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Untersuchungen zur Anwendung der Flüssigkeitschromatographie-Massenspektrometrie in der labormedizinischen Routinediagnostik

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1 Einleitung

1.1 Labormedizin und Massenspektrometrie

Labormedizinische Untersuchungen und Befunde sind meist ein wesentlicher Bestandteil des diagnostischen Prozesses. Ebenso werden Laboruntersuchungen im Rahmen der Prävention, zur Einschätzung eines Erkrankungsrisikos, zur Beurteilung der Prognose von Erkrankungen sowie zur Überwachung des Krankheitsverlaufes bzw. zur Kontrolle einer spezifischen Therapie durchgeführt. Häufig eingesetzte Untersuchungsmaterialien in der labormedizinischen Diagnostik sind Blut (Vollblut, Plasma, Serum) und Urin, es werden aber auch andere Matrices wie Speichel, Liquor, Knochenmark, Stuhl oder Gewebe verwendet. Der Schwerpunkt der Routineuntersuchungen in einem medizinischen Labor ist die Analyse endogener Substanzen als Biomarker. Zunehmend gewinnt jedoch auch die Quantifizierung von exogenen Substanzen (Xenobiotika, in der Regel Arzneistoffe) an Bedeutung. Mit der Bestimmung von Arzneistoffen kann nicht nur die Adherence (Compliance) des Patienten überwacht, sondern auch die individuelle Pharmakokinetik beurteilt werden. Diese kann unter anderem durch verschiedene Faktoren wie Alter, Geschlecht, Körpergewicht, Organfunktion, genetische Veranlagung und Komedikation beeinflusst werden. Im Rahmen eines Therapeutischen Drug Monitorings (TDM) kann nach der Bestimmung der Arzneistoffkonzentration und der Berechnung der individuellen Pharmakokinetik eine Dosisindividualisierung durchgeführt werden. Ein TDM ist sinnvoll, wenn der Therapieerfolg erhöht bzw. Nebenwirkungen vermieden werden können. Dies ist in der Regel der Fall, wenn ein therapeutischer Bereich bekannt ist und der Arzneistoff folgende Eigenschaften besitzt [1]:

- bestehende Korrelation zwischen Pharmakokinetik und Pharmakodynamik
- geringe therapeutische Breite
- hohe interindividuelle Variabilität
- Routinebestimmung geeigneter pharmakodynamischer Zielgrößen nicht möglich (wie Blutdruck und Blutzucker)

Eine Vielzahl von Laborparametern kann heute mittels vollautomatischer Analyzer, welche auf photometrischen und immunometrischen Methoden beruhen, bestimmt werden. Diese hochdurchsatztauglichen und bedienerfreundlichen Geräte leisten einen Hauptteil der Analysen in modernen klinischen Routinelabors. Ein Nachteil ist jedoch, dass die Etablierung neuer Verfahren auf diesen Analyzern in der Regel nur in Zusammenarbeit mit dem Hersteller des Analyzers erfolgen kann. Weitere Limitationen können eine eingeschränkte analytische Selektivität auf Grund von Kreuzreaktivitäten und eine mangelnde Übertragbarkeit der Ergebnisse zwischen verschiedenen Analyzern sein [2].

Im Gegensatz zu den vollautomatischen Analyzern ist der Einsatz der Massenspektrometrie (MS) in der Labormedizin nach wie vor auf wenige Spezial-laboratorien beschränkt. In den meisten dieser klinischen Laboratorien werden Tandem-Massenspektrometer (MS/MS) gekoppelt an die Hochleistungsflüssigkeitschromatographie (HPLC bzw. LC) genutzt [3]. Neben dem Neonatalscreening auf angeborene Stoffwechsel-erkrankungen kommt die HPLC-MS/MS vor allem für das TDM von Immunsuppressiva, Antikonvulsiva, Antipsychotika und die Bestimmung von Hormonen zur Anwendung. Folgende Stärken und Limitationen haben dazu beigetragen, dass die HPLC-MS/MS im Bereich der Labormedizin zwar an Bedeutung gewinnt, aber noch nicht flächendeckend eingesetzt wird [3, 4]:

Stärken:

- hohe Spezifität
- hohe Sensitivität
- breites Analytenspektrum
- relativ einfache und vom Hersteller unabhängige Methodenentwicklung für neue Analyte
- simultane Bestimmung mehrerer Parameter in einer Analyse
- Ergebnisse auf dem Niveau einer Referenzmethode im Routine-Setting
- gute Übertragbarkeit der Ergebnisse zwischen Assays und Laboratorien
- niedrige Kosten für Reagenzien

Limitationen:

