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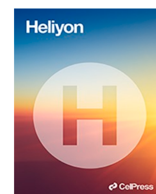

Research article

# A rapid and simple HPLC-MS/MS method for the therapeutic drug monitoring of six special-grade antimicrobials in pediatric patients

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ABSTRACT

Meropenem, linezolid, fluconazole, voriconazole, posaconazole, and vancomycin are six important antimicrobials used for severe infections in critically ill patients listed in special-grade antimicrobials in China. The six antimicrobials' highly variable pharmacodynamics and pharmacokinetics in critically ill pediatric patients present significant challenges to clinicians in ensuring optimal therapeutic targets. Therefore, therapeutic drug monitoring of these antimicrobials in human plasma is necessary to obtain their plasma concentration. A rapid, simple, and sample-saving high-performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) method was developed, which could simultaneously determine all six antimicrobials. It required only 10 μL of plasma and a one-step protein precipitation process. Chromatographic separation was achieved on a reversed-phase column (C18, 30 × 2.1 mm, 2.6 μm) via gradient elution using water and acetonitrile containing 0.1 % formic acid as mobile phase. The injection volume was 2 μL, and the total run time was only 2.5 min. Detection was done using a Triple Quad™ 4500MD tandem mass spectrometer coupled with an electrospray ionization (ESI) source in positive mode. The calibration curves ranged from 0.5 to 64 μg/mL for meropenem and fluconazole, 0.2–25.6 μg/mL for linezolid and voriconazole, 0.1–12.8 μg/mL for posaconazole and 1–128 μg/mL for vancomycin, with the coefficients of correlation all greater than 0.996. Furthermore, the method was validated rigorously according to the European Medicines Agency (EMA) guidelines, demonstrating excellent accuracy (from 93.0 % to 110.6 %) and precision (from 2.0 % to 12.8 %). Moreover, its applicability to various matrices (including serum, hemolytic plasma, and hyperlipidemic plasma) was evaluated. Thus, this method was successfully applied to routine therapeutic drug monitoring for critically ill pediatric patients and other patients in need.

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## 1. Introduction

Serious infection is a widespread clinical problem and represents the most common cause of morbidity and mortality in critical care medicine [1]. Meropenem, linezolid, fluconazole, voriconazole, posaconazole, and vancomycin are six special-grade antimicrobials commonly used for antimicrobial treatment in critically ill patients. Additionally, these drugs are sometimes used in combination to fight serious infections. Notably, the optimal dose of antimicrobials is one of the primary factors for the successful fight against severe infections. However, optimizing antimicrobial dosing is not easy in clinical practice, especially in pediatrics. The pharmacokinetic (PK) characteristics of critically ill patients may change considerably, making the treatment outcomes counterproductive [2–5]. In addition, PK parameters of pediatric patients may vary depending on age-related physiological factors [6]. Hence, an empiric fixed or standard antimicrobial dosing regimen may not be suitable for every intensive care unit (ICU) patient, especially for children. Meanwhile, inapposite plasma-drug concentrations may increase the risk of multiple drug resistance [7] and dangerous toxic side effects [8]. Furthermore, routine therapeutic drug monitoring (TDM) may guide individualized dose adjustment and conduce to maximum therapeutic effect and minimum adverse events produced by drugs [9].

In recent years, liquid chromatography-tandem mass spectrometry (LC–MS/MS) has been used more commonly in TDM due to its high selectivity, sensitivity, and throughput [10]. Some LC–MS/MS assays have also been published to quantify different antimicrobials [11–16]. However, the methods usually combine antimicrobials belonging to the same class, and none of them includes the six analytes simultaneously. Although these six antimicrobials are rarely given to patients simultaneously, in clinical practice, we have found that these drugs are often used in combination with each other to treat different infectious diseases. This means that different methods should be switched frequently in the routine determination to cover the six antimicrobials for TDM. Importantly, the established assay should be simple and have a high throughput to feed drug concentrations back to clinicians on time. Thus, developing a method of simultaneous determination of the above 6 antimicrobials for the TDM program is significant, which enables samples from different patients receiving different antimicrobials to be assayed together at the same time. Additionally, the total blood volume of children is less than that of adults, and their blood collection compliance is poor. Moreover, severely ill children often need frequent blood collection due to the need for treatment. Hence, sample-saving methods will be more friendly to pediatric patients.

This study aimed to develop a simple, fast, sample-saving HPLC–MS/MS method for the simultaneous determination of six special-grade antimicrobials in children's plasma, which are frequently used in severely infected children, including meropenem, linezolid, fluconazole, voriconazole, posaconazole, and vancomycin.

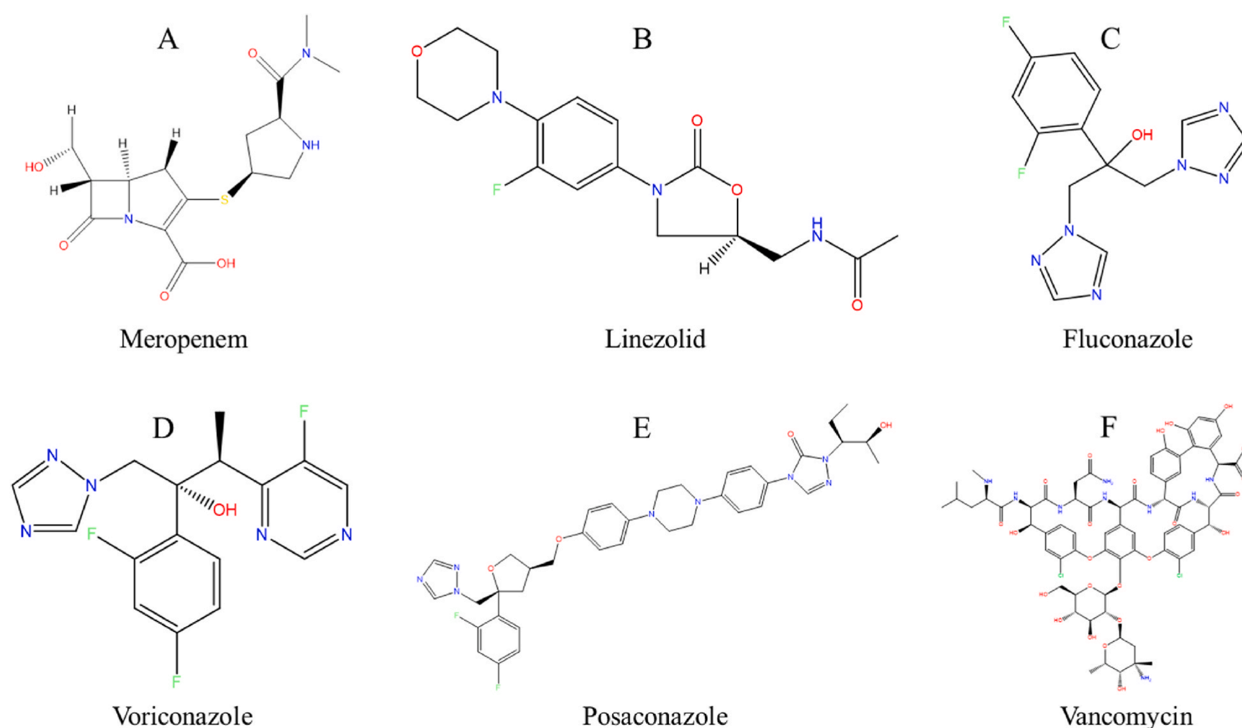


**Fig. 1.** Structural formula diagrams of meropenem (A), linezolid (B), fluconazole (C), voriconazole (D), posaconazole (E), and vancomycin (F).

