Validation and Clinical Application of a Multiplex High Performance Liquid Chromatography - Tandem Mass Spectrometry Assay for the Monitoring of Plasma Concentrations of 12 Antibiotics in Patients with Severe Bacterial Infections

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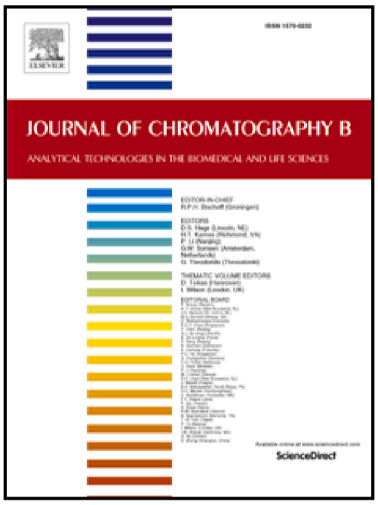

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Validation and Clinical Application of a Multiplex High Performance Liquid Chromatography - Tandem Mass Spectrometry Assay for the Monitoring of Plasma Concentrations of 12 Antibiotics in Patients with Severe Bacterial Infections

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ABSTRACT

Objective. Unpredictable pharmacokinetics of antibiotics in patients with life-threatening bacterial infections is associated with drug under- or overdosing. Therapeutic drug monitoring (TDM) may guide dosing adjustment aimed at maximizing antibacterial efficacy and minimizing toxicity. Rapid and accurate analytical methods are key for real-time TDM. Our objective was to develop a robust high -performance liquid chromatography-tandem mass spectrometry method (HPLC-MS/MS) for multiplex quantification of plasma concentrations of 12 antibiotics: imipenem/cilastatin, meropenem, ertapenem, cefepime, ceftazidime, ceftriaxone, piperacillin/tazobactam, amoxicillin, flucloxacillin, rifampicin, daptomycin.

Methods. A single extraction procedure consisting in methanol plasma protein precipitation and H_2O dilution was used for all analytes. After chromatographic separation on an Acquity UPLC HSS-T3 2.1 x 50 mm, 1.8 µm (Waters®) column, quantification was performed by electro-spray ionisation-triple quadrupole mass spectrometry with selected reaction monitoring detection. Antibiotics were divided in two pools of calibration according to the frequency of analyses requests in the hospital routine antibiotic TDM program. Stable isotopically-labelled analogues were used as internal standards. A single analytical run lasted less than 9 minutes.

Results The method was validated based on FDA recommendations, including assessment of extraction yield (96-113.8%), matrix effects, and analytical recovery (86.3-99.6%). The method was sensitive (lower limits of quantification 0.02-0.5 µg/mL), accurate (intra/inter-assay bias -11.3 to +12.7%) and precise (intra/inter-assay CVs 2.1-11.5%) over the clinically relevant plasma concentration ranges (upper limits of quantification 20-160 µg/mL). The application of the TDM assay was illustrated with clinical cases that highlight the impact on patients' management of an analytical assay providing information with short turn-around time on antibiotic plasma concentration.

Conclusion This simple, robust high-throughput multiplex HPLC-MS/MS assay for simultaneous quantification of plasma concentrations of 12 daily used antibiotics is optimally suited for clinically efficient real-time TDM.

Keywords : anti-bacterial agents; beta-lactams; carbapenems; penicillins; cephalosporins; imipenem; cilastatin; meropenem; ertapenem; cefepime; ceftazidime; ceftriaxone; piperacillin; tazobactam; flucloxacillin; amoxicillin; daptomycin; rifampicin; antibacterial; antibiotics; quantification; plasma; concentration; chromatography; mass spectrometry; assay; analysis; multiplex; therapeutic drug monitoring.

1. INTRODUCTION

The development of new antimicrobial agents does not keep the pace with rising bacterial resistance. Maximizing the efficacy and safety of the present antibacterial armamentarium is crucial for the management of severe bacterial infections. Unpredictable antibiotic distribution and elimination in critically ill patients may result in extremely variable antibiotic plasma concentrations despite adherence to the recommended dosing regimens [1]. The resulting drug under- or overdosing is associated with a risk of treatment failure or toxicity, respectively. Increase of antibiotic dosing following documentation of insufficient plasma concentrations, i.e. trough concentrations lower than the *in vitro* minimal inhibitory drug concentration (MIC) for the causative microbe, may improve response of life-threatening infections [2-5] and avoid selection of bacterial resistance. This scenario is frequently observed in ICU patients with augmented renal clearance (e.g. hyperfiltration)[6]. Patients with impaired or rapidly changing renal elimination are at risk for antibiotic accumulation. Dose decrease or therapy interruption may be crucial for prevention or management of drug-related neurotoxicity of beta-lactams (encephalopathy with coma, myoclonias, and seizures) [7, 8]. We found a 50% probability of toxic encephalopathy at trough cefepime plasma concentrations above 22 μg/mL in febrile neutropenic patients with mild impairment of renal function[9].

Therapeutic drug monitoring (TDM)-guided dosing adjustment aims at optimizing antibiotic therapy according to the individual clinical condition and *in vitro* susceptibility of the causative pathogen [10-13]. A key of clinically efficient TDM is measurement of plasma antibiotic concentrations with short turn-around time. Analytical techniques for quantification of multiple antibiotics using a unique extraction procedure, simultaneous calibrations and short turn-around analytical time may best fulfill these requirements. The method for multi-compound quantification of beta-lactams by Verdier *et al.*[14] used a chromatographic gradient over 22 minutes with ultraviolet detection. Tandem mass spectrometry (MS) detection qualifies for rapid, specific and sensitive quantification in plasma of structurally unrelated antimicrobial agents in a single analytical run. MS applications were published for the analysis of multiple beta-lactams [15-19] and/or other antimicrobials [20-22]. Quantification of antibiotic plasma concentrations may be perturbed by endogenous and exogenous compounds from very complex, highly variable plasma matrices influenced by renal

and/or hepatic dysfunction and simultaneous drug treatments. In their landmark ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) assays, Carlier *et al.*[23], Colin *et al.*[24], and Zander *et al.*[25] used isotope-labelled internal standards (I.S.) for correcting the potential interferences of plasma matrix variability. Subsequently, MS methods for antimicrobial agents included isotope-labelled I.S for most [26-31] or some [32-34] antibiotics.

