Dissertation

zum Erwerb des Doktorgrades der Humanbiologie

an der medizinischen Fakultät der

Ludwig-Maximilians-Universität München

Investigation and development of stable isotope dilution mass spectrometry methods for therapeutic drug monitoring of anti-infective drugs used in the critically ill

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Carina Schuster

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Kumulative Dissertation

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1 Introduction

1.1 Background

1.1.1 Therapeutic drug monitoring

Pharmacokinetics and pharmacodynamics

Pharmacological drugs and medicines to treat a wide variety of illnesses have been used since time immemorial. However, detailed understanding of the dose-response relationship between a drug and its effects arised in the 1960s for the first time [1].

Effect and effectiveness of an administered drug is dependent from pharmacokinetics (PK) and pharmacodynamics (PD). Pharmacokinetics characterize all processes a drug passes though the human body. These include adsorption, distribution, metabolism and excretion (ADME). Consideration of following individual pharmacokinetic variables is decisive:

- **A**: Knowledge of the correct time of administration, dosage and application type (e.g. oral or subcutaneous) together with the bioavailability of a specific drug influence adsorption.
- **D**: Both, physicochemical properties and varying milieus between different compartments affect distribution of a drug to its side of action, e.g. the epithelial fluid for pneumonia.
- **M**: Metabolism enzymatically alters drugs (e.g. by glucuronidation or sulphation) and thus first activates, so-called pro-drugs, or commonly inactivates drugs before elimination.
- **E**: Excretion of a drug is dependent from its physicochemical properties, its previous distribution in hydrophilic or lipophilic compartments and its metabolism.

Pharmacokinetics are balanced with pharmacodynamics: the doctrine of the interactions between drug and body. Drugs mainly interact through agonistic or antagonistic effects on receptors or receptor-like proteins, also including intracellular compounds (e.g. enzymes) within a body or microorganism. In the end, respective drug effects are dependent from related concentration levels. This relationship is commonly illustrated by a dose-response curve, where concentration levels are specified into sub-therapeutic, therapeutic and toxic levels. Sub-therapeutic levels result in poor drug effectiveness, whereas toxic concentrations potentially induce adverse events. Consequently, adequate dosage is crucial to attain and maintain drug concentration within its therapeutic range, without the occurrence of relevant side effects.

Accurate dose adjustment is both dependent from pharmacodynamics like drug target concentration and pharmacokinetics such as individual metabolism, in other words the PD/PK target. In particular, this applies to bacterial or fungal infections [2, 3].

The PD/PK target of anti-infectives

PK/PD target attainment is substantial to treat bacterial and fungal infections successfully. On the one hand, the pathogen species and potential resistance patterns contribute to microbial pharmacokinetics. On the other hand, the minimal inhibitory concentration (MIC) of a pathogen and the respective time- or dose-dependent susceptibility to anti-infectives are mandatory for the evaluation of pharmacodynamics. Equally, considering intra- and inter-individual pharmacokinetic variabilities such as altered metabolism

and clearance, inflammatory processes and co-medication along with pharmacogenomics (age, weight or gender) are decisive [4, 5].

Above all, challenging pharmacokinetics emerge in critically ill patients: including altered distribution of drugs in third space fluids (e.g. edemas) due to organ dysfunction and increased vascular permeability, compromised metabolism and clearance through renal and hepatic impairment or life-supportive artificial organs such as extra corporal membrane oxygenation that potentially affect drug levels inside the patient.

Detailed understanding of pharmacodynamics and pharmacokinetics result in pathogen- and patient-dependent PD/PK targets and are consequently required for adequate therapeutic drug monitoring (TDM) [2, 6-8].

Therapeutic drug monitoring in the clinical laboratory

The determination of a specific drug concentration in body fluids, so-called therapeutic drug monitoring, primarily aims to improve dosage of drugs and therefore to attain PD/PK targets. Accordingly, the patient is protected against sub-therapeutic and toxic drug concentrations. In addition, TDM provides a broad range of other applications such as compliance controlling, prevention and identification of drug-drug interactions or causal analysis of therapy failure [3, 8-10].

Besides prevention and therapy of various diseases by analysis of endogenous and exogenous substances in body fluids, TDM became an emerging focus in clinical laboratories, recently. An indispensable aspect of TDM is the total turnaround time, the time that is required from sampling until the analytical result. Apart from the pre-analytical phase like sample handling, the analytical method implies a major variable of the total turnaround time. Analysis of complex matrices such as blood or urine requires analytical systems with high selectivity and in addition, sufficient sensitivity to quantify low concentrations of target substances. In combination with multi-analyte quantification, the ideal analytical method provides accurate results with short acquisition times [11-13].

Almost every clinical laboratory is equipped with fully automatic analyzers. These immuno-photometric systems offer high throughput opportunities and therefore short turnaround times. However, the lack of multi-analyte assays and cross-reactivity between structurally related compounds represent major drawbacks of these systems. Indeed, auto-analyzers require marginal staff capacities, but individual assay development to comply clinics with innovative applications is barely practicable. Lastly, the lack of standardization still generates heterogeneous results between different systems and laboratories [13, 14].

In contrast, liquid chromatography mass spectrometry (LC-MS) bears the potential to overcome these drawbacks: a high level of selectivity plus sensitivity and the ability to develop multi-analyte assays in combination with individualized drug panels. Additionally, reliable quantification of structurally related compounds like isomers or metabolites as well as physicochemically diverse analytes is both feasible. Especially with respect to standardization, monitoring of inconsistent results is essential. Due to analytical metadata generated by the LC-MS system, respective results can be re-evaluated. To

summarize, LC-MS is an excellent quantitation method in the field of TDM but still poorly disseminated [15-17].

Until now, main applications of LC-MS in clinical laboratories are TDM of antidepressants, antipsychotics or immunosuppressive drugs. However, an increasing number of studies report on inadequate standard dosage of anti-infectives, accordingly TDM of these drugs using LC-MS is a logical consequence [5, 6, 18, 19].

1.1.2 Liquid chromatography mass spectrometry

Since the 1970s high performance liquid chromatography (HPLC) is used in laboratories to separate and analyze various compounds in solutions and mixtures [20]. Depending on their polarity, the analytes show different retention times due to varying adsorption to the stationary phase and are accordingly separated [21, 22]. Then, quantification of the target analytes is performed by transferring the eluate to a detector such as a mass spectrometer (MS) coupled to the HPLC system.

Tandem mass spectrometry

The MS is equipped with an ion source, where the solvent is vaporized and the analytes are transferred into gas phase as positively or negatively charged ions. Within the MS, the ions are carried stepwise from atmospheric pressure to a vacuum before they reach the mass analyzer, which separates them by their specific mass-to-charge ratio (*m/z*). In clinical laboratories, almost exclusively triple quadrupole or tandem mass spectrometry (MS/MS) is applied so far. A quadrupole consists of four parallel metal rods arranged in a square with opposite rods having the same potential. This potential is generated by alternating and direct currents. As soon as a periodic voltage is applied to these quadrupole rods, the ions are subject to a constant alternation of repulsion and attraction. Hence, only ions with a specific *m/z* and therefore a stable path may pass through the quadrupole arrangement (see Figure 1) [23, 24].

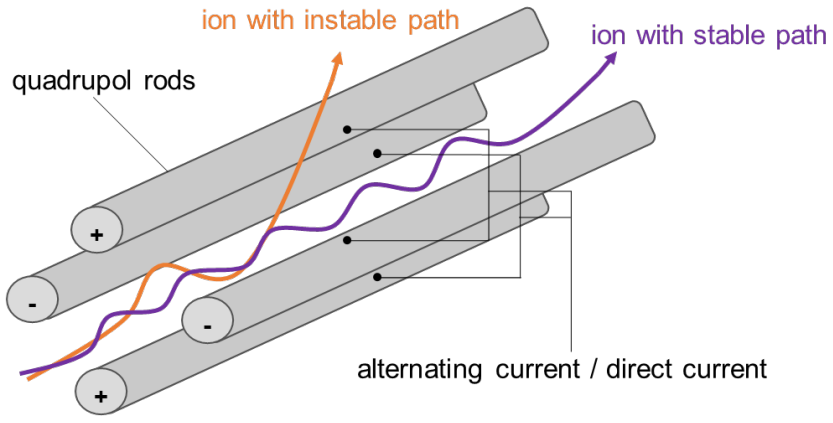

Figure 1: *Schematic representation of a quadrupole analyzer. Only ions with a specific mass-to-charge ratio (m/z) are able to pass through the quadrupole (purple line). Other ions with an irregular trajectory hit the metal rods, are discharged and therefore removed (orange line).*

Tandem MS, also called triple quadrupole MS, combines three quadrupoles in serial arrangement. Within this setting, multiple-reaction monitoring (MRM) mode is often used for quantification due to its

unique selectivity and sensitivity: The first and third quadrupoles act as mass filters. Whereas the second quadrupole represents a collision cell. The ions that traverse the first quadrupole, so-called precursor ions, are further fragmented in this cell into product ions by collision with inert gas (e.g. nitrogen). Lastly, specific product ions are selected by the third quadrupole and are able to pass to the ion detector, e.g. a photomultiplier [20, 24].

High-resolution mass spectrometry

A new type of mass analyzer is the Orbitrap-high-resolution mass spectrometer (HRMS). The Orbitrap-HRMS consists of a central electrode and an outer electrode that is divided by a ceramic ring (see Figure 2). Usage of a C-trap, which is a C-shaped quadrupole, is necessary to tangentially inject ions through high voltage pulses into the Orbitrap-HRMS [25]. An attracting electrostatic potential is applied to the central electrode inside the Orbitrap-HRMS, which counteracts the centrifugal force of the initial tangential velocity of the ions. The electrostatic field forces the ions to rotate around the central electrode with specific axial oscillations. The frequency of these axial oscillating ions is again dependent on their *m/z*, which is calculated using Fourier-transformation. Quantification based on frequencies generates high resolution and mass accuracy, which allows distinguishing between isobars without chromatographic separation. A distinctive characteristic of the Orbitrap-HRMS compared to MS/MS technology is its ability to act as mass analyzer and detector in combination. So far, Orbitrap-HRMS is predominantly used for analysis of proteins and metabolites or unknown screening [24, 26-28].

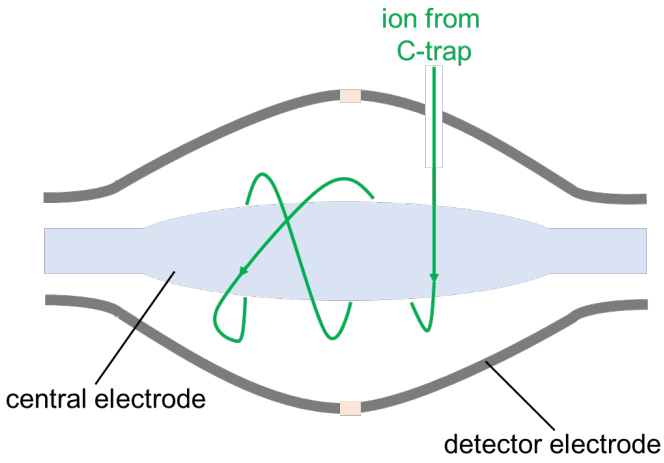

Figure 2: *Schematic representation of an Orbitrap-HRMS mass analyzer. Ions are tangentially injected by the C-trap and begin to oscillate axially around the central electrode. The frequency of the ion oscillation is specific for their respective mass-to-charge ratio (m/z).*

Isotope dilution technique

LC-MS applications for TDM mainly uses stable isotopically labeled internal standards (SIL-IS) to further improve its performance. Thereby, quantification is based on plotting the ratio of the analyte area to the SIL-IS area against respective concentrations. A SIL-IS differs in substitution of single atoms, typically exchange of hydrogen with deuterium or ^{12}C atoms with ^{13}C atoms, from the original analyte. Consequently both, analyte and SIL-IS, share the same physical and chemical properties and will behave almost identically regarding distribution within the matrix, retention time or ionization pattern.

Hence, usage of SIL-IS will correct variations during sample preparation or alterations inside the LC system. Furthermore, the suppression or enhancement of analyte ionization due to residual matrix components, so-called matrix effect, often compromises MS performance. Since analyte and SIL-IS share the same ionization pattern, usage of SIL-IS also fully compensates detrimental matrix effects [29-31].

1.1.3 Anti-infective drug application in patients with severe infections

Among any patient collective, intensive care unit (ICU) patients are at eminent risk to suffer from severe infections caused by invasive fungi or bacteria. The situation of ICU patients even exacerbates due to development of resistant bacterial and fungal strains [5, 6]. Extensive usage of antibiotics and antimycotics in agriculture and farming, inappropriate prescribing, missing identification of pathogens and resistance patterns leads to the emergence of antimicrobial resistance, which is even declared as one of the “ten threats to global health in 2019” by the World Health Organization [32]. In addition to their vulnerable health condition, altered pharmacokinetics in ICU patients through increased vascular permeability and volume of distribution, organ dysfunction and transplantation, extensive co-medications and co-morbidities or extracorporeal therapies, as mentioned in section *1.1.1*, facilitate invasion of pathogens and thus complicate PK/PD target attainment [33-36]. Treatment with sub-therapeutic dosages, resulting from failed PK/PD targets, triggers resistance and increases the risk of a negative outcome, especially in critically ill. To improve anti-infective treatment and attain PK/PD targets, TDM is a powerful tool [8, 37].

1.1.4 Aim and scope

In this thesis, the transversal objective was the investigation of the potential of complex MS-methods for application in the field of therapeutic drug monitoring using both tandem MS and Orbitrap-HRMS technology.

Besides analyte and technique specific requirements, methods intended for TDM application in clinical routine should comply with the following criteria: Practicability providing easy-to-use procedures and high-throughput as well as high sensitivity and selectivity for accurate quantification of target analyte at low concentrations in complex matrices. Furthermore, the capability of multi-analyte quantification in combination with wide dynamic ranges allows for broad performance spectra and quantitative working ranges covering sub-therapeutic up to toxic concentrations. Finally, method flexibility is crucial to integrate additional analytes for quantification and thus to provide innovative services. These method specifications for TDM in the clinical routine finally enable adequate control of serum levels, especially in critically ill patients.

The aim of the first project was to develop and to study a reliable method for the quantification of six most widely used antibiotics in critically ill using tandem mass spectrometry. A main aspect and technicolgical challenge of this study was the establishment of wide dynamic ranges to determine any relevant antibiotic serum concentration. In addition, a video was published to test an innovative

publication format and therefore to increase dissemination towards scientific colleagues and clinical laboratories.

The objective of the second project was to develop and to study a method to quantify the 11 most commonly used systemic antimycotics in ICU patients using high-resolution mass spectrometry. In this context, the broad *m/z* range of the physicochemically heterogeneous analyte panel required considerable efforts for accurate quantification. Moreover, a main goal was to evaluate suitability of high-resolution mass spectrometry in clinical routine of small molecule TDM by comparison of a panel of leftover routine samples with an established LC-MS/MS method.

1.2 Development and investigation of an isotope dilution LC-MS/MS method for therapeutic drug monitoring of antibiotics

Clear recommendations for the TDM of antibiotics from different substance classes are published [8, 36, 38]. Using LC-MS provides the ability to quantify analytes with different physicochemical properties in a single run and thus allows multi-analyte TDM. The beta-lactams cefepime, meropenem and piperacillin, the oxazolidinone linezolid, as well as the fluorochinolones ciprofloxacin and moxifloxacin are the most commonly used antibiotics in ICU. A major drawback of many analytical methods is the incomplete coverage of the clinically relevant range (sub-therapeutic and toxic concentrations) due to a complex combination of target measurement quantities [36, 39-42]. Despite these ambitious measurement quantities, broad calibration ranges in the original articles in this doctoral thesis were established to cover both peak levels, e.g. after bolus administration, and trough levels close to MIC of specific pathogens. Hence, measurements of concentrations from 0.05 mg/L ciprofloxacin to 400 mg/L piperacillin were practicable.

An additional challenge of the method development was to optimize analyte extraction protocol in order to provide adequate recovery and reproducibility results for all analytes. Therefore, methanol, ethanol, dichloromethane, dimethyl sulfoxide, acetonitrile, acetone and *tert*-butylmethylether were tested in different compositions and volumes for protein precipitation. Finally, a combination of methanol and *tert*-butylmethylether (90:10, v/v) was chosen for sample clean-up. Furthermore, samples were processed in cold environment whenever possible due to instability of piperacillin, meropenem and cefepime [43]. In the end, all analytes were chromatographically separated within 4 min, giving a total assay turnaround time of 25 min in order to provide short reporting times to the physicians.

The method was also tested thoroughly for analyte selectivity. Even though tandem MS technology is highly selective, as described in section *1.1.2*, interference with endogenous compounds from the specimen (the so-called matrix) and other exogenous interfering substances (e.g. patient medication and metabolites thereof) must be excluded during method validation. Especially, multimorbidity and polypharmacy introduce a high degree of complexity in the critically ill with the risk of unforeseen analytical interference. In addition, specimens showing interferences through hemolysate, bilirubin or lipids are common in clinical routine. Therefore, providing high selectivity is mandatory to obtain accurate results. For this purpose, blank specimens without the analytes in this study were investigated. To include the maximum amount of interfering elements, a highly diverse sample set was collected. The

sample set included various patient samples (both ICU and non-ICU) and samples spiked with hemolysate, bilirubin and lipids (HIL, hemolysis, icterus, lipemia). After thorough investigation, none of the samples showed interfering peaks at respective analytical retention times, so excellent method selectivity could be demonstrated.

The method was validated according to the *Guideline on bioanalytical method validation* from the European Medicines Agency (EMA). Guidelines for LC-MS methods quantifying xenobiotics are lacking and therefore the EMA guideline is commonly used for LC-MS method implementation and validation [44].

The method was not only published in the *Journal of pharmaceutical and biomedical analysis,* but also in the *Journal of visualized experiments* using the exact method protocol, which allows method-transfer with a video step-by-step guide. We decided to apply this innovative publication format to facilitate the dissemination of this technically demanding methodology.

To these publications, the author of this doctoral thesis contributed substantially in the processes of conceptualization, method development, formal evaluation and analysis of data, writing, review and editing.