- geringer Probendurchsatz
- hoher manueller Arbeitsaufwand
- Probenbestimmung im *Batch Modus* anstelle eines *Random Access Modus*
- Notwendigkeit von speziell geschultem Personal für Routineanalytik und erfahrenen Experten für Methodenentwicklung und Troubleshooting
- hohe Anschaffungs- und Instandhaltungskosten
- keine klar definierten Qualitätskriterien und Regularien für die MS im Bereich der Labormedizin
- geringe Anzahl an verfügbaren zertifizierten Kits

Den Einsatz dieser leistungsfähigen Technologie in der Labormedizin weiter auszubauen ist ein wichtiges Ziel der aktuellen labormedizinischen Forschung. Hier stehen vor allem die Automatisation der Methodik und die Erhöhung der Praktikabilität im Vordergrund.

Als übergeordnete Fragestellung dieser Promotionsarbeit sollte geklärt werden, welche Techniken dazu beitragen können, dass die HPLC-MS/MS-Analytik besser in den Ablauf

eines klinischen Routinelabors integriert werden kann und ob das Leistungsspektrum von klinischen Routinelabors – insbesondere um die Möglichkeit eines Antibiotika-TDMs bei kritisch kranken Patienten – erweitert werden kann. In einem ersten Teilprojekt sollte die Nichtunterlegenheit (Äquivalenz) einer alternativen Quantifizierungsmethode (Direkte Isotopenverdünnungsmethode, DIDA) gegenüber einer konventionellen externen Kalibration geprüft werden. Eine Äquivalenz von DIDA wäre für die Anwendung der HPLC-MS/MS in einem klinischen Routinelabor von beachtlicher praktischer Bedeutung, da in der Labormedizin eine kurze Turnaround-Zeit hohe Priorität besitzt und die Bestimmung mittels DIDA ohne zusätzliche Kalibratoren „*ad hoc*“ erfolgen kann (2.1). Um das Leistungsspektrum eines klinischen Routinelabors zu erweitern, sollten in weiteren Teilprojekten HPLC-MS/MS Methoden zur Quantifizierung von Antibiotika im Serum entwickelt werden, welche für die Anwendung in einem klinischen Routinelabor geeignet sind (2.2; 2.4). Neben der kurzen Turnaround-Zeit spielt in einem klinischen Routinelabor der Probendurchsatz eine enorme Rolle, deshalb sind Methoden mit geringem manuellen Arbeitsaufwand und einer kurzen Laufzeit wünschenswert. Aus diesem Grund haben wir es uns zum Ziel gesetzt, Techniken, die dies unterstützen – wie die Online-Festphasenextraktion (Online-SPE) oder die Ultrahochleistungsflüssigkeitschromatographie (UHPLC) – in die Methoden zu integrieren. In einem weiteren Teilprojekt wurden mit der entwickelten und erprobten Methode (2.2) Linezolid-Serumspiegel innerhalb einer prospektiv beobachtenden Studie bestimmt. Mit dieser Studie sollte eruiert werden, ob kritisch kranke Patienten, welche mit der Linezolid Standarddosierung behandelt werden, Spiegel innerhalb des therapeutischen Bereiches erreichen (2.3). Die einzelnen Teilprojekte werden im Folgenden näher dargestellt.

1.2 Direkte Isotopenverdünnungsmethode – Eine alternative Methode zur Quantifizierung

In der Labormedizin erfolgt die Quantifizierung eines Analyten mittels HPLC-MS/MS meist in einer abgeschlossenen Analysenserie (Batch), wobei die Konzentrationen in den untersuchten Proben über eine Mehrpunkt-Kalibrationsreihe bestimmt werden. Dazu werden mehrere Kalibratoren vor der eigentlich zu bestimmenden Probe analysiert. Dieser Modus ist gut geeignet, um Analyte zu bestimmen, die für die Krankenversorgung eher zeitlich unkritisch sind und bei denen daher die Proben gesammelt werden können. Für Analyte, deren Messwert jedoch schnell vorliegen muss, ist eine *ad hoc* Bestimmung rund um die Uhr aus klinischer Sicht sehr wünschenswert. Die Analyse mehrerer Kalibrationsproben vor der eigentlichen diagnostischen Probe kostet nicht nur Ressourcen, sondern ist auch zeitintensiv. Folglich kann die Quantifizierung über eine Mehrpunktkalibration ein Hindernis für den breiteren Einsatz der Massenspektrometrie in der Labormedizin bedeuten [3].