Our objective was to develop a robust analytical HPLC-MS/MS method for the quantification of the plasma concentrations of 12 daily used antibiotics, covering the entire clinically relevant ranges of concentrations. They were classified in two antibiotic pools i) constituted on the basis of the frequency of clinical requests in the hospital routine antibiotic TDM program, and ii) with respect to the maximum number of antibiotics that can be added into plasma calibration samples without drug solubility issues at the upper limits of clinically relevant concentrations. Simple unique extraction method, rapid turn-around time and use of stable isotope-labelled antibiotics analogues as internal standards aimed at providing an accurate, precise and rapid quantification of plasma concentrations for TDM-based drug dosing adjustment in patients with severe bacterial infections.

2. MATERIAL and METHODS

2.1. Chemicals, reagents and plasma

All chemicals were of analytical grade. For simplification, the 13 analytes will be designated as antibiotics throughout the text, even though cilastatin is a renal dehydropeptidase II inhibitor co-administered with imipenem to inhibit its enzymatic degradation in the kidney.

Piperacillin, tazobactam and cefazolin were purchased from Sigma-Aldrich Chemie GmbH (Buchs SG, Switzerland). The stable isotope-labelled internal standards (I.S.) cefepime-$^{13}CD_3$, ceftazidime-D_6, ceftriaxone-$^{13}CD_3$, ertapenem-D_4, flucloxacillin-$^{13}C_4{}^{15}N$, imipenem-D_4, piperacillin-D_5, were obtained from Alsachim (Strasbourg, France). Amoxicillin, ceftazidime, ceftriaxone, rifampicin and the I.S. amoxicillin-D_4, daptomycin-D_5 (major), meropenem-D_6 and rifampicin-D_8 were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). The pharmaceutical formulations of imipenem and cilastatin (Tienam® I.V., Merck, Zug, Switzerland), cefepime (Orpha Swiss, Küsnacht, Switzerland), meropenem (Meronem®, Astra Zeneca, Zug, Switzerland), daptomycin (Cubicin®, Novartis, Basel, Switzerland), ertapenem (Invanz®, Merck, Zug, Switzerland), flucloxacillin (Floxapen®, Actavis, Regensdorf, Switzerland) were used for the preparation of stock solutions, calibration samples and quality control samples (QCs). The procedures for the preparation of stock solutions, calibration samples, QCs, and I.S. solutions are reported in Table S1, Panel A and B. Chromatography was performed using Lichrosolv® HPLC-grade acetonitrile (MeCN) (Merck, Darmstadt, Germany). Ultrapure water was obtained using a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Ammonium formate (Fluka, Buchs SG, Switzerland) and formic acid (98-100%) (Merck, Darmstadt, Germany) were used for the solvents of the mobile phase in the gradient elution program.

Blank plasma used for the assessment of matrix effect and for the preparation of calibration samples and QCs was obtained according to institutional ethical standard from citrated blood from patients with *polycythemia vera*, who underwent regular phlebotomy at the Center of Transfusion Medicine, Lausanne University Hospital, Lausanne, Switzerland. The blood was centrifuged on a centrifuge Hettich® Rotanta 4600RF (2113g (3000 rpm), 10 min, +4 °C).

2.2. LC-MS/MS conditions

The liquid chromatography system consisted of Rheos Allegro quaternary pumps, equipped with an online degasser and a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) controlled by Janeiro-CNS 1.1 software (Flux Instruments, AG, Thermo Fisher Scientific, Waltham, MA, USA). Separations were done on a column Acquity UPLC HSS T3 2.1 x 50 mm, 1.8 μm (Waters®, Milford, MA, USA). The chromatographic system was coupled to a triple stage quadrupole (TSQ) Quantum Ultra mass spectrometer (MS) from Thermo Fisher Scientific, equipped with an electrospray ionization (ESI) (Ion Max Source Probe) interface and operated with Xcalibur 2.0.7 software (Thermo Fisher Scientific, Waltham, MA, USA).

The mobile phase was composed of 10 mM ammonium formate in ultrapure water + 0.4% formic acid (FA) (solvent A) and acetonitrile (solvent B). The mobile phase was delivered using an initial gradient elution followed by an equilibration step of the column to the initial solvent composition according to the sequences reported in Table S2. The temperature-controlled autosampler was maintained at +4°C.

The HPLC-MS/MS conditions were as follows: electrospray ionization (ESI) in positive mode; spray voltage 3800 V; vaporizer temperature 40°C; sheath gas and auxiliary gas (both nitrogen) pressure 60 and 10 arbitrary units, respectively; capillary temperature 350°C; collision energy (CE) from 5 eV to 53 eV; tube lens range voltage 57-104 V. The Q2 collision gas (argon) pressure was 1.5 mTorr (0.199 Pa). Mass spectra (MS) were acquired using the *selected reaction monitoring* (SRM) detection in the positive ionization mode. The selected *m/z* transitions and the collision energy for each analyte and I.S. are reported in Table 1.

The optimal instrument parameters and MS/MS transitions were determined by direct infusion of each compound solution separately into the MS/MS detector at a concentration from 1 to 10 μg/mL in a 50:50 mixture of MeOH and solution A, depending on their sensitivity.

The optimal instrument parameters for MS/MS analyses are as follows:

The first (Q1) and third (Q3) quadrupoles were set at 1 amu mass resolution (Full-Width Half-Maximum = 1.8-2.0 Da). Scan time and scan width were 0.02 s and 0.5 amu, respectively. Each chromatographic peak was the result of at least 20 scans. MS acquisitions were done in centroïd

mode. In *Pool 1* (see antibiotic list in Table S1, Panel A), three distinct segments of data acquisition were programmed in the positive mode: 0 - 2.4 min, 2.4 - 2.85 min, and 2.85 - 6.0 min. For *Pool 2* (see antibiotic list in Table S1, Panel A*),* the segments were as follows: 0 - 2.3 min, 2.3 - 3.3 min, and 3.3 - 6.0 min. Chromatographic data acquisition, peak integration and quantification were performed using the QUAL and QUAN browser of the Xcalibur software package (version 2.0.7, ThermoQuest, Thermo Fisher Scientific Inc, Waltham, MA, USA).

2.3. Calibration standard, quality control (QCs) and internal standard (I.S.) solutions for calibrations curves

The antibiotics imipenem/cilastatin, meropenem, ertapenem, cefepime, piperacillin/tazobactam, flucloxacllin, ceftazidime, ceftriaxone, amoxicillin, daptomycin and rifampicin, have been divided in two pools of calibration (*Pool 1 and 2*, respectively) according to the frequency of analyses requests in the hospital routine antibiotic TDM program. The preparation of two separate pools was also necessary because of solubility issues with multiple compounds spiked in plasma at their highest calibration levels (i.e. up to 100 µg/mL, 160 µg/mL for piperacillin). The preparation of calibration and QCs samples and of I.S. solutions used for calibration curves is reported in Table S1, Panel A and B.