1.3 Development and investigation of an isotope dilution LC-Orbitrap-HRMS method for therapeutic drug monitoring of antimycotics

Clinical relevance is also true for TDM of antimycotics. Adequate PK/PD target attainment equally leads to fewer side effects and development of resistances [5]. Azole antimycotics, echinocandins, 5-flucytosine (a cytosine-analogue) and amphotericin b (a polyene) are a heterogeneous and physicochemically more diverse panel with an *m/z* range from 129 – 1270 compared to the previously addressed antibiotics (*m/z* 331 – 517). Accordingly, LC-MS is the predestined method for quantification of this disparate, multi-analyte panel.

To achieve short turnaround times, online solid phase extraction using a turbulent flow extraction column was implemented for sample preparation: after the testing of several extraction columns with reversed phase properties, no adequate retention of the analyte 5-flucytosine could be achieved. Only, using an innovative extraction column with both reversed phase and ion exchange properties and mobile phases that were adapted to the necessary pH values, adequate retention of all analytes was possible. Applicability of clinical practice was further optimized using commercially available antimycotics TDM kit components. After protein precipitation by the manufacturer´s precipitation reagent and purification of the supernatant by the novel mixed-phase extraction column technology, the purified samples were separated within 4 min using an analytical C18 column and the eluent was transferred into the Orbitrap-HRMS.

Compared with precursor-to-product monitoring using MS/MS, Obritrap-HRMS detects non-fragmented precursor ions in full-scan mode. Selectivity of the Orbitrap-HRMS full-scan mode is ensured by high mass resolution and accuracy due to quantification based on frequencies, as described in section *1.1.2*. However, this principle was examined by analysis of more than 60 samples from ICU, non-ICU and TDM

panel QCs. No interfering peaks at analyte retention times could be detected after detailed investigation. Hence, selectivity of Orbitrap-HRMS using full-scan mode could be verified.

The method was comprehensively validated according to the *Guideline on bioanalytical method validation* from the European Medicines Agency (EMA) [38]. In addition, usage of three calibrators, as suggested by the kit manufacturer was compared to an extended range of six calibrators, as the EMA guideline recommends. The results of the quantifications were consistent using both a three-point and six-point calibration assay. Therefore, usage of only three calibrators is practicable in clinical routine. However, awareness is mandatory that invalid calibration curves may be generated using only three calibrators, if one calibrator fails the quality criteria (e.g. deviation from the nominal concentration ≥ 15%).

A main objective of this doctoral thesis was to evaluate whether Orbitrap-HRMS is suitable for TDM of small molecules, like the well-established MS/MS technology. So far, main application of Orbitrap-HRMS is predominantly in the field of proteomics and metabolomics, as mentioned in section *1.1.2*. Evaluation of Orbitrap-HRMS suitability for small molecule TDM was executed by quantification of leftover routine samples (containing itraconazole, OH-itraconazole, posaconazole and voriconazole) with a clinical routine LC-MS/MS method against the herein validated LC-Orbitrap-HRMS method. The results of both methods showed good correlation, regardless of using three or six calibrators. Furthermore, development of both, antibiotic and antimycotic TDM assays, revealed practicability of Orbitrap-HRMS besides MS/MS, especially with respect to efforts in system and tuning set-up, where Orbitrap-HRMS showed rapid feasibility. Additionally, time-consuming product ion search was dispensable due to usage of full-scan mode without loss of selectivity. On the other hand, the significant higher costs of an Orbitrap-HRMS system have to be taken into account. Purchasing this kind of system may be an economic challenge in a clinical laboratory, especially in cases without scientific scope besides the routine TDM. In those situations, usage of MS/MS is clearly superior, as these systems comply with any routine requirements and are considerably less expensive.

Hence, the capability of Orbitrap-HRMS for development of efficient methods targeting low molecular weight TDM alongside MS/MS was found. Thus, the extension of the Orbitrap-HRMS application in the field of TDM is justified. In addition, Orbitrap-HRMS technology allows for known and unknown screening or acquisition of metabolomic data in any specimen as well as retrospective sample analysis due to full-scan mode with the result of facultative information and broadened application [28].

The doctoral candidate was responsible for all parts of the project and publication respectively: project planning and conceptualization, experimental laboratory work (e.g. LC-MS method development in combination with all aspects of sample preparation), planning and realization of the method validation protocol, measurements, data analysis and evaluation, manuscript writing and submission.

1.4 Summary / Zusammenfassung

Severe infections caused by bacteria or fungi are a leading cause of morbidity and mortality in critically ill patients. Therapeutic drug monitoring is of uppermost importance for PK/PD target attainment and therapy control. Due to the structurally diverse antibiotic and antimycotic panels and the analytical

advantages over established immuno-photometric analyzers, LC-MS is the most suitable quantification technology, allowing both high selectivity and sensitivity.

In the first project of this doctoral thesis, an isotope dilution LC-MS/MS method was developed for quantification of six commonly used antibiotics in intensive care units. By protein precipitation with methanol and *tert*-butylmethylether (90:10, v/v) and rapid analytical separation within 4 min, a results-turnaround time of 25 min was achieved. In addition, wide dynamic ranges were established to cover serum concentrations from sub-therapeutic up to toxic. Good clinical suitability for everyday use as well as reliable and robust quantification of antibiotics was demonstrated by comprehensive validation.

The second project aimed at developing an isotope dilution LC- Orbitrap-HRMS method for quantification of eleven systemic antimycotics. By using a mixed-mode extraction column and subsequent analytical separation, a very clean sample was obtained before injection into the Orbitrap-HRMS system. The method provided accurate quantification of the heterogeneous analyte panel with broad *m/z* range. After validation, the method was compared to a routinely established LC-MS/MS method. This evaluation showed that novel Orbitrap-HRMS technology is appropriate for TDM of small molecules besides MS/MS.

Considering their respective assets and drawbacks, both MS/MS and Orbitrap-HRMS technologies enable the development of particularly efficient and reliable methods for small molecule TDM.

Eine der Hauptursachen für Morbidität und Mortalität bei kritisch kranken Patienten sind schwerwiegende bakterielle oder mykotische Infektionen. Um definierte PK/PD-Zielspiegel zu erreichen und Therapiekontrolle zu ermöglichen ist das therapeutische Drug-Monitoring von entscheidender Bedeutung. Durch die strukturelle Vielfalt von Antibiotika und Antimykotika sowie der analytischen Vorteile gegenüber etablierten immuno-photometrischen Messgeräten, ist die LC-MS die geeignetste Quantifizierungsmethode, die neben hoher Selektivität auch hohe Empfindlichkeit gewährleistet.

Im ersten Teil dieser Doktorarbeit wurde eine Isotopenverdünnungsmethode mittels LC-MS/MS entwickelt, die zur Quantifizierung von sechs, häufig auf Intensivstationen verwendeten Antibiotika, diente. Nach Proteinfällung mit Methanol und *tert*-Butylmethylether (90:10, v/v) und anschließender analytischer Trennung innerhalb von 4 min wurde eine effektive Gesamt-Turnaround Zeit von 25 min erreicht. Des Weiteren wurden breite dynamische Bereiche etabliert, um sowohl subtherapeutische als auch toxische Serumkonzentrationen zu erfassen. Gute klinische Alltagstauglichkeit sowie zuverlässige und robuste Quantifizierung der Antibiotika wurde hierbei im Rahmen einer umfassenden Validierung gezeigt.

Der zweite Teil der Arbeit hatte ebenso die Entwicklung einer LC-MS-basierten Isotopenverdünnungsmethode zum Ziel, jedoch unter Verwendung eines Orbitrap-HRMS zur Quantifizierung von 11 systemischen Antimykotika. Durch den Einsatz einer „*Mixed-Mode*“ Extraktionssäule konnte nach darauffolgender analytischer Trennung ein sauberes Eluat gewonnen werden, welches dann zum Orbitrap-HRMS weitergeleitet wurde. Die Methode ermöglichte die exakte Quantifizierung aller heterogenen Analyten, die eine breite *m/z*-Verteilung aufwiesen. Im Rahmen der Validierung wurde die Methode mit einer routinemäßig etablierten LC-MS/MS-Methode verglichen. Die

Auswertung ergab, dass die neuartige Orbitrap-HRMS-Technologie für das therapeutische Drug-Monitoring von kleinen Molekülen ebenso geeignet ist wie die MS/MS.

Unter Berücksichtigung ihrer jeweiligen Vor- und Nachteile erlauben sowohl MS/MS- als auch Orbitrap-HRMS-Technologien die Entwicklung besonders effizienter und zuverlässiger Methoden für das TDM von kleinen Molekülen.

2 Original articles

2.1 Paal, M., Zoller, M., Schuster, C., Vogeser, M., & Schuetze, G. (2018). Simultaneous quantification of cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid and piperacillin in human serum using an isotope-dilution HPLC-MS/MS method. *J Pharm Biomed Anal*, 152, 102-110

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Simultaneous quantification of cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid and piperacillin in human serum using an isotope-dilution HPLC–MS/MS method

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ABSTRACT

The aim of the current study was to develop and validate a robust multi-analyte high performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) method for simultaneous quantification of cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid and piperacillin, which are the most commonly used antibiotics in intensive care units.

Sample clean-up included a protein precipitation protocol, followed by chromatographic separation on a C_8 reverse phase HPLC column within 4 min, using a formic acid-ammonium formiate methanol step-elution gradient. All compounds were detected with electrospray ionization (ESI+) mass spectrometry in multiple reaction time monitoring. The method was validated according to the protocol from the European Medicines Agency and was thoroughly evaluated for interferences and quantification linearity.

Linear relationships between peak area responses and drug concentrations were obtained in the range of 0.25–200 mg/l for cefepime, 0.25–120 mg/l for meropenem, 0.05–10 mg/l for ciprofloxacin, 0.125–10 mg/l for moxifloxacin, 0.125–50 mg/l for linezolid and 0.5–400 mg/l for piperacillin with an $R^2 > 0.997$. Imprecision and inaccuracy values (both intra- and inter-assay) were $\leq$ 6.8% and $\leq$10.9% for all analytes in quality control samples, respectively. The assay proved to be selective for the study antibiotics, and the internal standards consistently compensated for matrix effects.

The described simple and reliable HPLC–MS/MS assay is a powerful tool for routine TDM of cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid and piperacillin in human serum in clinical laboratories. With a total process time of approximately 30 min, it allows for accurate and selective quantification up to the expected pharmacokinetic peak concentrations

1. Introduction

Severe infection is an important source of morbidity and mortality in intensive care unit (ICU) patients [1]. Timely administration of the antimicrobial substance with appropriate drug level and pathogen spectrum is of paramount importance for clinical success [2]. Achieving sufficiently high concentration levels is also relevant for preventing the development of antimicrobial resistance [3].

Antimicrobial therapy is usually based on standard dosing protocols. However, numerous studies point out that heterogeneous patient populations with growing complexity have pharmacokinetics that are subject to substantial inter-individual variation (e.g. the critically ill, obese, elderly, patients with severe comorbidities) [4–8]. Accordingly, traditional antibiotic dosing strategies are becoming increasingly problematic, as the pharmacokinetic/pharmacodynamic (PK/PD) target that is required for effective treatment of severe infections may not be reached [9–11]. Especially the prediction of drug exposure of hydrophilic antibiotics (e.g. ß-lactams) in ICU patients remains challenging, due to pathophysiological changes in distribution und drug clearance [12,13]. However, broad-spectrum antibiotics, such as piperacillin (usually prescribed with tazobactam) and meropenem, are widely used in intensive care [14,15]. For both substances inadequate PK/PD target attainments have been reported in several studies [9,16,17].

An individualized way to overcome variability and underdosing, is guided treatment with therapeutic drug monitoring (TDM) in an attempt to both maximize the antimicrobial efficacy and also to minimize toxic side effects, consequently improving the general clinical outcome from infections [18]. For the implementation

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of local and national antibiotic stewardship strategies there is an ever-increasing demand for antibiotic TDM [19], which has therefore received a lot of attention in recent years [20–24]. In clinics, TDM has been routinely used in the past for glycopeptide antibiotics and aminoglycosides, as these antibiotics have a narrow therapeutic index [18,25]. As commercial procedures are not available for generic antibiotic TDM in clinical routine, quantification methods for other antibiotic classes have to be developed and be validated in-house. To find application in clinics, TDM methods have to be robust and convenient, allowing for easy sample preparation, short assay run times and proper quantification in the clinical relevant range.

Various methods for multi-analyte antibiotic TDM have been published in the past [26–37]. Among these, several high-performance liquid chromatography (HPLC) procedures were combined with UV-detection [26–29]. These methods, however usually have rather long run times, low detection capabilities and they are not very selective, leading to the risk of interferences with co-medications and endogenous substances. By comparison, chromatographic separation coupled with tandem mass spectrometry (MS/MS) has greater detection capabilities and an improved selectivity [31–37]. Many of these published methods quantify structurally related substances from one antibiotic class only, especially ß-lactam antibiotics [38]. However, a TDM approach that analyzes the most relevant antibiotics from different classes can be more efficient in terms of high sample throughput and costs. It must be emphasized that several methods described in literature have rather narrow calibration ranges, which do not allow to measure peak concentrations and toxic levels without the requirement of sample dilution.

Summarized, there is a great demand for antibiotic TDM in many medical facilities on a day-to-day basis, allowing quick adaption of dosing with least possible turn-around times. Therefore, the purpose of the current work was to establish a simple and reliable multi-analyte LC–MS/MS method for quantification of the most utilized antibiotics in critical care that could be established in mass spectrometry laboratories with minimal expense. The assay required a broad concentration range, with a lower limit of quantification (LLOQ) as close to the minimal inhibitory concentrations (MIC) of susceptible microorganism and an upper limit of quantification (ULOQ) exceeding expected pharmacokinetic peak concentrations that may be obtained after bolus administration.

2. Materials and methods

2.1. Chemicals and reagents

A drug free serum pool was obtained from the blood donation center of the Bavarian Red Cross (Munich, Germany). Antibiotics cefepime hydrochloride, meropenem trihydrate, ciprofloxacin, moxifloxacin hydrochloride and piperacillin sodium salt, were provided by Sigma-Aldrich (St. Louis, Missouri, USA). Linezolid, and isotope-labelled antibiotics meropenem-D_6, ciprofloxacin-D_8, moxifloxacin hydrochloride-$^{13}C_1D_3$, linezolid-D_3, and piperacillin-D_5 were obtained from Toronto research chemicals (Toronto, Ontario, Canada). Isotope-labelled cefepime-$^{13}C_1{}^2D_3$ sulfate was from Alsachim (Straßbourg, Grand Est, France). HPLC-grade water, methanol, acetonitrile was from Baker (Jackson, Tennessee, USA), formic acid from Biosolve (Dieuze, Grand Est, France), ammonium formate from Sigma-Aldrich (St. Louis, Missouri, USA). Drugs used for the interference studies were obtained from Roche (Basel, Switzerland), Chromsystems (Gräfeling, Germany), Recipe (Munich, Germany) and Invicon (Munich, Germany). All used chemicals were of the highest purity available from the commercial suppliers.

2.2. Calibrators samples, quality control samples and internal standards

Stock solutions and corresponding ten-fold spike solutions for the antibiotics cefepime hydrochloride, meropenem trihydrate, linezolid and piperacillin sodium salt were prepared by separate weighting in a methanol-water solution (25:75, v/v). Stock solutions of moxifloxacin hydrochloride and ciprofloxacin were also prepared in a methanol-water solution (25:75, v/v), however with the addition of 20 mM acetate. These stock solution volumes were then combined to yield both the spike solutions for the calibrators and quality controls (QC). To preserve the physiological matrix, only one volume of 10x spike solution was admixed with nine volumes of drug free serum. A total of eight calibration standards and four quality control samples was prepared (see Table 1 for exact concentrations). After preparation, stock and spike solutions, calibrators and QC-samples were aliquoted and kept at −80 °C. The nominal concentrations of QC-samples were verified by comparative measurements with independent mono-analyte specific LC–MS/MS methods.

Stock solutions of internal standard (IS) with 1 mg/ml concentration were prepared by diluting cefepime-$^{13}C_1{}^2D_3$ sulfate in distilled water; meropenem-D_6, linezolid-D_3 and piperacillin-D_5 in a methanol-water solution (50:50, v/v); ciprofloxacin-D8 in a methanol-water solution (50:50, v/v) including 20 mM acetate and moxifloxacin hydrochloride-$^{13}C_1D_3$ in distilled water with 20 mM acetate. The internal standard stock solutions were then combined in a methanol-water mix (25:75, v/v) to prepare a IS standard mixture containing 30 mg/l cefepime-$^{13}C_1{}^2D_3$, 25 mg/l meropenem-D_6, 2.5 mg/l ciprofloxacin-D_8, 5 mg/l moxifloxacin hydrochloride-$^{13}C_1D_3$, 5 mg/l linezolid-D_3 and 25 mg/l piperacillin-D_5. Internal standard stock solutions and the mixture were temporarily stored at −20 °C until use.

2.3. Method development

Method development was initiated with a Reprosil PUR ODS-3 (100 mm × 2.1 mm, 5 μm particle size) column (Dr. Maisch, Ammerbuch-Entringen, Germany) using mobile phases A 10 mM ammonium formate in water-formic acid (99.9:0.1, v/v) and B acetonitrile. To establish an efficient extraction method for the antibiotics in this study, protein precipitants in different combinations were tested, including methanol, acetonitrile, dimethyl sulfoxide, methyl-*tert*-butyl ether, acetone, isopropanol, zinc-sulfate and trichloroacetic acid. The best results with regard do extraction yields and reproducibility were obtained with a methanol – methyl-*tert*-butyl ether (90:10, v/v) precipitation reagent.

To improve chromatography, various reverse phase C8 and C18 columns were tested with different mobile phases and gradients. Satisfactory chromatographic separation was obtained within 4 min using a Fortis 3 μm C_8 reverse phase column (100 mm × 2.1 mm, 3 μm particle size) (Neston, Cheshire, UK) selecting the mobile phases A 10 mM ammonium formiate in water-formic acid (99.9:0.1, v/v) and B methanol for a step-elution chromatographic protocol. Injecting varying volumes of processed QC A and D samples, identical responses (ratio of peak area analyte/peak area IS) were obtained for meropenem, moxifloxacin, piperacillin within 3–30 μl and for cefepime, ciprofloxacin, linezolid, within 15–50 μl, respectively.