Ein alternatives Verfahren zur Quantifizierung in der Massenspektrometrie ist die Direkte Isotopenverdünnungsmethode (direct isotope dilution analysis; DIDA). Die Bestimmung der Konzentration erfolgt bei DIDA nicht unter Einbeziehung einer Kalibrationsreihe, sondern wird direkt über die Konzentration des in die Probe zugegebenen isotopenmarkierten Internen Standard (IS) berechnet. Dieses DIDA-Prinzip beruht auf der Annahme, dass der native Analyt und sein isotopenmarkiertes Pendant nahezu gleiche physikalisch-chemische Eigenschaften besitzen. Da zu diesen Eigenschaften auch das Ionisationsverhalten zählt, sollten sich in einer HPLC-MS/MS Analyse bei gleicher Konzentration von Analyt (A) und IS gleiche Peak-Flächen (Area) ergeben. Ist dies erfüllt und ist die Peak-Fläche proportional zur Konzentration (c), kann die Konzentration des Analyten mit folgender Formel berechnet werden:

$$c_A = c_{IS} \frac{\text{Area}_A}{\text{Area}_{IS}}$$

Im Rahmen des Promotionsprojektes sollte das Prinzip der DIDA für einen exemplarischen Analyten (Cortisol) in einem klinischen Routinelabor getestet und die Resultate (c_{DIDA}) mit den Ergebnissen der konventionellen Methode (c_{Kon}) – Quantifizierung über 6-Punkt-Kalibrationsreihe – verglichen werden.

Es konnte gezeigt werden, dass DIDA tatsächlich eine valide Alternative zur Mehrpunktkalibration ist und dass *ad hoc* Analysen von Einzelproben mittels HPLC-MS/MS möglich sind (2.1).

1.3 Therapeutisches Drug Monitoring von Antibiotika

Eine adäquate frühzeitige antimikrobielle Therapie zählt zu den zentralen Maßnahmen für eine erfolgreiche Behandlung bei schweren bakteriellen Infektionen. Neben der Wahl eines geeigneten Antibiotikums ist eine ausreichende Dosierung entscheidend [5]. Eine Standarddosierung kann allerdings gerade bei kritisch kranken Patienten aufgrund einer veränderten und schwer vorhersehbaren Pharmakokinetik insuffizient sein bzw. auch zu stark erhöhten Spiegeln führen. [5, 6]. Während zu hohe Spiegel Nebenwirkungen hervorrufen können, sind zu niedrige Spiegel mit Therapieversagen und auch mit der Entwicklung von Resistenzen assoziiert [7]. Aus diesem Grund empfehlen verschiedene Expertengremien ein TDM von Antibiotika bei schwer kranken Patienten [7, 8]. Dies ist allerdings bisher nur für Aminoglykoside und Glykopeptide standardmäßig möglich, da für alle anderen Antibiotika keine kommerziell verfügbaren Tests existieren.

1.3.1 Linezolid

Linezolid ist gut wirksam gegen grampositive Bakterien, einschließlich hochresistenter Bakterien wie Methicillin-resistente Staphylococcus aureus Stämme (MRSA), Vancomycin-resistente Enterokokken (VRE) und Penicillin-G resistente Pneumokokken [9]. Die Standarddosierung beim Erwachsenen beträgt zweimal 600 mg Linezolid täglich. Eine Dosisanpassung ist laut Herstellerangaben weder bei Niereninsuffizienz noch bei Leberinsuffizienz nötig [10]. Tatsächlich ist es jedoch sehr schwierig vorherzusagen, ob die Linezolid Standarddosierung auch bei kritisch kranken Patienten ausreichend ist. Die derzeitige Studienlage ist diesbezüglich nicht einheitlich. Während in einigen Studien berichtet wird, dass eine Standarddosierung von Linezolid meist zu ausreichenden Wirkspiegeln führt [11, 12], berichten andere Studien über inadäquate Spiegel bei Standarddosierung [13 – 17]. Allerdings war in diesen Studien die Patientenzahl häufig gering oder es waren spezielle Subgruppen ausgeschlossen. Aus diesem Grund wurde eine monozentrische, prospektive und beobachtende Studie nach Genehmigung durch die Ethikkommission der Klinik der Universität München (Registriernummer: 428.12) durchgeführt.

