a,b,c], Jason A. Roberts[a,b,d,e], Steven C. Wallis[a], Suzanne L. Parker[a]

[a]Burns, Trauma and Critical Care Research Centre, UQ Centre for Clinical Research, The University of Queensland, Brisbane, Australia

[b]Department of Intensive Care Medicine, Royal Brisbane & Women's Hospital, Brisbane, Australia

[c]Faculty of Health, Queensland University of Technology, Brisbane, Australia

[d]Department of Pharmacy, Royal Brisbane & Women's Hospital, Brisbane, Australia

[e]Centre of Translational Pharmacodynamics, School of Pharmacy, The University of Queensland, Brisbane, Australia

*Corresponding author

Mailing Address: Burns, Trauma and Critical Care Research Centre (BTCCRC), Level 7, UQ Centre for Clinical Research, Royal Brisbane & Women's Hospital QLD 4029

Phone: +61 7 3346 1814

Email: s.naicker@uq.edu.au

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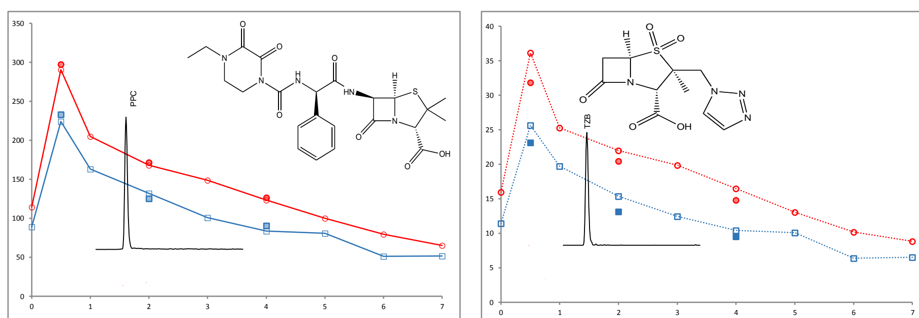
Graphical abstract



Highlights:

- Simultaneous measurement of piperacillin-tazobactam using UHPLC-MS/MS
- Validated for a range of clinically-relevant matrices
- Application of microsample volumes for analysis of plasma (total), urine and RRTE
- Method applied to a clinical pharmacokinetic study of a critically ill patient

Abstract

Piperacillin-tazobactam is a beta-lactam/beta-lactamase combination antibiotic used in patients with moderate to severe infection. Dosing of piperacillin-tazobactam requires an understanding of this patient group to maximise the effectiveness of this antibiotic and limit a further emergence of resistant pathogens. This is the first method that measures piperacillin and tazobactam simultaneously, across this range of clinically-relevant biological matrices. The calibration line was linear across the concentration range of 0.5 to 500 µg/mL for piperacillin and 0.625 to 62.5 µg/mL for tazobactam. All validation testing for matrix effects, precision and accuracy, specificity and stability were within 15%. A calibration equivalence study was performed to investigate the suitability of applying calibration curves prepared in an alternative matrix, with a mean bias of -10.8% identified for the application of a calibration line prepared for tazobactam in plasma only. Bias for all other calibration lines prepared in alternate matrices was within the 5% acceptance criteria. The method was successfully

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applied to a pharmacokinetic study of a critically ill patient receiving renal replacement therapy, with the results included.

Keywords: microsample, beta-lactam, mass spectrometry, chromatography

1. Introduction

Piperacillin-tazobactam is a beta-lactam/beta-lactamase combination antimicrobial therapy with broad-spectrum activity against both Gram-negative and Gram-positive bacteria [1]. Piperacillin-tazobactam is preferentially prescribed in hospital or critical care settings for the treatment of moderate to severe infections [2]. Administration of this combination results in bactericidal activity that includes *Pseudomonas aeruginosa* and *Enterobacteriaceae* [3, 4].

As with all beta-lactam antibiotics, piperacillin exhibits time-dependent antibacterial activity – where the time the antibiotic concentration remains above the minimum inhibitory concentration of the pathogen ($fT_{>MIC}$) correlates best with the efficacy of piperacillin. Similar pharmacodynamics likely exist for tazobactam, although less data exists for this. Traditional dosing of piperacillin-tazobactam in critically ill patients presents a challenge. Piperacillin is largely renally excreted and therefore augmented renal clearance (creatinine clearance > 130 mL/min) is associated with sub-therapeutic piperacillin concentrations [5, 6]. Similarly, patients requiring renal replacement therapy may also experience sub-optimal antibiotic exposures as recommended dosing regimens have not been validated in these patients. A study by Zander *et al* found high inter- and intra-patient variability of piperacillin concentrations, even in patients without severe renal dysfunction [7]. Dosing strategies that apply therapeutic drug monitoring may individualise piperacillin-tazobactam therapy [8].

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Giving the right dose is necessary to allow for maximum effectiveness of this antibiotic combination and suppress further emergence of resistant pathogens [9].

Piperacillin and tazobactam have been measured separately and simultaneously, with chromatographic methods employing ultra-violet detection [10-15] and mass spectrometry detection [16-21]. Di Giovamberardino *et al* describe the measurement of unbound piperacillin and tazobactam in plasma [11], Ocampo *et al* describe the measurement of piperacillin and tazobactam in urine [13], and Connor *et al* describe the measurement of piperacillin and tazobactam in renal replacement therapy effluent (RRTE) [22]. There are no methods that measure both piperacillin and tazobactam simultaneously, across this range of clinically-relevant biological matrices.

A method that can measure drugs in microsample volumes (less than 50 µL) can open opportunities to characterise drug disposition in small animals, patients with small blood volumes, or patients with challenging venous/arterial access. There are three methods that employ microsample volumes for the analysis of piperacillin and tazobactam in plasma [16, 17, 21], but none that apply microsample volumes to other biological matrices.

The aim of this work was to design a reliable method to simultaneously measure piperacillin and tazobactam in plasma (total and unbound), urine and RRTE. This method should be suitable for the characterisation of piperacillin-tazobactam in clinical studies and, where possible, employ microsample volumes.