2.4. Plasma extraction procedure

Plasma (clinical, calibration and control) samples and working solutions were kept on ice at +4°C during the entire processing. In a 1.5ml Eppendorf plastic vial placed on ice, a 100µl aliquot of plasma was mixed with 50µl of I.S. working solution (containing each I.S., i.e. for *Pool 1* imipenem-D_4, meropenem-D_6, cefepime-$^{13}CD_3$, and piperacillin-D_5 at 9µg/mL, and cefazoline at 18µg/mL and for *Pool 2* amoxicilin-D_4, ertapenem-D_4, flucloxacillin-$^{13}C_4{}^{15}N$, daptomycin-D_5 at 90µg/mL, ceftazidime-D_6 at 9µg/mL, ceftriaxone-$^{13}CD_3$ at 2µg/mL and rifampicin-D_8 at 1µg/mL; Table S1, Panel B) and 300µl of ice cold MeOH, vortexed for 5 sec. The mixture was centrifuged at +4°C for 10 minutes at 20'000 g (14'000 rpm) on a Mikro 200R benchtop centrifuge (Hettich GmbH, Tuttlingen, Germany). A 80µl aliquot of the supernatant was diluted without delay with 480µl of ultrapure H_2O, into 2mL glass HPLC vials, tightly closed with crimp seals and vortexed for 10 sec. A volume of 20µl was injected into UPLC-MS/MS for analysis.

2.5. Analytical method validation

The method validation procedure was based on : i) 2018 on-line recommendations of the Food and Drugs Administration (FDA)[35], ii) recommendations of the Conference Report of the Washington Conference on “Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic studies”[36], iii) Arlington Workshop “Bioanalytical Methods Validation - A revisit with a Decade of Progress”[37], iv) recommendations on methods validation from Matuzewski *et al.* [38, 39].

2.6. Accuracy and precision

Four concentrations of quality control samples (QCs) were used to cover the rather large analytical range of the calibration curves including the spectrum of antibiotic concentrations expected to be measured in clinical samples: low (L), intermediate (I), medium (M), and high (H) (Table S1, Panel A). Replicate analyses (n=6) of the four QCs were performed for assessment of intra-assay (within a single analytical run) and inter-assay (among different runs) accuracy and precision. The accuracy was calculated as the bias, or percentage of deviation, between measured and nominal concentrations. The precision was calculated as the coefficient of variation (C.V. %) of the measured concentrations.

2.7. Matrix effects, extraction yield and overall recovery

The same methodology was previously applied for the validation of the multiplex LC-MS/MS assay developed in our laboratory for antifungal drugs[40]. The matrix effects and their variability were examined qualitatively by the simultaneous post-column infusion of the antibiotics and corresponding I.S. into the MS/MS detector during the chromatographic analysis of 6 different blank plasma extracts. The chromatographic signals of each selected MS/MS transition were examined to check for any signal perturbation (drift or shift) of the MS/MS signal at the analytes’ retention time. The matrix effects , ME (i.e. ion suppression/enhancement of the MS/MS signal of drugs in presence of plasma matrix), the extraction yield and overall recoveries were quantitatively assessed based on recommendations by Matuszeski *et al.* [39] (Supplementary Material).

2.8. Lowest Limit of quantification

The lowest limit of quantification (LLOQ) for each drug was the minimal plasma concentration, which could be quantified with a ± 20% deviation of the measured from the nominal concentration, in accordance with FDA recommendations.

2.9. Antibiotics stability over time in plasma and whole blood at different storage conditions, method selectivity and anticoagulant's influence

These studies are described in details in Supplementary Material.

2.10 Clinical blood samples

Clinical samples from patients receiving antibacterial therapy for severe bacterial infections were analyzed with the new HPLC-MS/MS method as a quality control in the frame of the hospital routine antibiotics TDM program. According to Swiss Law and Institutional rules, ethical approval is not required for a retrospective case report involving anonymised clinical data (see the Federal Act on Research involving Human Beings [41]. Blood samples (2.7 ml) were collected in Monovettes® (Sarstedt, Nümbrecht, Germany), containing initially citrate, thereafter EDTA (which should be preferred, see below) as anticoagulant. Blood samples were centrifuged within 1 hour at 1850g (3000rpm) (Beckmann Centrifuge, Model J6B, Nyon, Switzerland), or at 2113g (3000rpm) on a Rotanta® 4600RF centrifuge (Hettich GmbH, Tuttlingen, Germany), for 10 min at +4°C. The plasma was separated and transferred into 5ml polypropylene test tubes, and directly analyzed (turn-around time within 6h), or frozen at -80°C and analyzed within 48h (e.g. external shipments from other hospitals).

3. RESULTS

3.1. Chromatograms

The multiplex HPLC-MS/MS method with an analytical run lasting less than 9 minutes quantified the plasma concentrations of 12 antibiotics *plus* cilastatin. Chromatographic profiles of calibration samples containing 25μg/ml of antibiotics from *Pool 1 and 2* are shown in Figure 1a and Figure 1b, using the gradient elution programs described in Table S2. The respective retention times and mass spectrometry conditions for antibiotics and their stable isotopically-labelled I.S. are reported in Table 1.

3.2. Internal standard and calibration curve

Deuterated (D)-, ^{13}C- or ^{15}N- analogues (and combinations thereof) of antibiotics were used as Internal Standards (I.S.). The I.S. imipenem-D_4 was of critical importance since the polar imipenem was eluted early on a reverse phase mode and its signal could be perturbed by endogenous hydrophilic components. In addition, imipenem-D_4 could correct for the irregular shape of imipenem signal due to the equilibrium between imipenem Z and E isomers [42] that may be partially resolved under the applied chromatographic conditions (see below).

Labelled internal standards for cilastatin or tazobactam were not available to us at the time of the initial setting-up of the method, but cefazoline was found to be a suitable I.S. (see Table S4) for these compounds which formally are not subjected to TDM, but are measured concomitantly for informative purposes.