Im Rahmen dieser Promotionsarbeit sollte im Kontext der oben genannten klinischen Studie untersucht werden, ob die Entwicklung einer robusten Isotopenverdünnungs-HPLC-MS/MS-Methode zur Quantifizierung von Linezolid im Serum möglich ist, da Linezolid bisher mit kommerziellen Methoden nicht bestimmt werden kann. Um eine spätere Integration in die Routineanalytik zu ermöglichen, sollte getestet werden, ob eine einfache und schnelle manuelle Probenvorbereitung sowie eine kurze Laufzeit mit einer guten analytischen Zuverlässigkeit vereinbar sind. Aus diesem Grund wurde eine semi-

automatische Probenvorbereitung gewählt, welche eine Proteinfällung mit der Online-SPE kombiniert. Diese Technik realisiert nicht nur eine Zeitersparnis gegenüber anderen manuellen Probenvorbereitungen (wie der Flüssig-Flüssig-Extraktion oder der klassischen Festphasenextraktion), sondern kann auch die Robustheit und die Sensitivität von HPLC-MS/MS Methoden erhöhen [18]. Trotz dieses zusätzlichen Schrittes lag die Laufzeit der Methode bei nur vier Minuten. Ein wesentlicher Bestandteil der Methodenevaluation war die parallele Erprobung der Methode an zwei unabhängigen Geräten.

Es konnte gezeigt werden, dass Linezolid mittels einer routinetauglichen HPLC-MS/MS Methode quantifiziert werden kann (2.2).

Somit konnte in der Studie für die Bestimmung von Linezolid die neu entwickelte HPLC-MS/MS Methode verwendet werden. Ziel dieser Studie war die Beurteilung, wie viele der kritisch kranken Patienten, die mit der Linezolid Standarddosierung behandelt wurden, Spiegel innerhalb des therapeutischen Bereiches erreichen. Der therapeutische Bereich wurde basierend auf Literaturdaten sowohl über die Fläche unter der Konzentrations-Zeit-Kurve (AUC: Area under the curve) (200 – 400 mg*h/L) wie auch über den Talspiegel (c_{min}) (2 – 10 mg/L) definiert [12, 19 – 21]. Die untere Grenze des therapeutischen Bereiches wurde in Anlehnung an *Rayner et al.* gewählt, die gezeigt haben, dass der Therapieerfolg bei schwer kranken Patienten größer ist, wenn die Linezolidkonzentration während eines Dosierungsintervalls die minimale Hemmkonzentration (MHK) nicht unterschreitet (c_{min} > MHK) bzw. das Verhältnis von AUC_{24}/MIC größer ist als 80 – 120 [12]. Als MHK wurde für diese Studie die MHK_{90} (Konzentration, die das Wachstum von mindestens 90% der getesteten Stämme hemmt) von klinisch relevanten infektiösen Pathogenen gewählt (*Staphylococcus aureus* und *Enterococcus species*) [22, 23].

In unserer Studie konnte eine erhebliche interindividuelle und intraindividuelle Variabilität von AUC und Talspiegel bei kritisch kranken Patienten, mit einer hohen Inzidenz von inadäquat niedrigen Spiegeln, festgestellt werden. Die Ergebnisse legen nahe, dass für kritisch kranke Patienten ein TDM für Linezolid sinnvoll sein kann (2.3). Auf Grundlage dieser Resultate ist eine prospektiv randomisierte Interventionsstudie anzustreben, um zu untersuchen, ob ein TDM von Linezolid das Outcome der Patienten tatsächlich verbessern kann.

1.3.2 Simultane Bestimmung von Piperacillin, Tazobactam, Cefepim, Meropenem, Ciprofloxacin und Linezolid

Die Empfehlung eines generellen TDM von Antiinfektiva bei schwer kranken Patienten ist derzeit auf Grund fehlender kommerziell verfügbarer Messmethoden schwer umzusetzen. Im Rahmen dieser Promotionsarbeit sollte nach erfolgreicher Entwicklung einer Methode zur Quantifizierung von Linezolid untersucht werden, ob das Angebot eines Antibiotika TDMs um weitere klinisch relevante Antibiotika aus unterschiedlichen Antibiotikaklassen ergänzt werden kann. Grundsätzlich können hier zwei Vorgehensweisen in Betracht gezogen werden: Die separate Entwicklung mehrerer Methoden für jeweils ein Antibiotikum (Monomethode) oder die Entwicklung einer Methode zur simultanen Quantifizierung aller Antibiotika (Multimethode). Im Hinblick auf die Anwendung in einem klinischen Routinelabor ist eine Multimethode zu favorisieren, da meist nur wenige HPLC-MS/MS Geräte in einem klinischen Routinelabor vorhanden sind und ein erforderlicher Wechsel zwischen Methoden nicht nur zeitintensiv ist, sondern auch dazu führt, dass andere Parameter gleichzeitig nicht angeboten werden können. Die Integration verschiedener Analyte in eine Multimethode kann eine Herausforderung sein – insbesondere wenn sich die Analyte physikalisch-chemisch unterscheiden, wie dies bei unterschiedlichen Antibiotikaklassen der Fall ist. Für die Auswahl der Antibiotika war neben der Studienlage bezüglich der Notwendigkeit eines TDMs [14 – 16, 20, 21, 24 – 29] die Anwendung bei kritisch kranken Patienten entscheidend. So zählen Ciprofloxacin, Linezolid, Meropenem und Piperacillin in Kombination mit Tazobactam auf einer Intensivstation zu den am häufigsten eingesetzten antibiotischen Substanzen ihrer jeweiligen Klassen [30].