2. Experimental

2.1. Chemicals and materials

Piperacillin-tazobactam was obtained from Aspen (St Leonards, Australia), [$^{2}H_{5}$] - piperacillin from Alsachim (Illkirch Graffenstaden, France) and sulbactam from Sigma Chemical Company

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(Sydney, Australia). The chemical structures for these compounds are shown in Figure 1. Acetonitrile was HPLC-gradient grade (Merck, Darmstadt, Germany), methanol was LCMS grade (Fisher Chemicals, Fairlawn, New Jersey, USA) and formic acid (Fisher Scientific, Victoria, Australia) was analytical grade. Ultrapure water was obtained using a Permutit system (resistivity at 25°C greater than 18 ΩM.cm). Drug-free human plasma was obtained from Innovative Research (Novi, Michigan, USA) and drug-free urine was obtained from healthy volunteers. Compound sodium lactate IV solution was obtained from Baxter (Old Toongabbie, Australia).

2.2. Instrument and conditions

The UHPLC-MS/MS used was a Shimadzu Nexera2 LC equipped with a Shimadzu 8030+ triple quadrupole mass spectrometer (MS) detector. An electro-spray ionization (ESI) source interface, switching between both positive-ion (for piperacillin and [$^{2}H_{5}$] - piperacillin) and negative ion mode (for tazobactam and sulbactam), was used for the selected reaction monitoring (SRM) UHPLC-MS/MS analysis. MS conditions for piperacillin, [$^{2}H_{5}$] - piperacillin, tazobactam and sulbactam are reported in Table 1. Nitrogen was used as the nebulizing gas, with interface setting consisting of the nebulizing gas flow of 3 L/min, a de-solvation line temperature of 250 °C, heat block temperature 400 °C and a drying gas flow of 15 L/min. A 100 ms dwell time was used. The collision gas was argon.

The LC analysis was performed on a Shimadzu Nexera2 system equipped with dual pumps, and an autosampler with a sample compartment set to 5°C. The column was a C18 Shimadzu Shim-pack XR-ODS III, 2.0 x 50 mm, 1.6 µm column (Shimadzu, Kyoto, Japan) used at room temperature. The mobile phase was a gradient of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in acetonitrile). The gradient was run from 7.5% to 95% (as %B) and back again over 4.5 min. A sample volume of 1.0 µL was injected.

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An Allegra 64R benchtop, temperature controlled centrifuge was sourced from Beckman Coulter (Lane Cove, Australia). Centrifree® ultrafiltration devices were sourced from Merck Millipore (Bayswater, Australia).

2.3. Stock and standard solution preparation

2.3.1. Drug-free matrix preparation

Preparation of ultrafiltered drug-free plasma was performed by pre-heating centrifree® ultrafiltration devices, centrifuge buckets and centrifuge to 37 °C. Drug-free plasma samples were thawed, vortexed and incubated at 37 °C. Clinical plasma sample (200 µL) was added to the pre-heated centrifree® ultrafiltration devices and incubated at 37 °C for 10 minutes. Immediately after 10 minutes warming the heated centrifree® ultrafiltration devices were placed into the heated centrifuge inserts and centrifuged at 37°C (for 5 minutes at 2400 g). The ultrafiltered plasma was subsequently used for the preparation of calibration standards for the quantification of unbound concentrations of piperacillin-tazobactam.

For the quantification of urine, the drug-free matrix was a dilution of 2% urine in 98% water. For the quantification of RRTE, the drug-free matrix was a dilution of 10% ultrafiltered plasma in 90% compound sodium lactate solution.

2.3.2. Calibration standard solutions

Piperacillin-tazobactam is co-formulated to contain piperacillin at concentrations eight-times higher than tazobactam. Aqueous stock solutions were prepared from the co-formulated standard material to contain 200, 500, 1000, and 40000 µg/mL of piperacillin and 25, 62.5, 125, and 5000 µg/mL of tazobactam, respectively. All aqueous stock solutions were stored at -80 °C.

On the day of assay, calibration standards were prepared by serial dilution with water to contain both piperacillin in concentrations ranging from 0.5 to 1000 µg/mL and tazobactam

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in concentrations ranging from 0.0625 to 125 µg/mL. Calibration standard solutions were prepared by combining these solutions with an equal volume of drug-free matrix (plasma, ultrafiltered plasma, urine or RRTE).

The calibration standards were processed alongside the clinical and quality control samples.

2.3.3. Internal standard solution

Internal standard stock solutions were prepared in water at 100 µg/mL for [$^{2}H_{5}$] – piperacillin and 100 µg/mL for sulbactam. A combined internal standard solution was then prepared containing both 10 µg/mL for [$^{2}H_{5}$] – piperacillin and 10 µg/mL for sulbactam in water. All solutions were stored at -20°C.

2.4 Quality control sample preparation

Quality control samples were prepared from a stock solution that contained both 40000 µg/mL of piperacillin and 5000 µg/mL of tazobactam in water, and were stored at -80°C. The stock solution was diluted with drug-free matrix (plasma, urine or RRTE). Quality control samples were prepared to contain both piperacillin and tazobactam in concentrations of 1.5, 15, 50 and 400 µg/mL and 0.1875, 1.875, 6.25 and 50 µg/mL, respectively. The quality control sample containing 1.5 µg/mL of piperacillin and 0.1875 µg/mL of tazobactam was used to quantify piperacillin only.

Quality control samples were stored at -80°C and processed alongside the clinical samples.

2.5 Sample preparation procedure

2.5.1. Plasma sample preparation (total)

Plasma samples (total) and quality control plasma samples were prepared by combining 2.5 µL of plasma sample with 2.5 µL of water (to match calibration standards). The following procedure was performed for all plasma calibration standards, drug-free samples, quality control samples and clinical samples.

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All samples were vortexed for 3 seconds, followed by the addition of 10 µL of internal standard solution, except for the drug-free plasma sample which received 10 µL of water. Samples were again vortexed for 3 seconds and 30 µL of acetonitrile added to all samples in the batch. Samples were vortexed for 3 seconds and then centrifuged (at 4000 g for 5 minutes) to remove precipitated proteins. The resulting supernatant was injected onto the UHPLC-MS/MS (injection volume of 1 µL).

2.5.2 Plasma sample preparation (unbound)

The unbound fraction of the clinical plasma samples and quality control samples was isolated from plasma following the same procedure for obtaining ultrafiltered plasma as described in Section 2.3.1 Drug-free matrix preparation.