## 2. Materials and methods

### 2.1. Experimental chemicals and reagents

Meropenem, linezolid, fluconazole, voriconazole, posaconazole, and norvancomycin (IS) were purchased from the National Institutes for Food and Drug Control (Beijing, China). Vancomycin was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Iss, except norvancomycin (meropenem-D6, linezolid-D3, fluconazole-D4, voriconazole-D3, and posaconazole-D4), were purchased from Toronto Research Chemicals (Toronto, Canada). Acetonitrile (ACN) and methanol (MeOH) were supplied by Fisher Scientific (Fairlawn, NJ, USA) in HPLC standard quality. Formic acid (FA, >96 %) for HPLC was purchased from MREDA Technology Inc (USA). Dimethyl sulfoxide (DMSO) was supplied by Tianjin Fuyu Fine Chemical Co., Ltd (Tianjin, China). Deionized water was purified with a Milli-Q IQ® Direct 8 system (Kenilworth, USA). Furthermore, a drug-free biological matrix used for method validation (including normal human plasma, serum, and whole blood) was obtained from Hebei Children's Hospital (Shijiazhuang, China). Structural formula diagrams of meropenem, linezolid, fluconazole, voriconazole, posaconazole and vancomycin are shown in Fig. 1.

### 2.2. Instrumentation and LC-MS/MS conditions

The LC-MS/MS system used for analysis included a Jasper™ HPLC system combined with a Triple Quad™ 4500MD mass spectrometer from AB SCIEX (Framingham, USA). A Genius 1024 nitrogen generator from Peak Scientific (Glasgow, UK) was also used to prepare nitrogen for MS. Analyst 1.6.3 and MultiQuant™ MD 3.0.2 software was used for instrument control, data acquisition, and data processing.

Chromatographic separations of the analytes and corresponding ISs were performed on a Kinetex® EVO C18 column (30 × 2.1 mm, 2.6 μm; Phenomenex, Torrance, CA, USA) using a binary gradient elution of water (A) and ACN (B) both containing 0.1 % (v/v) FA at a flow rate of 0.5 mL/min. The gradient elution program was as follows: 0–0.30 min, 98 % A; 0.30–1.20 min, 98%–5% A; 1.20–1.80 min, 5 % A; 1.80–1.81 min, 5%–98 % A and 1.81–2.50 min, 98 % A. Column and autosampler temperature were maintained at 40 °C and 5 °C, respectively. The sample injection volume was 2.0 μL.

All compounds were detected in positive mode with electrospray ionization and were analyzed using multiple-reaction monitoring (MRM) at an ion spray voltage of 5500 V; curtain gas of 20 psi; collision gas of 9 psi; temperature of 550 °C; ion source gas 1 of 50 psi and ion source gas 2 of 55 psi. The dwell time was also 20 msec for the six antimicrobials and ISs. Compound-specific parameters for each transition are given in Table 1.

### 2.3. Stock solutions, calibration standards, and quality control (QC) samples

Separate stock solutions were prepared by dissolving each powdered antimicrobial in its proper solvent: meropenem, vancomycin,

**Table 1**
Compound-specific instrument parameters.

| Drug | Corresponding IS | Mol wt (g/mol) | Form of ion | Transition (*m/z*) | Declustering potential (DP) (V) | Entrance potential (EP) (V) | Collision energy (CE) (V) | Collision cell exit potential (CXP) (V) |
|---|---|---|---|---|---|---|---|---|
| Meropenem | Meropenem -D6 | 383.5 | $[M+H]^+$ | 384.1 → 141.0 | 64 | 7 | 11 | 15 |
| Linezolid | Linezolid-D3 | 337.4 | $[M+H]^+$ | 338.0 → 296.0 | 96 | 11 | 15 | 20 |
| Fluconazole | Fluconazole-D4 | 306.3 | $[M+H]^+$ | 307.0 → 220.0 | 75 | 9 | 12 | 15 |
| Voriconazole | Voriconazole-D3 | 349.3 | $[M+H]^+$ | 350.0 → 281.1 | 50 | 7 | 6 | 15 |
| Posaconazole | Posaconazole-D4 | 700.8 | $[M+H]^+$ | 701.2 → 683.4 | 100 | 10 | 45 | 15 |
| Vancomycin | Norvancomycin | 1449.2 | $[M+2H]^{2+}$ | 725.5 → 144.2 | 99 | 5 | 19 | 10 |
| Meropenem-D6 | – | 389.5 | $[M+H]^+$ | 390.1 → 147.1 | 80 | 7 | 28 | 15 |
| Linezolid-D3 | – | 340.4 | $[M+H]^+$ | 341.0 → 297.0 | 96 | 9 | 31 | 20 |
| Fluconazole-D4 | – | 310.3 | $[M+H]^+$ | 311.0 → 223.0 | 75 | 9 | 25 | 15 |
| Voriconazole-D3 | – | 352.3 | $[M+H]^+$ | 353.2 → 284.0 | 50 | 7 | 15 | 15 |
| Posaconazole-D4 | – | 704.8 | $[M+H]^+$ | 705.2 → 687.4 | 100 | 10 | 48 | 15 |
| Norvancomycin | – | 1435.2 | $[M+2H]^{2+}$ | 718.5 → 144.2 | 100 | 5 | 20 | 3 |

meropenem-D6, and norvancomycin were dissolved in water/MeOH (1:1; v/v); fluconazole, voriconazole, posaconazole, fluconazole-D4, voriconazole-D3, and posaconazole-D4 were dissolved in MeOH; linezolid, and linezolid-D3 were dissolved in DMSO. Stock solution concentrations were prepared at 5 mg/mL (meropenem, linezolid, fluconazole, voriconazole, vancomycin and norvancomycin) and 1 mg/mL (posaconazole, meropenem-D6, linezolid-D3, fluconazole-D4, voriconazole-D3, posaconazole-D4). Furthermore, all prepared stock solutions were stored in 1 mL aliquots at −80 °C. Subsequently, the working solution (0.64 mg/mL for meropenem and fluconazole, 0.256 mg/mL for linezolid and voriconazole, 0.128 mg/mL for posaconazole, 1.28 mg/mL for vancomycin) was prepared by diluting the stock solutions in MeOH. Calibration standards and QC samples for all six compounds were also prepared using the appropriate working solution spiked with blank plasma, and exact concentrations are provided in Table 2.