Bei der Methodenentwicklung wurde der Fokus auf Routinetauglichkeit und Robustheit gelegt. Durch die Anwendung der UHPLC-Technologie ist eine simultane Bestimmung von sechs Analyten innerhalb von fünf Minuten möglich, obwohl auch in diese UHPLC Methode eine Online-SPE integriert wurde. Die UHPLC ist eine spezielle Form der HPLC. So werden bei der UHPLC Säulen mit sehr kleinem Partikeldurchmesser (< 2 µm) und kleinem Säulendurchmesser verwendet. Dies führt zu einer erhöhten Trennleistung und damit zu einer höheren Analysengeschwindigkeit, Empfindlichkeit und Auflösung. Ein Nachteil ist allerdings der extrem hohe Rückdruck (> 400 bar), welcher unter anderem spezielle Pumpen nötig macht [31].

Unsere ausführliche Erprobung konnte zeigen, dass mit der im Rahmen dieser Promotionsarbeit entwickelten Methode klinisch relevante Antibiotika aus unterschiedlichen Antibiotikaklassen richtig und präzise bestimmt werden können (2.4).

1.4 Zusammenfassung/Summary

Als übergreifende Fragestellung dieser Promotionsarbeit sollte geklärt werden, welche Techniken dazu beitragen können, dass die HPLC-MS/MS-Analytik besser in den Ablauf eines klinischen Routinelabors integriert werden kann und ob das Leistungsspektrum von klinischen Routinelaboratorien – insbesondere um die Möglichkeit eines Antibiotika-TDMs – erweitert werden kann. In einem ersten Teilprojekt wurde eine alternative Quantifizier-methode, die direkte Isotopenverdünnungsmethode (DIDA), hinsichtlich ihres Einsatzes in einem klinischen Routinelabor getestet. Diese Untersuchung konnte zeigen, dass die mittels DIDA erzeugten Resultate vergleichbar mit den Ergebnissen sind, die über eine Mehrpunkt-Kalibrationsreihe ermittelt wurden. Somit kann DIDA eine mögliche Option zur Quantifizierung in einem klinischen Routinelabor sein und vor allem die Möglichkeit bieten *ad hoc* Analysen durchzuführen (2.1). In weiteren Teilprojekten konnte gezeigt werden, dass Linezolid (2.2) und verschiedene Antibiotika (2.4) mit routinetauglichen HPLC-MS/MS Methoden bestimmt werden können. Bei beiden dargestellten Methoden wurde die Routinetauglichkeit durch die Integration einer semi-automatischen Probenvorbereitung (Proteinfällung und Online-SPE) und eine kurze Laufzeit erreicht. Die kurze Laufzeit wurde bei der Multimethode durch die Anwendung der UHPLC-Technologie realisiert. Die neu entwickelte Methode zur Quantifizierung von Linezolid (2.2) wurde innerhalb einer klinischen Studie angewendet. Das Ergebnis dieser Studie legt nahe, dass ein Linezolid-TDM bei kritisch kranken Patienten sinnvoll sein könnte (2.3).

Summary

The main aspect of this doctoral thesis was to evaluate which techniques could be helpful to integrate HPLC-MS/MS better in the work flow of a routine clinical laboratory. Furthermore, the possibility of extending the spectrum of routine clinical laboratories should be explored by developing HPLC-MS/MS methods for TDM of antibiotics. In the first project an alternative quantification method (direct isotope dilution analysis; DIDA) was tested regarding its application in a routine clinical laboratory. The finding of this study demonstrated that results obtained with DIDA were comparable with those obtained by employing a multi-point calibration curve. Therefore DIDA is a possible option for quantification in a routine clinical laboratory especially to facilitate *ad hoc* analyses. The results of further sub-projects have shown that linezolid (2.2) and various antibiotics (2.4) can be measured with HPLC-MS/MS methods. In both presented methods suitability for routine use was achieved through the integration of a semi-automatic sample preparation (protein precipitation combined with online-SPE) and a short run time. To realize a short run time for the multi-method UHPLC-chromatography was applied. The newly developed method for determination of linezolid (2.2) was employed in a clinical study. The findings of this study suggest that TDM of linezolid might be useful in critically ill patients (2.3).

2 Originalpublikationen

2.1 Maier B, Vogeser M.

Target analyte quantification by isotope dilution LC-MS/MS directly referring to internal standard concentrations – validation for serum cortisol measurement.