Plasma samples (unbound) were then processed following the same procedure as described in Section 2.5.1 Plasma sample preparation (total). Calibration standards and drug-free samples were prepared in drug-free ultrafiltered plasma, prepared as described in Section 2.3.1 Drug-free matrix preparation.

2.5.3 RRTE sample preparation

RRTE samples were processed following the same procedure as described in Section 2.5.1 Plasma sample preparation (total). Calibration standards, quality control samples and drug-free samples were prepared in drug-free RRTE, prepared as described in Section 2.3.1 Drug-free matrix preparation.

2.5.4 Urine sample preparation

Urine samples were filtered using a 0.45 µm filter into a clean microfuge tube, with samples then diluted 1:50 with water. Diluted urine samples were then processed following the same procedure as described in Section 2.5.1 Plasma sample preparation (total). Calibration

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standards, quality control samples and drug-free samples were prepared in drug-free diluted urine, prepared as described in Section 2.3.1 Drug-free matrix preparation.

2.6 Data analysis

For both piperacillin and tazobactam the concentration of each clinical sample and quality control sample was obtained using the data from the calibration curve prepared from standards within each batch. A linear regression with peak area ratio (analyte/internal standard area response) against concentration (x) with a $1/x^2$ weighting was used as the mathematical basis for quantification.

2.7 Method of Validation

The validation was performed in accordance to the guidelines provided by the U.S. Food and Drug Administration (USFDA) and assessed against the prescribed acceptance criteria [23]. The validation for all matrices was assessed for linearity, matrix effects, recovery, selectivity, lower limit of quantification (LLOQ), stability and inter-day and intra-day precision and accuracy.

2.7.1 Linearity

Calibration curves were prepared in each matrix, and across a suitable concentration range to investigate linearity.

2.7.2 Matrix effects

Matrix effects were evaluated to identify any suppression or enhancement of signal from an interfering substance around the retention times of piperacillin and tazobactam by applying the matrix factor test. Five blank matrix samples, for each matrix, were assayed at spiked low and high concentration levels with internal standard. The resulting area was compared to those produced following the same sample preparation procedure using water instead of matrix. The precision of the matrix factor (normalized against internal standard) was used to

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determine if any concentration level demonstrated unacceptable variability from the expected result.

2.7.3 Selectivity

The selectivity of the method was evaluated to identify the ability of the method to differentiate and quantify piperacillin and tazobactam in the presence if other components in the sample. This was achieved by analysing drug-free plasma and drug-free urine for interference from different sources and containing different anticoagulant compounds, including lithium heparin, ethylenediaminetetraacetic acid, and sodium citrate. This analysis was also performed at the lower limit of quantification.

2.7.4 Limit of quantification and detection

The lower limit of quantification for piperacillin and tazobactam were evaluated by analysis of replicate standards (n = 5), for all matrices assessed. The LLOQ was tested at the lowest concentration of the calibration standards 0.5 uq/mL. The lower limit of detection for piperacillin and tazobactam in all matrices were calculated based on its definition as being the lowest peak reliably distinguished from the background noise and calculated as ≥ three-times the noise of the blank sample.

2.7.5 Recovery

The recovery of piperacillin and tazobactam was evaluated in plasma by comparing the peak area of samples spiked with analyte prior to extraction to the peak area of samples spiked with analyte after extraction. By adjusting the concentration and volume of calibration standard in the extraction the injection matrix was kept identical in comparable samples.

2.7.6 Stability

The stability of piperacillin and tazobactam was assessed in frozen storage (at -80 °C), at room temperature (for 4 hours at 24 °C), and across three freeze-thaw cycles (from -80 °C to

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ambient temperature). This was performed using three replicates of quality control samples prepared at four concentrations for piperacillin and three concentrations for tazobactam and comparing the results to nominal concentrations. Stability of processed samples while stored on the autosampler was tested by comparing stored quality control samples concentrations to original concentrations when reinjected alongside the original standard curve. Stability of stock solutions prepared in water and stored at -80 °C was assessed by comparing the analyte area to that obtained for freshly prepared solutions.

2.7.7 Precision and Accuracy

Quality control samples in all matrices at four concentrations for piperacillin and three concentrations for tazobactam were assayed alongside freshly prepared standard curves. Concentrations of each quality control sample was obtained by application of the calibration regression line, and precision and accuracy calculated against nominal quality control sample concentrations.

2.7.8 Calibration Equivalence: alternate matrix preparation of the calibration line

The suitability of quantifying clinical samples from calibration standards prepared in an alternative matrix was tested by extracting sets of quality control samples prepared in drug-free plasma, ultrafiltered plasma, urine, RRTE and water. To achieve this, 2.5 µL of a piperacillin-tazobactam calibration standard solution was combined with 2.5 µL of matrix, 10 µL of internal standard and 30 µL of acetonitrile, and prepared in accordance with Section 2.5.1. Plasma sample preparation (total).

The suitability of using an alternate matrix for the preparation of the calibration line was performed by comparing the response factor of the calibration standard in one matrix to the response factor of the calibration standards in the other matrices. The response factor was calculated as the area of analyte divided by the area of the internal standard, divided by the

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nominal concentration of the solution. The bias was calculated as the percentage deviation in the quantitation response factor relative to the calibration response factor, averaged over all concentration levels. Criteria were pre-established as a bias of 5% or less being acceptable.

2.8 Pharmacokinetic Application

This method was applied to the analysis of plasma (total and unbound), urine and RRTE samples from a pharmacokinetic study for critically ill patients, receiving concomitant piperacillin-tazobactam during treatment with renal replacement therapy in an Intensive Care Unit.

A critically ill patient receiving renal replacement therapy was administered a dose of piperacillin-tazobactam, as prescribed by the treating physician. A blood sample (3 mL) was collected from an indwelling central-line cannula prior to the patient receiving a dose (0 h) and then at 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 hours, with the dose being administered as an infusion from 0 to 0.5 hours. Blood samples were collected using heparinised vacuum tubes (Greiner Bio-One, Vacuette® lithium heparin). Blood samples were centrifuged at 1400 g for 10 minutes to obtain plasma samples. The plasma samples were subsequently transferred into 2 mL polypropylene tubes and stored at -80 °C.

