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Simultaneous measurement of piperacillin, tazobactam and meropenem in patient samples using LC-MS/MS to support β-lactam therapeutic drug monitoring

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Background: accurate therapeutic drug monitoring (TDM) of β-lactam antibiotics such as meropenem and piperacillin–tazobactam is essential in critically ill patients, where profound pharmacokinetic variability may lead to subtherapeutic exposure or toxicity. Practical, high-throughput analytical workflows are needed to support timely dose optimisation in routine clinical settings. *Methods*: we developed and fully validated a unified LC-MS/MS method for the simultaneous quantification of meropenem, piperacillin, and tazobactam in human serum. Samples were prepared by protein precipitation and analysed on a C18 column using a water/methanol gradient with 0.1% formic acid. Detection was performed by multiple reaction monitoring with isotopically labelled internal standards. *Results*: the assay demonstrated excellent linearity (R^2 > 0.998), high sensitivity (LLOQ 0.02 mg L^{-1}), and robust intra- and inter-day precision (<10%), with a 10 min run time and no detectable carry-over. The method demonstrates high analytical sensitivity, with a lower limit of quantification (LLOQ) of 0.02 mg L^{-1} for all analytes, which is among the lowest reported for simultaneous quantification of meropenem, piperacillin, and tazobactam. Application to >40 clinical samples from critically ill patients revealed wide concentration ranges (meropenem 0.2–270 mg L^{-1}; piperacillin 1–580 mg L^{-1}; tazobactam 0.1–60 mg L^{-1}), with unbound fractions consistent with published data. *Conclusions*: this streamlined, low-volume LC-MS/MS workflow enables rapid, accurate quantification of key β-lactam antibiotics and supports routine TDM in critically ill and paediatric populations, facilitating more individualised antimicrobial dosing in clinical practice. Since our method already accommodates β-lactams and a β-lactamase inhibitor with highly heterogeneous physicochemical properties, it provides a robust basis for extending the approach to additional antibiotics within a multi-analyte framework.

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Introduction

Antimicrobial resistance is a global concern in hospitals and is partially driven by suboptimal antibiotic use, especially in intensive care units where broad-spectrum antibiotics are used widely.[1,2] Achieving the right antibiotic exposure in critically ill patients is not straightforward. Their clinical condition can change rapidly, drug distribution and clearance can be altered due to factors such as sepsis, multi-organ failure and renal replacement therapy.[3,4] Accordingly, blood antibiotic concentrations can differ widely between patients receiving the same dose. If antibiotic concentrations are too low, this can cause a treatment failure and contribute to the development of drug resistance. If the levels are too high, it could cause toxicity.[5,6] Therapeutic Drug Monitoring (TDM) can help clinicians to individualise dosing based on measured drug levels rather than taking a one-size-fits-all approach to antibiotic dosing.[7]

Piperacillin–tazobactam and meropenem are among the most prescribed β-lactam antibiotics for serious infections in critically ill patients.[8] Piperacillin, a broad-spectrum penicillin, is combined with tazobactam, a β-lactamase inhibitor, in a fixed dose combination to provide cover against a wide range of aerobic and anaerobic Gram-positive and Gram-negative bacteria. Meropenem, a carbapenem antibiotic, is typically used to treat multidrug-resistant Gram-negative bacterial

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infections, such as extended-spectrum β-lactamase (ESBL) producing organisms.[9] Both piperacillin–tazobactam and meropenem show time-dependent bactericidal activity, meaning that maintaining blood concentrations above the minimum inhibitory concentration (MIC) for an adequate period (fT > MIC) is key to achieving therapeutic success.

In critically ill patients, we observe wide pharmacokinetic variability for piperacillin–tazobactam and meropenem with standard dosing regimens frequently failing to achieve pharmacokinetic/pharmacodynamic (PK/PD) targets.[10–14] TDM for β-lactam antibiotics can help to address individual patient-level PK variability to improve PK/PD target attainment.[15–17] Optimising PK/PD target attainment improves clinical outcomes, reduces adverse events, and minimises selective pressure on antimicrobial resistance by helping achieve therapeutic serum concentrations.[17,18]

Despite international consensus guidelines recommending routine TDM for critically ill patients treated with β-lactams, this has not yet become standard practice in most institutions.[19] The main barrier is analytical with few laboratories having access to rapid, reliable assays capable of measuring different β-lactams simultaneously.[19] Analytical approaches using immunoassays and sensors are limited and often overestimate the measurements due to lack of specificity.[20] Liquid chromatography-tandem mass spectrometry (LC-MS/MS) can offer high sensitivity and selectivity and can quantify multiple antibiotics from a small sample volume, making it ideal for clinical applications.[21] Several LC-MS/MS methods have been reported for β-lactam therapeutic drug monitoring; however, achieving high analytical sensitivity while maintaining a simple and rapid workflow remains challenging. Many existing methods report higher limits of quantification or require more complex sample preparation to achieve sufficient sensitivity. In addition, not all methods enable simultaneous quantification of multiple β-lactam antibiotics within a single assay. Therefore, there remains a need for analytical approaches that combine high sensitivity with simplified sample preparation and suitability for high-throughput clinical workflows.

The aim of this study was to develop and validate a single LC-MS/MS method that can quantify simultaneously piperacillin, tazobactam, and meropenem in human serum. The LC-MS/MS method was designed to be simple, fast, and robust, making it suitable for high throughput testing with minimal sample volume. The method was applied to clinical samples to measure both the total and free concentrations from blood samples collected from hospitalised patients.

Materials and methods

Chemicals and reagents

Human serum (heat-inactivated, male AB plasma, USA origin), meropenem pharmaceutical secondary standard, piperacillin sodium salt analytical standard, formic acid (98–100%, HPLC grade), and Centrifree® ARTM Ultrafiltration Centrifugal Filters, MWCO 30A kDa, regenerated cellulose membrane were obtained from Merck Life Science (UK). Tazobactam sodium salt and isotopically labelled tazobactam-15N3 were purchased from Fisher Scientific. Isotopically labelled meropenem-d_6 (>90%) and piperacillin-d_5 were supplied by molecular dimensions. LC-MS grade water, methanol, and acetonitrile were purchased from VWR International. All reagents were used as received.