Hemolytic plasma was prepared as follows: whole blood was fully frozen at −80 °C for 1 h and vortexed for 30 s after being thawed to obtain fully hemolytic plasma. Full hemolytic plasma was added to nonhemolytic blank plasma at volume ratios of 5:95 and 2:98 and then mixed well to simulate hemolytic human plasma. QC samples in hemolytic plasma were treated as mentioned above.

For hyperlipidemic plasma, 20 % medium/long-chain fat emulsion injection and blank human plasma were mixed evenly in a ratio of 1:9 (v/v) to simulate hyperlipidemic human plasma with triglycerides >300 mg/dL. QC samples in hyperlipidemic plasma were also prepared, as described above.

ISs working solution was prepared by diluting IS stock solutions in MeOH to obtain final concentrations of 4 μg/mL for meropenem-D6, fluconazole-D4, and voriconazole-D3, 2 μg/mL for linezolid-D3, 0.4 μg/mL for posaconazole-D4, 15 μg/mL for norvancomycin, respectively.

### 2.4. *Sample processing*

A 10 μL ISs working solution was added to a 10 μL aliquot plasma sample (patient samples, calibration standards, quality controls). Double-blank samples were prepared by spiking 10 μL of blank plasma with 10 μL of methanol, and blank samples were prepared by spiking blank plasma with 10 μL of IS working solutions. Samples were vortexed for 30 s, and 30 μL of methanol was added for protein precipitation. Then, the mixture was vortexed for 1 min and centrifuged at 13,680 *g* for 10 min at 4 °C. Following this, the supernatant of each sample was transferred to LC-MS vials for analysis.

### 2.5. *Method validation*

Validation procedures were carried out according to EMA guidelines for bioanalytical method validation [17] in terms of selectivity, LLOQ, calibration curve, carry-over, accuracy, precision, dilution integrity, matrix effect, stability, evaluation of the applicability to various matrices (including serum, hemolytic plasma, and hyperlipidemic plasma), and incurred samples reanalysis.

#### 2.5.1. *Selectivity*

The method's selectivity was ascertained by analyzing six different sources of blank samples (blank plasma, hemolytic plasma, hyperlipidemic plasma, and serum). The chromatograms of the blank samples were compared to those of the lower limit of quantification (LLOQ) standards. The response of the interfering substances should generally be less than 20 % of the LLOQ of the target compound and less than 5 % of the response of the IS.

#### 2.5.2. *LLOQ, calibration curve, and carry-over*

The LLOQ is the lowest calibration standard. The analyte signal of the LLOQ sample should be at least 5 times the signal of a blank sample. A standard curve was plotted by plotting the peak area ratio of IS against the corresponding analyte concentration in three sets of calibration curves run on three different days. Different regression modes, in combination with different weighting approaches, were tested to select the best fit for the data. Back-calculated concentrations should be within 15 % of the nominal value for all calibrators and within 20 % for the LLOQ. Carry-over was assessed by injecting blank samples after the upper limit of quantification (ULOQ) to ensure that it did not influence precision and accuracy. Carry-over should not exceed 20 % of the peak area at the LLOQ and 5 % of the peak area of the IS.

#### 2.5.3. *Within-run and between-run accuracy and precision*

Accuracy and precision were determined by analyzing the LLOQ and three QC levels at low, medium, and high concentrations for

**Table 2**
Concentrations of calibrators and QCs (in μg/mL).

| Drug | Calibration Curve | | | | | | | | QC | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | LLOQ | Low | Medium | High |
| Meropenem | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 0.5 | 1.5 | 6 | 48 |
| Linezolid | 0.2 | 0.4 | 0.8 | 1.6 | 3.2 | 6.4 | 12.8 | 25.6 | 0.2 | 0.6 | 2.4 | 19.2 |
| Fluconazole | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 0.5 | 1.5 | 6 | 48 |
| Voriconazole | 0.2 | 0.4 | 0.8 | 1.6 | 3.2 | 6.4 | 12.8 | 25.6 | 0.2 | 0.6 | 2.4 | 19.2 |
| Posaconazole | 0.1 | 0.2 | 0.4 | 0.8 | 1.6 | 3.2 | 6.4 | 12.8 | 0.1 | 0.3 | 1.2 | 9.6 |
| Vancomycin | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 1 | 3 | 12 | 96 |

three days. A new calibration curve was prepared for each batch for quantification. For investigation of within-run (n = 5) and between-run (n = 15) accuracy, the mean concentration should be within 15 % of the nominal values for the QCs, except for the LLOQ, which should be within 20 % of the nominal value. For evaluation of within-run (n = 5) and between-run (n = 15) precision, the coefficient of variation (CV) value should not exceed 15 % for the QCs, except for the LLOQ, which should not exceed 20 %.

#### *2.5.4. Dilution integrity*

Dilution integrity was demonstrated by 4-, 8-fold dilution of the spiked samples above ULOQ (128 μg/mL for meropenem and fluconazole; 51.2 μg/mL for linezolid and voriconazole; 25.6 μg/mL for posaconazole; 256 μg/mL for vancomycin) in replicates of five. Furthermore, accuracy and precision should be within ±15 %.

#### *2.5.5. Matrix effect*

The matrix effect was evaluated using six biological plasma matrices from different sources, including 2 % hemolytic and hyperlipidaemic plasma samples. The matrix effect factor (MF) was calculated by comparing the peak areas of six analytes (at QC low and QC high) existing in the extracted blank plasma with the peak areas of six compounds diluted in a pure solution in the corresponding concentration. Moreover, the IS-normalized MF was calculated by the ratio of $MF_{analyte}$ and the appropriate $MF_{IS}$. The CV of the IS-normalized MF should not be greater than 15 %.

#### *2.5.6. Stability*

Stability was extensively investigated using low and high QC samples (n = 4) in different conditions for all analytes. To study the short-term stability of the analytes, QCs were maintained unprocessed at room temperature (RT) for 4 h and at 4 °C for 72 h. For post-extracted stability (autosampler stability), four samples for each QC level were stored in the autosampler at 5 °C for 6 h. Freeze-thaw stability was also evaluated on spiked plasma samples after 3 freeze-thaw cycles (−80 °C to ambient temperature), and QC samples were frozen for at least 12 h between cycles. Furthermore, long-term stability covering 4 weeks of storage at −80 °C was also estimated. For all the above tests, stability was claimed if the mean concentration at each level was within ±15 % of the nominal concentration. Stability of stock and working solutions was tested at −80 °C for 3 weeks. For this evaluation, the comparison was made between the results from the fresh solutions and the stored solutions, and the differences should be within ±15 %.

For whole-blood stability of six antimicrobials, samples were prepared in drug-free human whole blood collected in $K_2$-EDTA tubes with low and high QC levels for each analyte. They were determined at RT at 4 different time points (15 min and 1, 2, and 4 h). To allow for adequate balance, the tube containing spiked whole blood was gently inverted 10 times and kept on the bench for 15 min. Subsequently, plasma was separated by centrifugation at 1610 *g* for 5 min (4 °C) at each presupposed time point and then assayed in four replicates. Acceptance criteria were defined as the mean IS-normalized peak areas for each analyte at 1, 2, and 4 h, consistent with the mean area ratio at 15 min (not exceeding 15 %).