Calibration curves over the clinically relevant concentrations ranges reported in Table S1, Panel A were best described by $1/x^2$ weighted quadratic log-log regression of the peak-area ratios of the antibiotic to its I.S. in each standard sample, which were referred to the respective nominal concentrations. This model of calibration was used in order to obtain a minimum bias for each single calibrator over the entire calibration range (data not shown). The regression coefficients r^2 of all calibration curves were higher than 0.99 with back-calculated concentrations of the calibration samples within ±15% (±20% at LLOQ) of the nominal concentrations.

3.3. LLOQ, accuracy, and precision

The lower levels of quantification (LLOQs) corresponded to the lowest levels of calibration reported in Table S1, Panel A. For *Pool 1* antibiotics, the LLOQs were within 0.05-0.08μg/mL. For *Pool 2*, the LLOQs were 0.02μg/mL (rifampicin), 0.1μg/mL (amoxicillin, flucloxacillin, ceftazidime and ertapenem), and 0.5μg/mL (ceftriaxone and daptomycin). The accuracy and precision of the LLOQs for all antibiotics were within the ±20% limit recommended by the FDA. Accuracy and precision for the four QC samples are summarized in Table S3. The intra-assay and inter-assay deviation (bias) from the nominal concentrations of QCs ranged -7.1 to +12.7%, and -11.3 to +6.8%, respectively. The intra-assay and the inter-assay precision (CV) ranged from 1.4 to 11.5%, and from 2.1 to 11.3%, respectively. All the measured intra- and inter-assay accuracy and precision parameters lied within the ±15% range recommended by the FDA. Our laboratory participates to inter-laboratory (external) QC proficiency programs for antibiotics [43-45] which confirmed analytical accuracies and precisions within the recommended validation ranges (data not shown).

3.4. Matrix effect and recovery

The matrix effects were analyzed qualitatively by the simultaneous post-column infusion of all analytes and I.S. into the MS/MS detector during the chromatographic analysis of blank plasma extracts from six different batches of blood donors. As illustrated in Figure S1 (Supplementary material) signals at the *m/z* transition showed a similar pattern for all traces: no significant drifts or shifts of selected transition signals (overlapping traces in offset) were apparent at the retention time of each antibiotic, except for piperacillin in *Pool 1*, for which a significant drop was observed, that was reproduced in quantitative assessment (Table S4), albeit compensated by its I.S. (see below). The inter-subject variations in matrix effects have been studied quantitatively in Table S4. Using the protein precipitation with MeOH and supernatant dilution 1+6 in purified H_2O, the variability in matrix effect (ME) of the 13 analytes in 6 different plasma matrices was below 11.8% and 12.8%, for *Pool 1* and *Pool 2*, respectively (column ME%, Table S4).

A comprehensive assessment of the ME, extraction recovery, and overall method recovery for all antibiotics is reported in **Table S4**. The mean ME was around 100% for imipenem, meropenem, cefepime, tazobactam, cilastatin, amoxicillin, ceftazidime, ertapenem and rifampicin. ME values for

piperacillin, daptomycin and flucloxacillin were 82.5%, 88.7% and 87.3%, respectively, indicating a weak (around -15%) negative matrix effect, whereas the ME was 165.2% for ceftriaxone, reflecting an important positive matrix effect. However, the B2/A2 ratio (i.e. ME normalized with stable isotope-labelled I.S. signals) for piperacillin, daptomycin, flucloxacillin, and ceftriaxone were 103%, 93.7%, 98.7%, and 101.7%, respectively, demonstrating that the use of a labelled I.S. successfully corrected the ME (either negative or positive) observed for these four antibiotics. The mean extraction yield ranged between 96% (imipenem) to 113.8% (daptomycin). The mean overall process efficiencies (PE) were comprised between 82.8% (piperacillin) and 106.3% (cilastatin), with the exception of a PE at 165.2% for ceftriaxone due to a positive ME, fully corrected by the use of the I.S. ceftriaxone-$^{13}CD_4$. For all antibiotics the variability of the overall PE was low (< 15%) (Table S4).

3.5. Carry-over (memory) effect

No carry-over was observed in blank samples analyzed immediately after two injections (i.e. an extreme case scenario) of the highest calibration levels, except for ceftriaxone, daptomycin, flucloxacillin, and rifampicin, whereby the first following blank sample showed a signal corresponding to a concentration of 0.34, 0.39, 0.12 and 0.04 µg/ml, respectively, unlikely to influence to a clinically significant extent the analytical results. However, it is advised to program the analysis of one blank sample (MeOH/H_2O 50:50) immediately after the highest calibration level to eliminate a potential memory effect for these antibiotics.

3.6. Stability studies

The quantification over time of QCs of antibiotics from *Pool 1* and *Pool 2*, in *plasma* and *whole blood* at RT and +4°C, is reported in Table 2, left and right column, respectively.

In plasma at RT, the measured concentrations were within 85-115% of the nominal concentrations up to 4h for imipenem, up to 8h for cefepime, meropenem, and piperacillin/tazobactam, up to 24h for amoxicilin, ceftazidime, and ertapenem, and up to 48h for ceftriaxone, flucloxacillin,

daptomycin, rifampicin, and cilastatin. All antibiotics remained stable up to 48h in plasma stored *at* +4 °C, except imipenem that was stable (-14.3%) up to 24h.

In citrated *whole blood* samples, all antibiotics, with exception of meropenem and imipenem, were stable up to 24h and 48h, when stored at RT and +4°C, respectively. Imipenem and meropenem remained within 85-115% of the initial concentration in whole blood at RT up to 2h and 8h, respectively. Storage of whole blood samples at +4° slowed a degradation of imipenem and meropenem (-13% and -15% from T_0 values after 8 and 24h, respectively).

The concentrations of antibiotics/cilastatin in plasma after three freeze-thaw cycles were within 85-115% of those measured at T_0, except for the L level of imipenem after the third cycle (-17%, data not shown).

Quantification of QCs of antibiotics at -80°C was within 85-115% of the values at T_0 up to 2 months and 3-4 months, for *Pool 1* and *Pool 2*, respectively (data not shown).

3.7. Influence of anticoagulants

The method has been validated using citrated plasma for the preparation of matrix-matched calibration samples and QCs. The validation was completed by analyzing four series of the four (L, I, M, H) QCs prepared in serum, citrated, heparinized or EDTA plasma, using calibration curves in citrated plasma. In heparinized plasma samples, the measured imipenem, tazobactam, and meropenem concentrations exceeded 115% of the nominal values, especially for the L QCs, indicating that, in our assay, heparin is not an appropriate anticoagulant for blood sampling. For all other antibiotics, the concentration deviations using serum, heparinized or EDTA plasma were comprised within 85-115% of the nominal concentrations. When blood is collected in tubes containing 10% volume of citrate solution, such dilution implies a correction of the measured plasma concentration by a factor of 1.1, which is correct if the blood collection tube is completely filled.