Instrumentation and chromatographic conditions

Chromatographic analyses were performed using an Agilent 1290 Infinity II LC system coupled to an Ultivo triple quadrupole mass spectrometer (Agilent Technologies). Data acquisition and processing were conducted using MassHunter software (v10). Chromatographic separation was achieved using an Eclipse Plus C18 column (4.6 × 50 mm, 3.5 μm; Agilent Technologies).

The mobile phases consisted of:

- *A*: Water + 0.1% formic acid.
- *B*: Methanol + 0.1% formic acid.

The flow rate was maintained at 0.20 mL min^{-1}, the injection volume was 4 μL, and the total run time was 10 min. The column temperature and the autosampler temperature were maintained at 30 °C and 6 °C, respectively. The gradient program is summarized in Table 1.

Mass spectrometry conditions

Analytes were detected using electrospray ionisation (ESI) operated in positive ion mode with multiple reaction monitoring (MRM) on the Ultivo triple quadrupole mass spectrometer. Optimised transitions were selected for both quantifier and qualifier ions for each compound, together with their respective isotopically labelled internal standards.

Meropenem. Meropenem was monitored using the transitions *m*/*z* 384 → 68.1 (quantifier) and 384 → 141.1 (qualifier). The internal standard, meropenem-d_6, was monitored at *m*/*z* 389.5 → 68.3, 389.5 → 260.1, and 389.5 → 114.5.

Tazobactam. Tazobactam was monitored using the transition *m*/*z* 301.1 → 168.1, with the internal standard tazobactam-$^{15}N_3$ monitored at *m*/*z* 304.2 → 168.1.

Piperacillin. Piperacillin was monitored using transitions *m*/*z* 518.2 → 160.3 (quantifier) and 518.2 → 143.3 (qualifier), and its internal standard piperacillin-d_5 was monitored at *m*/*z* 523.3 → 148.3.

The ion source settings were as follows: gas temperature 340 °C, drying gas flow 9 L min^{-1}, nebuliser pressure 40 psi,

Table 1 Liquid chromatography gradient program

Time (min)	% *A*	% *B*	Flow (mL min^{-1})	Max pressure (bar)
0.00	99.5	0.5	0.20	1300
0.20	99.5	0.5	0.20	1300
2.00	50.0	50.0	0.20	1300
3.00	50.0	50.0	0.20	1300
4.00	40.0	60.0	0.20	1300
5.00	0.0	100.0	0.20	1300
8.00	0.0	100.0	0.20	1300
9.00	99.5	0.5	0.20	1300
10.00	99.5	0.5	0.20	1300

sheath gas temperature 250 °C, and sheath gas flow 12 L min^{-1}. The capillary voltage was set to +5000 V with a nozzle voltage of 1000 V.

Clinical samples

Serum samples were collected as part of an ethically approved prospective, observational PK/PD study (REC reference 21/HH/7287). Written informed consent was obtained from the patient or their personal consultee. Participants were hospitalised patients admitted to the Intensive Care Unit receiving either piperacillin–tazobactam (4.5 g three to four times daily) or meropenem (1–2 g three times daily) for clinical indications. A total of 32 patients receiving piperacillin–tazobactam and 25 patients receiving meropenem were included in the study.

Venous blood samples were collected from a central venous catheter or by phlebotomy at three predefined timepoints: T1 (30 minutes after infusion), T2 (mid-interval), and T3 (trough, immediately prior to the next dose). Whole blood was collected into serum-separating tubes, allowed to clot, and centrifuged according to standard laboratory procedures. The resulting serum was aliquoted and stored at −80 °C until analysis. The clinical samples were used to demonstrate the analytical applicability of the developed LC-MS/MS method in real-world patient matrices and were not intended to support pharmacokinetic or pharmacodynamic conclusions.

All clinical samples were processed using the same protein precipitation protocol described.

Matrix for method validation

All validation experiments, including calibration standards and quality control samples, were prepared using blank human serum obtained from a commercial source (heat-inactivated, male AB plasma, USA origin). This matrix was selected to ensure consistency and to avoid interference from residual antibiotics present in patient samples. Blank serum was processed in the same way as clinical samples (protein precipitation or ultrafiltration, where applicable) to maintain matrix equivalence during validation. Haemolysed and lipaemic matrices were not specifically evaluated in this study, as validation was performed using standard serum matrices; however, the use of isotopically labelled internal standards is expected to minimise potential matrix-related variability.

Sample preparation and preparation of calibration and quality control samples

For each analytical run, 100 μL of serum (clinical or blank) was mixed with 200 μL of acetonitrile in a microcentrifuge tube. The mixture was vortexed, allowed to stand for 10 min at room temperature, and centrifuged at 14 000 g for 10 min. An aliquot of the resulting supernatant was diluted 1 : 1 with water containing isotopically labelled internal standards, and the final extract was transferred to LC-MS/MS vials for analysis.

All calibration standards and quality control (QC) samples were prepared using blank human serum obtained from a commercial source to ensure the absence of endogenous β-lactam antibiotics. Primary stock solutions of piperacillin, tazobactam, and meropenem were spiked into blank serum and serially diluted (1 : 3) to generate calibration ranges of:

- Meropenem: 0.020–45,000 mg L^{-1}.
- Tazobactam: 0.020–45,000 mg L^{-1}.
- Piperacillin: 0.020–135,000 mg L^{-1}.

The internal standards piperacillin-d_5 and tazobactam-$^{15}N_3$, were added to all calibration and QC levels at a final concentration of 1.25 mg L^{-1}. While meropenem-d_6 was added at a final concentration of 2.5 mg L^{-1}.