2.4. Sample preparation

First, 100 μl of either calibrator, QC or patient serum samples were mixed with 25 μl of the internal standard mixture in 1.5 ml polypropylene cups (Eppendorf, Hamburg, Germany) for 5 min at

Table 1
Concentrations of calibrators and quality controls for the study antibiotics.

Analyte	Calibration standards (mg/l)							Quality controls (mg/l)			
	1	2	3	4	5	6	7	A	B	C	D
Cefepime	0.25	0.75	2.5	10.0	50.0	125.0	200.0	0.75	50.0	100.0	150.0
Meropenem	0.25	0.75	2.50	10.0	40.00	80.0	120.0	0.75	30.0	60.0	90.0
Ciprofloxacin	0.05	0.1	0.3	1.0	3.0	6.5	10.0	0.150	2.5	5.0	7.5
Moxifloxacin	0.125	0.25	0.75	2.0	5.0	7.5	10.0	0.375	2.5	5.0	7.5
Linezolid	0.125	0.25	0.75	3.0	12.5	27.5	50.0	0.375	12.5	25.0	37.5
Piperacillin	0.5	1.5	6.0	25.0	100.0	225.0	400.0	1.5	100.0	200.0	300.0

room temperature using a vortex shaker (Eppendorf, Hamburg, Germany). 150 μl methanol – methyl-*tert*-butyl ether precipitation solution (90:10, v/v) were added, followed by shaking for another 5 min. After centrifugation at 20.000*g* for 10 min at 4 °C, 75 μl supernatant were diluted 1:3 with HPLC grade water in a glass vial with microinsert that was then loaded to an autosampler (10 °C sample cooling, ±5 °C) ready for injection.

2.5. HPLC conditions

Chromatography was performed on a Waters 2795 Alliance HT HPLC system that was coupled to a Waters Quattro micro API Tandem Quadrupole System (Waters, Milford, Massachusetts, USA). Control of all system components and monitoring of the chromatographic run was performed with the Mass Lynx V4.1 software (Waters, Milford, Massachusetts, USA).

Chromatographic separation was performed with a total run time of 4 min using a Fortis 3 μm C_8 reverse phase column (100 mm x 2.1 mm) with a 3 μm particle (Fortis, Cheshire, UK) and a 2 μm pre-column filter (Chromsystems, Gräfeling, Germany). The column chamber was held at 30 °C. Mobile phases A 10 mM ammonium formiate in water-formic acid (99.9:0.1, v/v) and B methanol, were delivered at a flow rate of 0.5 ml/min in step dilution mode. Starting at 7% B for 0.1 min, mobile phase B was suddenly increased to 65% and held for another 0.5 min. Afterwards the column was washed with 95% B for 1.5 min and finally re-equilibrated to starting conditions for approximately 2 min with 7% mobile phase B. The generic sample injection volume was 15 μl in a 100 μl loop (partial loop filling mode, air prefill: pre- and post-sample 2.0 μl). Retention times were: 1.66 min for cefepime, 1.81 min for meropenem, 2.04 min for ciprofloxacin, 2.16 min for moxifloxacin, 2.31 min for linezolid and 2.38 for piperacillin. A typical MRM chromatogram for calibrator 3 is depicted in Fig. 1. Autosampler wash conditions were as follows: the wash solvent was methanol, the seal and purge solvent a methanol-water-formic acid solution (7:92.9:0.1, v/v/v), the wash sequence was: 1x purge-wash-purge with 600 μl purge solvent replacement volume.

2.6. Mass spectrometry conditions

Electrospray ionization in the positive mode (ESI+) was used for all analytes with following ion source settings: capillary voltage 1.5 kV; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas flow rate, 600 l/h; RF lens voltage 0.1 V.

The best mass transition-specific parameters, including the collision energy and cone voltage were manually tuned for each antibiotic substance and its corresponding internal standard by infusion of neat substance at 10 μl/min. Quantifier and qualifier product ions were selected for optimal mass spectrometry detection. Multiple reaction monitoring transitions (MRM) for each antibiotic and internal standard (quantifier and qualifier) with the corresponding cone voltage and collision energy are shown in Table 2. The dwell time was set to 80 ms for each channel. Data evaluation was performed with the Waters QuanLynx 4.1 software (Waters, Milford, Massachusetts, USA).

2.7. Method validation

Assay validation was conducted according to the guideline of bioanalytical method validation from the European Medicines Agency (EMEA), 21 July 2011 [39]. The procedure was evaluated in terms of limits of quantification, calibration curve, accuracy and precision, carry-over, selectivity, dilution integrity, recovery, matrix effect and stability. Further assay robustness experiments were conducted, with regard to interference from drugs and endogenous serum compounds, and linearity.

2.7.1. Limits of quantification

According to the EMA guideline the lower limit of quantification (LLOQ) represents the lowest concentration of an analyte in a sample that can be quantified reliably. The lowest calibrator with an inter-day imprecision and inaccuracy both <20% and a signal to noise ratio ≥ 5 is considered the LLOQ. Imprecision was expressed with the coefficient of variation (CV), inaccuracy with relative bias ($\delta_{\downarrow r}$)

Meeting the clinical requirements of antibiotic TDM, LLOQís were as close to the lowest minimal inhibitory concentrations (MIC) of susceptible pathogens from the EUCAST breaking point tables [40]. The upper limit of quantification (ULOQ) was defined as the calibration standard with the highest concentration of analytes.

2.7.2. Calibration curves

The clinical relevant TDM range was covered with seven calibrators containing the antibiotics that were processed together with a blank (without analyte and internal standard) and zero samples (including the internal standard only). According to the EMA guidelines back calculated concentrations of the calibration standards including the ULOQ should be within ±15% of the nominal value, except for the LLOQ for which it should be within ± 20%. The following calibration model was used: curve type, linear; origin, included; weighting function, 1/x; axis transformation, none.

2.7.3. Inaccuracy and imprecision

Inaccuracy und imprecision was tested by replicate analysis (n = 10 for intra-assay, n = 5 for inter-assay on five different days) using four different QC-samples A-D and the LLOQ, covering low, medium and high concentration levels. Acceptable criteria were deviations of the CV and δ_r within ±15%, except ±20% for the lowest calibrator. In addition, the total error was assessed in form of the relative root-mean-square-error (% RSME) that should also not exceed 15% according to the guidelines of the German Federal Medicine Council (RiliBÄK) [41]. The RMSE was calculated as follows:

$$\%\mathbf{RMSE} = \frac{\sqrt{\frac{\sum_{i=1}^{n}(\mu_i-\mu)^2}{n}}}{\mu}$$

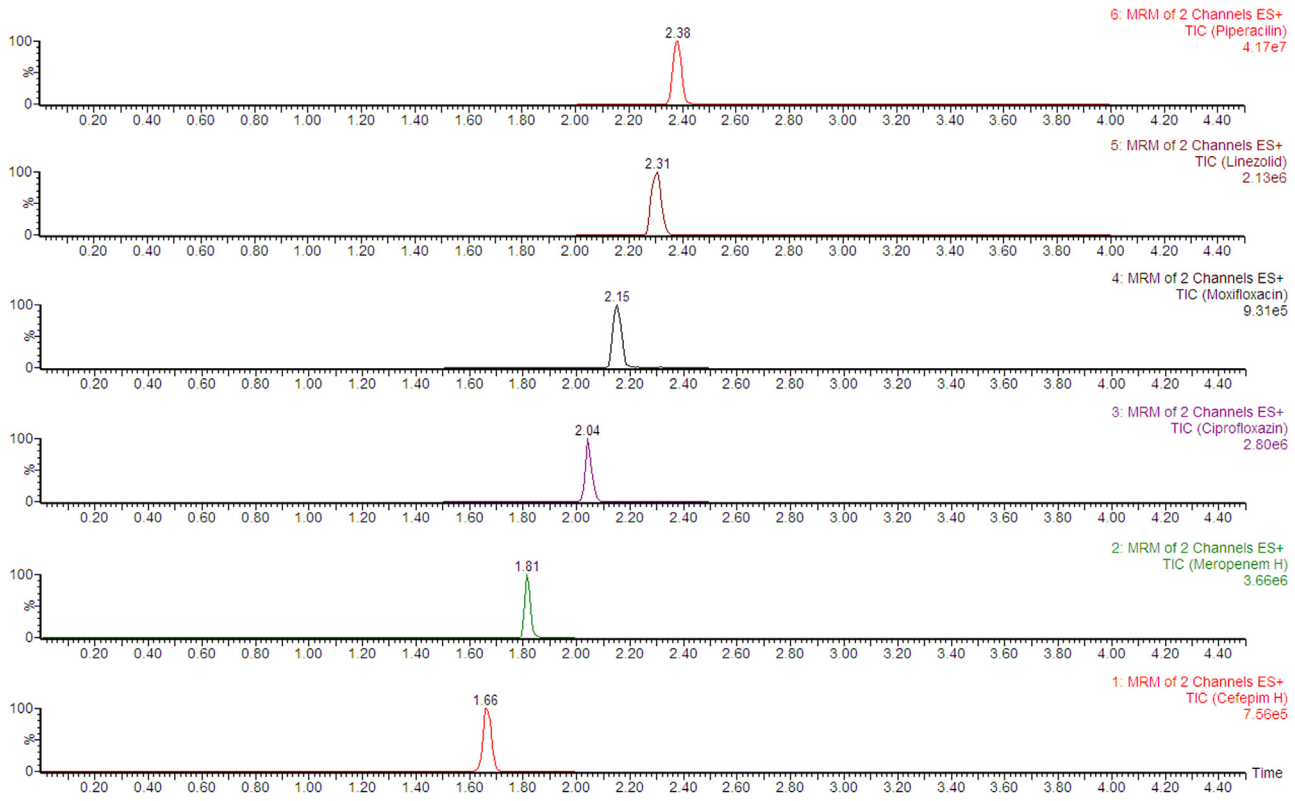

Fig. 1. Multiple reaction monitoring (MRM) chromatograms of analytes with calibrator 3 concentrations. Analytic retention times are given in minutes.

Table 2
Mass spectrometry parameters: multiple reaction monitoring transitions with parent and daughter ions for quantifier and qualifier.

Analyte	Rt (min)	Quantifier				Qualifier			
		Parent (*m*/*z*)	Daughter (*m*/*z*)	CV (kV)	CE (eV)	Parent (*m*/*z*)	Daughter (*m*/*z*)	CV (kV)	CE (eV)
Cefepime	1.66	481.0	167.0	20	22	481.0	395.7	20	13
Meropenem	1.81	384.1	114.0	20	25	384.1	141.0	20	16
Ciprofloxacin	2.04	332.0	231.0	30	35	332.0	245.0	30	25
Moxifloxacin	2.15	402.0	261.0	25	27	402.0	383.9	25	25
Linezolid	2.31	338.0	235.0	30	22	338.0	296.0	30	19
Piperacillin	2.38	518.0	143.0	25	20	518.0	358.9	25	10
Cefepime-$^{13}C_1{}^2D_3$	1.66	485.1	167.1	20	22	485.1	400.0	20	13
Meropenem-D_6	1.81	390.1	114.0	20	25	390.1	147.2	20	16
Ciprofloxacin-D_8	2.04	340.1	235.1	30	35	340.1	249.3	30	25
Moxifloxacin- $^{13}C_1D_3$	2.15	406.1	265.1	25	27	406.1	388.0	25	25
Linezolid-D_3	2.31	341.1	235.1	30	22	341.1	297.1	30	19
Piperacillin-D_5	2.38	523.1	148.2	25	20	523.1	364.1	25	10

Retention time, RT; cone voltage, CV; collision energy, CE.

where μ_i is the measured value of a sample that is compared to the target nominal value μ.

2.7.4. Carry-over

To test for carry-over, blank samples were injected after the highest calibration sample (the ULOQ). According to the EMA guideline the peak area in blank samples following the ULOQ should not exceed 20% of the peak area at the LLOQ and 5% of the peak area of the internal standard. In addition the carry-over was assessed injecting the ULOQ twice, followed by three injections of the LLOQ and quantifying the carry-over ratio with following formula using the peak area response signal [42]:

$$\mathbf{Carry-over}\ \% = [\frac{(\mathbf{LLOQ_{\#1}} - \mathbf{LLOQ_{\#3}})}{(\mathbf{ULOQ_{\#2}} - \mathbf{LLOQ_{\#3}})}] \times \mathbf{100}$$

where the symbol # refers to the injection number.

2.7.5. Selectivity

Selectivity was tested by spiking drug-free patient serum samples with varying degree of hemolysis, icterus and lipemia (HIL) with the antibiotics in this study and quantifying the concentration to be expected (n = 5). In addition, ten different serum samples were used from both non-ICU and ICU patients (n = 20) not treated with the antibiotics in this study, but receiving a broad range of other drugs that were maintained in their respective therapeutic range. Medications included other antimicrobials, psychotropic drugs, antihypertensives, diuretics, cardiac drugs, anti-inflammatories, analgesics and many other pharmaceutical drugs. Quality controls from various routine TDM panels in our laboratory were also tested for any interference. The substances investigated and their concentrations tested are given in the Supplemental Data, Table 1 and 2.

In accordance with the EMA guideline, the absence of interfering components is accepted when the response of an antibiotic and its

standard is <20% of the LLOQ for the analyte and <5% for the internal standard at the analytic retention time.

2.7.6. Dilution integrity

Dilution integrity was tested to ensure that analyte concentrations above the ULOQ in real samples (e.g. reaching toxic levels) could produce original concentrations by back calculation. Antibiotics with 75% and twice the ULOQ concentration were spiked to drug-free sera on two separate days, including hemolytic, lipemic and icteric (HIL) patient specimen (n = 5) for each concentration tested. Following serial dilution with distilled water (1:2, 1:3, 1:5), samples were prepared as described above and the initial concentration determined. According to the EMA guideline imprecision and inaccuracy should not exceed ±15%.

2.7.7. Matrix effects and recovery

Ion suppression and matrix effects were evaluated with post-column infusion experiments using neat calibrator 7 spike solution in methanol-water [25/75 (v/v)] with a flow rate of 10 μl/min.

Internal standard (IS) normalized matrix effects and the recoveries were examined according to the EMA guideline using three sample sets that had identical final analyte and internal standard concentrations. Antibiotic-free normal and hemolytic, icteric, lipemic (HIL) patient sera (n = 6) were spiked before (set B) and after sample preparation (set C) with low (3× LLOQ, 20% ULOQ) and high antibiotic concentrations (75% ULOQ) on three separate days and peak areas compared to ones derived from neat antibiotic samples in aqueous solution (set A). IS were added at the same stage to the specimen as the antibiotics.

Matrix effects are defined as the ratio of the peak area of antibiotics spiked after extraction (presence of matrix) to the peak area of neat analyte solution (absence of matrix). Since the internal standard is also affected by matrix effects, an IS normalized matrix factor was calculated as the ratio of the matrix effect of a specific analyte and the matrix effect of the corresponding internal standard. According to the EMA guideline both the coefficient of variation for the IS normalized matrix effect and the CV among the concentrations should not exceed 15%. The recovery is defined as the mean ratio between the peak area of the analyte spiked before extraction and the corresponding peak area of antibiotics spiked after extraction. According to the CLSI-IFCC-guideline [43] deviations of the mean value should be ≤15%.

2.7.8. Stability experiments

Stability experiments were performed with quality control samples QC B and QC D that were stored up to 6 h at RT, 24 h at 4 °C and 14 days at −20 °C. On-instrument stability of processed samples on the autosampler was also tested for 24 h. Freeze and thaw stability (−80 °C and RT, freeze time ≥12 h) of analyte in matrix was also tested. According to the EMA decreases in nominal concentrations ≥15% when compared to fresh counterparts that were immediately analyzed after preparation were considered relevant. In accordance with the EMA protocol no long-term stability experiments were performed as pre-analytical long-term stability at −80 °C has been addressed in previous works [44,45].

3. Results

3.1. Method validation

3.1.1. Limits of quantification

Each lowest calibrator concentration of the antibiotics in this study fulfilled the EMA criteria of the LLOQ with an (both intra- and inter-assay) imprecision, inaccuracy and RSME of ≤12.5%, ≤9.6% and ≤11.2% (summarized in Table 3). All analyte signals of the LLOQ were at least ≥ × 30 the signal of blank samples at corresponding antibiotic retention times.

3.1.2. Calibration curves

A linear regression model using weighting factor of 1/X was used for antibiotic quantification using seven calibrators. The method was linear over the whole concentration range with R^2 ≥0.997 for all antibiotics in this study.

3.1.3. Inaccuracy and imprecision

The data for intra- and inter-day inaccuracy and imprecision and the relative root-mean-square-error is summarized in Table 3. The coefficient of variation (CV), relative bias (δ_r) and RSME estimated at four concentrations with quality controls A–D never exceeded 6.8%, 10.8% and 10.9% in the validated concentration range, respectively.

3.1.4. Carry-over

Initially, the peak-area at the retention times of ciprofloxacin and moxifloxacin in blank calibration samples (without internal standards and analyte) after measurement of the highest calibrator considerably exceeded the criteria proposed by the EMA. Respective percentage carry-over ratios calculated according to Haeckel [42] were 0.98% for ciprofloxacin and 1.90% for moxifloxacin. Various strategies were employed to reduce the HPLC carry-over, including changes of the rinsing solution and the wash program [46]. Acidifying the purge solution with 0.1% formic acid and using a purge-wash-purge sequence considerably reduced the autosampler carry-over of the fluorchinolones.

Peak-areas observed in blank calibration samples after measurement of the highest calibrator were accordingly ≤5.5%, ≤4.5%, ≤26.0%, ≤9.6%, ≤ 4.9.% and ≤ 19.4% of the LLOQ for cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid and piperacillin. The peak-area of the respective internal standard did not exceed 5%. The percental carry-over ratio calculated according to Haeckel [42] was ≤0.03%, ≤0.03%, ≤0.21%, ≤0.18%, ≤0.02% and ≤0.07% for cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid and piperacillin. Although pH alterations and different additives in the wash and purge buffer were tested, the carry-over could not further be reduced for the fluorchinolone ciprofloxacin.