#### *2.5.7. Evaluation of applicability to serum, hemolytic plasma, and hyperlipidaemic plasma*

Bioanalytical methods are generally developed, optimized, and validated for the same matrix. In severe patients, due to the influence of pathology, drugs, etc., the blood sometimes presents abnormalities inevitably, such as hemolysis and hyperlipemia [18]. To confirm the applicability of this method, QCs (LQC, MQC, and HQC) samples were prepared in 2 % hemolyzed plasma, 5 % hemolyzed plasma, hyperlipidaemic plasma, and serum according to D'Cunha et al. [19]. Furthermore, replicate analyses (n = 15) of three different concentrations of QCs from three runs were analyzed on two separate days to confirm between-run accuracy, and precision. The acceptance criteria require accuracy and the CV % should be within ±15 %.

#### *2.5.8. Incurred samples reanalysis*

Forty-three previously analyzed patient samples were randomly selected and reanalyzed separately on different days to perform the incurred samples reanalysis (ISR). All of the selected samples were stored at −80 °C until reanalysis. Moreover, the concentration obtained for the initial analysis and the concentration obtained by reanalysis should be less than 20 % of their mean concentration for at least 67 % of the repeats.

### *2.6. Analysis of patient samples*

Clinical samples were collected from pediatric patients receiving anti-infective treatment for severe infections in Hebei Children's Hospital between December 2022 and June 2023. Blood samples were collected only after informed consent was obtained from all patients, and this study was approved by the Medical Research Ethics Committee of Hebei Children's Hospital (No. YYLS2020-11). Blood samples were collected in $K_2$-EDTA tubes and immediately centrifuged at 1610 *g* for 5 min. The extracted plasma was then stored at −80 °C before being assayed.

## 3. Results and discussion

### *3.1. Method development*

#### *3.1.1. MS/MS analysis*

The six antimicrobials and ISs showed a higher response in the positive ion mode with the forms of $[M+H]^+$ (all analytes except for

vancomycin and norvancomycin) and $[M+2H]^{2+}$ (for vancomycin and norvancomycin), in accordance with previous investigations [12,14,20]. Declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) of most ions were optimized to obtain optimal ion response (Table 1). DPs and CEs of meropenem, linezolid, fluconazole, voriconazole and posaconazole were reduced appropriately from the optimal values in order to acquire proper responses. The dwell time of each analyte was 20 msec to have at least 15 points per peak.

*3.1.2. Chromatographic separation*

Chromatographic conditions were optimized for a good symmetrical peak shape and appropriate retention times. Compared with MeOH, ACN was more suitable as mobile phase B under the gradient elution mode in this study. Some studies indicated that adding of

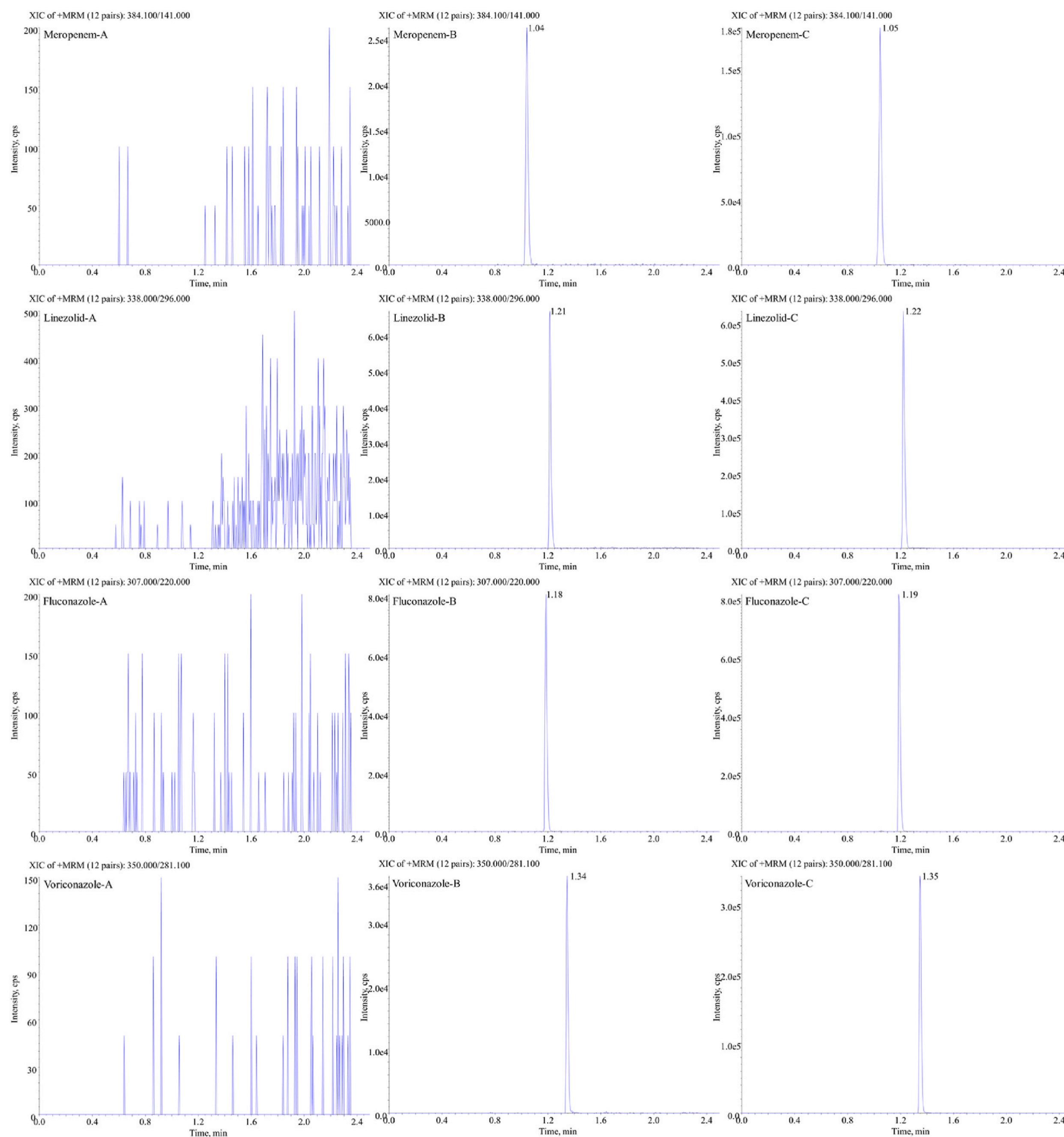


**Fig. 2.** Representative chromatograms of six analytes and ISs in human plasma: (A) blank plasma sample; (B) blank plasma sample spiked with analytes (LLOQ); (C) clinical plasma sample.