Internal standards were evaluated both before and after the protein precipitation step during method development. No significant difference in analytical performance was observed between the two approaches. Internal standards were therefore added after the protein precipitation step to minimise variability associated with differential protein binding and precipitation efficiency between analytes and internal standards. This approach ensures that the internal standards primarily correct for matrix effects during ionisation, which represent the dominant source of variability in LC-MS/MS analysis.

Serum protein binding

Because meropenem is minimally protein-bound (∼98% unbound), total serum concentrations accurately reflect pharmacologically active levels. However, protein binding may still vary in critically ill patients due to factors such as hypoalbuminaemia, renal replacement therapy, and altered physiology. In this study, only total meropenem concentrations were measured, which represents a limitation and should be considered when interpreting the results.[22]

Unbound piperacillin and tazobactam were quantified using ultrafiltration devices of 30 KDa MWCO. The ultrafiltration procedure was optimised during method development, and the potential influence of temperature, pH, and relative centrifugation force (RCF) on protein binding. Analyte recovery was evaluated to ensure reliable measurement of the free drug fraction.

Calibration standards for unbound piperacillin and tazobactam were prepared using serum processed through the same ultrafiltration procedure applied to clinical samples to ensure matrix equivalence. Each filter was loaded with 950 μL of blank human serum and centrifuged at 2000×g for 20 min at 25 °C. The resulting ultrafiltrate was pooled separately for each analyte.

Primary standards for piperacillin and tazobactam were prepared in the pooled ultrafiltrate, and serial 1 : 3 dilutions were prepared from the highest concentration level. Isotopically labelled internal standards (piperacillin-d_5 or tazobactam-$^{15}N_3$) were added to all calibration levels to achieve a final IS concentration of 1.25 mg L^{-1}. The final calibration ranges were 0.00508–100 mg L^{-1} for piperacillin and 0.0152–100 mg L^{-1} for tazobactam.

Bioanalytical method validation

This study presents a fully validated LC-MS/MS method for the simultaneous quantification of meropenem, piperacillin, and tazobactam in human serum, designed for clinical therapeutic

drug monitoring (TDM). Method validation was performed in accordance with ICH M10 bioanalytical guidance and in line with our previously reported analytical method validation.[22–24]

Selectivity

Selectivity of the LC-MS/MS method was evaluated to ensure the absence of significant endogenous interferences at the retention times of piperacillin ($R_T = 7.4$ min, Fig. S1), tazobactam ($R_T = 4.6$ min, Fig. S2), meropenem ($R_T = 4.6$ min, Fig. S3), and their corresponding internal standards (IS) (Fig. S4, S5, and S6 respectively). Six independent blank human serum samples were processed using the complete sample preparation procedure without the addition of an analyte or IS. Each blank matrix extract was examined for potential co-eluting peaks or interferences at the characteristic multiple reaction monitoring (MRM) transitions of the analytes and IS. The method was considered selective if the response at the analyte retention times in blank samples did not exceed 20% of the mean peak area obtained at the lower limit of quantification (LLOQ) and did not exceed 5% of the IS response.

Linearity

The linearity of the method was assessed by preparing calibration standards spanning a wide concentration range to cover the expected clinical concentrations of the analytes. For meropenem and tazobactam, calibration curves were constructed over the concentration range of 0.020–45 000 mg L^{-1}, while for piperacillin the range was extended to 0.020–135 000 mg L^{-1}. The upper limit of the calibration range was selected to encompass the wide variability in β-lactam concentrations observed in critically ill patients, including high post-infusion levels. No samples exceeded the upper calibration limit, and no evidence of detector saturation was observed within the validated range.

Calibration standards were prepared by serial three-fold dilution of independently prepared stock solutions in blank human serum. Internal standards were added post-precipitation for consistency with clinical sample processing and to ensure comparable correction of matrix effects across all samples.

Linearity was evaluated using weighted ($1/x$) least-squares linear regression of analyte-to-internal standard peak area ratios against nominal concentrations. Calibration curves were considered acceptable when the coefficient of determination (r^2) was ≥0.99 and back-calculated concentrations of calibration standards were within ±15% of nominal values (±20% at the lower limit of quantification). Results were obtained using four independent calibration curves prepared on different days, with each calibration standard analysed in triplicate.

LLOQ and ULOQ

The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve that could be quantified with acceptable accuracy (within ±20% of nominal concentration) and precision (coefficient of variation ≤20%) based on six replicate measurements. Similarly, the ULOQ was established as the highest concentration meeting the acceptance criteria for precision and accuracy (≤15%).

Accuracy and precision

The quality controls (QCs) for accuracy and precision runs were prepared at four concentration levels within the calibration curve range: the LLOQ, within three times of the LLOQ (low QC), around 30–50% of the calibration curve range (medium QC) and at least 75% of the ULOQ (high QC). The medium QC for piperacillin (QC5) was selected at ∼15 000 mg L^{-1} to provide a representative concentration near the midpoint of the calibration curve on a logarithmic scale. Although the 30–50% range of the curve corresponds to ∼40 500–67 500 mg L^{-1}, the calibration standards were prepared using a serial three-fold dilution scheme, resulting in discrete, log-spaced concentrations. Choosing 15 000 mg L^{-1} ensures even distribution of QC samples across the full dynamic range, maintains consistency with the prepared standards, and minimizes additional dilution errors. This approach supports robust assessment of accuracy and precision across the entire calibration range. Five replicate samples at each concentration level were analysed in three separate analytical runs performed on different days.

Accuracy was expressed as the percentage difference between the mean measured concentration and the nominal concentration of the QC samples. Intra-run (within-run) and inter-run (between-run) precision were evaluated as the coefficient of variation (CV%). Acceptance criteria were set at ±15% for accuracy and ≤15% for precision across all QC levels, except at the LLOQ, where ±20% was considered acceptable.