3.1.5. Selectivity

Quantification of spiked patient specimen (n = 5) was not disturbed by hemolysis, icterus and lipemia (HIL) as the quantified concentrations to be expected were 95.9–105.2%. None of the investigated substances, such as other antimicrobials, psychotropic drugs, cardiac drugs, analgesics, immunosuppressants (for further detail see Supplemental Data, Table 1) and none of the compounds in non-ICU and ICU patient sera (n = 20) were found to interfere with the quantification of the antibiotics in this study (see Supplemental Data, Table 2). For all samples tested no interfering peaks were present. Responses at the retention times were ≤7.9%, ≤4.0%, ≤10.2%, ≤3.3%, ≤0.6%, ≤6.6% of the LLOQ for cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid, piperacillin and ≤1% for all internal standards.

3.1.6. Dilution integrity

Inaccuracy and imprecision values for HIL sample dilution integrity are summarized in Table 4). They were found to be ≤13.7% and ≤9.1% for an initial concentration of 75% of the ULOQ (n = 5), and ≤13.8% and ≤8.4% for an initial concentration twice the ULOQ (n = 5), respectively.

3.1.7. Matrix effects and recovery

Post-column infusion experiments did not indicate relevant ion suppressions at the expected retention time of each analyte (see Fig. 2 in the online Data Supplement).

Table 3
Intra-day (n = 10) and inter-day (n = 5) inaccuracy and imprecision obtained from measurement of quality control (A–D) and LLOQ (calibrator 1) samples.

Analyte		QC A		QC B		QC C		QC D		LLOQ	
		Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Inter-assay
Cefepime											
	$\bar{x}$ (mg/l)	0.78	0.79	51.66	53.8	99.22	100.4	137.36	146.9	0.25	0.24
	CV (%)	5.6	4.8	3.9	4.0	4.5	5.7	3.4	6.8	8.1	12.5
	δ_r(%)	4.4	4.8	3.3	7.7	0.8	0.4	8.4	2.1	0.4	2.4
	RSME (%)	7.1	6.7	5.1	8.7	4.3	5.3	8.9	6.5	4.7	11.2
Meropenem											
	$\bar{x}$ (mg/l)	0.72	0.72	31.49	31.54	55.47	56.31	83.40	90.35	0.26	0.25
	CV (%)	3.5	6.5	3.2	3.9	2.8	2.6	2.2	4.8	7.1	4.5
	δ_r(%)	3.84	3.4	5.0	5.1	7.5	6.1	7.3	0.4	4.4	1.6
	RSME (%)	4.9	6.7	5.9	6.4	8.0	6.5	7.6	4.5	8.0	4.4
Ciprofloxacin											
	$\bar{x}$ (mg/l)	0.15	0.15	2.56	2.56	4.94	4.90	7.32	7.20	0.05	0.05
	CV (%)	5.0	3.8	2.9	4.9	2.4	2.3	3.6	3.0	3.7	4.5
	δ_r(%)	0.7	0.0	2.4	2.4	1.3	1.9	2.5	4.0	3.0	4.0
	RSME (%)	4.7	3.6	3.7	5.2	2.5	2.9	4.1	4.8	4.5	4.8
Moxifloxacin											
	$\bar{x}$ (mg/l)	0.35	0.37	2.57	2.63	4.68	4.95	6.69	7.38	0.11	0.13
	CV (%)	2.3	4.7	3.7	3.5	3.4	3.1	1.8	4.1	4.3	3.5
	δ_r(%)	6.7	1.3	2.6	5.2	6.5	0.9	10.8	1.5	9.6	2.4
	RSME (%)	6.9	4.5	4.4	6.2	7.2	3.0	10.9	4.1	10.3	4.0
Linezolid											
	$\bar{x}$ (mg/l)	0.37	0.37	11.94	12.73	23.74	24.55	34.12	35.74	0.12	0.13
	CV (%)	4.4	3.8	2.4	3.5	1.7	3.2	3.4	2.7	3.9	6.3
	δ_r(%)	1.9	2.1	4.5	1.9	5.0	1.8	9.0	4.7	4.0	5.6
	RSME (%)	4.5	4.0	5.0	3.8	5.3	3.5	9.5	5.3	5.4	8.2
Piperacillin											
	$\bar{x}$ (mg/l)	1.47	1.51	98.77	101.69	189.03	197.66	302.36	298.77	0.51	0.50
	CV (%)	5.5	4.0	2.9	3.4	4.3	2.5	2.7	3.5	2.3	7.3
	δ_r(%)	1.9	0.4	1.2	1.7	5.5	1.2	8.1	0.4	2.0	0.0
	RSME (%)	5.4	3.7	3.0	3.6	5.9	2.6	2.7	3.2	3.0	6.6

Observed mean concentration, $\bar{x}$; coefficient of variation, CV; relative bias, δ_r; relative root-mean-square-error, RSME; quality control samples, QC.

Table 4
Dilution integrity experiments spiking hemolytic, icteric and lipemic samples (n = 5) with antibiotic concentrations of 75% ULOQ and twice the ULOQ.

Analyte	Dilution factor											
	1:2				1:3				1:5			
	μ (mg/l)	$\bar{x}$ (mg/l)	CV (%)	δ_r(%)	μ (mg/l)	$\bar{x}$ (mg/l)	CV (%)	δ_r(%)	μ (mg/l)	$\bar{x}$ (mg/l)	CV (%)	δ_r(%)
High concentration (75% ULOQ)												
Cefepime	62.5	65.3	4.1	4.4	41.7	43.6	3.0	4.7	25.0	25.1	3.6	0.4
Meropeneme	40.0	43.8	4.0	9.4	26.7	30.3	3.4	13.7	16.0	18.2	3.2	13.6
Ciprofloxacin	3.3	3.3	9.1	2.2	2.2	2.2	4.2	1.9	1.3	1.4	3.7	5.1
Moxifloxacin	3.8	3.8	8.0	0.6	2.5	2.6	2.7	3.0	1.5	1.5	5.1	0.8
Linezolid	13.8	13.8	2.9	0.2	9.2	9.4	3.6	2.9	5.5	5.8	3.4	4.8
Piperacillin	112.5	118.3	4.3	5.2	75.0	81.0	3.6	8.0	45.0	47.8	3.1	6.1
Concentration out of range (2x ULOQ)												
Cefepime	200.0	196.6	7.9	1.7	133.3	128.1	2.8	4.0	80.0	84.7	6.8	5.8
Meropeneme	120.0	126.8	4.1	5.6	80.0	83.1	2.6	3.9	48.0	50.8	1.9	5.9
Ciprofloxacin	10.0	9.8	7.5	2.4	6.7	6.2	1.9	7.3	4.0	4.1	5.0	1.6
Moxifloxacin	10.0	10.0	1.7	0.1	6.7	7.0	2.4	5.0	4.0	4.1	3.5	1.4
Linezolid	50.0	49.3	7.8	1.4	33.3	31.3	3.2	6.0	20.0	20.8	8.4	3.9
Piperacillin	400.0	442.5	5.8	10.6	266.7	303.4	0.9	13.8	160.0	181.2	1.2	13.2

Expected nominal concentration, μ; observed mean concentration,$\bar{x}$; coefficient of variation, CV; relative bias, δ_r.

For the three concentration tested (3x LLOQ, 20% ULOQ, 75% ULOQ) using 6 samples the recovery rates for all analytes were found to be 77.9–110.3% with deviations of the mean value ≤13.4%. The internal standard (IS) constantly compensated for matrix effects, given by the IS normalized matrix factors in between 94.6 and 105.4% with a CV ≤8.3%. Imprecision of analyte peak area responses never exceeded 9.9% for all concentrations tested.

3.1.8. Stability experiments

All antibiotic concentrations tested were stable with an absolute deviation (%D) from the nominal concentration of fresh counterparts that was consistently lower than 13.0%. Stability was therefore given for serum samples stored 6 h at RT, 24 h at 4 °C, two weeks at −20 °C and up to three repeated freeze-thaw cycles (−80 °C and RT). Processed specimen at autosampler temperature (10 °C, ±5 °C) was also stable for 24 h (absolute%D value ≤ 11.1%).

4. Discussion

In the present study we developed and validated a HPLC–MS/MS multi-analyte method for simultaneous quantification of cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid and piperacillin using corresponding isotope-labelled internal standards. Commercially available human serum was used as matrix for the preparation of calibrators and quality control samples, guaranteeing absence of interfering drugs. Of all protein precipitating agents tested, best extraction yields and reproducibility were obtained with a methanol-methyl-*tert*-butyl ether (90:10, v/v) mixture. Analyte extraction was performed within 25 min, followed by a 4 min step-elution HPLC chromatographic separation using a C_8 reverse phase column. The major advantages of the method in this study are the simple sample preparation and instrumentation setting, which should allow antibiotic TDM in laboratories with limited mass spectrometry capabilities.

Although mere protein precipitation is not the best procedure to prevent matrix effects, our sample preparation protocol provided acceptable internal standard normalized matrix factor results with a CV ≤8.3%. For the analytes in this study, imprecision and inaccuracy (both intra- and interassay) were ≤12.5% for all quality control samples and the lowest calibrator (LLOQ), complying with the EMA requirements. Extensive testing with TDM quality controls from our routine laboratory and patient samples (non-ICU and ICU) gave no significant signals at analytic retention times and therefore confirmed assay selectivity. The method was also not disturbed by severe hemolysis, icterus and lipemia.

Our short-term stability experiments gave different results when compared with previously published work [44]. Given that precautions should be taken to prevent beta-lactam degradation *in vitro*, we recommend immediate extraction after blood collection or temporary storage up to one week at −20 °C if immediate processing is not possible.

Various multi-analyte LC–MS/MS methods have been published in the recent years [28,33–37,47,48], however many of these approaches quantify structurally related antibiotics from the beta-lactam class only (extensively reviewed in [38]). In contrast to other studies, our TDM panel allows quantification of substances with different physico-chemical properties, including ß-lactam antibiotics, as well as the oxazolidinone linezolid and fluorchinolone drugs. The rationale for quantifying cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid and piperacillin in a single multi-analyte assay is that these antibiotics are most frequently used in ICUs and are of uttermost importance for routine TDM in critical illness [49,50]. A TDM panel including relevant antibiotics from different classes can therefore be more efficient in terms of high sample throughput, minimal turn-around time and costs when compared to mono-analyte and class-specific quantification methods that require multiple instruments and different chromatographic solvents.

For the implementation of local and national antibiotic stewardship programs, routine TDM is becoming increasingly important in the clinical setting [19]. Still, common concentrations required for maximal antimicrobial efficacy and dose adjustement strategies are controversially discussed [10,18,20,51]. Uniform therapeutic ranges are not defined for a specific antibiotic substance; the central component of the PK/PD target is the minimal inhibitory concentrations (MIC) of the causative pathogen. For time-dependant antibiotics, such as ß-lactams, the efficacy is related to the time of the free (or unbound) concentration above the MIC ($fT_{>MIC}$). In this context maximal bactericidal activity is reported for through concentrations that exceed the MIC four to five times ($fT_{>4\text{-}5xMIC}$) [16,52]. When quantifying total ß-lactam concentrations, including both the free and bound antibiotic fraction, the target range has to be increased. This is in particular relevant for piperacillin, with the high average protein binding fraction of approximately 30% [53]. According the European Committee on Antimicrobial Susceptibility Testing (EUCAST) the threshold for the treatment of wild-type *Pseudomonas spp* is therefore 64 mg/l for unbound piperacillin, which corresponds to 91.4 mg/l total piperacillin [40]. For the oxazolidinone linezolid maintenance of the through level above the MIC ($fT_{>MIC}$), preferably ≥2 mg/l, is considered the most relevant marker for efficacy [22]. Contrary, fluorchinolones do act as concentration-dependant antimicrobials with a certain degree of time dependence, whose efficacy is mainly related to the ratio of the area under the concentration time curve (AUC) and the MIC ($AUC_{0\text{-}24}$/MIC). As quantification of the AUC requires multiple blood sampling, quantification of the peak concentration C_{max} is generally preferred. In this context, the target ratio of a C_{max}/MIC of ≥ 10 can be used as universal therapeutic threshold for fluorchinolones [54,55].

In contrast to other approaches our TDM assay has a broad calibration range. It allows quantification of concentrations close to the MIC of susceptible pathogens and also pharmacokinetic peak levels that may be obtained after bolus administration in standard dosage regimen. Consequently, our procedure allows quantification of through, as well as peak levels and also calculation of the AUC, using a uniform extraction protocol without the necessity of specimen dilution. Should the antibiotic concentration of a sample still exceed the ULOQ of our assay it may be diluted with distilled water (up to 1:5) prior to processing.

Carry-over was negligible for all antibiotics, except for ciprofloxacin at the LLOQ of 0.05 mg/l. Analytic carry-over was unavoidable, presumably due to inherent properties of our autosampler system. When using ciprofloxacin as antimicrobial the lowest target peak concentration needed for treatment is 0.3 mg/l for *Neisseria spp* (MIC of 0.03 mg/l) according to EUCAST breakpoint tables [40]. As the carry-over of 0.21% would increase this threshold merely by 5% when following a sample with a high peak concentration of 7.5 mg/l, it can be considered clinically non-relevant.

In conclusion, we report a simple and fast method for simultaneous quantification of cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid and piperacillin in serum that covers the clinical useful concentration ranges. Being robust with regard to analytic selectivity, linearity, matrix effects, and stability the described method allows routine antibiotic TDM in clinical laboratories with limited HPLC–MS/MS capacity.

Author contributions

All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements:(a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Conflicts of interest

Upon manuscript submission, all authors declare no conflict of interest

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jpba.2018.01.031.

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2.2 Schuster, C., Sterz, S., Teupser, D., Bruegel, M., Vogeser, M., & Paal, M. (2018). Multiplex Therapeutic Drug Monitoring by Isotope-dilution HPLC-MS/MS of Antibiotics in Critical Illnesses. *J Vis Exp* (138)

Video Article

Multiplex Therapeutic Drug Monitoring by Isotope-dilution HPLC-MS/MS of Antibiotics in Critical Illnesses

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Abstract

There is an ever-increasing demand for the therapeutic drug monitoring of antibiotics in many clinical facilities, particularly with regard to the implementation of hospital antibiotic stewardship programs.

In the current work, we present a multiplex high-performance liquid chromatography-tandem mass spectrometry (HPCL-MS/MS) protocol for the quantification of cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid, and piperacillin, commonly used antibiotics in intensive care units. The method was previously comprehensively validated according to the guideline of the European Medicines Agency.

After a rapid sample cleanup, the analytes are separated on a C8 reverse-phase HPLC column within 4 minutes and quantified with the corresponding stable isotope-labeled internal standards in electrospray ionization (ESI+) mass spectrometry in multiple reaction time monitoring (MRM). The presented method uses a simple instrumentation setting with uniform chromatographic conditions, allowing for the daily and robust antibiotic therapeutic drug monitoring in clinical laboratories. The calibration curve spans the pharmacokinetic concentration range, thereby including antibiotic amounts close to the minimal inhibitory concentration (MIC) of susceptible bacteria and peak concentrations (C_{max}) that are obtained with bolus administration regimens. Without the necessity of the serum dilution before the sample cleanup, the area under the curve for an administered antibiotic can be obtained through multiple measurements.

Video Link

The video component of this article can be found at https://www.jove.com/video/58148/

Introduction

Although antibiotics have revolutionized the practice of medicine, severe bacterial infections remain a leading cause of morbidity and mortality in critical illnesses[1]. In this regard, the prompt administration of a suitable anti-infective in an adequate dosage is of the uppermost importance for disease control[2].

A growing body of evidence demonstrates that the empirical treatment with broad-spectrum antibiotics is becoming increasingly problematic with the complexity of patient populations. This is especially true for intensive care units (ICU), where a tremendous inter-individual variability of key pharmacokinetic (PK) parameters is frequently observed[3,4]. Accordingly, ICU patients are at imminent risk of sub-therapeutic levels with the danger of an insufficient therapeutic success[5,6]. Then again, patients are unnecessarily exposed to excessively high antibiotic concentrations that may result in serious adverse events with no clinical benefits[7]. Both the antibiotic misuse and the insufficient dosing have also fueled the dissemination of antibiotic resistance, which is becoming an ever-growing threat to public health[8].

To improve the use of antibiotics and to preserve their effectivenessas long as possible, the World Health Organization has launched a global action plan on antimicrobial resistance in 2015[9]. Antibiotic stewardship programs constitute an essential cornerstone of prudent antimicrobial use in national public health strategies[10], helping clinicians to improve the quality of patient care[11] and, at the same time, significantly reducing the antibiotic resistance[12]. Antimicrobial dosing in individual patients through the application of therapeutic drug monitoring (TDM) is a key instrument in this context[13].

To date, commercially available TDM assays are only available for the glycopeptide antibiotics and aminoglycosides. The quantification of substances from other classes commonly requires an in-house method development or validation that can be cumbersome. We, therefore, present in detail the protocol for a robust mass spectrometry-based assay that can be used for the quantification of the most relevant antibiotics in ICU within their clinical relevant concentration ranges[14]. The method was recently established in our mass spectrometry facility and has been applied for the routine TDM in ICU since then. The procedure uses a straightforward and simple analytical setting with a uniform sample cleanup, allowing for the rapid implementation of antibiotic TDM in many facilities with mass spectrometry capabilities.

The protocol described here was optimized for the quantification of cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid, and piperacillin in human serum, using isotope dilution liquid chromatography (LC) in combination with a tandem mass spectrometry (MS/MS). For the isotope dilution LC-MS/MS methodology, stable isotope-labeled compounds are added to a sample of interest with a specific matrix (*e.g.*, serum). Isotope-labeled standards can be distinguished from their unlabeled counterpart, namely the analyte of interest, due to different molecular weights of the natural molecule and their fragmentation products, termed a parent-ion-to-daughter-ion transition. As isotope-labeled compounds have an almost identical overall physicochemical behavior compared to their unlabeled counterpart, they are ideal internal standards for the MS/MS, allowing a nearly matrix-independent analyte quantification with a high degree of accuracy[15]. Nowadays, many stable isotope-labeled internal standards that can be used for small-molecule quantification, including the TDM of antimicrobials, are commercially available.