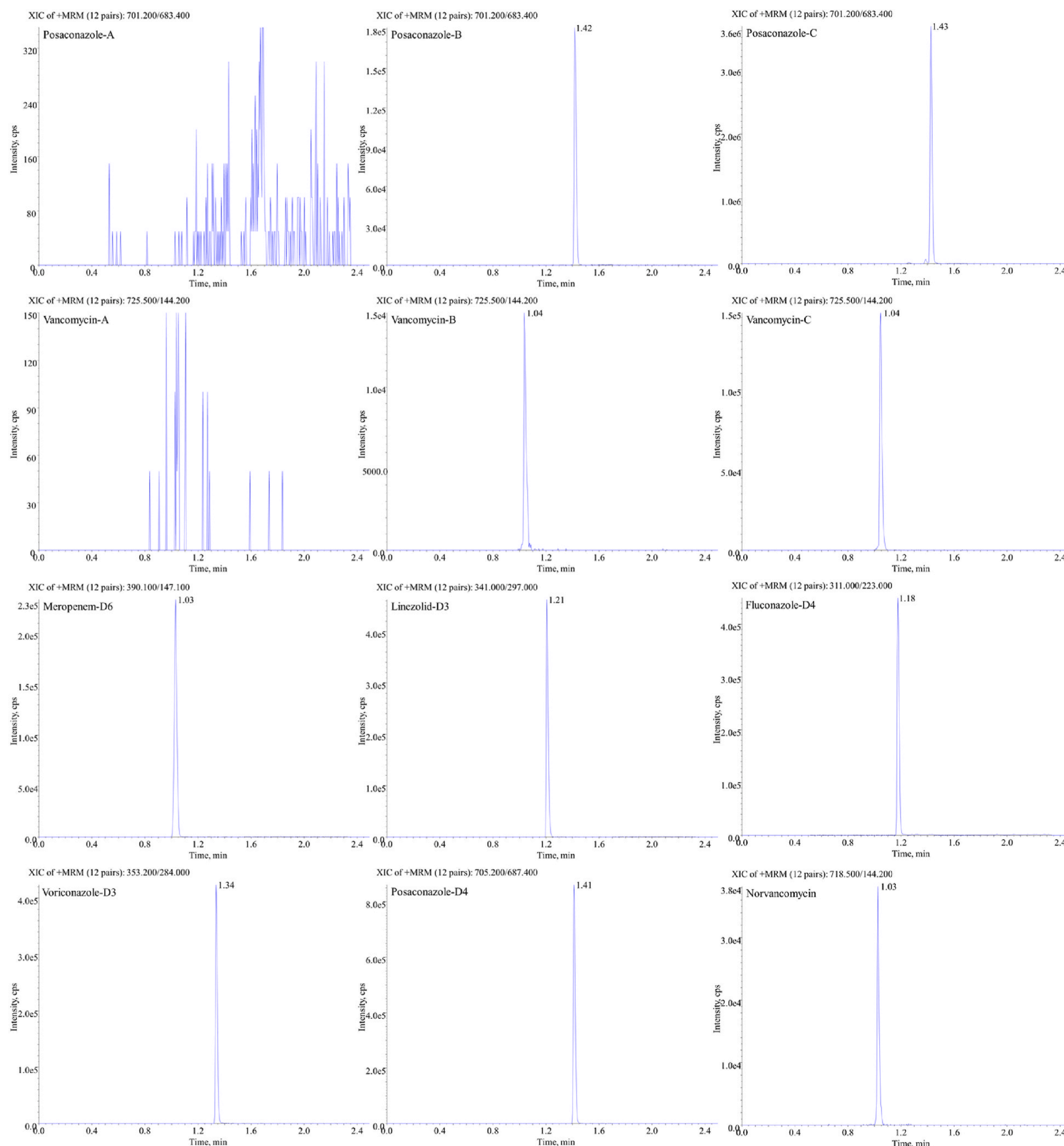


**Fig. 2.** (*continued*).

0.1 % FA could improve peak shapes, enhance the ionization efficiency of analytes, and reduce degradation for some β-lactams, particularly for meropenem [21,22]. Hence, 0.1 % FA was used as an additive in the mobile phase. Stabilizers (e. g. MES) were added in biological samples to carbapenems' stability, since carbapenems are universally unstable, especially at room temperature [23–25]. However, Lefeuvre [26] reported that MES buffer would strongly clog the mass spectrometer's ion source, requiring recurring and time-consuming cleaning. This assay was successfully executed without any stabilizing buffers.

Protein precipitation was applied in this method due to its time-saving and easy operation. According to previous reports of percent protein precipitation, 10 % (w/v) trichloroacetic acid (TCA) in a 0.5:1 ratio, MeOH in a 2:1 ratio, and ACN in a 2:1 ratio showed comparable protein precipitation efficiency [27,28]. However, it was demonstrated that 10%TCA affected the stability of the meropenem [27]. Due to one-step protein precipitation, MeOH/ACN accounted for a high proportion of the supernatant. It also showed that ACN had a pronounced solvent effect when used as a protein precipitant, seriously affecting the peak shape of vancomycin and

meropenem. Thus, MeOH was chosen as a protein precipitant in this assay.

Sufficient re-equilibration is necessary to make the chromatogram repeatable. Notably, the short column requires a shorter re-equilibration time. Therefore, a Kinetex® EVO C18 column (30 × 2.1 mm, 2.6 μm) was applied in our study, and the column provided excellent peak shapes for all analytes in only 2.5 min.

Representative chromatograms are shown in Fig. 2. The retention times of meropenem, linezolid, fluconazole, voriconazole, posaconazole, and vancomycin were 1.04, 1.21, 1.18, 1.34, 1.42 and 1.04 min, respectively. Complete chromatographic separation was not necessary for MS/MS analysis in MRM mode since each compound's ion transitions were unique.

## 3.2. *Method validation*

### 3.2.1. *Selectivity*

No interference was found in the blank (Fig. 2-A), spiked (Fig. 2-B), and patient (Fig. 2-C) samples at the retention time windows for each compound.

### 3.2.2. *LLOQ, calibration curve, and carry-over*

The LLOQ was 0.5 μg/mL for meropenem and fluconazole, 0.2 μg/mL for linezolid and voriconazole, 0.1 μg/mL for posaconazole and 1 μg/mL for vancomycin. The S/N ratio at the LLOQ for all compounds was much greater than 5:1. The presupposed calibration curves for meropenem, linezolid, and fluconazole were established by weighted ($1/x^2$) linear regression analysis. The calibration curves showed that quadratic regression with $1/x^2$ best described the data set generated for voriconazole, posaconazole, and vancomycin in human plasma. We hypothesized that the MS/MS detector was very sensitive to this phenomenon, and its response evolved rapidly between very low and high concentrations. On the other hand, we may have aimed for large calibration ranges for all analytes. Likewise, some studies have successfully applied quadratic regression models with excellent results [11,19,27]. This study's mean regression coefficients (*r*) for all standard curves were greater than 0.996. All back-calculated concentrations were in accord with the accepted criteria. Details of LLOQ and linearity are summarized in Table 3. Blank plasma samples injected after the ULOQ showed inapparent signals for all analytes and their corresponding ISs.

### 3.2.3. *Accuracy, precision*

Within-run and between-run accuracy and precision for LLOQ and QC samples are presented in Table 4. Notably, results were within the accepted criteria for all antimicrobials. This demonstrates that this assay possesses satisfactory accuracy and precision.