Carryover

Potential carryover effects were investigated by injecting a blank serum sample immediately following the highest concentration calibration standard (ULOQ). The resulting chromatograms were examined for residual analyte and internal standard signals at the relevant retention times and MRM transitions. Carryover was considered acceptable if the response in the blank sample did not exceed 20% of the analyte signal at the LLOQ and 5% of the IS signal.

Recovery

Extraction recovery was evaluated to determine the efficiency and reproducibility of the sample preparation procedure. Recovery was assessed by comparing the analytical response (peak areas) of extracted QC samples at low, medium, and high concentrations with those of non-extracted reference standards prepared in the mobile phase or appropriate solvent at equivalent concentrations.

Recovery experiments were performed in triplicate for each QC level. The percentage recovery was calculated by dividing the mean peak area of the extracted samples by that of the non-extracted samples.

Matrix effects

Matrix effects were assessed to determine the influence of endogenous serum components on the ionization of the analytes and internal standards. The evaluation was conducted at multiple QC concentration levels by comparing the responses of

analytes spiked into post-extracted blank serum with those of neat (aqueous) standard solutions.

The matrix effect for each analyte was calculated as the percentage ratio of the peak area in the presence of matrix to that in the absence of matrix. Matrix effect was evaluated in triplicate at multiple concentration levels using a representative serum matrix. While the results demonstrated minimal ion suppression or enhancement, it is acknowledged that evaluation across a larger number of individual donor matrices would further strengthen assessment of inter-individual variability, in line with regulatory expectations. Nevertheless, the use of isotopically labelled internal standards ensures robust correction for matrix-related variability during ionisation.

Short-term stability

Short-term (bench-top and autosampler) stability of piperacillin, tazobactam, and meropenem was evaluated to ensure the integrity of the samples during routine handling and analysis. Working and stock solutions were kept at room temperature for 4 h and 24 h, while processed samples were stored in the autosampler at 6 °C for up to 12 h prior to injection.

Stability was assessed at high and low QC levels in triplicate and determined by comparing the measured concentrations of stability samples with freshly prepared QC samples. The analytes were considered stable if the deviation from initial concentrations was within ±15%.

Freeze–thaw stability

Freeze–thaw stability was investigated to assess the effect of repeated freezing and thawing on analyte integrity. In accordance with the ICH M10 guideline, stability experiments are required to include a minimum of two QC concentration levels to adequately assess analyte stability. In this study, low and medium QC levels were selected for freeze–thaw stability assessment. The low QC represents the lower end of the quantifiable range, where variability and instability are most likely to be observed. The medium QC provides coverage of the central portion of the calibration range, which is typically representative of the majority of study samples. QC samples at low and medium concentration levels were subjected to three complete freeze–thaw cycles, consisting of storage at −80 °C followed by thawing at room temperature. For all three cycles, the samples were analysed in triplicate and compared to freshly prepared QC samples. The analytes were considered stable if the deviation from initial concentrations was within ±15%.

Results and discussion

This study aimed to develop a rapid and reliable LC-MS/MS method for the quantification of piperacillin, tazobactam, and meropenem in human serum, with sufficient sensitivity to support pharmacokinetic analysis across the full dosing interval, including the low concentrations observed toward the end of a dosing cycle. Method development focused on achieving simultaneous measurement of all three analytes while maintaining short run times, robust peak shapes, and consistent analytical performance.

During method development, multiple chromatographic conditions were evaluated to achieve optimal separation and signal intensity for all analytes. Different organic modifiers, including acetonitrile and methanol, were assessed in combination with water and different buffers containing 0.1% formic acid. Methanol-based mobile phases provided improved peak shape and signal intensity, particularly for tazobactam. Gradient profiles were further optimised to ensure adequate retention and separation of all analytes within a short analytical run time. Key parameters such as column temperature, injection volume, and autosampler conditions were also systematically adjusted to maximise sensitivity and reproducibility. The final conditions were selected based on overall chromatographic performance, including peak symmetry, retention stability, absence of interfering signals, and analyte's response.

This comprehensive optimisation process enabled the identification of chromatographic conditions that resulted in strong, reproducible responses with minimal matrix interference. A weighted $(1/x)$ regression model was applied to ensure accurate fitting across the wide concentration range, particularly at lower concentration levels where precision is most critical for therapeutic drug monitoring.

The method demonstrates a notable improvement in analytical sensitivity, operational simplicity, and clinical applicability compared with previously reported assays.

A key advancement is the exceptionally low LLOQ of 0.02 mg L^{-1} for all analytes, which is substantially lower than values reported for existing UHPLC-MS/MS methods (typically ≥0.1 mg L^{-1}) and far below those achieved using HPLC-UV approaches (≈1 mg L^{-1}).[25,26] To the best of our knowledge, this represents the lowest reported LLOQ for these β-lactam antibiotics, enabling reliable quantification of subtherapeutic concentrations that are increasingly relevant in critically ill populations with altered pharmacokinetics.[27–30]

The chromatographic runtime of 10 min is comparable to established multi-analyte assays, although marginally longer than some optimised methods.[31–33] However, this is offset by the substantial improvement in sensitivity and the absence of carryover, alongside excellent intra- and inter-day precision (<10%), supporting robust high-throughput batch analysis.

Application to >40 clinical samples from critically ill patients confirmed wide interindividual variability in β-lactam exposure, consistent with previous pharmacokinetic studies. Such variability has been linked to difficulty in achieving pharmacodynamic targets against organisms such as *Pseudomonas aeruginosa*, particularly under standard dosing regimens.[27,34] The wide dynamic range and high sensitivity of the present method therefore make it well suited to clinical TDM and dose individualisation in this patient population.

Overall, the proposed method provides a strong balance between analytical sensitivity, simplicity, and clinical utility. While not the fastest reported assay, it offers a superior combination of ultra-low LLOQ and streamlined sample preparation, making it highly suitable for routine implementation in

clinical pharmacokinetic and antimicrobial stewardship programmes.