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Target analyte quantification by isotope dilution LC-MS/MS directly referring to internal standard concentrations – validation for serum cortisol measurement

Abstract

Background: Isotope dilution LC-MS/MS methods used in the clinical laboratory typically involve multi-point external calibration in each analytical series. Our aim was to test the hypothesis that determination of target analyte concentrations directly derived from the relation of the target analyte peak area to the peak area of a corresponding stable isotope labelled internal standard compound [direct isotope dilution analysis (DIDA)] may be not inferior to conventional external calibration with respect to accuracy and reproducibility.

Methods: Quality control samples and human serum pools were analysed in a comparative validation protocol for cortisol as an exemplary analyte by LC-MS/MS. Accuracy and reproducibility were compared between quantification either involving a six-point external calibration function, or a result calculation merely based on peak area ratios of unlabelled and labelled analyte.

Results: Both quantification approaches resulted in similar accuracy and reproducibility.

Conclusions: For specified analytes, reliable analyte quantification directly derived from the ratio of peak areas of labelled and unlabelled analyte without the need for a time consuming multi-point calibration series is possible. This DIDA approach is of considerable practical importance for the application of LC-MS/MS in the clinical laboratory where short turnaround times often have high priority.

Keywords: cortisol; direct isotope dilution analysis (DIDA); liquid chromatography-tandem mass spectrometry (LC-MS/MS); stable isotope labelled internal standard.

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Introduction

Today liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods employed in the clinical laboratory are routinely performed as batch analyses, involving a multi-level calibrator series followed by "unknowns". This batch mode of analysis is applicable for analytes like 25-hydroxyvitamin D which do not require reporting within short time frames. However, it is likely that LC-MS/MS will be increasingly used in fields, such as clinical toxicology where often single samples have to be analysed instead of batches and it is desirable to obtain results rapidly. This may also apply to measuring serum cortisol at a high level of accuracy in the context of diagnosing adrenocortical dysfunction in septic shock [1]. Thus, analysing a calibration series in the conventional and time consuming operation mode of LC-MS/MS analysis is a substantial drawback in clinical mass spectrometry, which indeed makes a more widespread application of LC-MS/MS questionable in this setting.

The current practice to perform multi-point calibration for clinical LC-MS/MS analyses is adopted, on the one hand, from standard biomedical method validation protocols and, on the other hand, from traditional, non-automated ligand binding assays. In these latter tests, typically complex and non-linear calibration functions are observed which definitely require in most cases at least five calibration points for the quantification of unknowns. In LC-MS/MS, in contrast, in the majority of assays, linear response is observed over a wide range of concentrations and the need for multi-point calibration is indeed questionable. Notably, the relation between the number of calibration samples and the analytical reliability of routine LC-MS/MS methods or the "stability" of a calibration is hardly studied systematically in the methodological literature of clinical mass spectrometry so far.

Direct isotope dilution analysis (DIDA) is an alternative quantification method. The principle of DIDA is based on the assumption (and precondition) that the

physico-chemical behaviour of the native analyte molecule is very similar to that of its isotope labelled counterpart used as the internal standard. For these labelled compounds very similar ionisation yields and consequently LC-MS/MS-peak areas are observed as with native (unlabelled) analyte molecules. Therefore, when adding the stable isotope labelled internal standard compound solution in a strictly quantitative manner to a biological sample as the first step of sample preparation, it is possible to determine the analyte concentration in the sample from the LC-MS/MS-peak area of the native, unlabelled analyte molecule in relation to the peak area of the labelled internal standard compound, by simple calculation of an individual sample.

The aim of our study was to investigate the feasibility of this DIDA principle in the field of routine clinical laboratory application of LC-MS/MS where DIDA is not used so far. We tested the hypothesis that applying DIDA is not inferior to the standard approach of multi-calibrator isotope dilution LC-MS/MS quantification with respect to accuracy and reproducibility in the quantification of serum cortisol as an exemplary analytical system.

Materials and methods

A comparative validation protocol was applied including four analytical series: In these series cortisol was determined as described previously [2] in spiked quality control (QC) samples, in human serum pools and in 25 patients' samples using LC-MS/MS involving a six-point set of calibration samples and three-fold deuterated ([9,12, 12-^{2}H]cortisol) as the internal standard in a conventional approach, using a standard chromatography software program (MassLynx, Waters). Unlabelled cortisol was purchased from Sigma Aldrich (Steinheim, Germany; purity 98.7%) and labelled cortisol was purchased from Cambridge Isotope Laboratories (Andover, USA; purity: 98%). According to the MS-scan investigation experiments, no three-fold deuterated cortisol was detectable in the native cortisol formulation, nor was unlabelled cortisol found in the formulation of labelled cortisol. Serum pools were prepared from leftover samples sent for clinical chemistry investigations to our laboratory. Twenty-five leftover individual patients' samples sent for cortisol testing to our laboratory were included in the series after anonymisation. Calibrators were prepared by spiking a solution of bovine serum albumin (70 g/L) in phosphate buffered saline to the following cortisol concentrations: 12.3, 24.7, 49.4, 98.7, 197 and 395 µg/L. QC samples were prepared in the same way (49.4, 148 and 296 µg/L).