A single urine sample was collected as a pooled sample of urine collected from 0 to 8 h, post-administration of the piperacillin-tazobactam dose. An aliquot of urine was transferred into a urine specimen vial and stored at -80 °C. Similarly, an aliquot of RRTE was removed from the effluent bag at 3, 4, 6 and 8 hours post-administration of the piperacillin-tazobactam dose. New effluent bags were attached immediately after each sample collection. The RRTE sample was transferred into a 2 mL polypropylene tube and stored at -80 °C.

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3. Results and Discussion

3.1 Mass Spectrometry

This method employs both positive and negative selected reaction monitoring (SRM). Fragmentation ions were manually selected, followed by software-directed auto-optimization routines which were conducted to identify any alternative fragmentation patterns and also to optimize voltages. Rapid ionization-switching on the mass spectrometer allowed simultaneous monitoring of all analytes.

The piperacillin fragmentation pattern to form an ion at m/z 143 involves the loss of CO_2 and C_5H_7NO typical to penicillin beta-lactam antibiotics [24, 25]. The same fragmentation pattern is observed for the internal standard, [$^2H_5$] – piperacillin, to form an ion at m/z 148. The tazobactam fragmentation pattern to form an ion at 138 involves the loss of $C_2H_4O_2S$, typical to penicillin beta-lactam antibiotics in negative ionisation mode [25]. The same fragmentation pattern for negative ionisation mode is observed for the internal standard sulbactam, to form an ion at 140.

3.2 Chromatography

Chromatograms for samples in plasma (total and unbound), urine, RRTE are shown in Figure 2. The chromatography employed replicates the conditions used by Parker *et al* to measure ampicillin and sulbactam [26]. The retention time for piperacillin and its internal standard, [$^2H_5$] – piperacillin, was 2.7 minutes. The retention time for tazobactam and its internal standard, sulbactam, was 2.4 minutes. While piperacillin is retained longer than ampicillin and demonstrated base-line separation from ampicillin it is unlikely these antibiotics would be co-administered in a clinical setting.

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The total run time for analysis was 7 min, which included time to re-equilibrate the column to the starting gradient mobile phase conditions. The mobile phase was delivered at 0.3 mL/min and generated a backpressure of approximately 5500 psi. A sample volume of 1 µL was injected.

Blank samples, as well as zero samples (blank matrix samples spiked with internal standard only) were analysed within the batch run order for validation and analysis of clinical samples to elucidate evidence of a carryover effect, with all resulting chromatograms inspected for interfering peaks. No carryover effect was observed.

3.3 Sample Preparation

A very small sample volume of 2.5 µL was selected for this assay. This microsample volume can allow less-invasive collection of clinical samples, such as using a skin-prick with collection into a capillary tube, rather than venepuncture. This volume is also useful for analysing samples from pre-clinical studies in small animals. A reduction in sample volume has the potential to improve study participation in 'high burden' clinical studies, for example studies were multiple samples are required across short time intervals, or where infants or paediatric patients are involved [27].

The unbound fraction of piperacillin and tazobactam in plasma was isolated by centrifuging 200 µL of plasma in centrifree® ultrafiltration devices, as described in Section 2.3.1. To prevent perturbation of the equilibrium the time of centrifugation was selected to allow filtration of approximately 40% of the plasma volume.

All urine samples and drug-free urine were filtered and diluted 1 in 50 v/v with water. This step was employed to ensure the urine samples could be analysed within the calibration range used for the other matrices, and within the linear range of the mass spectrometer.

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RRTE calibration standards and quality controls were prepared in a solution containing 10% ultrafiltered plasma and 90% compound sodium lactate IV solution (v/v). This solution was prepared to mimic the likely components of a clinical RRTE sample.

3.4 Validation

The lower limit of quantification was validated for precision and accuracy in plasma (total and unbound), urine, and RRTE and all met acceptance criteria. The validation results for the lower limit of quantification and limit of detection are reported in Table 2.

A linear regression with a 1/concentration^2 weighting provided an adequate calibration equation within the concentration range. The calibration range, mean correlation coefficient (r^2) and the percentage of maximum deviation (inaccuracy) of the standards of calibration curves for all matrices are presented in Table 3.

The intra- and inter- batch precision and accuracy of the plasma (total and unbound), urine and RRTE are reported for piperacillin in Table 4a and for tazobactam in Table 4b. All precision and accuracy results met the acceptance criteria. Intra- and inter- assay batch results for unbound plasma concentrations were used to calculate the unbound fractions of the quality control samples. The mean unbound fraction in plasma and across the concentration range was 87 ± 5% and 96 ± 8% for piperacillin and tazobactam, respectively.

The matrix test indicated that there was no unacceptable variability in the response of piperacillin or tazobactam in plasma (total or unbound), these results are reported in Table 5. The mean recovery from the processing for total plasma concentrations was 81.5% for piperacillin and 82.0% for tazobactam. Recovery results are also reported in Table 5. Recovery testing was not performed for urine or RRTE as these sample preparations involved direct injection only.

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The selectivity of the method was tested in different donor matrices; the donor plasma containing lithium heparin, sodium oxalate or ethylenediaminetetraacetic acid. At the lower limit of quantification for piperacillin in plasma (total) the selectivity result was 0.508 µg/mL with a precision of 5.4% (n = 6), and in urine was 0.485 µg/mL with a precision of 2.7% (n = 5). At the lower limit of quantification for tazobactam in plasma (total) the selectivity result was 0.688 µg/mL with a precision of 10.7% (n = 6), and in urine was 0.743 µg/mL with a precision of 5.9% (n = 5). The accuracy of tazobactam in urine is high, but within the 20% acceptance criteria for the lower limit of quantification. No peaks were detected in any of the drug-free matrices. Selectivity testing was not performed for RRTE as the variable constituent of the RRTE was ultrafiltered plasma, and this has been tested as plasma (total).