3.8. Clinical application of the multiplex UPLC-MS/MS method for TDM-guided patients' management

The application of the multiplex UPLC-MS/MS method is illustrated with clinical cases from the hospital routine antibiotic TDM program (Figure 2, Panel A to I) highlighting the impact on patients' management of an assay that provides real-time information on antibiotic plasma concentrations.

Figure 2, Panel A shows the chromatographic profile of plasma collected at steady state in a 3-year old child (15 kg) receiving 350mg imipenem + cilastatin (Tienam®) i.v. over 60 min every 6h for suspected knee osteomyelitis. The trough plasma concentration of imipenem was 0.2μg/mL (cilastatin < 0.1μg/mL) (Figure 2, Panel A), which was below the target of 1μg/mL recommended for appropriate antibacterial activity when the MIC of the causative pathogen is unknown [3]. This prompted dosing increase to 500mg administered as a 2h-infusion every 6h. After 24 hours, the trough level was 1.6μg/mL (cilastatin 0.3μg/mL) (Figure 2, Panel B). The signal peak for imipenem in Figure 2, Panel A and Panel B was broadened (such as in Figure 1A) due to the presence of E/Z isomers, which were not separated with the analytical conditions used for TDM (see below Figure 3A).

Figure 2, Panel C illustrates the chromatographic profile of plasma collected in a 85-year old patient (72kg, 166μM serum creatinine) treated with cefepime 2g bid for foot osteomyelitis. During antibiotic therapy he developed a delirium. TDM showed a cefepime plasma trough concentration of 61.5μg/ml, while the recommended trough level should not exceed 15μg/ml [9]. This result suggested a cefepime accumulation due acute renal failure complicated by a toxic encephalopathy. The documentation of cefepime overdosing with concurrent neurological signs prompted treatment interruption, which resulted in complete clinical resolution within 24h. Cefepime therapy was resumed at an adapted dosing of 1g bid.

Figure 2, Panel D and E represent the chromatographic profiles of plasma from a 33- year old patient (72kg, 53μM serum creatinine, with a Cl_{crt} 182ml/min calculated according to Cockroft & Gault) with small bowel perforation and polymicrobial peritonitis (*Enterobacter cloacae, Pseudomonas* sp., alpha-hemolytic *Streptococcus*, *E. faecium*, *S. epidermidis, S. haemolyticus*). He received high-dose meropenem (2g tid) as undetectable imipemen C_{min} plasma concentrations had been measured during a previous treatment course with 750mg 4 times a day (qid). His

creatinine plasma levels decreased to 33μM, with a corresponding Cl_{crt} of 287ml/min and a meropenem plasma trough concentration of 0.2μg/ml (Figure 2, Panel D), reflecting insufficient antibiotic exposure. The detection of low meropenem blood concentrations in this patient with renal hyperfiltration was followed by dose adjustment to 2g qid as 2-hour infusion resulting in meropenem trough concentrations at 3.7μg/ml (Figure 2, Panel E).

Figure 2, Panel F represents the chromatographic profile of plasma from a 81-year old patient (75kg, Cl_{crt} 55ml/min calculated according to Cockroft & Gault) treated with daptomycin for methicillin-resistant *Staphylococcus epidermis* post-surgical mediastinitis with bloodstream infection. He received daptomycin (Cubicin®) 500mg (6mg/kg) i.v. once daily, in association with oral rifampicin 300mg BID. The patient was discharged on parenteral antibiotic therapy and 24 hours later he complained of myalgias. His creatine kinase (CK) concentration was 2012 U/L (normal range: 25-195 U/L), and daptomycin trough plasma concentration was 28.5μg/ml (Figure 2, Panel F) [10μg/ml 48 hours and 0.3μg/ml 5 days after the last dose, data not shown]. The mean C_{min} reported for daptomycin at this dosing regimen is 6.7μg/ml [46]. These findings reflected a toxic rhabdomyolysis due to daptomycin accumulation resulting in drug overdosing. After daptomycin interruption, myalgias resolved and CK values normalized. In this patient with mild impairment of renal function, TDM documented daptomycin accumulation resulting in muscular toxicity that required treatment interruption and switch to an alternative antibiotic therapy.

A 88-year old woman (60kg) with chronic renal failure (150μmol/L serum creatinine; eGFR 27ml/min/1.73 m^2) was diagnosed with *E. cloacae* and *P. aeruginosa* surgical wound infection after peripheral arterial by-pass grafting. She received cefepime 2g bid. Two days after treatment initiation she developed aphasia followed 24 hours later by myoclonias, along with deterioration of the Glasgow Coma Scale (GCS) score to 12/15. The EEG diagnosed a status epilepticus, treated with clonazepam, levetiracetam and valproate. The chromatographic profile of plasma showed a cefepime trough concentration of 83.3μg/ml, (Figure 2, Panel G), an extremely high level when compared to the recommended therapeutic range 2-15μg/ml [9]. This cefepime overdosing due to chronic renal failure resulted in severe neurotoxicity. Hemodialysis was initiated and cefepime pre-filter and post-filter concentrations were monitored (Figure 2, Panel H and I): 57.5μg/ml and

8.3μg/ml, respectively, were measured 55min after starting hemodialysis. This allowed decreasing the cefepime trough concentration in plasma to 24.1μg/ml after the first dialysis, and to 2.5μg/ml after the second dialysis 24 hours later. The patient fully recovered from the cefepime neurotoxicity after 72 hours. Of note, the 7-epimer of cefepime [47] (see below) was detected in all samples (plasma, pre- and post-dialysis column) collected from this woman with renal failure (Figure 2, Panel G, H, and I).