### 3.2.4. *Dilution integrity*

The CV and bias values of the 4- and 8-fold diluted samples were less than 6.2 % and 11.3 %, respectively. This finding illustrates that if the sample concentrations are higher than the ULOQ, the samples can be 4- fold or 8-fold diluted and quantified accurately.

### 3.2.5. *Matrix effect*

The data for the IS-normalized matrix effect is presented in Table 4. There was no notable variation among the six batches of plasma (including 2 % hemolytic and hyperlipidaemic plasma) with less than 8.4 % CV of the mean matrix effect for each compound. These findings suggest that ion enhancement or suppression from normal, 2 % hemolytic, and hyperlipidaemic plasma is negligible for the six analytes with the current method.

### 3.2.6. *Stability*

Table 5 summarizes the percent accuracy of stability for QCs under different storage conditions. In summary, all antimicrobials in QC samples were stable under the conditions tested, with the percent accuracy and CV falling in the acceptable ranges. The whole-

**Table 3**
Summary of standard curves.

| Drug | Linear equation | | Quadratic equation | | | *r* | Range |
|---|---|---|---|---|---|---|---|
| | Slope (mean ± SD) | Intercept (mean ± SD) | Quadratic coefficient (mean ± SD) | Linear coefficient (mean ± SD) | Intercept (mean ± SD) | (mean ± SD) | (μg/mL) |
| Meropenem | 0.18626 ± 0.00297 | 0.00203 ± 0.00045 | – | – | – | 0.99859 ± 0.00067 | 0.5–64 |
| Linezolid | 0.69905 ± 0.02010 | 0.02049 ± 0.00496 | – | – | – | 0.99803 ± 0.00095 | 0.2–25.6 |
| Fluconazole | 0.28317 ± 0.00937 | 0.00967 ± 0.00349 | – | – | – | 0.99892 ± 0.00048 | 0.5–64 |
| Voriconazole | – | – | −0.00431 ± 0.00025 | 0.36423 ± 0.00334 | 0.01093 ± 0.00074 | 0.99914 ± 0.00020 | 0.2–25.6 |
| Posaconazole | – | – | −0.05616 ± 0.00607 | 2.82951 ± 0.18512 | −0.00176 ± 0.00254 | 0.99915 ± 0.00051 | 0.1–12.8 |
| Vancomycin | – | – | −0.00117 ± 0.00013 | 0.44040 ± 0.01912 | 0.04015 ± 0.04406 | 0.99689 ± 0.00175 | 1–128 |

**Table 4**
Summary of accuracy, precision and matrix effect.

| Drug | Concentration (μg/mL) | Within-run (n = 5) | | Between-run (n = 15) | | Matrix factor | |
|---|---|---|---|---|---|---|---|
| | | Mean accuracy (%) | CV (%) | Mean accuracy (%) | CV (%) | Mean (%) | CV (%) |
| Meropenem | 0.5 | 101.0 | 4.1 | 103.1 | 3.7 | – | – |
| | 1.5 | 99.0 | 4.8 | 100.6 | 4.5 | 109.3 | 4.2 |
| | 6 | 101.4 | 5.3 | 100.9 | 5.0 | – | – |
| | 48 | 103.9 | 2.7 | 103.5 | 2.3 | 101.8 | 4.4 |
| Linezolid | 0.2 | 103.5 | 5.5 | 102.8 | 8.2 | – | – |
| | 0.6 | 103.9 | 2.0 | 103.0 | 4.3 | 110.0 | 3.7 |
| | 2.4 | 107.6 | 3.2 | 105.5 | 6.6 | – | – |
| | 19.2 | 99.2 | 4.0 | 100.1 | 6.6 | 103.3 | 6.3 |
| Fluconazole | 0.5 | 99.4 | 4.3 | 99.6 | 4.7 | – | – |
| | 1.5 | 104.5 | 2.8 | 103.2 | 4.4 | 104.5 | 6.7 |
| | 6 | 100.7 | 3.6 | 101.6 | 3.6 | – | – |
| | 48 | 97.9 | 3.6 | 98.4 | 4.8 | 98.0 | 4.4 |
| Voriconazole | 0.2 | 99.2 | 6.4 | 97.6 | 5.4 | – | – |
| | 0.6 | 103.1 | 7.0 | 101.6 | 4.9 | 95.9 | 7.5 |
| | 2.4 | 101.1 | 4.3 | 101.0 | 3.3 | – | – |
| | 19.2 | 107.1 | 4.6 | 104.3 | 6.4 | 93.3 | 4.1 |
| Posaconazole | 0.1 | 106.5 | 5.5 | 99.9 | 7.2 | – | – |
| | 0.3 | 93.0 | 5.3 | 97.0 | 4.8 | 100.1 | 6.6 |
| | 1.2 | 95.3 | 6.6 | 97.5 | 5.0 | – | – |
| | 9.6 | 100.2 | 6.1 | 98.2 | 4.6 | 94.8 | 2.8 |
| Vancomycin | 1 | 104.5 | 12.8 | 103.6 | 12.5 | – | – |
| | 3 | 110.6 | 7.9 | 105.2 | 7.8 | 104.8 | 8.4 |
| | 12 | 102.1 | 7.1 | 99.8 | 8.4 | – | – |
| | 96 | 105.5 | 11.4 | 99.6 | 9.9 | 107.0 | 3.7 |

**Table 5**
Stability for QCs. Values represent mean % accuracy (% CV), n = 4.

| Drug | QC level | Room temperature for 4 h | 4 °C for 72 h | −80 °C for 4 week | Autosampler stability | Freeze-thaw Stability |
|---|---|---|---|---|---|---|
| Meropenem | Low | 90.8 (6.6) | 91.7 (1.2) | 100.6 (7.0) | 105.8 (2.8) | 96.9 (7.5) |
| | High | 96.2 (3.7) | 90.9 (1.9) | 106.9 (6.1) | 104.3 (6.4) | 105.8 (8.6) |
| Linezolid | Low | 96.9 (2.3) | 93.1 (5.0) | 101.6 (2.7) | 108.9 (1.9) | 95.7 (4.5) |
| | High | 92.3 (3.8) | 93.6 (2.4) | 96.7 (3.7) | 102.0 (3.4) | 93.9 (2.1) |
| Fluconazole | Low | 95.3 (4.1) | 96.2 (6.8) | 96.2 (7.0) | 101.7 (5.7) | 94.6 (3.7) |
| | High | 95.1 (1.9) | 91.4 (5.3) | 98.2 (1.7) | 99.2 (2.2) | 95.6 (1.7) |
| Voriconazole | Low | 96.1 (4.9) | 92.3 (4.4) | 99.2 (5.0) | 99.6 (4.1) | 95.7 (2.3) |
| | High | 96.4 (2.8) | 94.9 (5.1) | 99.1 (1.6) | 94.3 (5.6) | 94.0 (1.2) |
| Posaconazole | Low | 89.4 (4.2) | 99.3 (5.1) | 98.9 (6.7) | 90.0 (7.0) | 96.8 (8.8) |
| | High | 95.1 (1.4) | 89.9 (3.0) | 94.0 (1.9) | 93.6 (3.5) | 94.1 (0.9) |
| Vancomycin | Low | 97.4 (10.2) | 94.8 (6.9) | 107.0 (4.6) | 93.5 (11.7) | 98.4 (7.1) |
| | High | 89.3 (5.8) | 95.3 (5.9) | 96.3 (3.1) | 88.5 (5.7) | 102.5 (11.3) |