Testing at room temperature for four hours, for three freeze-thaw cycles and long term storage for at least 16 months, found that both piperacillin and tazobactam were stable within acceptable limits. The results are reported in supplementary tables 7a and 7b. Testing of stock solutions containing piperacillin for 22 months at -80 °C showed acceptable deviations of -1.7% for piperacillin and 1.1% for tazobactam. Testing of the storage of processed samples at 4 °C was performed by comparing the deviation between paired samples from prior to storage and then when reinjected after storage. For piperacillin in plasma a mean deviation of -0.3% was found after 6 days (n = 8 pairs), in urine 3.6% after 7 days (n = 12 pairs), and in RRTE -2.4% after 4 days (n = 8 pairs). For tazobactam in plasma a mean deviation of -5.7% was found after 6 days (n = 6 pairs), in urine 4.9% after 7 days (n = 9 pairs), and in RRTE 2.1% after 4 days (n = 6 pairs).

An incurred sample reanalysis of clinical plasma samples was performed on 18 samples and resulted in a mean deviation from the original result of -4.7 ± 6.1% for piperacillin and -5.8 ± 8.8% for tazobactam. All of the 18 samples tested for piperacillin and all but one of the 18

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samples tested for tazobactam (representing 94% of the total tested for each analyte) were within 20% deviation of the mean result. An incurred sample reanalysis of clinical plasma samples for unbound concentrations was performed on 6 samples and resulted in a mean deviation of 14.1 ± 3.9% and -12.3 ± 8.2% of piperacillin and tazobactam, respectively. All but one of the 6 samples tested for tazobactam (representing 83% of the total tested) were within 20% deviation of the mean result; all 6 samples tested for piperacillin were within 20% deviation of the mean result. These results meet the acceptance criteria.

Investigations into the requirement to matrix-match calibration standards for the quantification of samples (calibration equivalence) found the magnitude of bias was within the pre-established acceptance criteria of 5% for all matrices tested, except for plasma. Based on these results, calibration standards prepared in either drug-free ultrafiltered plasma, urine, RRTE or water may be used to quantifying clinical samples in ultrafiltered plasma, urine and RRTE by this method.

3.5 Pharmacokinetic application

This method was successfully applied to the analysis of samples in a clinical pharmacokinetic study. The plasma concentration – time profile from a critically ill patient receiving renal replacement therapy is presented in Figure 3. The peak concentration (C_{max}) measured in plasma (total) was 291 µg/mL and 36.2 µg/mL for piperacillin and tazobactam, respectively. The lowest concentration (C_{min}) measured in plasma (total) was 51.4 µg/mL and 6.52 µg/mL for piperacillin and tazobactam, respectively. The mean unbound plasma concentration for piperacillin was 102 ± 4% (n = 6) of the total plasma concentration, and for tazobactam was 90 ± 3% (n = 6) of the total plasma concentration. The mean unbound fractions of both piperacillin and tazobactam are higher than previously found in a study of patients receiving renal replacement therapy by Wong *et al* (82.5%, n = 94 [28]). The results may reflect both

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altered protein binding and pharmacokinetic variability found in critically ill patients receiving renal replacement therapy [29, 30]. However, the study by Wong *et al* also found calculated unbound drug concentrations were a poor predictor of measured unbound drug concentrations, for beta-lactam antibiotics in critically ill patients [28]. Based on the uncertainty in prediction of unbound concentrations therapeutic drug monitoring to optimize beta-lactam dosing is gaining popularity, although this may require measuring unbound rather than total concentrations.

No urine samples were collected for this patient. The concentration in RRTE ranged from 47.2 to 123 μg/mL and 7.99 to 20.6 μg/mL of piperacillin and tazobactam, respectively.

4. Conclusion

The developed analytical method met all pre-established validation criteria for the simultaneous determination of piperacillin and tazobactam in plasma (total and unbound), urine and RRTE. As demonstrated in this work, the microsample volumes used in this methodology may be applied to the analysis of samples from clinical pharmacokinetic studies. The use of these microsample volumes may lead to improved clinical study participation for patient groups with challenging phlebotomies or where the collection of small volumes of sample can reduce the burden of study participation.

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Ethical Conduct of Research

The clinical trial was performed in accordance with the principles laid down by the Australian guidelines for Good Clinical Practice and the applicable local regulatory requirements. The trial was approved by the Human Research & Ethics Committee Royal Brisbane and Women's Hospital for the project (HREC/13/QRBW/1).

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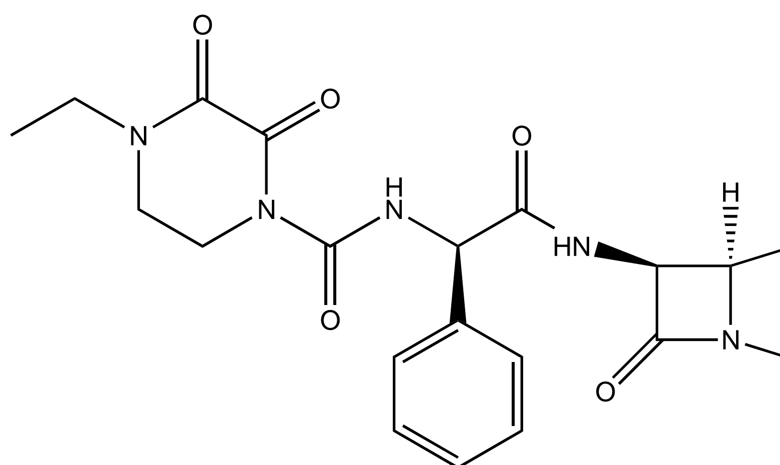

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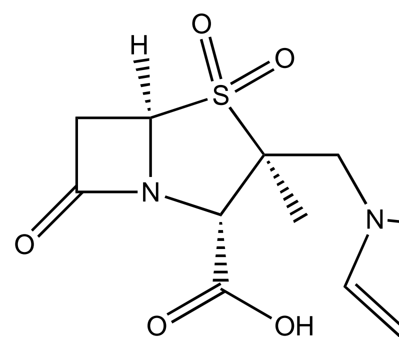