The following sections describe the full validation results together with the application of the assay to measure antibiotic concentration in clinical serum samples.

Method validation

The method demonstrated acceptable linearity, accuracy, precision, and stability across the validated concentration ranges. Following, %Bias and %CV values are reported.

Selectivity

No significant response was observed at the retention times corresponding to either the target analytes or the internal standard in any of the samples, confirming the absence of interfering matrix components and demonstrating the selectivity of the method under the established chromatographic conditions.

Linearity

Calibration curves for all three analytes showed strong linearity ($R^2 > 0.998$) over the validated ranges (Table S1 and Fig. S7, S8 and S9), indicating excellent proportionality between signal and concentration. Back-calculated concentrations for all calibration standards were within ±15% of nominal values (±20% at the lower limit of quantification), satisfying generally accepted analytical performance criteria. Piperacillin showed %Bias between −7.72% and 6.84% with %CV values up to 7.11%. Tazobactam exhibited higher inaccuracy at low levels (−14.96% Bias), but precision remained within acceptable limits. Meropenem showed excellent linearity, with %Bias between −12.59% and 1.41% and %CV < 3.7%.

The broad calibration range, particularly for piperacillin, was designed to capture the substantial variability in concentrations observed in critically ill patients, including occasional high peak levels following dosing. However, most clinical samples fell within a narrower concentration window, indicating that the extended upper range primarily serves to ensure analytical robustness rather than reflecting typical exposure levels. The use of weighted regression and dilution of samples exceeding the calibration range ensures accurate quantification across clinically relevant concentrations.

LLOQ and ULOQ

For all analytes six replicate measurements yielded a lower limit of quantification of 0.02 mg L^{-1} for meropenem and piperacillin–tazobactam, demonstrating high sensitivity of the method. The upper limit of quantification was 45 000 mg L^{-1} for meropenem and tazobactam, and 135 000 mg L^{-1} for piperacillin (Table S2) indicating a broad linear dynamic range spanning over six orders of magnitude. This wide quantification range supports the applicability of the method across both trace-level and highly concentrated samples without the need for extensive dilution.

Within-run (intra-day) accuracy and precision

Mean accuracy at all QC levels was within ±15% of nominal concentrations. No systematic bias was observed across concentration levels or runs. Within-run precision was consistently ≤15% for all QC levels (Table S3). These findings demonstrate that the method provides reproducible measurement performance across multiple days and independent calibrations, even over the broad, validated dynamic range.

Between-run (inter-day) accuracy and precision

Across all three analytes, the method demonstrated good overall performance, with high precision (%CV 1.07–5.33%) and acceptable accuracy (%Bias within ±15%) for all QC levels. Piperacillin showed the most balanced behaviour, with low variability and minimal, non-directional bias (−7.38% to +5.53%), indicating stable and well-controlled quantification. Tazobactam exhibited generally acceptable precision but showed a slight tendency toward negative bias at several QC levels (down to −9.16%), suggesting minor systematic underestimation. Meropenem showed the greatest deviation from nominal values, with a clear concentration-dependent negative bias (up to −11.54%) despite consistently low %CV (1.45–2.47%), indicating a systematic rather than random analytical effect (Table S4). Overall, the method is precise and reliable for all three compounds, with minor accuracy trends observed particularly for meropenem and tazobactam.

Carryover

Carryover was evaluated by monitoring analyte and internal standard (IS) responses in post-injection blanks relative to the lower limit of quantification (LLOQ).

For piperacillin, the mean analyte response in the post-injection blank represented 9.6% of the LLOQ response, while the IS carryover was negligible at 0.004%. Tazobactam exhibited minimal carryover, with analyte and IS responses of 0.7% and 0.009% of their respective LLOQ signals. For meropenem, analyte carryover was slightly higher at 3.52%, although the IS response remained minimal at 0.0028%. Importantly, for all or three analytes carryover remained well within commonly accepted bioanalytical criteria, not exceeding 20% of the analyte LLOQ response and 5% of the IS response. These findings demonstrate that the method provides adequate control of carryover, ensuring that residual signal does not compromise quantification accuracy in subsequent injections.

Recovery

For piperacillin, mean recovery value was exceeding 100% (mean 119.69%) across QC1, QC5, and QC9, with %CV values between 0.79% and 2.78%. This is likely due to matrix-induced ion enhancement rather than true extraction efficiency. This effect was consistent and corrected using isotopically labelled internal standards, indicating a controlled, systematic matrix effect rather than variability. The results highlight the importance of distinguishing recovery from matrix effects in LC-MS/MS, with internal standards ensuring accurate quantification.

Tazobactam recovery showed a mean value of 89.95% at QC1, QC4, and QC8, with analyte %CVs between 4.02% and 6.06%. Meropenem showed a mean recovery value of 96.74% with analyte %CVs between 2.51% and 3.32%, confirming precise and reproducible recovery even at the lowest concentrations. Collectively, these results demonstrate that the extraction procedure provides consistent, precise, and concentration-independent recovery for all three β-lactam antibiotics, supporting reliable quantification across low, medium, and high QC levels.

Matrix effect

For piperacillin, %Bias ranged from −1.27% to 3.48%, with % CV values between 1.91% and 4.68%. For tazobactam, %Bias ranged from −2.91% to −1.93%, with %CV values between 1.61% and 5.36%. For meropenem, %Bias ranged from −5.31% to −0.06%, with %CV values between 0.68% and 2.93%. All values are well within the ICH M10 acceptance criteria (%Bias ≤ ±15% and %CV ≤ 15% for QC levels, ≤20% at LLOQ), indicating negligible matrix effects (Table S5). These findings confirm that the developed LC-MS/MS method is robust and reliable for accurate and precise quantification of all three antibiotics in aqueous matrices.