**Table 6**
Whole blood stability. Unacceptable values are typed in bold and underline.

| Drug | QC level | 15 min ($T_0$) | 1 h ($T_1$) | | 2 h ($T2$) | | 4 h ($T3$) | |
|---|---|---|---|---|---|---|---|---|
| | | CV (%) | Difference from $T_0$ (%) | CV (%) | Difference from $T_0$ (%) | CV (%) | Difference from $T_0$ (%) | CV (%) |
| Meropenem | Low | 3.8 | −4.6 | 3.7 | −9.9 | 11.6 | **<u>−18.4</u>** | 7.1 |
| | High | 2.8 | −1.9 | 2.0 | −2.1 | 1.3 | 0.1 | 3.0 |
| Linezolid | Low | 6.8 | −2.3 | 3.9 | −3.9 | 1.3 | 5.3 | 5.8 |
| | High | 5.0 | 5.0 | 1.9 | 10.4 | 3.1 | 10.9 | 2.9 |
| Fluconazole | Low | 2.4 | −0.7 | 5.4 | −0.9 | 5.8 | −1.2 | 4.3 |
| | High | 3.0 | −2.0 | 2.2 | 1.1 | 2.4 | 0.7 | 2.5 |
| Voriconazole | Low | 4.9 | −5.7 | 3.6 | −1.5 | 3.7 | −4.7 | 4.0 |
| | High | 3.0 | −9.1 | 4.2 | −3.8 | 3.7 | −6.7 | 4.5 |
| Posaconazole | Low | 2.7 | −2.2 | 1.4 | −2.4 | 2.0 | −10.6 | 3.7 |
| | High | 2.7 | −0.5 | 2.0 | −1.7 | 4.1 | −3.9 | 1.6 |
| Vancomycin | Low | 7.0 | 3.8 | 10.3 | 8.5 | 12.4 | 4.5 | 2.3 |
| | High | 11.3 | −0.7 | 9.2 | −6.5 | 2.7 | 0.9 | 6.0 |

blood samples of all drugs were also stable for 4 h except meropenem, which should be sent to the laboratory within 2 h after blood collection according to the stability results (Table 6). Stock and working solutions were stable for at least 3 weeks at −80 °C (absolute values of differences ≤9.4 %).

#### 3.2.7. Evaluation of applicability to serum, hemolytic, and hyperlipidaemic plasma

The data of applicability evaluation for serum, hemolytic, and hyperlipidaemic plasma is displayed in Table 7. It shows that this assay is appropriate for the determination of the six analytes in normal plasma and serum, hemolytic plasma, and hyperlipidaemic plasma. One thing to note is that the hemolysis degree, measured by colorimetry [29], should be no more than 2 % when collecting blood samples containing linezolid because linezolid in 5 % hemolytic plasma showed more than 120 % accuracy. Thus, we speculate that endogenous interferences enhanced the ionization efficiency of linezolid in 5 % hemolytic plasma.

#### 3.2.8. Incurred samples reanalysis

The concentration obtained for the initial analysis and the concentration obtained by reanalysis were less than 14.7 % of their mean concentration for all 20 selected samples, which is within the EMA guideline limits.

### 3.3. Analysis of patient samples

A total of 54 samples, including multiple antimicrobial drug combinations and the concentration of 22 meropenem, 17 linezolid, 3 fluconazole, 12 voriconazole, 17 posaconazole, and 25 vancomycin, were determined using the HPLC–MS/MS method described above. Among them, drug combinations were present in 32 samples. All TDM samples were collected before the next dose at a steady state ($C_{min}$). The recommended concentration ranges for meropenem, linezolid, fluconazole, voriconazole, posaconazole, and vancomycin were fT > MIC100 % [30], 2.0–7.0 μg/mL [15], at least 1.9–6.7 μg/mL [31], 1.0–5.5 μg/mL [15], 1–6 μg/mL [31], 10.0–20.0 μg/mL [12], respectively. As illustrated in Table 8, $C_{min}$ of 6 special-grade antimicrobials varied considerably among individuals and many $C_{min}$ values were outside the recommended concentration ranges, including 36.4 % (8/22) of meropenem, 64.7 % (11/17) of linezolid, 33.3 % (1/3) of fluconazole, 66.7 % (8/12) of voriconazole, 41.2 % (7/17) of posaconazole, and 68.0 % (17/25) of vancomycin samples. Then, these results were provided to help clinicians and clinical pharmacists formulate individualized TDM-guided dosage regimen for each patient. After using individualized TDM-guided dosage regimen, the majority of patients achieved a target concentration compared with the standard dosing regimen. All above demonstrate that routine TDM of these six drugs is essential for optimizing drug therapy and minimizing adverse effects in clinical trials.

### 3.4. Comparison with reported methods

Several LC–MS/MS methods have been published to determine different antibacterials, summarized in Table 9 and compared with our method. Most reported methods usually targeted the same class of antimicrobials, e.g., beta-lactams or azoles [11–16]. This means

**Table 7**
Applicability to serum, hemolytic plasma and hyperlipidaemic plasma (n = 15). Unacceptable values are typed in bold and underline.