After this conventional analysis and quantification, the peak areas (of analyte and labelled internal standard) recorded for QC samples, pool samples and patients' samples were re-assessed separately for each sample. Based on their raw peak areas (Figure 1) – as a second and independent quantification process – cortisol concentrations were determined using an Excel sheet according to the above described DIDA principle, not taking into account the calibration samples analysed in the respective series at all. In this way for any of the four analytical series two sets of quantitative data were obtained for each validation material and individual patient's sample (conventional quantification vs. DIDA). Accuracy (% deviation from target concentration for spiked samples) and reproducibility (% relative standard deviation) were comparatively assessed for the two different quantification processes. The FDA criteria for bioanalytical method validation were applied [3, 4]. Individual patient's sample results were plotted for comparison.

Results

The data of the validation experiment are summarised in Table 1. For the three spiked QC samples, analytical accuracy realised using the DIDA quantification was judged to be not inferior when compared to the conventional approach of calibration with an external series of calibrants. Regarding reproducibility, a total of seven samples (spiked QC samples and pool samples) were assessed, and in all samples lower CVs were observed for DIDA when compared to conventional calibration. Applying a t-test for paired observations, CVs of DIDA were significantly lower compared to conventional calibration ($p<0.05$). Very close agreement was found for paired results of patients' samples ($r>0.999$; Figure 2).

Both methods met the requirements of the FDA guidance for bioanalytical method validation (CV and bias <15%; see Table 1) [3].

Discussion

Our study demonstrates that isotope dilution-LC-MS/MS can allow – besides traditional batch analysis – reliable "ad-hoc" analyses of single samples without the need to perform a multi-point calibration when the DIDA approach is used. In the comparative imprecision study, DIDA even showed superior reproducibility when compared to conventional calibration. Potentially multi-point calibration introduces a somewhat higher degree of "noise" into the analytical system, resulting from the unavoidable variation in preparing and analysing six individual calibration samples. Regarding the degree of analytical accuracy, however, both principles were judged equal (Table 1). The reliability of DIDA quantification was further confirmed by the analysis of a set of individual patients' samples, displaying fully commutable results.

In newborn screening for inborn metabolic diseases by LC-MS/MS, the principle of estimating analyte concentrations derived from the concentration of the added internal standard is already used. This use, however, is in

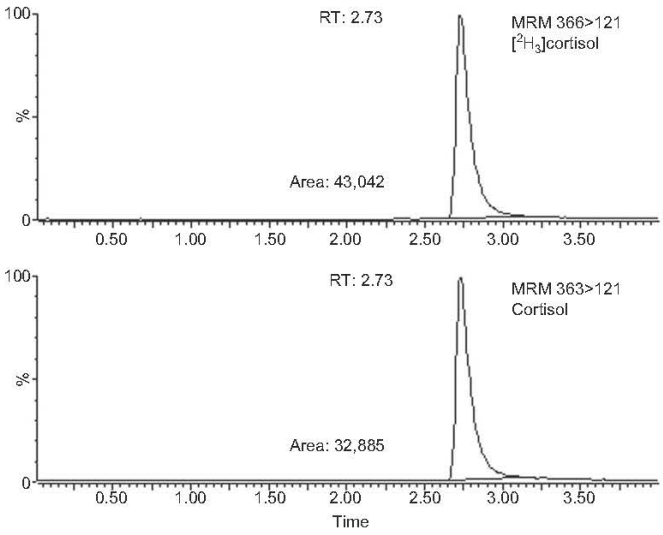

Figure 1 Example for the quantification of a target analyte concentration based on the LC-MS/MS peak area of a stable isotope labelled internal standard compound.
Peak area of the target analyte cortisol, 32,885 counts, and of the internal standard [$^2H_3$]cortisol, 43,042 counts. Since the ionisation behaviour of both cortisol molecules is assumed to be very similar and since the volume of the serum aliquot and the internal standard solution applied during sample preparation are identical, the concentration of native cortisol is calculated as follows: Concentration of cortisol = area of cortisol * (concentration of [$^2H_3$]cortisol/area of [$^2H_3$]cortisol). For the serum sample with the shown LC-MS/MS chromatogram, a cortisol concentration of 150 µg/L is calculated (concentration of [$^2H_3$]cortisol in the internal standard solution, 196 µg/L; 98% isotopic purity).

a semi-quantitative approach and in a very high analyte concentration range, using flow injection instead of HPLC separation [5]. A similar approach is also described for the quantification of inorganic elements [6]. Here, we demonstrate the applicability of this approach also for the quantification of small molecule analytes in a low concentration range and applying chromatographic separation.