Short-term stability

Piperacillin remained stable for up to 24 h at room temperature, in both working and stock solutions, showing %Bias values between −14.8% and 2.69%; stability was fully maintained for 12 h at 6 °C on the autosampler (%Bias of −9.69% and −3.42% for high and low QC, respectively) (Fig. 1a). Tazobactam showed consistently reliable behaviour, with both working and stock solutions remaining stable for at least 24 h at room temperature (%Bias between −4.86% and 12.7%) and for 12 h at 6 °C on the autosampler (%Bias between −8.62% and −4.47%) (Fig. 1b). Meropenem demonstrated shorter solution stability compared with the other analytes: working solutions were stable for 4 h at room temperature (%Bias of −11.73% and 5.46% for low and high QC respectively) but exhibited marked degradation at 24 h, particularly at the lowest concentration (QC8, %Bias −25.1%). Stock solutions of meropenem were similarly stable only at higher concentrations, as QC8 exceeded the ±15% criterion after 24 h at room temperature (%Bias −16.44%) or when stored for 12 h at 6 °C on the autosampler (%Bias −16.91%) (Fig. 1c). Overall, piperacillin and tazobactam displayed robust short-term solution stability across tested conditions, while meropenem required more restrictive handling and shorter benchtop and autosampler residence times to remain within acceptable Bias limits.

Freeze–thaw stability

Stability was considered acceptable when results remained within ±15% Bias and ≤15% CV. Piperacillin showed minimal deviation from nominal concentrations, with QC1 and QC5 exhibiting %Bias values of 2.10% and −0.30% and corresponding precision values of 6.79% and 2.71%, indicating strong stability and reproducibility. Tazobactam displayed greater negative Bias, with QC1 and QC4 yielding −14.21% and −14.88% Bias, respectively, although precision remained within 6% CV, keeping both levels within acceptable accuracy limits. Meropenem showed good stability with %Bias values of −2.60% and 14.58% and precision ≤5.32% CV. Collectively, the findings confirm that the sample handling and storage conditions used in this study are appropriate for bioanalytical workflows requiring multiple freeze–thaw cycles, and that all three compounds meet the freeze–thaw stability acceptance criteria specified in ICH M10 (Table S6).

Clinical application of the LC-MS/MS assay: pharmacokinetic results for meropenem, piperacillin, and tazobactam

The validated LC-MS/MS assay was applied to clinical samples from critically ill patients to quantify total meropenem concentration, and total and unbound piperacillin and tazobactam concentrations across the dosing interval. The clinical dataset presented here is intended to demonstrate the analytical applicability of the developed LC-MS/MS method in real-world patient samples. The study was not designed to evaluate pharmacokinetic/pharmacodynamic relationships or to draw definitive conclusions regarding dosing strategies.

The method proved capable of accurate measurement over a wide dynamic range, enabling reliable detection of high post-infusion concentrations as well as low trough values. Together, the datasets illustrate substantial inter-patient variability in β-lactam exposure, highlighting the importance of direct measurement in settings where pharmacokinetic is highly unpredictable. A PK/PD target of 40–50% fT > MIC has demonstrated effectiveness for penicillin and carbapenems, however higher targets such as 100% fT > MIC are required for critically ill patients.[35,36] We evaluated serum concentrations and target attainment based on 100% fT > MIC, using an MIC of 2 mg L^{-1} for meropenem, and 8 mg L^{-1} for piperacillin. For contextual interpretation only, a reference concentration of 4 mg L^{-1} was used for tazobactam, corresponding to the fixed inhibitor concentration employed in antimicrobial susceptibility testing.[37]

Meropenem. Meropenem demonstrated the expected concentration-time profile, with high post-infusion peaks followed by rapid elimination; however, considerable inter-patient variability was observed (Fig. 2a). These data reflect samples obtained under routine clinical dosing regimens (1 or 2 g three times daily), with dose selection and renal adjustment determined by treating clinicians. When concentrations were visualised relative to a reference MIC of 2 mg L^{-1}, 7 of 25 pre-dose (T3) samples (28%) were below this threshold, indicating potential risk of subtherapeutic exposure in some patients under routine dosing conditions. These observations underscore the potential value of therapeutic drug monitoring, particularly in patients with augmented renal clearance where rapid elimination may compromise sustained exposure.[38]

Piperacillin. Piperacillin displayed pronounced variability, with total concentrations spanning more than two orders of magnitude at each sampling point (Fig. 2b). When concentrations were visualised relative to a reference MIC of 8 mg L^{-1}, 17