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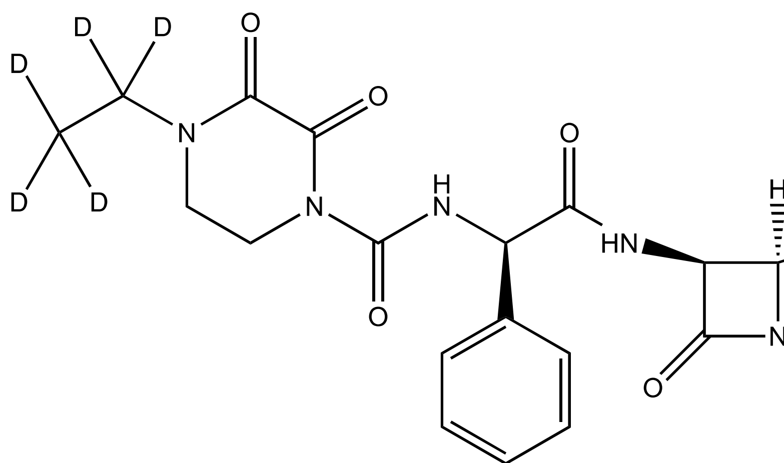

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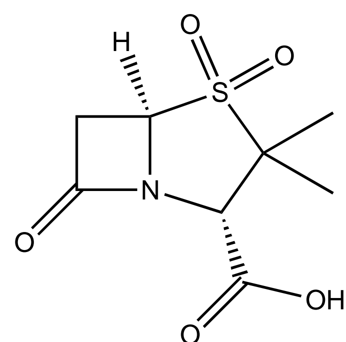

Figure 1: Structure of piperacillin (A), [$^{2}H_{5}$] – piperacillin (B), tazobactam (C) and sulbactam (D)

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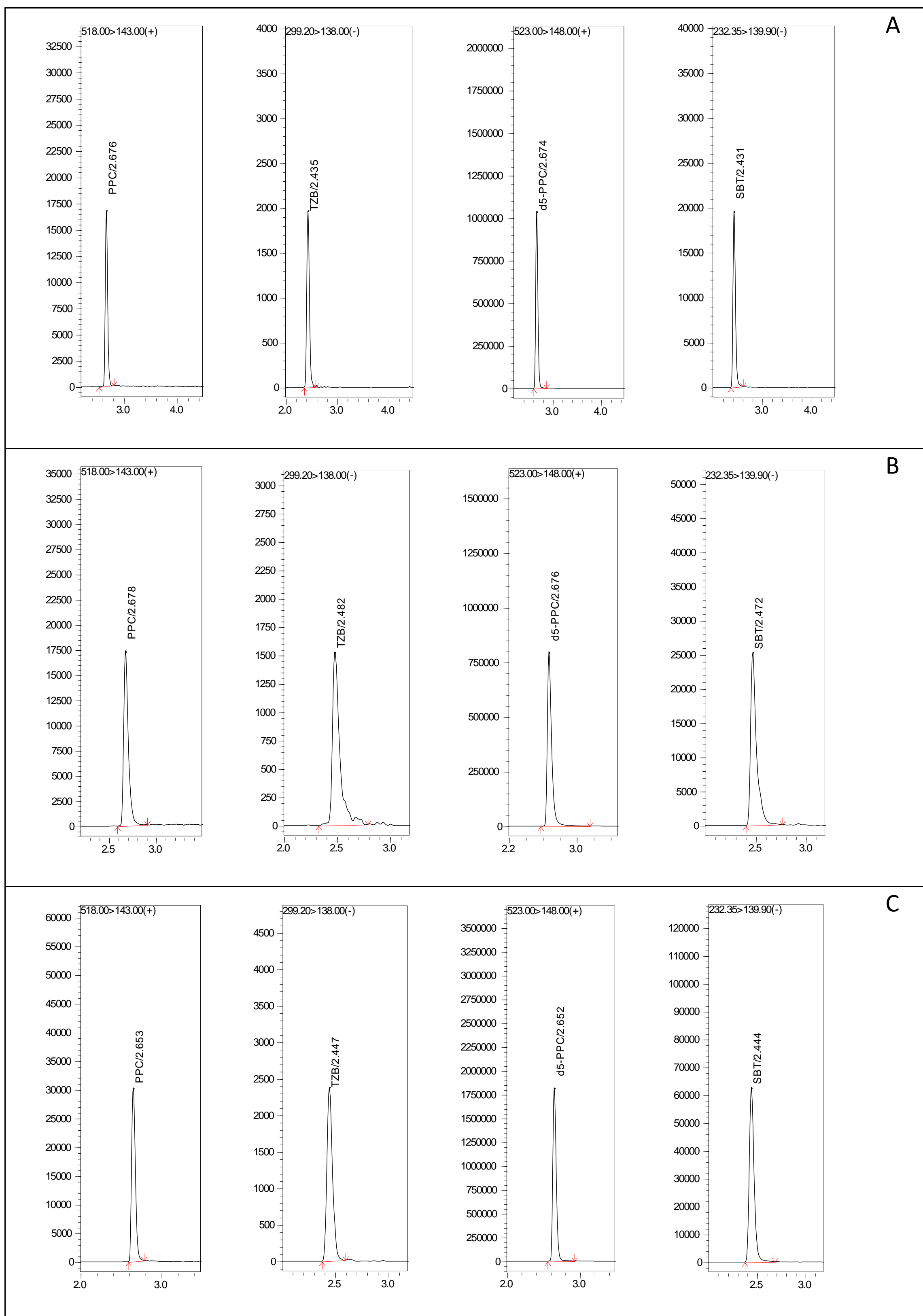