The chromatographic separation of the antibiotic analytes in the described protocol is performed with an analytical C8 alkyl-chain-length reverse-phase column (100 mm x 2.1 mm, 3 µm particle-size). During the method development, the internal standard normalized matrix factors for all analytes was between 94.6% and 105.4%, with a coefficient of variation of ≤8.3%[14].

Protocol

NOTE: It is recommended to work in a fume hood when handling organic solvent, such as methanol. Prepare all buffers and mobile phases in volumetric flasks. If not otherwise specified, the solutions can be stored at room temperature for up to 1 month after preparation.

1. Preparation of the Calibrators and Quality Control Samples

NOTE: A corresponding data analysis sheet for the preparation of stock and spike solutions is given in the **Supplemental File**. For reasons of traceability, insert the manufacturer, catalog number, and a lot number of each antibiotic in the corresponding columns. Dissolve all antibiotics in a cold storage at 4 °C and keep the working time as short as possible.

1. Prepare 100 mL of 25% methanol in water: prefill a 100 mL volumetric flask with 25 mL of absolute methanol and fill it up to 100 mL with distilled water.
2. Prepare 10 mL of 200 mM acetic acid in water: prefill a 10 mL volumetric flask with 9 mL of HPLC grade water, add 115 µL of glacial acetic acid (99.5% purity, 17.4 M), and add distilled water up to 10 mL.
3. Prepare 25 mL of 25% methanol in water with 20 mM acetic acid: prefill a 25 mL volumetric flask with 2.5 mL of the aqueous 200 mM acetic acid solution, add 6.25 mL of absolute methanol, and fill up the flask to 25 mL with distilled water.
4. Use a precision scale to weigh the proper amounts of antibiotics in 15-mL conical tubes as described in the **Supplemental File** in the column *initial weight*.
5. Prepare stock solutions of the fluoroquinolones, ciprofloxacin, and moxifloxacin in the 25% methanol-water including 20 mM acetic acid. To do this, add the corresponding volume to the weighted quantities as described in the **Supplemental File** in the column "final volume". Rapidly dissolve the fluoroquinolone antibiotics in an ultrasound bath for 2 min and by intense vortexing.
6. Prepare stock solutions of cefepime, meropenem, linezolid, and piperacillin in the 25% methanol-water. To do this, add the corresponding volume to the weighted quantities as described in the **Supplemental File** in the column *final volume* and rapidly dissolve the antibiotics by intense vortexing. Dissolve meropenem as the last substance.
7. Combine the stock solutions of all the antibiotics as described in the *corresponding volume of stock solution* chart in the **Supplemental File** to yield tenfold concentrated spike-solutions.
8. Spike nine volumes of drug-free serum with one volume of the tenfold concentrated spike solutions to obtain the serum calibrators 0–7 and quality controls (QC) A–D. For example, add 0.5 mL of spike solution to 4.5 mL of serum in a 10-mL polypropylene tube and incubate it for 15 min in the cold storage at 4 °C on a roller mixer at 50 rpm.
9. Use a repetitive pipette to generate 100 µL aliquots of the calibrators and QCs in 1.5 mL polypropylene tubes.
10. Store the calibrators, quality controls, and antibiotic stock solutions at -80 °C for up to six months.
11. For each antibiotic, also prepare a neat solution containing 1,000 mg/L of a single antibiotic. Dilute the corresponding stock solution with an appropriate diluent (*e.g.*, for ciprofloxacin, use 25% methanol-water including 20 mM acetic acid).
    NOTE: The neat antibiotic solutions are required for the instrument-tuning only.

2. Preparation of the Internal Standards Mix

NOTE: Internal standards are isotope-labeled counterparts of the analytes of interest that are added to a sample during sample cleanup. As the internal standards have almost identical overall physicochemical properties to their unlabeled counterparts, they compensate for the matrix effects of a given sample.

1. Prepare 10 mL of 50% methanol in water by adding 5 mL of absolute methanol to a 10 mL shake flask and fill it up to 10 mL with distilled water.
2. Prepare 10 mL of 50% methanol in water including 20 mM acetic acid. To do this, add 1 mL of 200 mM acetic acid to a 10 mL flask, add 5 mL of absolute methanol, and fill it up to 10 mL with distilled water.
3. Generate stock solutions of internal standards (IS) with 1,000 mg/L directly in the vials provided by the manufacturer. Dissolve cefepime-$^{13}C_1{}^2D_3$ sulfate in distilled water, meropenem-D_6, linezolid-D_3, and piperacillin-D_5 in a 50% methanol-water solution. Dissolve ciprofloxacin-D_8 in 50% methanol-water with 20 mM acetate and moxifloxacin hydrochloride-$^{13}C_1D_3$ in distilled water with 20 mM acetate.
4. Combine the IS stock solutions in a 1.5 mL polypropylene tube to yield a fivefold concentrated internal standard mix. Add 10 µL of cefepime-$^{13}C_1{}^2D_3$, 10 µL of meropenem-D_6, 1 µL of ciprofloxacin-D_8, 2 µL of moxifloxacin hydrochloride-$^{13}C_1D_3$, 2 µL of linezolid-D_3, and 10 µL of piperacillin-D_5 to 965 µL of 25% methanol-water.
5. Store the internal standard stock solutions and the fivefold concentrated IS mix at -80 °C.

3. Patient Sample Storage

NOTE: Ensure that the serum is obtained as fast as possible and that the cold chain of frozen samples is maintained.

1. Collect the whole blood in the serum collection tubes.
2. Let the blood clot for 20–30 min at room temperature.
3. Separate the serum from the blood by centrifugation at 2,000 x *g* for 10 min.
4. Transfer the supernatant to a clean polypropylene tube.
5. Store the serum up to six months at -80 °C until it is assayed. Alternatively, store the samples up to 3 days at -20 °C.

4. Buffer Preparation for Chromatography

1. To prepare 1 M ammonium formate in water, dissolve 6.306 g of ammonium formate in 100 mL of HPLC grade water using a 100 mL shake flask. Store the solution up to 1 month at 4 °C.
2. Prepare the mobile phase A [10 mM ammonium formate in water-formic acid (99.9:0.1 v/v)]. Prefill a 1,000 mL volumetric flask with approximately 500 mL of HPLC grade water, add 1 mL of formic acid and 10 mL of the 1 M ammonium formate solution, and fill it to 1,000 mL with HPLC grade water. Transfer mobile phase A to a clean glass bottle and connect it to the HPLC system. Store mobile phase A up to 2 weeks at room temperature.
3. Prepare the mobile phase B. Transfer HPLC-grade absolute methanol into a clean glass bottle and connect it to the HPLC system.
4. Use absolute methanol as the needle wash solvent and connect the corresponding tube to the glass bottle containing mobile phase B.
5. Generate the seal and a purge solvent of methanol-water-formic acid (7:92.9:0.1, v/v/v). Prefill a 1,000 mL volumetric flask with approximately 500 mL of distilled water, add 70 mL of absolute methanol, 1 mL of formic acid, and add distilled water to 1,000 mL. Transfer the solvent to a clean glass bottle and connect it with the HPLC system.
   Note: Various autosampler systems use both a strong and a weak needle wash solvent. In such a case, prepare the wash solutions according to the manufacturer's recommendations. For example, do the strong wash with methanol-water-isopropylic alcohol (70:20:10, v/v/v) and the weak wash with water-methanol (95:5, v/v).

5. Instrument Tuning

NOTE: This step is performed for the set-up of the method on a specific mass spectrometer.

1. Dilute the neat 1,000 mg/L analyte and the internal standard solutions 1:10 or 1:100 in a mixture of mobile phase A and B (50:50, v/v), depending on the detector signal intensities. Tune the mass spectrometer with the autotune function or do a manual tuning for the following parent-to-daughter ions transitions[14]: cefepime (481.0 > 167.0/395.7), cefepime-$^{13}C_1{}^2D_3$ (485.1 > 167.1/400.0), meropenem (384.1 > 114.0/141.0), meropenem-D_6 (390.1 > 114.0/147.2), ciprofloxacin (332.0 > 231.0/245.0), ciprofloxacin-D_8 (340.1 > 235.1/249.3), moxifloxacin (402.0 > 261.0/383.9), moxifloxacin-$^{13}C_1D_3$ (406.1 > 265.1/388.0), linezolid (338.0 > 235.0/296.0), linezolid-D_3 (341.1 > 235.1/297.1), piperacillin (518.0 > 143.0/358.9), and piperacillin-D_5 (523.1 > 142.8/364.1).
2. For instruments with autotuning, use the autotune function to automatically adjust the voltage and settings of the MS inlet through the detectors.
3. For instruments with manual tuning, adjust the settings (*e.g.*, collision voltage and collision energy) until the optimum (usually the maximum) signal intensity is obtained at the detector for each parent and daughter ion. For example, plug a mixing tee, deliver mobile phase A and B (50:50, v/v) at 0.5 mL/min, and continuously infuse the neat antibiotic or internal standard with a flow rate of 0.1 mL/min.

6. HPLC-MS/MS Set-up

NOTE: Features of the mass spectrometer, HPLC system (including the autosampler), and the corresponding software depend on the manufacturer. Adapt the mass spectrometer parameters and the wash procedure according to the manufacturer's recommendations.

1. Store the mass spectrometer parameters in a corresponding '*MS tune file*'. Use electrospray ionization in the positive mode (ESI+) for all analytes. Adapt the ion source settings for the instrument used (*e.g.*, a capillary voltage of 1.5 kV, a source temperature of 120 °C, a desolvation temperature of 400 °C, a desolvation gas flow rate of 600 L/h, an RF lens voltage of 0.1 V, and a dwell time of 80 ms).
2. Specify the analyte and internal standards tune parameters (*e.g.*, capillary voltage, collision energy) in an '*MS file*'.
3. Set the autosampler conditions as follows in the '*inlet file*': the sample temperature at 10 °C with a limit of ± 5 °C; the wash sequence at 1x purge-wash-purge with a 600 µL purge volume replacement.
4. In the above-mentioned '*inlet file*', set the flow rate to 0.4 or 0.5 mL/min, the run time to 4 min, the pressure high limit to 345 bar, and the column temperature to 30 °C with a limit of ± 5 °C. Add the solvent name of mobile phases A and B and set them to 7% B/93% A, respectively.
5. Program the chromatographic gradient in the '*inlet file*' as follows: 0.00–0.10 min with 7% mobile phase B/93% A, 0.11–0.60 min with 65% mobile phase B/35% A, 0.61–2.10 min with 95% mobile phase B/5% A, 2.11–4.00 min with 7% mobile phase B/93% A.
   NOTE: Calculate the extra-column volume, the hold-up volume for the instrumental platform, and the analyte retention factors as described in the USP <621> Chromatography guideline[16].

7. Sample Measurement Master File

NOTE: With the '*sample measurement master file'*, the patient samples are specified, the HPLC-MS/MS analysis is started, and the data evaluation is performed. Two separate template files including a low- and high-quality control pair are generated; one template includes QC pair A and C, the other one QC pair B and D.

1. Create a new '*sample measurement master file'*. Select the above-mentioned '*MS tune file'*, '*MS file'*, and *'inlet file'* (section 6), insert them in each sample line, and specify the injection volume with 15 µL.
2. In ascending order, add the “sample text” for calibrators 0–7 and quality control (QC) pair A/C or QC pair B/D.
3. Specify the sample type. Select the sample type “standard” for the calibrators and “QC” for the quality control pairs.
4. Specify the concentration of each antibiotic substance for the corresponding calibrators and quality controls (see the spreadsheet, concentration [µg/mL] Cal 7–Cal 0, QC A/C or B/D,).
5. Program the '*data evaluation method'*. Use the transitions that were optimized during the instrument tuning (section 5). Match each antibiotic with the corresponding isotope-labeled standard (*e.g.*, meropenem - meropenem-D_6).

8. Sample Cleanup and HPLC-MS/MS Analysis

NOTE: For each sample batch, a paired quality control set with a low and high antibiotic concentration (QC A/C or QC B/D) is processed and analyzed. Between different batches, the paired QC samples are used in an alternate sequence (*e.g.*, on day 1, select the '*sample measurement master file'* including QC pair A/C; on day 2, select the one including QC pair B/D. The processing of the serum samples is illustrated in **Figure 1**.

1. Prepare the precipitation agent 10% methyl-*tert*-butyl ether in methanol (10:90, v/v) (*e.g.*, prefill a 25-mL volumetric flask with 2.5 mL of methyl-*tert*-butyl ether and fill it to 25 mL with absolute methanol).
2. Place the C8 reverse phase into the column chamber. Connect it to the HPLC and mass spectrometer in the direction of the flow.
3. Generate the sample list. Open the corresponding '*sample measurement master file'* template and add the patient samples meant to be processed to the list. Generate groups of up to 20 patient samples and flank them with the corresponding quality control pair.
4. Wet-prime the HPCL system using the '*inlet file'* control software: set the “wet prime” function to 50% mobile phase A/50% B, and wet-prime for 2 min with a flow rate of 1 mL/min.
5. Refresh the syringe. To do this, execute 6 strokes of 600 µL in the control software.
6. Equilibrate the C8 reverse phase column. Using the software, turn on the flow in the '*inlet file'* and flush it with 7% mobile phase B/93% A for a minimum of 5 min, using a flow rate of 0.5 mL/min. Verify the column temperature of 30 °C.
7. Thaw the patient samples, one aliquot of calibrators 0–7, and a quality control pair (either A/B or C/D).
8. With a repetitive pipette, add 25 µL of the internal standard mix to the 100 µL calibrator, QC sample, or patient serum in a 1.5 mL polypropylene tube, and vortex the tube for a few seconds.
9. Incubate the mixture for 5 min at room temperature on a benchtop shaker (*e.g.*, at 1,200 rpm).
10. With a repetitive pipette, add 150 µL of a precipitation reagent to the sample-internal standard mix.
11. Again, vortex the tube for a few seconds and incubate it for 5 min at room temperature on a benchtop shaker (*e.g.*, at 1,200 rpm).
12. Centrifuge the suspension at 20,000 x *g* in a tabletop centrifuge for 10 min at 4 °C.
13. Dilute the supernatant 1:3 with HPLC grade water using a glass vial with a micro-insert and load it as processed samples to the autosampler.
14. Manually start the HPLC-MS/MS analysis in the '*sample measurement control file'*.
    NOTE: For prolonged storage, thoroughly flush the analytical column according to the manufacturer’s recommendation [*e.g.*, 0.5 mL/min methanol-water (50:50, v/v)] to prevent phase collapse.

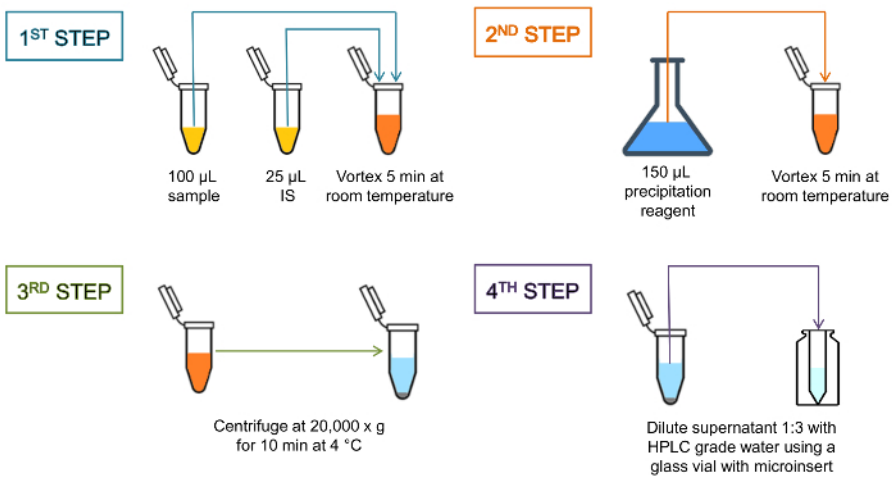

Figure 1: Schematic representation of the sample cleanup. Protein precipitation at the high centrifugal force gives a dense pellet and clear supernatant, indicating that protein precipitation was complete. The entire processing time is approximately 30 min, including the sample cleanup, the chromatographic separation, and the MS/MS analysis. Please click here to view a larger version of this figure.

9. Quality Assessment and Quantification

1. To process the samples, open the corresponding '*sample measurement control file'*, select the calibrators, quality controls, and patient samples, and evaluate them with the '*antibiotics quantification method'*.
2. Check whether the peaks for a specific analyte are properly integrated. Inspect the peaks for each calibrator, QC, and patient sample, and manually reintegrate them at the baseline if necessary.
3. Study the calibration curve and examine whether it fulfills the following quality criteria: a) linearity over the entire calibration range, b) a calibration coefficient $r^2 > 0.995$, c) the deviation of each calibration standard within ± 15% of the nominal value, except for the lower limit of quantification (LLOQ), where ± 20% is required.
4. Reject a calibration standard not complying with the above-mentioned criteria and re-evaluate the calibration curve, including the regression analysis.
5. Study the quality controls and examine whether the deviations are within ± 15% of the nominal value.
6. If the concentration of a patient sample exceeds the concentration of the highest calibrator, dilute the sample with distilled water, up to 1:5 (*e.g.*, 100 µL of serum plus 400 µL of distilled water) before the sample cleanup. Reperform steps 8.8–8.14 for that specific sample and reprocess it.

Representative Results

Using the described protocol, a typical chromatogram is depicted in **Figure 2**. According to the United States Pharmacopeia (USP) chromatography guidelines[16], the column dead volume in the present system was determined with ~0.22 mL and the extra-column volume (including the injector, tubing, and connectors) with ~0.08 mL, giving a hold-up volume of ~0.30 mL. The calculated retention factors for all analytes were 2.8 (for cefepime) - 4.2 (for piperacillin).

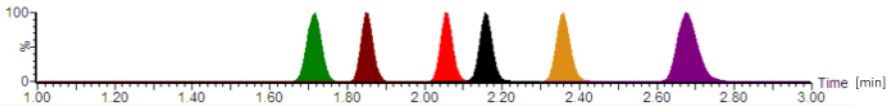

Figure 2: Typical analytical chromatogram with normalized signal intensities. The antibiotics are eluting in the following order: cefepime (green), meropenem (brown), ciprofloxacin (red), moxifloxacin (black), linezolid (orange), and piperacillin (purple). The retention times, which are given in minutes, and the analyte peak symmetries vary, depending on the exact composition of the mobile phases, the flow-rate, the chromatography tubing, and the analytical column age. Please click here to view a larger version of this figure.