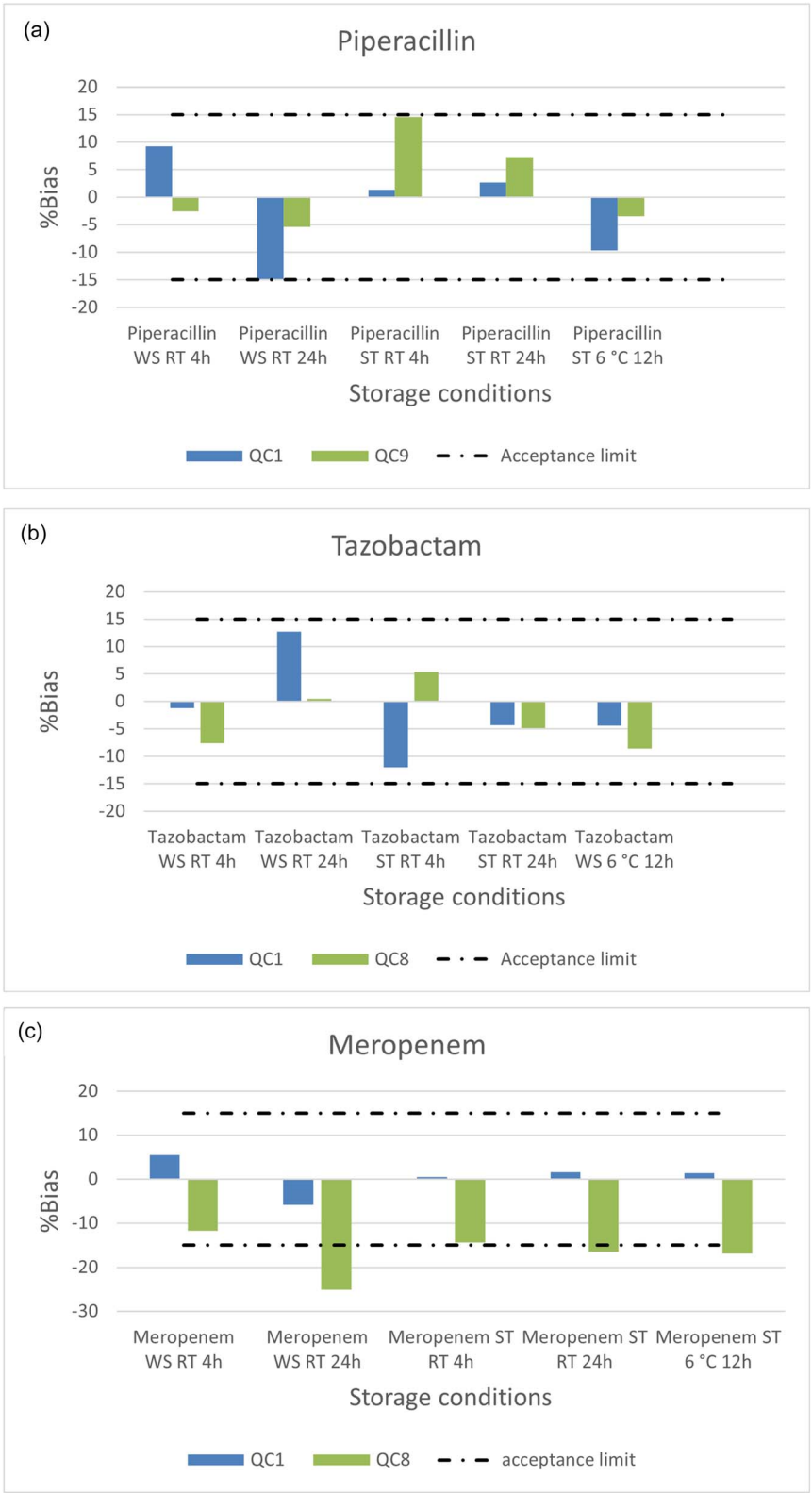

Fig. 1 (a, b, and c): Short-term stability of β-lactam antibiotics. Percentage bias (%Bias) of (a) piperacillin (QC1 and QC9), (b) tazobactam (QC1 and QC8) and (c) meropenem (QC1 and QC8) in working solutions (WS) and stock solutions (ST) following storage at room temperature (RT) for 4 and 24 h, and at 6 °C for 12 h in the autosampler. Dashed lines indicate the acceptance limits (±15%), within which values are considered analytically acceptable.