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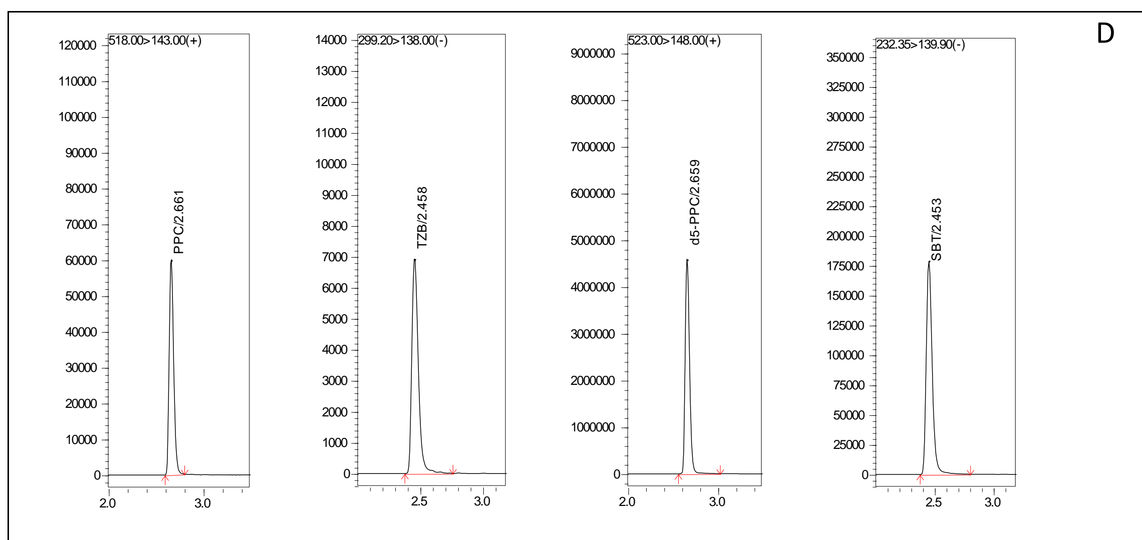

Figure 2: Chromatograms for the lower limit of quantification for piperacillin (PPC; 0.5 µg/mL) and tazobactam (TZB; 5 µg/mL) and internal standards [2H5]-piperacillin (d5-PPC) and sulbactam (SBT) in plasma (total; A), plasma (unbound; B), urine (C), and renal replacement therapy effluent (D)

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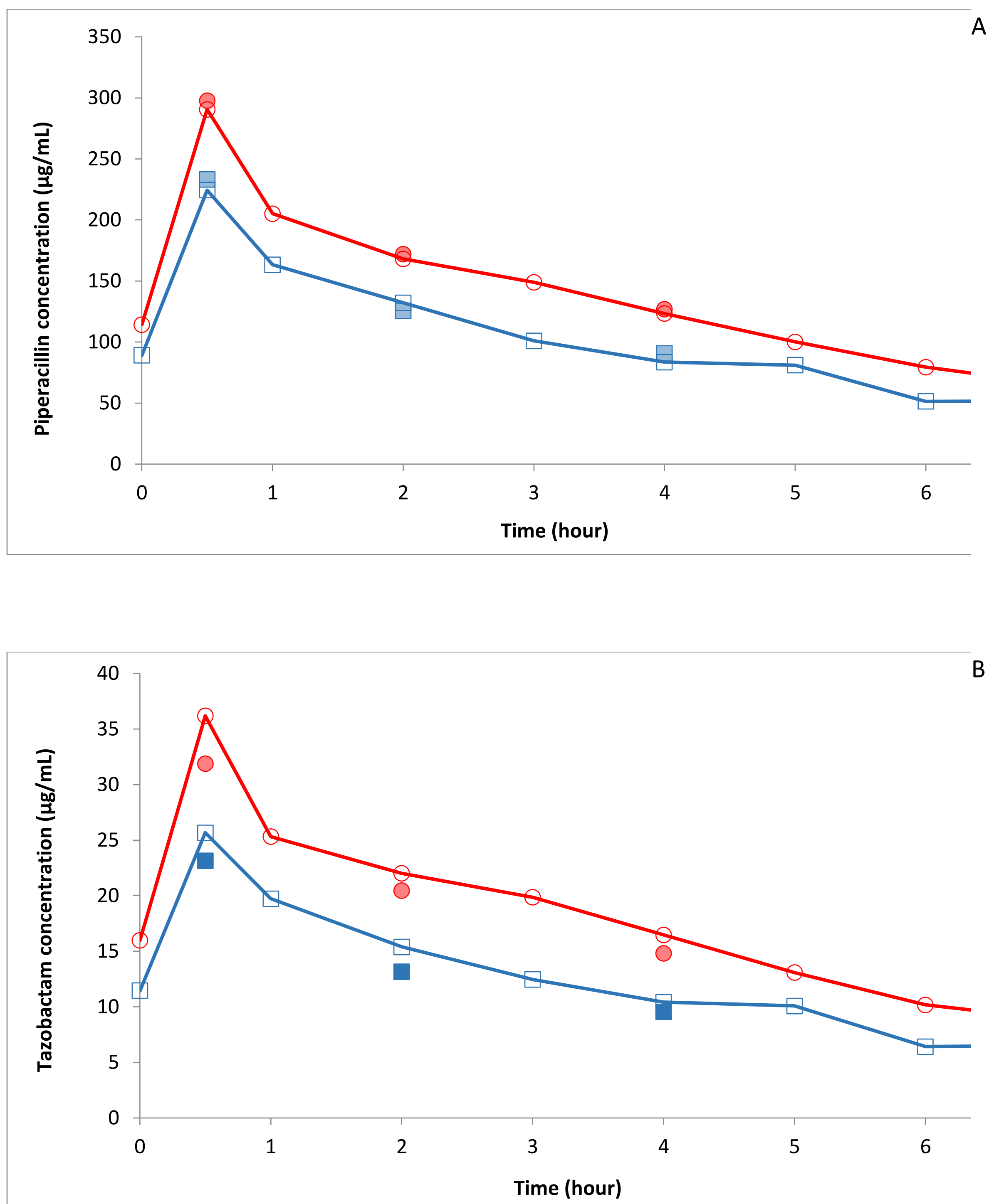

Figure 3: Plasma piperacillin concentrations (A; µg/mL) and tazobactam concentrations (B; µg/mL) versus time (hour) for a critically ill patient receiving renal replacement therapy (RRT); samples were collected pre-RRT filter (circle) and post-RRT filter (square) with total plasma concentrations (unfilled) and unbound plasma concentrations (filled) included in the plot.