Figure 3A contains a sample chart list for the processed samples, including the calibrators 0 - 7 ("Kalibrator 0" - "Kalibrator 7"), quality controls, and patient sera, that are indicated with the injection number (#); the sample identification text (*Sample Text*); the measured concentration in mg/L (*Conc.*); the sample type that is either a blank, standard, quality control, or patient sample (*Type*); the nominal concentration of the calibrators in mg/L (*Std. Conc*); the analytical retention time (*RT*); the response that is the ratio of the peak area of the analyte/peak area IS (*Response*); the deviation from the nominal concentration value (*%Dev*); the vial position (*Vial*); and the acquisition time (*Acq.Time*). The key parameter used for the quantification is the *Response*, gradually increasing with the analyte concentration, due to the constant amount of added isotope-labeled internal standard.

Figure 3B shows the calibration curve. In regression, the coefficient of determination r^2 should be > 0.995. The following calibration model is used for all analytes described in this method: curve type = linear; origin = included; weighting = 1/x; axis transformation = none. In the given example, the calibration curve and quality controls fulfill all quality criteria: $r^2 > 0.995$ for the calibration curve and the deviation of the calibrators (including the LLOQ) and the QC samples is within ± 15% of the nominal value.

The measured parent-to-daughter ion transitions (MRM) are given in **Figure 3C**, showing four peaks at the same retention time: the two upper peaks depict two transitions that are measured for the analyte of interest, the lower two peaks represent the transitions for the corresponding isotope-labeled internal standard. For the quality assessment, the analyte peaks in the respective retention time windows are visually checked and manually reintegrated at the baseline, when necessary.

The minimally inhibitory concentration (MIC) is the central component of the antimicrobial TDM, defining the pharmacokinetic exposure that is required to achieve a target pharmacokinetic/pharmacodynamic (PK/KD) ratio[13,17]. Accordingly, the target antibiotic TDM concentration levels are expressed in relation to the MIC of the causative pathogen. Given that the action of beta-lactam antibiotics is time-dependent, their efficacy is maximized through the achievement of the therapeutic concentrations that exceed the MIC 4x -5x ($fT > {}_{4\text{-}5x\ MIC}$).When facing unknown infectious pathogens, the target trough concentration range of free (protein-unbound) piperacillin is, therefore, 64 mg/L, corresponding to approximately 90 mg/L total piperacillin[18].

The first patient (sample #11) has a satisfactory high serum trough level of 83.4 mg/L piperacillin that is also sufficient for problem pathogens, such as *Pseudomonas aeruginosa*. The second patient (sample #12) has a concentration of approximately 0.2 mg/L, which is below the lowest calibrator (LLOQ). Perhaps the patient has recovered, and the administration of piperacillin was discontinued. The result "< 0.5 mg/L" is, therefore, reported in the hospital information system. The third patient (sample #13) has a low piperacillin trough concentration of only 5.3 mg/L that is not sufficient for the clear majority of pathogens. For effective antimicrobial chemotherapy, the dosage should be increased by the physician.

A

#	Name	Sample Text	Conc.	Type	Std. Conc	%Dev	RT	Response	Vial	Acq.Time
1	180206_Antibiotika_005	Kalibrator 0							1:1,C	10:17:30
2	180206_Antibiotika_006	Kalibrator 1	0.550	Standard	0.500	10.1	2.49	0.092	1:1,D	10:23:04
3	180206_Antibiotika_007	Kalibrator 2	1.417	Standard	1.500	-5.5	2.49	0.256	1:1,E	10:28:08
4	180206_Antibiotika_008	Kalibrator 3	5.761	Standard	6.000	-4.0	2.49	1.077	1:1,F	10:33:13
5	180206_Antibiotika_009	Kalibrator 4	25.533	Standard	25.000	2.1	2.49	4.813	1:2,A	10:38:20
6	180206_Antibiotika_010	Kalibrator 5	99.484	Standard	100.000	-0.5	2.49	18.786	1:2,B	10:44:08
7	180206_Antibiotika_011	Kalibrator 6	213.453	Standard	225.000	-5.1	2.49	40.321	1:2,C	10:49:13
8	180206_Antibiotika_012	Kalibrator 7	411.802	Standard	400.000	3.0	2.50	77.800	1:2,D	10:54:17
9	180206_Antibiotika_014	9951	96.537	QC	100.000	-3.5	2.49	18.229	1:2,E	11:04:26
10	180206_Antibiotika_015	9953	296.630	QC	300.000	-1.1	2.49	56.038	1:2,F	11:09:30
11	180206_Antibiotika_033	0002720456	83.378				2.49	15.743	1:5,A	12:50:01
12	180206_Antibiotika_034	0002720665	0.223				2.49	0.031	1:5,B	12:55:49
13	180206_Antibiotika_035	0002720692	5.298				2.49	0.989	1:5,C	13:00:53

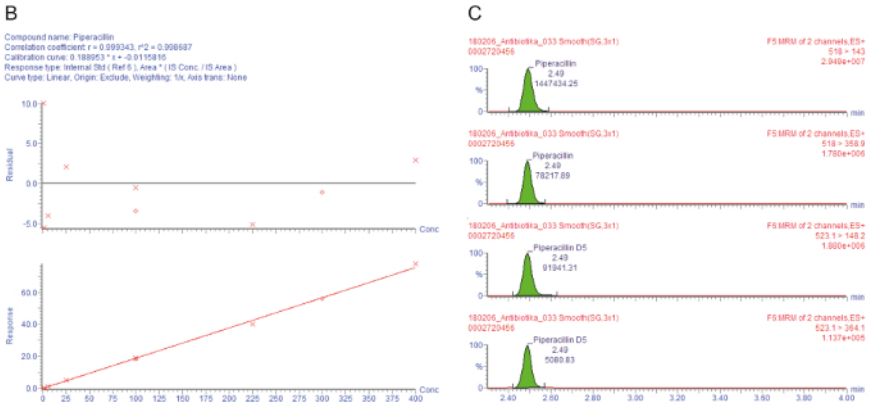

Figure 3: Exemplary quality assessment and quantification for the analyte piperacillin. These panels represent the mass spectrometry data analysis. (**A**) This panel shows the sample list, including the calibrators (*Standard*, samples #1 - #8), quality controls (*QC*, samples #9 and #10), and patient sera (samples #11 - #13). Calibrator 0 refers to the blank without analyte, but with the addition of an internal standard. 9951 represents QC B, 9953 represents QC D. (**B**) This panel shows the calibration curve for piperacillin. The percentage deviations from the nominal calibrator concentrations are given in the upper graph (*y*-axis: *residual*), the lower graph depicts the linear calibration range. (**C**) This panel shows the multiple reaction time monitoring (MRM) for piperacillin and the corresponding internal standard piperacillin-D_5 for patient serum sample #12. Two parent-to-daughter ion transitions are presented with their retention time and respective signal intensities. Please click here to view a larger version of this figure.

Supplemental File. Please click here to download this file.

Discussion

In this manuscript, we report the protocol for a simple and robust tandem mass spectrometry-based method for the quantification of frequently used antibiotics in ICU[19], namely cefepime, meropenem, ciprofloxacin, moxifloxacin, linezolid, and piperacillin[14]. A spreadsheet accompanies the manuscript for the preparation of antibiotic stock solutions, calibrators, and quality controls, taking into account the purity of the antibiotics and the molecular weight of their counterions. Given that the concentrations of the antibiotics are rather high, their quantification should be no particular challenge from an analytical perspective. Accordingly, we are confident that this protocol is applicable to various MS instrumental platforms. For a method transfer, users are encouraged to quantify the extra-column volume and hold-up volume of their chromatographic system and to adapt the gradient start time accordingly[16]. During the method set-up, the system should also be evaluated for carry-over and, if necessary, a blank sample must be injected after the highest calibrator and patient samples with high antibiotic concentrations. Users must also consider the possibility of detector saturation that occurs when too many ions enter a tandem mass spectrometer. Relevant detector saturation can be eliminated with smaller injection volumes, a higher analyte dilution during the sample cleanup, and/or a detuning of a target analyte (*e.g.*, downgrading the optimal voltage settings).

Contrary to other methods, the calibration range allows both a quantification of concentrations close to the MIC of susceptible pathogens, as well as peak concentrations (c_{max}) that are obtained with a bolus administration. The highest C_{max}-values for adults are reported in the corresponding professional information sheets on the FDA drug safety database as follows: 163.9 mg/L for cefepime[20], 112 mg/L for meropenem[21], 4.6 mg/L for ciprofloxacin[22], 4.1 mg/L for moxifloxacin[23], 21.2 mg/L for linezolid[24], and 298 mg/L for piperacillin[25]. Antibiotic concentration monitoring in the patient's blood circulation allows a dose adjustment to the susceptibility of the involved pathogens, but the pharmacokinetic area under the curve can also be obtained through multiple blood sampling with the given protocol.

Many antibiotics (especially beta-lactam meropenem) are chemically unstable once dissolved. The most critical step in this protocol is, therefore, the preparation of the stock solutions, calibrators, and quality controls under cold conditions[26,27]. In that respect, it is also essential to freeze patient samples as quickly as possible. Although serum storage at -80 °C is recommended[26], our stability experiments show that samples can also be stored up to 3 days at -20 °C without any significant decrease of antibiotics concentrations (even at the trough levels).

We recommend performing a system suitability test before each HPLC-MS/MS analysis of patient samples (*e.g.*, with calibrator 3). Generally, a system suitability test is used to verify the repeatability of the LC-MS/MS system and to see if it is also adequate for the analysis to be done. Thus, for instance, decreasing signal intensities are caused by a contamination of the MS sweep cone, which, then, requires its cleaning with an organic solvent. To keep the MS source clean, a divert valve can be introduced after the chromatography column, directing "analyte-free"

portions of the mobile phase to the waste before they reach the mass spectrometer. On the other hand, an overall increase of the pressure can indicate column clogging over time. To increase the column longevity usage of a cost-effective precolumn filter is recommendable. If the pressure still continues to be a problem, a flow rate of 0.4 mL/min can also be used with the chromatographic gradient in this protocol.

A minor limitation of this technique is that it requires three separate manual steps for sample clean-up, resulting in a total turnaround time of approximately 30 min. Adding the isotope-labeled internal standards to the precipitation agent may save some processing time. However, this should only be done for high sample throughput rates and with the precipitation agent being stored in the cold (*e.g.*, at -20 °C), as the internal standards also degrade *in vitro* at elevated temperatures.

The described protocol has been developed for sample processing in standard 1.5 mL polypropylene tubes. Should a higher throughput rate be required for antibiotic TDM, the procedure can be upgraded to the multi-well plate format using adequate centrifuge inserts or filter plates with a vacuum manifold.

Disclosures

The authors have nothing to disclose.

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2.3 Schuster, C., Paal, M., Lindner, J., Zoller, M., Liebchen, U., Scharf, C., & Vogeser, M. (2019). Isotope dilution LC-orbitrap-HRMS with automated sample preparation for the simultaneous quantification of 11 antimycotics in human serum. *J Pharm Biomed Anal*, 166, 398-405

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Isotope dilution LC-orbitrap-HRMS with automated sample preparation for the simultaneous quantification of 11 antimycotics in human serum

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ABSTRACT

Introduction: The aim of this project was to develop and validate an isotope-dilution liquid chromatography high resolution mass spectrometry (LC-HRMS) method for the quantification of the 11 most widely used systemic antimycotics and to study whether HRMS is a feasible alternative for therapeutic drug monitoring (TDM) when compared to tandem MS (MS/MS) technology.
Methods: After protein precipitation, followed by automated online sample clean-up the analytes were separated within 4 min on a C18 column using an acetonitrile-water gradient. Eleven antimycotics, namely 5-flucytosine, amphotericin B, anidulafungin, fluconazole, isavuconazole, itraconazole, ketoconazole, micafungin, OH-itraconazole, posaconazole and voriconazole were finally quantified in full MS scan mode using positive electrospray ionization (ESI +) with a mass range from *m/z* 110–1300 using HRMS. The method was comprehensively validated on the basis of the European Medicines Agengy (EMA) method validation protocol using commercially available IVD kit components.
Results: Good linear relationship between peak area responses and drug concentrations ($R^2 > 0.995$) and excellent selectivity were observed for all antimycotics in this study. Inaccuracy and imprecision of all quality controls were consistently below ± 12.6% and ± 8.1%, respectively. Quantification results were in agreement with an IVD LC–MS/MS method.
Conclusion: HRMS was shown to be suitable for TDM of small molecules when compared to tandem mass spectrometry. The novel HRMS method is quickly installed and may be a robust and reliable tool for routine TDM of antimycotics in clinical laboratories.

1. Introduction

Invasive fungal infections by *Candida*, *Aspergilli* and *Cryptococci* species are a leading cause of morbidity and mortality in patients with compromised immunity [1]. Invasive pulmonary diseases caused by *Aspergillus* spp. are still associated with high mortality rates up to 82% [2]. Early diagnosis, pathogen identification and therapy initiation with appropriate antimycotic drugs are of uppermost importance for therapeutic success. However, in addition to immune-compromising conditions, altered pharmacokinetic variabilities with inadequate antimicrobial exposure at the site of infection and drug interactions are contributing to poor prognosis in critically ill patients [3,4]. Therapeutic drug monitoring (TDM) of antimicrobials is therefore helpful to guide therapy in these vulnerable patients [5–7].

Several systemic antimicrobial drugs are routinely administered for severe invasive fungal infections such as azole antimycotics or echinocandins. Quantification of these analytes is mainly conducted using LC with UV detection (LC-UV) or LC-mass spectrometry (LC–MS) [8–14]. Although mass spectrometers have high purchasing and maintenance costs, LC–MS assays are generally favored over LC-UV analyses in several respects. Usage of MS-detection provides a high degree of selectivity and sensitivity, especially when samples from patients receiving extensive co-medication are analyzed. In addition LC–MS analysis can be scaled

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up in simpler fashion to simultaneous quantification of many small molecules and their metabolites in a single analytical run [15–17].

Orbitrap high resolution mass spectrometry (HRMS) analysis is recognized as an adequate technology for protein and metabolic profiling due to the enormous output of high resolution spectral data. However, mass spectrometry based small molecule TDM is so far the domain of tandem mass spectrometry (MS/MS) [18–20]. Whether Oribtrap-HRMS with its analytical features is also applicable for the quantitative analysis of small molecules, such as anti-infective drugs, remains a subject of ongoing research [21,22]. Even though, LC–MS/MS methods were published for simultaneous quantification of azole antifungals and some echinocandins [11,14,23], to our knowledge no LC-HRMS method which addresses the previously mentioned 11 antimycotics was published yet. The main goal of the present study was therefore to develop a quantitative antimycotics LC-HRMS test procedure and to compare it to a routine IVD LC–MS/MS assay. Accordingly, the LC-HRMS method in this study was implemented using commercially available components from an antimycotics TDM IVD kit.

2. Materials and methods

2.1. Chemicals and reagents

The optimization-mix 1 and 2 (containing different amounts of analytes), the calibrators and the quality controls (QC) sets containing 5-flucytosine (5-FC), amphotericin B (AM-B), anidulafungin (ANF), fluconazole (FCZ), isavuconazole (IVZ), itraconazole (ITZ), ketoconazole (KTZ), micafungin (MCF), OH-itraconazole (OH-ITZ), posaconazole (PSZ), voriconazole (VRZ) and the corresponding isotope labelled internal standards $^{13}C^{15}N_2$-5-fluyctosine ($^{13}C^{15}N_2$-5-FC), d_4-fluconazole (d_4-FCZ), $^{13}Cd_4$-isavuconazole ($^{13}Cd_4$-IVZ), d_5-itraconazole (d_5-ITZ), d_8-ketoconazole (d_8-KTZ), d_5−OH-itraconazole (d_5−OH-ITZ), d_4-posaconazole (d_4-PSZ), d_3-voriconazole (d_3-VRZ), as well as the precipitation reagent (precipitant P) are part of the LC–MS/MS ClinMass® TDM kit system that was obtained from RECIPE (Munich, Germany). Furthermore a neat 5-FC (1.14 mg/mL) in absolute methanol was provided by RECIPE (Munich, Germany). Missing isotope labelled internal standards $^{13}C_6$-micafungin ($^{13}C_6$-MCF) and $^{13}C_6$-anidulafungin ($^{13}C_6$-ANF) were purchased from Alsachim (Illkirch-Graffenstaden, France). The isotope labelled internal standard d_3-amphotericin B (d_3-AM-B) was from Toronto Research Chemicals (North York, Canada). Drug-free serum was purchased from the blood donation service of the Bavarian Red Cross (Wiesentheid, Germany).

Water, acetonitrile, methanol and formic acid 99% (each HPLC grade) were obtained from Biosolve-chemicals (Dieuze, France). Ammonium acetate (HPLC grade) was purchased from Sigma-Aldrich (Missouri, USA). Ammonia solution 32% was from Merck (Massachusetts, USA). All chemicals were of the highest purity available from commercial providers.

2.2. Calibrators, quality control samples and internal standard solution

Calibrators, quality controls (QC) and internal standard (ISTD) solutions were prepared according to the manufacturerś instructions: Calibrator and QC lyophilisates provided in vials by the manufacturer were directly re-suspended in 1.0 ml water and mixed for 15 min at room temperature (RT) using a vortex shaker (IKA, Staufen, Germany). The IVD kit ISTD solution was prepared by adding 5.0 ml precipitant P, followed by solubilization for 15 min at RT using an ultrasonic bath. The missing ISTD of ANF, AM-B and MCF were dissolved in methanol and added to IVD kit precipitation reagent P (1:8 v/v) directly before sample preparation. Final concentrations were 7.0 mg/L $^{13}C^{15}N_2$-5-FC, 0.80 mg/L d_3-AM-B, 1.6 mg/L $^{13}C_6$-ANF, 2.1 mg/L d_4-FCZ, 1.4 mg/L $^{13}Cd_4$-IVZ, 0.52 mg/L d_5-ITZ, 1.5 mg/L d_8-KTZ, 4.0 mg/L $^{13}C_6$-MCF, 0.66 mg/L d_5−OH-ITZ, 0.70 mg/L d_4-PSZ, 0.87 mg/L d_3-VRZ. For simplicity the precipitation reagent including all ISTD is referred to as “precipitation mix”.