3.9. Isomers profiles and metabolites identification studies for imipenem and cefepime

Given the reduced elution time of the analytes with UPLC, it was important to check for interferences with drug quantification by beta-lactams isomers. The chromatographic elution pattern of isomers was studied for imipenem and cefepime. Imipenem is eluted as a broad peak (Figure 1, Panel A) that can resolve into a nearly base-line separation of two peaks existing at a 7:3 ratio when applying a more prolonged gradient program (0 min to 4min: 98% → 60% Solvent A; followed by column re-equilibration to the initial solvent composition (98% Solvent A)) (Figure 3, Panel A). Imipenem has been shown to exist as a mixture of two rotational Z and E formamidinium isomers that are in equilibrium in solution, and that can be separated chromatographically (41). Preparative HPLC isolation of one single isomer was reported to give an enriched solution of one isomer, which rapidly re-equilibrates to the original 7:3 composition within minutes at pH 7 and at RT (41). For imipenem quantification, a base-line separation of Z and E isomers was not considered, since under physiological pH and temperature conditions, the equilibrium is expected to be dynamic without any influence on imipenem pharmacological properties. The use of the stable isotopically-labelled I.S. imipenem-D_4 was however essential for normalizing the imipenem peak shape feature which is changing over the concentration range, and for correction of the matrix effect of polar endogenous compounds possibly co-eluting with imipenem.

In patients receiving cefepime, a second small peak could be observed in some plasma samples at the SRM transitions (*m/z* 481.2→125; 481.2→396.2) selected for cefepime, close after the main peak of cefepime eluted at 2.3-2.5 min (Figure 2, Panel G, H, and I). Base-line resolution of

cefepime and its isomer was obtained using the following gradient adaptation: 0 min to 4min: 98% → 60% Solvent A, which allows a recording of the mass spectrum of cefepime and its isomer (Figure 3, Panel B). The mass spectrum and fragmentation pattern of this metabolite in patients was virtually identical to that of standard cefepime spiked into blank plasma. Addition of cefepime pure standard to patient's plasma samples containing both cefepime and its isomer, increases uniquely the cefepime signal, indicating that, unlike imipenem, the presence of this additional peak is not due to an isomeric equilibrium. We hypothesized that this second peak is related to the 7-epimer of cefepime (Figure 3, Panel B) that has been identified *in vivo* (46), and has been reported to correspond to 2.5% of a 1000 mg dose of (radiolabelled) cefepime excreted in urine by healthy subjects. In the majority of plasma samples from patients receiving cefepime, the level of this metabolite, if present, was very low (Figure 2, Panel C), except in patients with impaired renal function (Figures 2, Panel G, H, and I). In these patients, the concentrations of the metabolite (calculated using the IS cefepime-^{13}CD3 and cefepime calibration) correspond up to 40.5% (mean 14.7% (range 3.5-40.5%, n= 50)) of that of cefepime. In the absence of information on a potential influence of stereochemistry on cefepime's antibiotic activity (which should *a priori* be irrelevant, given the intact beta-lactam cycle), the cefepime signal was integrated as a single peak for the calculation of the plasma concentration for routine TDM (Figure 2, Panel G, H, and I).

4. DISCUSSION AND CONCLUSION

This new multiplex HPLC-MS/MS method was developed to meet laboratory and clinical requirements for an efficient TDM: i) simple workup with a unique procedure of sample extraction and processing for 12 antibiotics; ii) excellent extraction yield with MeOH precipitation, iii) high sensitivity and specificity (lower LOQs 0.02-0.5μg/ml with isotope-labelled antibiotic analogues as I.S.); iv) extended analytical range over a factor 200 to 2000 (higher LOQs 20-160μg/ml) directly covering the entire clinically relevant concentrations spectra; v) multiplex approach with establishment of 13 calibration curves for simultaneous quantification of the corresponding analytes; vi) short analytical turn-around time (single analytical run lasting less than 9 minutes) providing results within 6h and allowing TDM-based dosing adjustments in real-time.

The use of stable isotope-labelled (^{13}C, ^{15}N, deuterium) analogues as I.S. for the simultaneously quantified antibiotics was a major strength of this new method, when compared with previous assays, which simultaneously quantified a smaller number of compounds and/or did not use I.S. for all the tested antibiotics [26-34]. Stable I.S. played a key role to circumvent the potential influence on antibiotics ionization of highly variable matrices in plasma samples collected from patients with impaired renal and/or hepatic function and simultaneously receiving multiple medications. The I.S.-guided correction also allows an accurate quantification of antibiotics concentrations in matrices other than plasma such as ultrafiltrate fluid from continuous renal replacement therapy (CRRT), urine, cerebrospinal fluid, and other biological fluids.

The use of a unique plasma extraction and a single supernatant dilution step providing simultaneously the best analytical sensitivity over the entire clinically relevant concentrations ranges of all analytes was a major achievement in the optimization of the pre-analytical plasma processing. Of note, the more polar antibiotics were found to have a limited solubility in MeOH, and therefore the plasma/organic solvent ratio was limited to 1+2 for the plasma proteins precipitation. In such conditions, the use of *ice cold* MeOH added to plasma kept at +4°C was found suitable for maximizing the plasma proteins precipitation. Yet, the plasma protein precipitation step with ice cold MeOH yielded a plasma supernatant with a relatively high organic content of 67% MeOH, in which the more polar antibiotics have a limited solubility. It was therefore important to dilute without delay the supernatant with purified H_2O to maintain the polar antibiotics in an aqueous medium ensuring stability in the auto-sampler at +4°C during the entire analytical sequence.

The existence of isomers of imipenem [42] and cefepime [47] had not been accounted for or only partially investigated [14, 32] in previous analytical developments. The present chromatographic studies support the existence of a dynamic equilibrium between the Z/E formamidium rotational isomers of imipenem in both calibration and patients' samples. This chemical characteristic had probably no clinical relevance. The presence of the 7-epimer of cefepime had only been observed in clinical samples, with especially high proportions in plasma from patients with renal failure. To which extent this epimer contributed to the neurotoxic effects of cefepime overdosing remains to be determined.

Several pre-analytical and clinical requirements had been considered in the present development. A plasma volume of 100μL was needed for analysis, which was particularly suitable for TDM in neonates and infants. If necessary, the LLOQ allowed this volume to be reduced to 50μL. The definition of clinically relevant calibration ranges was based on concentrations expected according to pharmacokinetic studies and *in vitro* bacterial susceptibility, by prioritizing upper limits of quantification over clinically irrelevant sensitivity.

The conditions for handling of blood samples by nursing personnel and for pre-analytical processing and storage in the laboratory had been investigated. For standardizing conditions of routine antimicrobial TDM, we recommend that blood is centrifuged within 30 minutes after venipuncture and plasma immediately stored at -80°C. For TDM in samples received from other hospitals, plasma needs to be sent frozen on dry ice. Method validation has been carried out using blank plasma from citrated blood collection. Yet, we have found that EDTA and serum can be used instead indifferently, and we recommend for optimal quantification of antibiotic concentrations to use EDTA as anticoagulant in TDM blood sampling (no need for correction (f =1.1) for the volume of anticoagulant solution contained in the tube *and* the volume of blood collected, as for citrate). Serum is a valid alternative**.**

Participation to external quality proficiency programs [43-45] provided the demonstration of the reliability and robustness of our analytical method and its compliance with the criteria of quality recommended for routine clinical application of TDM [12].