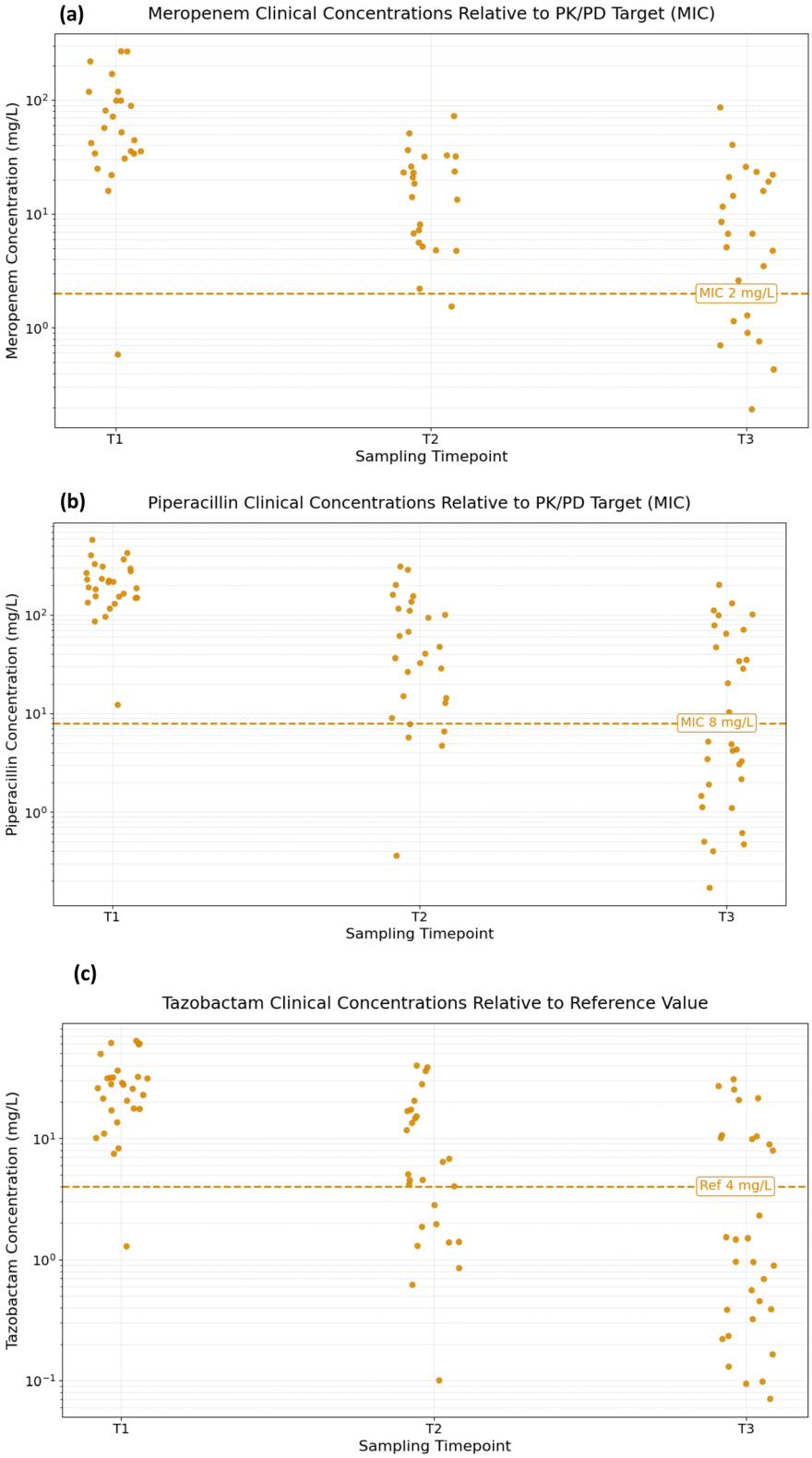

Fig. 2 (a, b, and c): LC-MS/MS-measured β-lactam concentrations in critically ill patients.

of 32 pre-dose (T3) samples (53.1%) were below this threshold, indicating a substantial risk of subtherapeutic exposure. Unbound piperacillin quantification, achieved using ultrafiltration, showed a generally high free fraction (typically 70–90%), though several patients demonstrated divergence between total and unbound levels due to reduced protein binding in critical illness. These findings reinforce the importance of assessing unbound concentrations when interpreting

piperacillin exposure, as total concentrations alone may not reliably represent active drug levels.

Tazobactam. Tazobactam exhibited lower overall concentrations and narrower variability than piperacillin (Fig. 2c). Total and unbound concentrations tracked closely across all timepoints, reflecting its low protein-binding characteristics. The mean unbound fraction across samples was approximately 75%, consistent with rapid elimination and minimal influence of binding on overall exposure.

For contextual interpretation, concentrations were visualised relative to a reference value of 4 mg L^{-1}, corresponding to the fixed tazobactam concentration used in antimicrobial susceptibility testing. As definitive PK/PD targets for tazobactam are not well established, this value should be interpreted with caution and does not represent a validated pharmacodynamic threshold. At the pre-dose (T3) timepoint, 20 of 32 samples (62.5%) were below this reference value, indicating variability in inhibitor exposure at trough.

Interpretation and analytical relevance

Across all three agents, the LC-MS/MS assay provided high-resolution characterisation of β-lactam exposure. The assay's analytical sensitivity and wide dynamic range enabled measurement of concentrations spanning several orders of magnitude, supporting accurate assessment of peak, mid-interval, and trough levels. The combined PK profiles demonstrate that fixed dosing often fails to account for substantial inter-patient variability, particularly in critical care, where renal function, inflammation, fluid status, and organ support therapies can markedly alter drug clearance.

A proportion of samples were observed below commonly used PK/PD targets, illustrating variability in drug exposure and highlighting the potential utility of quantitative measurement. Tazobactam concentrations, although lacking direct PK/PD thresholds, further illustrate the importance of quantifying inhibitor exposure to ensure adequate support for piperacillin activity. Collectively, these data highlight the capability and clinical utility of the LC-MS/MS method for delivering precise, quantitative assessment of β-lactam concentrations and supporting dose optimisation strategies in critically ill patients. Beyond critical care, additional populations that may benefit from β-lactam therapeutic drug monitoring include obese patients, paediatric populations, and patients with augmented renal clearance or renal dysfunction, where pharmacokinetic variability can be pronounced.[30,39]

In summary, this work establishes a single, fully validated LC-MS/MS assay for the simultaneous quantification of piperacillin, tazobactam, and meropenem in human serum with high analytical sensitivity and a wide dynamic range, enabling reliable measurement of both peak and trough concentrations in critically ill patients. To our knowledge, the combination of very high analytical sensitivity with very low serum volume requirements within a single unified workflow has not been reported previously. The clinical component of this study is observational in design and intended to demonstrate the analytical applicability of the method in real-world patient samples. Therefore, the results should be interpreted as an illustration of analytical feasibility rather than evidence of pharmacotherapeutic outcomes.

The analytical performance and clinical applicability of the method support its potential implementation in routine clinical and translational pharmacokinetic studies.

Concentration-time profiles for meropenem, piperacillin, and tazobactam at post-infusion (T1), mid-interval (T2), and pre-dose (T3). Dashed lines represent PK/PD targets (MIC 2 mg L^{-1}, 8 mg L^{-1}, and 4 mg L^{-1} for meropenem, piperacillin, and tazobactam, respectively). All three drugs show marked inter-patient variability, particularly at trough, highlighting the need for therapeutic drug monitoring in critical illness. *Y*-axis is shown on a log 10 scale.

Ethical statement

Ethical approval was granted by the Brighton and Sussex Research Ethics Committee (22/LO/0063). All experiments involving human samples were performed in accordance with relevant institutional guidelines and the principles of the Declaration of Helsinki. Prior informed written consent was provided by all study participants before entry into the study.

Author contributions

Conceptualization: SR, SW, RCW, PA, MJG, AHH, TMR, and AR. Formal analysis: SR and AR. Investigation: SR, SW, RCW, PA, MJG, AHH, TMR, and AR. Methodology: SR and AR. Project administration: TMR, AHH and AR. Validation: SR and AR. Visualization: SR, RCW, TMR and AR. Writing – original draft: SR, RCW, TMR and AR. Writing –review & editing: SR, RCW, AHH, TMR and AR.

Conflicts of interest

T. M. R. received honoraria for consultancy from Sandoz (2020), honoraria for the delivery of educational activities from BioMerieux (2021–2022), and speakers fees from Roche Diagnostics Ltd (2021). All other authors have no potential conflicts of interest to declare.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Supplementary information (SI) is available. See DOI: https://doi.org/10.1039/d6ay00331a.

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