The kit contained 3 calibrator levels and 2 QC levels. To obtain 6 different calibrator levels the high and low level calibrator level were diluted 1.5-fold and the medium calibrator level was diluted 2.5-fold with drug-free serum, respectively. The lower QC level from the kit was diluted with drug-free serum (1:3 v/v) to obtain concentrations within 3-fold of the concentration of the lowest limit of quantification (LLOQ) (QC 1). For simplicity calibrators and QCs are named numeric Cal 1 - Cal 6 and QC 1 - QC 3. Final concentrations are displayed in Table 1. All calibrators and QCs were aliquoted to 50 μL and stored at −20 °C, according to the kit manufacturerś instructions.

2.3. Sample preparation

First, 100 μL precipitation mix, containing all internal standards for the analytes in this study, were added to 50 μL of Calibrator, QC or serum sample. The samples were vigorously mixed for 5 min at RT using a Thermomixer (Eppendorf, Hamburg, Germany). After centrifugation for 5 min (15 °C, 21,255 *g*) the supernatants were transferred into a glass vial with micro insert (Chromatographie Handel Müller, Fridolfing, Germany) and placed into the autosampler ready for injection.

2.4. Method development

According to the manufacturerś kit instructions internal standards were dissolved in precipitant P. The ISTD vial from manufacturerś kit included corresponding isotope labelled analogs of all analytes except for AM-B, ANF and MCF. As a consequence these missing ISTD were supplemented to the IVD kit ISTD solution giving the final precipitation mix (see chapter 2.2).

To separate all antimycotics within a single chromatographic run usage of a turbulent flow technique extraction column with mixed-phase properties was necessary. Separation of all analytes wasnt́ feasible by four tested silica or polymer based extraction columns exhibiting only reversed-phase properties. Due to reversed phase and cationic-exchange properties of the extraction column all analytes could be retained and concentrated before analytical separation, even the most challenging and small analyte 5-flucytosine.

The usage of a mixed-phase extraction column needed adjustment of mobile phases: Mobile phase C was used to load the extraction column with the analytes providing acidic conditions. Buffer wasnt́ used to guarantee the absence of ammonium ions, taking into account the cationic exchange mode. After the loading phase the loop was included with a mixture of mobile phase B1 and D to elute the analytes using basic conditions with ammonium acetate buffer and subsequently to re-equilibrate the ion exchanger. To elute all analytes it was necessary to increase the ammonia content to 2% since complete elution was not possible with less ammonia. Only a small amount of ammonia (0.1% ammonia solution) was added to mobile phase D to change the pH to basic conditions but still preserve the system from too much ammonia. In summary mixed-phase Cyclone MCX-2 (0.5 x 50 mm) column in combination with mobile phases C: water-formic acid (99.8:0.2 v/v), B1: water-acetonitrile-ammonium acetate (500 mM)-ammonia solution (32%) (5:92:1:2 v/v/v/v) and D: water-ammonium acetate (500 mM)-ammonia solution (32%) (98.9:0.1:1 v/v/v) were used.

Table 1
Calibrator (Cal) and quality control sample (QC) concentrations [mg/L] for the antimycotics in this study.

Analyte	Cal 1*	Cal 2	Cal 3**	Cal 4	Cal 5*	Cal 6	QC 1***	QC 2	QC 3
5-Flucytosine	3.41	5.12	15.9	39.7	78.0	117	7.23	21.7	50.9
Amphotericin B	0.056	0.084	0.688	1.72	3.49	5.23	0.288	0.864	2.59
Anidulafungin	0.307	0.461	1.27	3.17	5.99	8.98	0.607	1.82	4.11
Fluconazole	0.376	0.564	1.66	4.14	8.40	12.6	0.763	2.29	5.40
Isavuconazole	0.321	0.482	1.40	3.49	7.07	10.6	0.640	1.92	4.55
Itraconazole	0.089	0.133	0.382	0.955	1.96	2.94	0.176	0.528	1.26
Ketoconazole	0.271	0.406	1.16	2.89	5.56	8.34	0.543	1.63	3.68
Micafungin	1.41	2.12	5.96	14.9	30.9	46.3	2.75	8.25	19.1
OH-Itraconazole	0.109	0.164	0.476	1.19	2.40	3.60	0.218	0.654	1.56
Posaconazole	0.155	0.232	0.672	1.68	3.34	5.01	0.303	0.910	2.18
Voriconazole	0.177	0.265	0.772	1.93	3.93	5.90	0.357	1.07	2.53

* 1.5× dilution of Cal 2/Cal 6 from the kit.
** 2.5× dilution of Cal 4 from the kit.
*** 3× dilution of QC 2 from the kit.

2.5. Liquid chromatography-high resolution mass spectrometry conditions

Chromatography was performed on a Thermo Scientific Ultimate 3000 system (Sunnyvale, CA, USA) provided with a quaternary RS pump for online SPE (loading pump) and a binary RS pump for analytical separation (eluting pump) with 2 six-port switching valves. The LC system was coupled to a Thermo Scientific Q Exactive Focus Orbitrap mass spectrometer. The Thermo Scientific CTC HTS PAL autosampler was kept at 10 °C, the injection volume was 5 μL.

Online sample extraction was performed on a mixed-phase properties Cyclone MCX-2 (0.5 x 50 mm) column from Thermo Scientific. The analytes in this study were then separated on a C18 Hypersil GOLD (50 x 2.1 mm, 1.9 μm) column (Thermo Scientific) that was kept at 30 °C. Instrument controlling, data acquisition and processing were performed using the Tracefinder 4.1 software (Thermo Scientific). Schematic representation of the valve-switching and corresponding chromatographic gradients is given in Fig. 1. The total run time was 4.0 min.

Optimized parameters of the HRMS system using optimization-mix 1 were: sheath gas and auxiliary gas flow rate 50 AU and 15 AU respectively, spray voltage 3.50 kV, S-lens 85.0 V, capillary and auxiliary gas heater temperature 350 °C and 400 °C respectively. The eluate was analyzed in full MS mode from *m/z* 110–1300 with a resolution of 70,000 (FWHM) and an automatic gain control (AGC) target of $1e^6$ in positive electrospray ionization (ESI+) mode. The maximum injection time was 50 ms and mass tolerance was set to 5 ppm. Analytes and internal standards with respective *m/z* and retention times are shown in Table 2.

2.6. Method validation

Assay validation was performed on the basis of the *Guideline of bioanalytical method validation* from the European Medicines Agency (EMA), 21 July 2011 [24]. The method was evaluated regarding calibration curve, inaccuracy, imprecision, limits of quantification, carry-over, dilution integrity, matrix effect, selectivity and stability.

2.6.1. Calibration curve

The manufacturerś antimycotic kit contains 3 calibrators covering the clinically relevant range from sub-therapeutic to toxic concentrations. Three calibrators were introduced by dilution of the kit calibrators using drug-free serum as described in section *2.2*.

All calibrators were processed together with a blank (serum without analyte and ISTD) and zero sample (serum without analyte, but added ISTD). According to the EMA guideline back calculated concentrations of all calibrators should be within ±15% of the nominal value, except for the LLOQ (calibrator 1), which should be within ±20%. The calibration curve parameters were: curve type: linear; origin: ignore; weighting: 1/X.

2.6.2. Limits of quantification

According to the EMA guideline the lower limit of quantification (LLOQ) was defined as the lowest calibrator with a signal-to-noise ratio ≥ 5 and imprecision/inaccuracy ≤20%. The LLOQ was prepared by 1.5-fold dilution of the lowest calibrator level of the antimycotics TDM IVD kit (see section *2.2*). The upper limit of quantification (ULOQ) was defined as the highest assay calibrator.

2.6.3. Inaccuracy and imprecision

Inaccuracy and imprecision were tested using calibrator 1 (LLOQ) and QC samples 1–3. Within-run inaccuracy and imprecision were determined by replicate analysis of 5 individually prepared QCs. Between-run inaccuracy and imprecision were evaluated by analyzing 5 individually prepared QC samples per day, on 3 different days. Mean back calculated concentrations of all QCs should be within ±15% of the nominal value, except for the LLOQ where it should be within ±20%.

2.6.4. Carry-over

Carry-over was investigated by injecting blank serum samples (without the analytes in this study and ISTD) from different donors (n = 8) after the highest calibrator (ULOQ). According to the EMA guideline the peak area in the drug-free serum sample should not exceed 20% of the LLOQ peak area and 5% of the ISTD peak area.

2.6.5. Matrix effect

Matrix effects were tested by adding the analytes at 3 different concentration levels (using optimization-mix 2 and the 5-FC solution) to drug-free serum samples from different donors (n = 6) and to absolute methanol. After sample extraction the matrix factor (MF) was calculated for each analyte and ISTD forming the ratio of the peak area in presence of the matrix and the peak area in absence of the matrix. The ISTD normalized matrix factor was calculated as the ratio of the analyte MF and the MF of the corresponding ISTD. The coefficient of variation (CV) of the ISTD normalized MF should not exceed 15%.

To additionally evaluate potential matrix effects a post-column infusion experiment was performed according to Bonfiglio et al. [25]. The neat IVD kit optimization-mix 1, containing all analytes with a concentration of 4 mg/L in methanol, was continuously infused into the HRMS using a T-piece and a syringe pump while processed blank drug-free sera were injected to the chromatographic system. Corresponding chromatograms were compared to the chromatogram obtained by injection of absolute methanol.

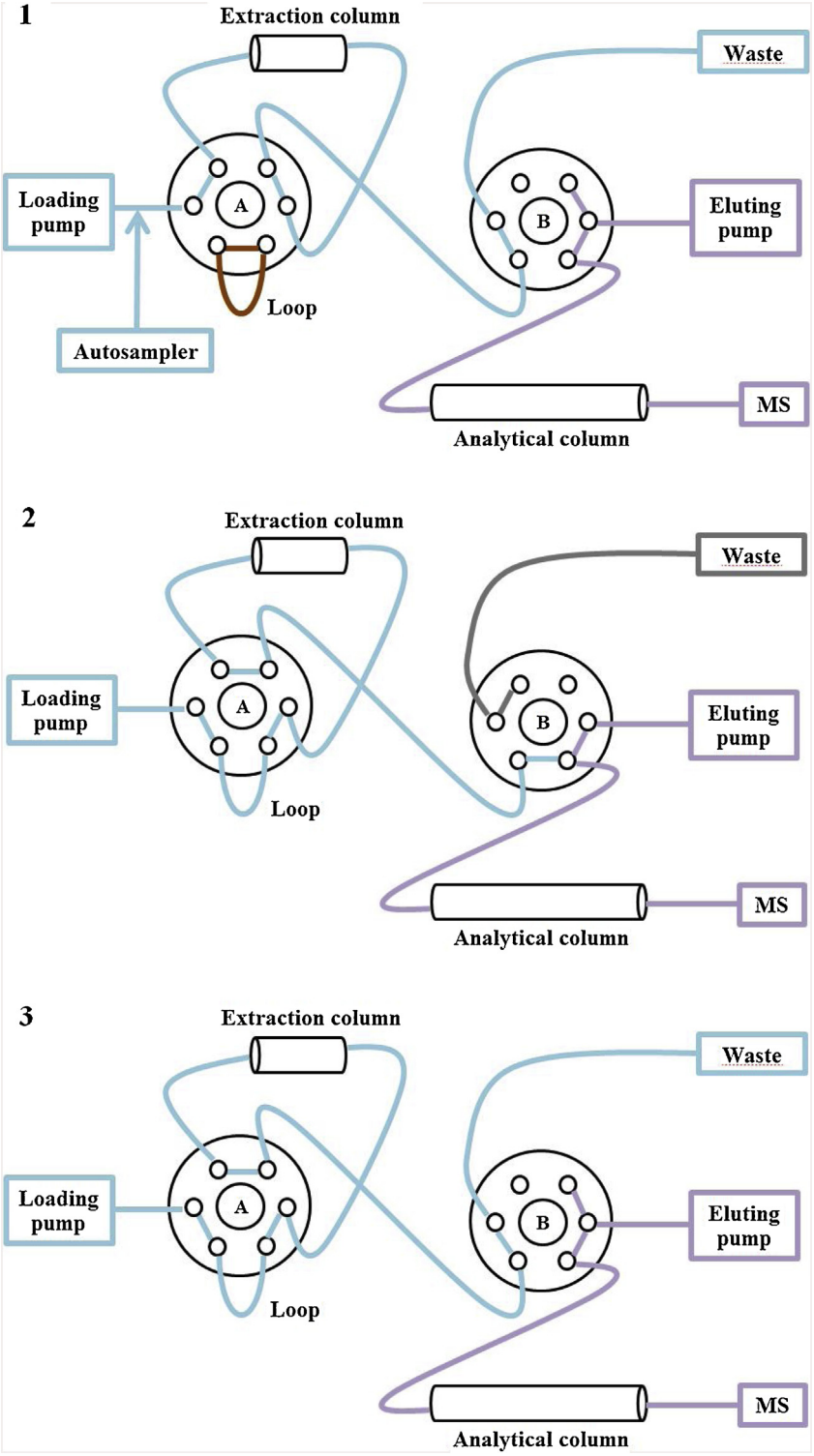

Fig. 1. (A) Switching of valves (1) Loading and extraction step: 5 μL sample was loaded onto the extraction column with mobile phase C. Interfering non-retained serum components were directed into waste. At the same time the analytical column was equilibrated with mobile phase A and B2. (2) Transfer step: After switching of valves and inclusion of the loop, containing mobile phase B1 and D the analytes were eluted from the extraction column by backflush and transferred onto the analytical column. To compensate the flow from the extraction column to the analytical column the flow rate of the eluting pump was reduced accordingly. (3) Analytical separation step: After re-switching of the valves, the analytes were separated on the analytical column using the gradient shown in Fig. 1B. Meanwhile the extraction column was washed with mobile phase B1 and the loop was filled with conditions mentioned previously. Solvents from the extraction column were sent to waste again. (B) Corresponding solvent flow rates of the automated online extraction and analytical separation. (A): water-ammonium acetate (500 mM)-formic acid (98.9:0.1:1 v/v/v), B1: water-acetonitrile-ammonium acetate (500 mM)-ammonia solution (32%) (5:92:1:2 v/v/v/v), B2: water-acetonitrile-ammonium acetate (500 mM)-formic acid (5:93.9:1:0.1 v/v/v/v), C: water-formic acid (99.8:0.2 v/v), D: water-ammonium acetate (500 mM)-ammonia solution (32%) (98.9:0.1:1 v/v/v).

Turboflow™ system										Analytical system			
Step	Start (min)	Duration (sec)	Flow rate (ml/min)	Gradient	%B1	%C	%D	Tee	Loop	Flow rate (ml/min)	Gradient	%A	%B2
1	0.00	30	2.0	Step	-	100	-	-	Out	0.5	Step	95	5
2	0.30	30	0.2	Step	100	-	-	Tee	In	0.3	Step	95	5
3	1.00	60	1.0	Step	100	-	-	-	In	0.5	Ramp	-	100
4	2.00	60	1.0	Step	40	-	60	-	In	0.5	Step	-	100
5	3.00	60	1.0	Step	-	100	-	-	Out	0.5	Step	95	5

Fig. 1. (*Continued*)

Table 2
Retention times and *m/z* of analytes and corresponding isotope labelled internal standards.

Analyte	[M+H]	r.t. (min)	Corresponding internal standard	[M+H]	r.t. (min)
5-flucytosine	130.0411	1.10	$^{13}C^{15}N_2$-5-fluycotsine	133.0385	1.10
Amphotericin B	906.4815*	2.06	d_3-amphotericin B	923.5170*	2.06
Anidulafungin	1140.5136	2.31	$^{13}C_6$-anidulafungin	1146.5337	2.31
Fluconazole	307.1113	1.92	d_4-fluconazole	311.1364	1.92
Isavuconazole	438.1194	2.36	$^{13}Cd_4$-isavuconazole	443.1479	2.36
Itraconazole	705.2466	2.44	d_5-itraconazole	710.2780	2.44
Ketoconazole	531.1560	2.10	d_8-ketoconazole	539.2062	2.10
Micafungin	1270.4456	2.22	$^{13}C_6$-micafungin	1276.4658	2.22
OH-itraconazole	721.2414	2.31	d_5-OH-itraconazole	726.2728	2.31
Posaconazole	701.3369	2.29	d_4-posaconazole	705.3620	2.29
Voriconazole	350.1223	2.18	d_3-voriconazole	353.1411	2.18

* [M-H+H_2O].

Break downs of analyte signal intensities would indicate relevant ion suppression.

2.6.6. Selectivity

To investigate selectivity, leftover sera from intensive care unit (ICU) patients (n = 18), non-ICU patients (n = 40) and various TDM panel QCs from the clinical routine laboratory (n = 8), not including the antimycotics in this project, were analyzed. Especially ICU patients received a broad range of medications in therapeutic regimen, including analgesics, anaesthetics, antibiotics, antidepressants, antivirals, anticoagulants, antiemetics, cardiovascular drugs, hypnotics and sedatives. Absence of interfering substances was accepted if the response at respective analytical retention times was ≤ 20% of the LLOQ and ≤5% for the ISTD.

2.6.7. Stability

Stability was tested using QC samples 1–3 that were stored up to 6 h at RT and at 4 °C, up to 5 weeks at −20 °C and −80 °C. Autosampler stability was tested up to 24 h. Freeze-thaw stability was determined in 3 cycles at −20 °C and −80 °C (freeze time > 12 h, thawing at RT). Stored QC samples were analyzed using freshly prepared calibration samples. According to the EMA guideline stability is given if mean concentration changes are within ± 15% of the nominal concentration.

2.6.8. Dilution integrity

To test dilution integrity drug-free serum samples (n = 5) were spiked with an analyte concentration approximately 50% above the ULOQ and then diluted 5-fold with drug-free serum. The measured concentration was back calculated and compared to the nominal concentration. Inaccuracy and imprecision should not exceed ± 15%.