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Table 1: Mass spectrometry settings

	Piperacillin[a]	Tazobactam[b]	[$^{2}H_{5}$]-piperacillin[a]	Sulbactam[b]
Precursor Ion	518	229	523	232
Product Ion	143	138	148	140
Q1 (V)	-24	22	-24	25
CE (V)	-20	15	-20	14
Q3 (V)	-28	24	-28	24

a Precursor and product ion measured as [MH+]

b Precursor and product ion measured as [MH-]

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Table 2: Lower limit of quantification (n = 5) and detection limits

	Piperacillin				Tazobactam			
Matrix	Mean (µg/mL)	Precision (%)	Accuracy (%)	Detection Limit (µg/mL)	Mean (µg/mL)	Precision (%)	Accuracy (%)	Detection Limit (µg/mL)
Plasma	0.460	5.5	92.0	< 0.01[a]	0.640	5.1	102	< 0.01[a]
Unbound	0.518	3.0	104	0.01	0.629	7.7	101	0.04
RRTE	0.488	2.3	97.6	0.01	0.614	4.3	98.2	< 0.01[a]
Urine	0.513	4.0	103	< 0.01[a]	0.576	13.7	92.2	0.01

a Noise level was too low to be determined

RRTE Renal replacement therapy effluent

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Table 3: Linearity analysis

	Piperacillin			Tazobactam		
Matrix	Calibration range (μg/mL)	Correlation coefficient* (mean)	Maximum deviation** (%)	Calibration range (μg/mL)	Correlation coefficient* (mean)	Maximum deviation** (%)
Plasma	0.5 to 500	0.9984	± 9.3%	0.625 to 62.5	0.9974	± 11.1%
Unbound	0.5 to 500	0.9981	± 12.5%	0.625 to 62.5	0.9928	± 14.8%
RRTE	0.5 to 500	0.9998	± 13.1%	0.625 to 62.5	0.9993	± 14.6%
Urine	0.5 to 500	0.9990	± 7.7%	0.625 to 62.5	0.9973	± 13.0%

* Mean (n = 3)

** Reported maximum deviation from nominal (%) across all standard curves and all concentration levels.

RRTE Renal replacement therapy effluent

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Table 4a: Intra- and inter- assay precision and accuracy for piperacillin in plasma (total and unbound), RRTE and urine

Piperacillin												
	Plasma			Unbound			RRTE			Urine		
Study	Mean (µg/mL)	Precision (%)	Accuracy (%)	Mean (µg/mL)	Precision (%)	Fu (%)	Mean (µg/mL)	Precision (%)	Accuracy (%)	Mean (µg/mL)	Precision (%)	Accuracy (%)
Intra-	1.51	5.8	101	1.21	5.2	80.7	1.42	2.2	94.7	1.41	1.7	94.0
	15.0	3.6	100	12.2	2.1	81.3	14.3	2.4	95.3	14.3	3.4	95.3
	55.0	1.8	110	41.2	1.7	82.4	47.5	3.3	95.0	49.4	2.2	98.8
	408	3.0	102	351	6.7	87.8	388	4.1	97.0	385	2.8	96.3
Inter-	1.35	2.2	90.0	1.32	7.4	88.0	1.30	6.1	86.7	1.38	2.0	92.0
	13.6	1.8	90.7	13.8	2.2	92.0	13.7	1.6	91.3	14.0	1.1	93.3
	46.2	2.6	92.4	44.8	5.1	89.6	45.9	3.9	91.8	47.8	2.5	95.6
	369	3.0	92.3	378	2.8	94.5	376	6.6	94.0	387	1.7	96.8

Fu fraction unbound

RRTE Renal replacement therapy effluent

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Table 4b: Intra- and inter- assay precision and accuracy for tazobactam in plasma (total and unbound), RRTE and urine

Tazobactam												
	Plasma			Unbound			RRTE			Urine		
Study	Mean (µg/mL)	Precision (%)	Accuracy (%)	Mean (µg/mL)	Precision (%)	Fu (%)	Mean (µg/mL)	Precision (%)	Accuracy (%)	Mean (µg/mL)	Precision (%)	Accuracy (%)
Intra-	1.91	3.3	102	1.83	2.3	97.6	1.79	3.1	95.5	1.86	2.7	99.2
	6.73	7.0	108	5.96	2.1	95.4	6.10	2.1	97.6	6.13	2.5	98.1
	50.6	3.1	101	49.4	8.5	98.8	47.2	4.7	94.4	48.0	2.7	96.0
Inter-	1.69	7.0	90.1	1.77	8.7	94.4	1.73	1.8	92.3	1.87	2.6	99.7
	5.71	7.4	91.4	6.07	8.7	97.1	6.10	2.9	97.6	6.19	2.6	99.0
	46.1	3.9	92.2	48.3	8.0	96.6	48.3	4.0	96.6	47.4	3.0	94.8

RRTE Renal replacement therapy effluent

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Table 5: Matrix effect and recovery studies for piperacillin and tazobactam

Analyte	Piperacillin				Tazobactam			
Study	Matrix	Concentration (µg /mL)	Mean	Precision (%)	Matrix	Concentration (µg /mL)	Mean	Precision (%)
Matrix Factor ($MF_{normalised}$)	Plasma	50	0.99	2.3	Plasma	6.25	1.02	5.2
		100	1.03	6.2		12.5	1.04	5.8
		200	0.98	5.2		25	0.98	6.3
	Unbound	25	0.98	2.3	Unbound	25	1.04	6.9
		50	1.03	3.6		50	1.10	9.3
		100	1.00	4.8		100	1.07	11.1
Recovery (%)	Plasma	20	81.0	4.6	Plasma	2.5	82.1	2.4
		100	81.0	2.1		12.5	79.3	0.7
		200	82.4	1.2		25	84.6	1.4

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Table 6: Calibration equivalence testing

Calibration Matrix	Bias (%) for Piperacillin quantified in matrix of interest					Bias (%) for Tazobactam quantified in matrix of interest				
	Total Plasma	Unbound Plasma	Urine	RRTE	Water	Total Plasma	Unbound Plasma	Urine	RRTE	Water
Total Plasma	NA	3.9	-0.1	0.8	0.4	NA	11.1	13.8	11.8	12.7
Unbound Plasma	-3.7	NA	-3.8	-3.0	-3.3	-9.9	NA	2.4	0.6	1.4
Urine	0.1	4.0	NA	0.9	0.5	-11.9	-2.2	NA	-1.7	-0.8
RRTE	-0.8	3.1	-0.9	NA	-0.4	-10.4	-0.5	1.8	NA	0.9
Water	-0.4	3.5	-0.5	0.4	NA	-11.1	-1.3	1.1	-0.8	NA

RRTE Renal replacement therapy effluent
NA Not applicable