| Drug | Concentration (μg/mL) | Serum | | 2 % hemolytic plasma | | 5 % hemolytic plasma | | hyperlipidaemic plasma | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean accuracy (%) | CV (%) | Mean accuracy (%) | CV (%) | Mean accuracy (%) | CV (%) | Mean accuracy (%) | CV (%) |
| Meropenem | 0.5 | 98.8 | 4.6 | 92.1 | 10.3 | 112.8 | 6.2 | 101.1 | 6.3 |
| | 1.5 | 93.6 | 5.0 | 95.0 | 4.8 | 106.3 | 5.8 | 101.5 | 5.6 |
| | 6 | 94.7 | 4.1 | 95.9 | 5.3 | 103.8 | 4.9 | 101.1 | 4.3 |
| | 48 | 100.3 | 4.3 | 99.9 | 5.8 | 107.2 | 3.4 | 107.8 | 5.4 |
| Linezolid | 0.2 | 101.4 | 6.7 | 91.9 | 9.4 | **<u>134.0</u>** | 8.8 | 97.5 | 6.1 |
| | 0.6 | 102.7 | 4.0 | 98.0 | 5.9 | **<u>127.3</u>** | 10.1 | 97.7 | 5.3 |
| | 2.4 | 105.6 | 6.3 | 102.2 | 3.9 | **<u>126.5</u>** | 6.0 | 103.8 | 11.2 |
| | 19.2 | 104.5 | 6.3 | 104.2 | 8.5 | **<u>123.9</u>** | 6.8 | 98.6 | 9.5 |
| Fluconazole | 0.5 | 99.2 | 6.0 | 93.3 | 4.4 | 105.7 | 5.1 | 93.0 | 7.3 |
| | 1.5 | 100.6 | 5.4 | 90.8 | 3.8 | 105.9 | 6.3 | 94.8 | 4.3 |
| | 6 | 102.3 | 3.4 | 92.5 | 4.1 | 106.2 | 3.9 | 100.9 | 4.2 |
| | 48 | 102.6 | 3.5 | 94.5 | 3.7 | 101.8 | 3.4 | 99.8 | 5.7 |
| Voriconazole | 0.2 | 90.4 | 7.6 | 85.6 | 7.9 | 98.5 | 9.3 | 91.1 | 8.2 |
| | 0.6 | 93.2 | 5.1 | 88.7 | 5.3 | 99.1 | 6.2 | 95.3 | 4.2 |
| | 2.4 | 95.1 | 4.2 | 87.9 | 2.6 | 99.9 | 4.8 | 98.4 | 4.2 |
| | 19.2 | 99.6 | 4.8 | 96.3 | 4.9 | 100.6 | 5.3 | 101.2 | 6.7 |
| Posaconazole | 0.1 | 108.4 | 3.8 | 89.5 | 7.6 | 106.4 | 4.1 | 108.0 | 3.8 |
| | 0.3 | 103.8 | 2.9 | 89.0 | 3.4 | 101.2 | 4.3 | 104.7 | 4.9 |
| | 1.2 | 105.4 | 5.2 | 91.6 | 3.8 | 102.7 | 5.3 | 106.8 | 3.7 |
| | 9.6 | 109.4 | 3.8 | 92.7 | 4.5 | 102.4 | 3.9 | 101.8 | 5.9 |
| Vancomycin | 1 | 94.1 | 9.0 | 98.3 | 10.6 | 115.1 | 11.8 | 99.3 | 12.9 |
| | 3 | 101.3 | 7.7 | 95.6 | 5.1 | 107.7 | 8.1 | 101.7 | 6.6 |
| | 12 | 100.5 | 7.8 | 95.0 | 7.2 | 108.2 | 7.3 | 99.5 | 7.4 |
| | 96 | 106.0 | 12.9 | 92.5 | 7.9 | 102.8 | 10.1 | 102.4 | 12.0 |

**Table 8**
Results of the measurement of 6 special-grade antimicrobials from patients' samples.

| Drug | Samples (n) | Mean concentration (range, μg/mL) | Reference Values | % of Concentrations outside of reference Values |
|---|---|---|---|---|
| Meropenem | 22 | 11.90 (0.47–54.26) | fT > MIC100 % | 36.4 (8/22) |
| Linezolid | 17 | 9.97 (0.22–27.87) | 2.0–7.0 μg/mL | 64.7 (11/17) |
| Fluconazole | 3 | 4.25 (0.72–7.22) | at least 1.9–6.7 μg/mL | 33.3 (1/3) |
| Voriconazole | 12 | 3.36 (0.51–11.40) | 1.0–5.5 μg/mL | 66.7 (8/12) |
| Posaconazole | 17 | 1.33 (0.29–2.99) | 1–6 μg/mL | 41.2 (7/17) |
| Vancomycin | 25 | 10.79 (1.81–36.85) | 10.0–20.0 μg/mL | 68.0 (17/25) |

**Table 9**
Representative comparison between previous LC-MS methods and the present study.

| Article | Sample volume (μL) | Extraction methodology | Total run time (min) | Hemolytic/hyperlipidaemic effect evaluation | Whole blood stability |
|---|---|---|---|---|---|
| Current manuscript | 10 | PP | 2.5 | Yes | Yes |
| Y Qi et al. [13] | 100 | mSPE | 3 | No | No |
| KY Beste et al. [16] | 100 | PP and concentration by SPE | 3 | No | No |
| T Ohmori et al. [32] | 50 | SPE | 8 | No | No |
| D Wu et al. [37] | 70 | PP and dilution | 5.5 | No | Yes |
| J Zander et al. [34] | 50 | PP and on-line SPE | 4 | No | No |
| H Mei et al. [35] | 50 | PP | 9 | No | No |
| M Zhang et al. [36] | 100 | PP | 5 | No | No |
| FB Sime et al. [27] | 300 | PP and dilution | 7 | No | No |

PP, protein precipitation; mSPE, magnetic solid phase extraction; SPE, solid phase extraction.

that several methods should be applied in the routine TDM to cover different antimicrobials mentioned in this article. The assay that can satisfy the simultaneous determination of meropenem, linezolid, fluconazole, voriconazole, posaconazole and vancomycin is more advantageous and time-saving, especially for samples with a drug combination. Meanwhile, the developed assay is based on one-step protein precipitation without diluting/concentrating supernatant, which is much less labor-intensive and more economical than the solid-phase extraction procedure used in many other methods [13,16,32]. In addition, each sample's total chromatographic run time is only 2.5 min, more time-saving than even some mono-analyte assays [33–37]. All these advantages ensure that the method is high-throughput and more suitable for routine TDM.

Most importantly, only 10 μL plasma was required for sample treatment, and only 2 μL of extracted sample was used for sample injection. The sample volume is much smaller than the former reported LC-MS/MS methods [13,16,27,37]. Notably, the small sample volume interests pediatric patients because limited sample volumes can be collected. Moreover, a smaller sample makes converting venous blood sampling to peripheral blood sampling possible to minimize sampling injury.

This method is verified as applicable to various biological matrices, including serum, hemolytic plasma, and hyperlipidaemic plasma. Additionally, the widespread applicability makes the technique beneficial to patients in case of abnormal plasma (hemolytic or hyperlipidaemic plasma) or incorrect sampling (e.g., changing anticoagulated tube to procoagulant tube by mistake). Furthermore, the data on whole-blood stability provides a time window regarding the isolation of plasma from whole blood. Thus, the validation results are also instructive for sample collection.

## 4. Conclusion

A simple, robust, and sensitive LC–MS/MS method is described for simultaneously quantifying meropenem, linezolid, fluconazole, voriconazole, posaconazole, and vancomycin in human plasma. The method is fully validated and demonstrates many advantages in routine analysis, such as small sample volume, simple sample process, short analytical time, and wide applicability. Furthermore, this new assay was successfully applied to routine TDM of the 6 special-grade antimicrobials in pediatric patients.

## Ethics statement

This study was approved by the Medical Research Ethics Committee of Hebei Children's Hospital (No. YYLS2020-11).

## Data availability statement

Data will be made available on request.

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## CRediT authorship contribution statement

**Xijuan Jiang:** Writing - original draft, Methodology, Funding acquisition, Formal analysis. **Yabin Qin:** Writing - review & editing, Validation, Conceptualization. **Rong Lei:** Writing - review & editing, Investigation. **Yu Han:** Data curation. **Jing Yang:** Resources. **Guying Zhang:** Supervision, Project administration. **Jianfang Liu:** Writing - review & editing, Supervision.

## Declaration of competing interest

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

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