The simultaneous analysis of different compounds with short turn-around time has a substantial impact on timing of clinical decisions. Antibiotic dosing adjustment in patients undergoing CRRT could be guided by TDM results following online algorithms. We illustrated some clinical situations from the hospital routine antibiotic TDM program in which detection of drug over- or underdosing associated with toxicity or non-response was key for appropriate patient management. In patients presenting neurological symptoms and signs during cefepime therapy, documentation of plasma concentrations above the toxic threshold was crucial for prompt interruption of antibiotic therapy. For daptomycin, TDM-guided dose adjustment in patients with renal insufficiency may prevent toxic rhabdomyolysis. Real-time information on insufficient plasma concentrations of imipenem and

meropenem allows dose adjustment for improving antibiotic exposure, especially in ICU patients with renal hyperfiltration who are at high risk for drug underdosing [6].

In conclusion, this robust, sensitive and selective multiplex UPLC-MS/MS analytical method accurately and precisely quantifies plasma concentrations of 12 daily used antibiotics with high throughput, i.e. with a unique plasma extraction step and a 9-minute analytical run. This new assay providing analytical results within 6 hours offers an efficient tool for TDM aimed at individualizing drug dosing for optimal efficacy and minimal toxicity. These characteristics are of key importance for establishing new standards of treatment individualization in patients with life-threatening infections.

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6. TRANSPARENCY DECLARATIONS

None to declare

7. AUTHORS CONTRIBUTIONS

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8. FIGURES INDEX AND LEGENDS

Figure 1: Multiplex analysis by UPLC-MS/MS for quantification of the plasma concentrations of 12 antibiotics with corresponding internal standards. Chromatographic profile of a plasma calibration sample at 25μg/ml for Pool 1 (**Panel A:** imipenem, cilastatin, meropenem, cefepime, piperacilline, tazobactam, cefazoline) and Pool 2 (**Panel B**: amoxicilline, flucloxacillin, ceftazidime, ceftriaxone, ertapenem, daptomycin, rifampicin), constituted on the basis of the frequency of clinical requests for the respective antibiotics in the hospital routine antibiotic TDM program.

Figure 2: Chromatographic profiles by UPLC-MS/MS of samples from patients receiving: imipenem/cilastatin (**Panel A and B**); cefepime (**Panel C**); meropenem (**Panel D and E**); daptomycin (**Panel F**); cefepime (**Panel G, H, and I**). The internal standards used for these antibiotics were their stable isotope-labelled analogues (see details in the text).

Figure 3: Isomers profiles and metabolites identification studies for imipenem and cefepime.
Panel A. Imipenem extended gradient for formamidinium E and Z isomers separation.
Panel B. Cefepime and 7-epimer, in a patient sample.

9. TABLES INDEX AND LEGENDS

Table 1: LC-MS/MS method for quantification of plasma concentrations of antibiotics and their stable isotope-labelled analogues used as internal standards. Antibiotics are listed according to their inclusion in *Pool 1* or *Pool 2*.

Table 2. *Left column.* Evolution over time of antibiotic concentrations in plasma at room temperature (RT) and +4°C. Each time point represents the mean % difference from the initial nominal concentrations (analysed at T_0) of the 4 QC levels (low (L), intermediate (I), medium (M) and high (H) at 0.5, 2.5, 7.5, and 75 µg/ml, respectively, with exception of piperacillin at 0.4. 4, 12, and 120 µg/ml, and rifampicin at 0.1, 0.5, 1.5, and 15 µg/ml.

Right column. Evolution over time at room temperature and at +4°C of antibiotic concentrations in plasma obtained from spiked citrated whole blood. Each time point represents the mean % difference from the initial nominal concentrations (analyzed at T_0) for of the 4 QC levels.

Table 1

ANALYTES	Mean RT [min]	Transitions [m/z] Parent → product	CE [eV]	Tube Lens [V]	Segment
Pool 1					
Imipenem	2.1*	300.20 → 98.00	37	57	1, 2
		300.20 → 142.10	28	57	1, 2
Imipenem-D_4		304.20 → 142.10	34	58	1
Cefepime	2.3	481.20 → 125.00	47	65	1, 2
		481.20 → 396.2	11	65	1, 2
Cefepime-$^{13}CD_3$		485.30 → 125.00	53	71	1, 2
Tazobactam	2.4	301.38 → 94.10	22	104	1, 2
		301.38 → 207.10	15	104	1, 2
Meropenem	2.6	384.20 → 68.10	35	59	2, 3
Meropenem-D_6		390.70 → 68.10	35	77	2, 3
Cilastatin	2.9	359.30 → 97.00	22	67	2, 3
Cefazolin	3.1	384.20 → 68.10	35	59	2, 3
Piperacillin	3.8	518.20 → 114.00	46	93	3
		518.20 → 115.10	48	93	3
		518.20 → 143.10	29	93	3
Piperacillin-D_5		523.30 → 116.20	53	94	3
		523.30 → 148.30	21	94	3
Pool 2					
Amoxicillin	2.0	366.10 → 114.10	25	61	1
		366.10 → 134.10	30	61	1
		366.10 → 349.30	7	61	1
Amoxicillin-D_4		371.20 → 354.30	6	65	1
Ceftazidime	2.3	547.20 → 468.30	11	58	3
Ceftazidime-D_6		553.20 → 474.80	13	69	3
Ceftriaxone	2.6	555.10 → 396.10	13	69	3
Ceftriaxone-$^{13}CD_3$		559.20 → 400.20	11	61	3
Ertapenem	2.75	476.20 → 67.80	37	69	2
		476.20 → 114.10	23	69	2
		476.20 → 432.40	6	69	2
Ertapenem-D_4		480.10 → 436.40	5	75	2
Daptomycin	4.2	811.20 → 159.00	37	88	1, 2
		811.20 → 313.20	28	88	1, 2
Daptomycin-D_5 (major)		813.70 → 161.90	44	100	1, 2
Flucloxacillin	4.6	454.10 → 114.10	36	74	1, 2
		454.10 → 182.10	33	74	3
		454.10 → 295.10	15	74	3
Flucloxacillin-$^{13}C_4$,^{15}N		459.10 → 300.10	13	69	3
Rifampicin	4.7	823.40 → 791.70	16	80	3
Rifampicin-D8		831.40 → 799.70	16	80	3