2.6.9. Comparison commercial kit and extended kit

To meet the EMA guideline requirements all calibrator levels and 1 QC level were diluted to extend the number of calibrators and QCs (see section *2.2*). Quantification conditions using 3 calibrator levels and 2 QCs as suggested by the kit manufacturer were compared to a calibration curve generated using 6 calibrators and 3 QCs. Correlation coefficients (R^2) of the calibration curves as well as inaccuracy and imprecision of QCs were then compared to each other. An aberration of these parameters between 6/3 calibrators/QCs and 3/2 calibrators/QCs would indicate that usage of 6 calibrator levels and 3 QCs is more reliable when assessing unknown samples.

2.6.10. Comparison of LC–MS/MS and LC-HRMS measurement

Analytical performance of the LC-HRMS method developed in this study was compared to a LC–MS/MS IVD Kit (Chromsystems, Gräfelfing, Germany) by replicate analysis of leftover routine TDM samples including ITZ, OH-ITZ, PSZ and VRZ.

Both methods had very similar sample clean-up with protein precipitation, but the chromatographic separation and quantification approaches differed significantly (usage of the exact analyte mass in HRMS vs. parent → daughter transitions in tandem MS). Only patient samples with concentrations within the calibration ranges of both assays were included in the comparative analysis.

3. Results

3.1. Method validation

3.1.1. Calibration curve

The calibration curve was generated using 6 calibrators with a linear regression model and weighting factor 1/X. The linearity of the method could be shown over the whole calibration range with R^2 > 0.995 for all analytes. A representative analytical chromatogram is shown in Fig. 2.

3.1.2. Lower limit of quantification (LLOQ)

The LLOQ signal-to-noise ratio of all analytes was at least ≥ x7 when compared to the blank samples at respective antimycotic retention times. For back calculated LLOQ concentrations both

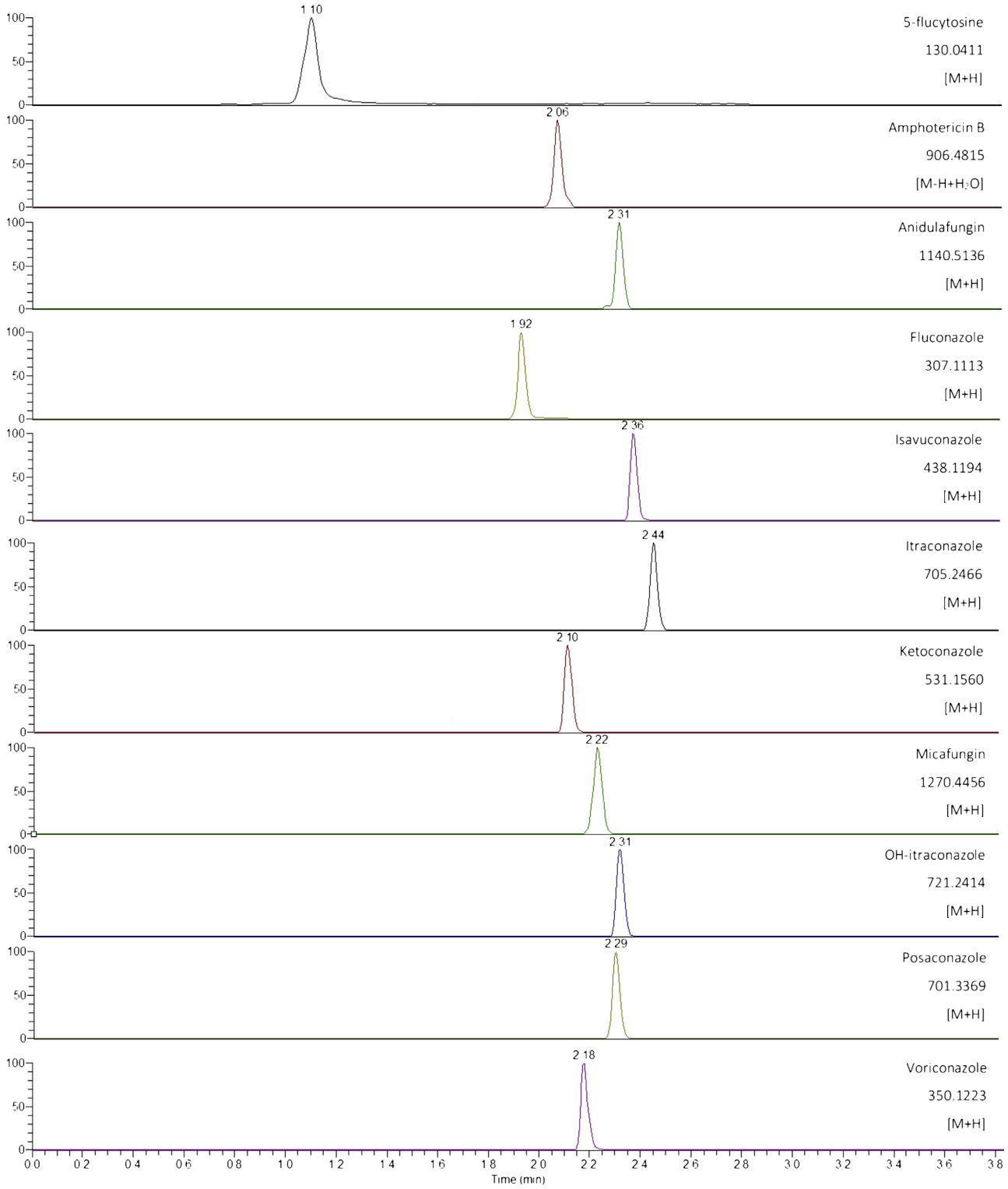

Fig. 2. Representative chromatograms of analytes (calibrator 5 concentration). Retention times are shown in minutes.

between- and within-run inaccuracy and imprecision were ≤4.6% and ≤ 16.6%, respectively (see Table 3).

3.1.3. Inaccuracy and imprecision

For all analytes within-run inaccuracy was ≤12.0%, within-run imprecision was ≤8.1%, between-run inaccuracy was ≤9.0% and between-run imprecision was ≤7.1% in QC samples 1–3. The respective results are summarized in Table 3.

3.1.4. Carry-over

Peak areas in 8 different drug-free serum samples injected after a highest calibrator sample were consistently below 20% of the LLOQ for all analytes and below 5% for the respective ISTD. The highest peak area of all analytes was 12.7% of the LLOQ and 1.1% of the ISTD, respectively.

Table 3
Mean results for inaccuracy and imprecision of the LLOQ (= Cal 1) and QCs. calculated using 6 calibrator levels.

	5-FC	AM-B	ANF	FCZ	IVZ	ITZ	KTZ	MCF	OH-ITZ	PSZ	VRZ
Within-run inaccuracy (run 1) (n = 5) [%]											
LLOQ	−0.18	4.64	2.28	−1.12	−0.75	0.45	1.33	4.18	0.55	0.26	0.34
QC 1	3.06	−6.81	9.79	4.95	5.69	4.89	1.73	3.25	1.74	4.69	2.97
QC 2	−2.31	−8.38	−11.99	0.06	−0.53	−3.98	−6.87	−8.54	−3.33	−1.49	−1.44
QC 3	−1.57	−1.37	−0.59	0.92	−0.80	−2.89	−1.68	−4.38	−1.83	−0.68	−0.23
Between-run inaccuracy (n = 15) [%]											
LLOQ	−0.31	3.33	2.52	−0.76	−0.75	−0.15	0.71	3.95	−0.43	0.56	0.08
QC 1	2.43	−7.99	3.76	3.23	4.32	2.46	0.16	−2.23	2.54	1.28	2.00
QC 2	−2.63	−7.45	−8.96	−1.11	−0.60	−2.65	−4.99	−7.39	−1.92	−0.65	−1.60
QC 3	−2.17	2.62	−3.92	−0.85	−1.33	−3.29	−1.82	−2.97	−2.12	−1.72	−1.76
Within-run imprecision (run 1) (n = 5) [%]											
LLOQ	3.05	11.86	12.10	1.39	1.76	1.87	1.20	16.57	2.29	2.35	1.62
QC 1	2.26	3.07	5.99	2.49	2.01	1.98	2.42	8.09	1.73	2.39	2.18
QC 2	1.58	5.65	2.28	1.66	0.81	1.40	1.07	7.66	1.42	0.61	1.33
QC 3	0.37	3.33	3.41	1.52	0.75	0.81	0.34	4.16	1.43	0.61	0.59
Between-run imprecision (n = 15) [%]											
LLOQ	2.78	11.40	8.42	1.98	1.66	3.58	1.79	12.57	3.30	2.95	2.25
QC 1	2.96	6.58	6.87	2.17	2.06	4.27	4.04	6.85	3.07	5.02	2.30
QC 2	3.27	4.79	4.79	3.03	3.19	2.07	2.04	5.98	4.44	1.91	3.59
QC 3	1.97	7.09	6.06	3.16	1.39	2.94	1.97	7.12	2.38	3.21	2.35

LLOQ: lower limit of quantification. QC: Quality control. 5-FC: 5-flucytosine. AM-B: amphotericin B. ANF: anidulafungin. FCZ: fluconazole. IVZ: isavucaonazole. ITZ: itraconazole. KTZ: ketoconazole. MCF: micafungin. OH-ITZ: OH-itraconazole. PSZ: posaconazole. VRZ: voriconazole.

3.1.5. Matrix effect

The post column infusion experiment did not indicate any relevant matrix.

Evaluation of matrix effects according to the EMA guideline showed constant compensation of potential matrix effects by the ISTD for all analytes at 3 tested concentration levels. ISTD normalized matrix factors were as follows: 102.3% 5-FC, 103.7% AM-B, 98.6% ANF, 101.8% FCZ, 100.5% IVZ, 99.0% ITZ, 101.6% KTZ, 93.5% MCF, 99.1% OH-ITZ, 98.2% PSZ and 100.4% VRZ. The variation coefficients of the ISTD normalized MF for all analytes was ≤ 9.2% and therefore complied with the EMA guideline requirements.

3.1.6. Selectivity

The method showed excellent selectivity where the maximum response was ≤3.0% of the LLOQ for all analytes and ≤0.38% for the ISTD in all ICU, non-ICU patient samples and assayed clinical laboratory routine TDM quality controls.

3.1.7. Stability

All analytes were stable for at least 5 weeks at −20 °C and −80 °C. Freeze-thaw stability was given for all analytes, except for amphotericin B where QC 1 slightly exceeded the EMA guideline criteria with a deviation of -16.3% for the 3rd freeze-thaw cycle.

Benchtop stability (up to 6 h at RT) was given for all analytes except for anidulafungin where QC 2 and QC 3 showed a deviation of -16.6% and -18.2% after 4 h and -25.2% and -21.4% after 6 h, respectively. Additionally, micafungin did not meet the requirements with a deviation of -18.5% in QC 2 after 6 h at RT. All analytes were stable during 6 h at 4 °C. Autosampler stability was proven for all analytes within 24 h at 10 °C.

3.1.8. Dilution integrity

Dilution integrity was shown for all analytes. Inaccuracy ranged from 9.6% to 13.1% and imprecision ranged from 1.1% to 4.1%. However, amphotericin B did not meet the suggested EMA guideline requirements of inaccuracy and imprecision ranging ±15% where inaccuracy was -20.5% and imprecision 16.5%.

3.2. Method comparison

3.2.1. Commercial kit and extended kit

To evaluate potential impairments using 3 instead of 6 calibrators three calibration curves and respective QCs were compared. Linearity was shown for all calibration ranges with $R^2 > 0.995$ using 6 calibrators and $R^2 > 0.997$ using 3 calibrators. Additionally calculated % CV of all R^2 values was < 0.2% (n = 6). Inaccuracy of QC 2 and QC 3 was ≤ 13.9% calculated with 6 calibrators and ≤ 12.9% calculated with 3 calibrators. Imprecision was ≤7.8% and ≤9.2% for QC 2 and ≤8.1% and ≤11.7% for QC 3 calculated using a calibration curve with 6 calibrators and 3 calibrators respectively.

3.2.2. LC-HRMS and LC–MS/MS

Comparable results were obtained between the HRMS and MS/MS method when assaying anonymized trough serum samples from patient receiving treatment with ITZ, PSZ and VRZ according to clinical guidelines. The mean difference was 1.2% (n = 14), 2.5% (n = 15), 13.2% (n = 10) and 6.8% (n = 8) for ITZ, OH-ITZ, PSZ and VRZ respectively.

4. Discussion

The developed method provides rapid sample preparation, followed by clean-up with a mixed-phase extraction column in order to minimize matrix interference. The cationic exchange mode of this column mixed with reversed phase properties required mobile phases with different amounts of acidic and basic components, which made concentration and purification of all analytes possible. Usage of commercially available kit components supported easy and rapid set up of the described method on an Orbitrap-HRMS, although the kit was designed for MS/MS.

Notably 5-fluyctosine showed highest deviations regarding carry-over (≤12.7%), which is still below the EMA guideline requirements of ≤20%. This can be due to its early elution where residual matrix components can co-elute, since 5-flucytosine is a very small and challenging analyte with *m/z* 130.0411. These findings indicate that mere protein precipitation without on-line sample extraction or other secondary sample preparations wouldnt́ sufficiently eliminate relevant matrix effects. After optimization of the extraction protocol HRMS analysis showed good LLOQ results (inaccuracy ≤ 4.6%, imprecision ≤16.6%), insignificant carry-over, as well as excellent selectivity for all analytes in this study.

Concentrations of antimycotics above the calibration curve can be reliably quantified by 5× dilution using drug-free serum with exception of amphotericin B. Yet, observed deviations may result

from the instability of the analyte amphotericin B, especially when samples are exposed to light during processing.

EMA guideline within-run and between-run inaccuracy and imprecision requirements complied with all analytes. Furthermore no relevant matrix effect was shown due to compensation by the ISTD with a CV of the internal standard normalized matrix factor ≤9.2%.

Stability experiments showed good results when QCs were stored at 4 °C, −20 °C and −80 °C or processed QCs were cooled in the autosampler at 10 °C. Freeze-thaw stability was excellent for all analytes except of the 3rd cycle for amphotericin B, which could again be explained by its light instability. Problematic benchtop stability was proven for anidulafungin and micafungin after 4 h and 6 h respectively. Therefore it would be reasonable to avoid freeze-thaw cycles and long storage at room temperature by producing aliquots and keeping the benchtop sample preparation time as short as possible or storing samples at 4 °C with light protection until they are processed.

The comparison of 3 calibrators, as suggested by the MS/MS kit manufacturer, against 6 calibrator levels showed that using 3 calibrators is sufficient to generate a calibration curve. No major deviations could be observed regarding inaccuracy and imprecision of QCs or correlation coefficient of the calibration curve. However, awareness that no calibration curve can be generated is mandatory if one calibrator out of three would be rejected from analysis.

Although a limited number of ITZ (including OH-ITZ), PSZ and VRZ samples were quantified with the clinical routine LC–MS/MS method and the herein described LC-HRMS method, the measured results of both methods were comparable to each other with a percentage deviation ≤13.2%.

We believe that HRMS can be a feasible alternative for TDM of small molecules besides MS/MS. Even though higher costs are related with Orbitrap HRMS the technology provides many advantages. At first method development can be executed rapidly since there is no need for time-consuming optimization of tuning parameters (e.g. collision energy of product ions) with Full MS scan mode. Also retrospective adding of analytes to an existing method is practicable, as well as unknown screening of complex matrices. Furthermore usage of HRMS for protein and metabolomic analysis, besides TDM is a common procedure. At last quantification of analytes or metabolites with very similar *m/z* using HRMS is feasible without extensive LC separation.

Conflicts of interest

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3 Appendix

3.1 List of abbreviations

ADME	absorption, distribution, metabolism, excretion
EMA	European Medicines Agency
HRMS	high-resolution mass spectrometry
ICU	intensive care unit
LC-MS	isotope dilution liquid chromatography mass spectrometry
MIC	minimal inhibitory concentration
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
PD	pharmacodynamics
PK	pharmacokinetics
SIL-IS	stable isotopically labeled internal standard
TDM	therapeutic drug monitoring

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3.3 Publications

Articles

- Schuster, C., Paal, M., Lindner, J., Zoller, M., Liebchen, U., Scharf, C., & Vogeser, M. (2019). Isotope dilution LC-orbitrap-HRMS with automated sample preparation for the simultaneous quantification of 11 antimycotics in human serum. *J Pharm Biomed Anal*, 166, 398-405
- Schuster, C., Sterz, S., Teupser, D., Bruegel, M., Vogeser, M., & Paal, M. (2018). Multiplex therapeutic drug monitoring by isotope-dilution HPLC-MS/MS of antibiotics in critical illnesses. *J Vis Exp*(138)
- Paal, M., Zoller, M., Schuster, C., Vogeser, M., & Schuetze, G. (2018). Simultaneous quantification of cefepime, meropenem, ciprofloxacin moxifloxacin, linezolid and piperacillin in human serum using an isotope-dilution HPLC-MS/MS method. *J Pharm Biomed Anal*, 152, 102-110

Posters

- Schuster, C., Ritter-Sket, C., Zander, J., & Vogeser, M., Evaluation of external quality assessment for vancomycin, MSACL 2017 EU, Salzburg, Sept 10th - 14th, 2017, Poster H03
- Schuster, C., Ritter-Sket, C., Zander, J., & Vogeser, M., Evaluation of external quality assessment for vancomycin, 14th Annual Meeting of the German Society for Clinical Chemistry and Laboratory Medicine, Oldenburg, Oct 11th – 14th, 2017, Poster P057
- Schuster, C., Paal, M., Lindner, J., & Vogeser, M., Isotope dilution Turboflow™ LC-HRMS for the simultaneous quantification of 12 antimycotics in human serum, MSACL 2018 EU, Salzburg, Sept 9th – 13th, 2018, Poster E16
- Schuster, C., Paal, M., Lindner, J., & Vogeser, M., Isotope dilution Turboflow™ LC-HRMS for the simultaneous quantification of 12 antimycotics in human serum, 15th Annual Meeting of the German Society for Clinical Chemistry and Laboratory Medicine, Mannheim, Sept 26th – 28th, 2018, Poster P080

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