Polarity mode: Positive; **CE**: collision energy ; **RT** :Retention Time; **MS acquisition time (min)** = 5.0
Q2 Collision gas pressure (mTorr) = 1.00 ; **Polatity mode :** Positive

Segment =	1	2	3	4
Duration (min) =	2.00	0.90	0.90	1.30

* Imipenem exists as 2 formamidinium E and Z isomers in equilibrium that may be separated with extended gradient chromatographic conditions (see text and Figure 3A)

Table 2

Pool 1						Pool 2					
		Plasma		Whole Blood				Plasma		Whole Blood	
		RT	+4°C	RT	+4°C			RT	+4°C	RT	+4°C
	Time [h]	Percentage of initial (nominal) levels [%]					Time [h]	Percentage of initial (nominal) levels [%]			
Imipenem	0	1.8	1.8	0	0	**Amoxicillin**	0	0.8	0.8	0.0	0.0
	1	-3.1	-3.0	-5.5	-2.1		1	2.5	2.1	0.1	0.6
	2	-8.3	0.2	-8.7	-3.3		2	-0.6	-0.2	2.1	-1.4
	4	-13.2	-2.7	-17.7	-7.6		4	6.2	-0.4	-4.1	2.9
	8	-19.6	-1.3	-30.2	-12.9		8	-2.9	5.6	-3.2	-5.5
	24	-60.9	-14.3	-63	-29.9		24	-9.7	1.4	-9.5	0.6
	48	-88.2	-23.2	-84.8	-44.6		48	-24.5	-2.3	-14.1	-4.5
Cefepime	0	-1.4	-1.4	0	0	**Ceftazidime**	0	-1.2	-1.2	0.0	0.0
	1	-0.7	0.3	-1.1	1.5		1	2.3	-7.1	3.8	-4.7
	2	-5.5	-6.5	-2.4	0.4		2	-3.2	-1.5	3.4	-2.1
	4	-8.1	-3.5	1.4	3		4	0.0	-1.6	-0.4	0.8
	8	-11.0	-5.3	1.8	-0.7		8	-6.6	6.5	-6.4	-7.8
	24	-35.2	-1.1	-7.2	0.5		24	-10.5	-0.7	-2.4	-2.2
	48	-66.9	-12.2	-8.9	-2.7		48	-27.0	2.1	-10.8	-5.7
Tazobactam	0	-2.1	-2.1	0	0	**Ceftriaxone**	0	0.6	0.6	0.0	0.0
	1	2.3	-6.0	-2.9	-0.2		1	0.5	3.9	-0.2	0.2
	2	-5.0	-0.3	-1.5	-1.1		2	8.0	-6.7	3.9	-0.3
	4	-5.7	-1.9	-4.4	-3.8		4	2.3	-4.2	1.4	0.2
	8	-8.1	-3.9	-4.8	-5.2		8	2.4	0.0	-1.0	-4.1
	24	-20.4	-9.1	-12.4	-2.9		24	-6.5	-1.6	2.5	3.0
	48	-31.6	-8.7	-19.3	-5.9		48	-3.1	3.4	-1.3	-3.4
Meropenem	0	-5.2	-5.2	0	0	**Ertapenem**	0	1.1	1.1	0.0	0.0
	1	-3.0	-5.0	-2.4	-2.8		1	3.1	-2.8	3.7	-4.3
	2	-6.7	-1.3	-6.2	-2.8		2	3.6	-0.4	1.4	-1.1
	4	-6.1	-0.6	-8.5	-4.2		4	1.6	-2.9	-1.2	1.0
	8	-5.4	-4.7	-13.8	-6.2		8	0.7	4.8	-3.5	-3.0
	24	-24.5	-4.6	-33.6	-14.5		24	-10.1	0.7	-8.4	-2.1
	48	-43.6	-9.0	-54.2	-20.6		48	-18.9	-3.4	-11.3	-6.9
Cilastatine	0	2.3	2.3	0	0	**Daptomycin**	0	-6.4	-6.4	0.0	0.0
	1	8.0	-0.1	-3.6	-3		1	2.0	-5.3	-3.8	6.3
	2	2.7	4.6	-2.7	-2		2	0.1	-7.7	-0.1	4.8
	4	1.3	-3.3	-3.3	-2		4	1.8	-1.0	-6.3	6.5
	8	6.9	0.9	-0.5	-1.9		8	0.9	-2.3	-0.3	3.3
	24	0.9	6.1	-2	-0.7		24	2.2	-3.6	1.2	2.7
	48	0.1	6.2	-1	-1.1		48	-2.3	-0.1	6.4	5.5
Piperacillin	0	0.8	0.8	0	0	**Flucloxacillin**	0	-0.1	-0.1	0.0	0.0
	1	-0.2	-1.8	-1.2	-2.4		1	6.0	-1.5	-2.4	6.2
	2	0.2	-3.2	-3	0.1		2	4.3	0.8	-1.5	-4.0
	4	-7.7	-6.3	-3.5	-1.5		4	-1.1	0.3	-1.9	7.3
	8	-8.8	-2.3	-4.3	-3.2		8	3.7	2.0	-6.7	-5.5
	24	-38.2	-4.2	-8.2	-4.2		24	2.2	3.2	-3.4	2.1
	48	-66.7	-6.6	-15.3	-3.2		48	-2.9	1.5	-14.3	-4.9
						Rifampicin	0	-3.7	-3.7	0.0	0.0
							1	-2.2	-6.7	5.4	-1.1
							2	-2.4	-5.4	5.2	-1.9
							4	-5.7	-5.8	2.4	1.8
							8	-6.2	-1.2	3.9	-0.6
							24	-5.0	-3.7	-1.3	-0.2
							48	-7.0	-2.8	-10.0	-0.4

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Declaration of Interests

☒ 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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Highlights

- Robust HPLC-MS/MS quantification of plasma levels of 12 daily-used antibiotics
- Directly covering the entire clinically relevant ranges of concentrations
- Simple extraction, short turn-around time, stable isotope-labeled internal standards
- In vivo studies on isomers of imipenem and cefepime
- Suitable for adjusting antibiotics dosage for optimal efficacy and minimal toxicity