We demonstrated for one exemplary analyte that DIDA is feasible for clinical chemistry application of LC-MS/MS. It must be noted, however, that the reliability of DIDA is determined by the accuracy of the declared concentration of the internal standard compound. The content and entire isotopic purity of the internal standard preparation employed is crucial and should be specified on the level of a reference material if DIDA is used in clinical diagnostic – similar to the specification of calibrator samples in standard tests of laboratory medicine. Similar to the conventional application of stable isotope labelled compounds for internal standardisation, compounds should be labelled in at least three positions of the molecule in order to avoid spectral overlap with naturally occurring molecules. Furthermore, labelling has to be chemically stable under storage and mass spectrometric conditions with no Deuterium-Hydrogen exchange.

	Target concentration, µg/L	Mean concentration, µg/L		Accuracy, %		Imprecision, CV%	
		CC	DIDA	CC	DIDA	CC	DIDA
QC 1	49.4	50.4	50.3	102	102	3.9	3.6
QC 2	148	145	149	98.0	101	5.7	2.8
QC 3	296	284	293	95.9	99.0	4.7	1.8
Pool 1		28.3	27.3			4.1	2.6
Pool 2		283	292			5.0	2.9
Pool 3		37.9	37.3			4.6	4.5
Pool 4		202	207			6.8	3.1

Table 1 Quantification of serum cortisol concentrations by isotope dilution LC-MS/MS using conventional six-point calibration (conventional calibration, CC) and using direct isotope dilution analysis (DIDA): Results of a validation study including 20-fold determination of quality control (QC) samples and human serum pool samples analysed in four independent series over a period of 14 days.

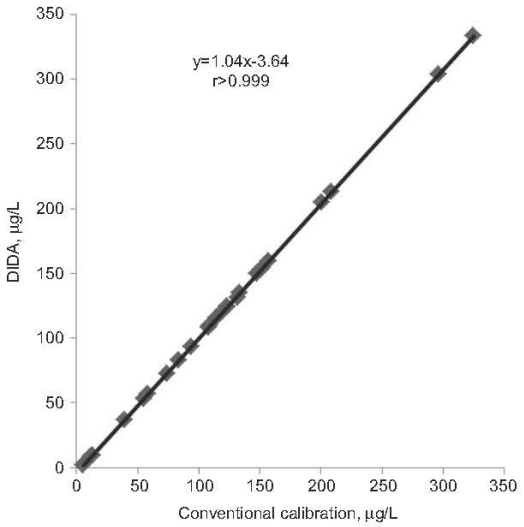

Figure 2 Comparison of serum cortisol results in 25 clinical samples obtained applying conventional calibration and DIDA, respectively.

DIDA – by principle – only can lead to reliable results if the ionisation behaviour of target analyte and stable isotope-labelled internal standard compound is very similar within the relevant concentration range (corresponding to linearity in the conventional calibration approach). We studied serum cortisol measurement for a proof of concept of DIDA-LC-MS/MS, considering the relevance of reliable quantification of this marker for intensive care medicine [1]. When considering applying this principle for other analytes in different sample matrices, of course a careful analyte-individual validation of this approach of quantification is required, in order to exclude differential isotope effects and non-linearity in the ionisation behaviour or chromatographic characteristics of stable isotope labelled molecules compared to the respective unlabelled counterparts [7, 8]. In this context, for DIDA methods the limits of minimum and maximum isotope ratios have to be established, thereby describing the measuring range. In our present study on cortisol, reliable and consistent results were observed for isotope ratios (native cortisol/labelled cortisol) from 0.14 to 1.5.

The widespread application of LC-MS/MS in clinical laboratories offers substantial potential advantages over standard technologies, such as photometry and immunoassays: flexible and straightforward method development for innovative small molecule analytes; highest analytical specificity; the potential to record metabolites patterns; no interferences from heterophilic antibodies; low running costs for analytical consumables instead of antibody-based reagents; and reliable standardisation and traceability realising assay-independent reference ranges. Thus, LC-MS/MS can address many of the unmet needs of clinical diagnostics and enables substantial innovation in clinical chemistry [9]. Given the workflow of today's typical clinical laboratories, comprehensive realisation of these potentials requires the development of fully automated random-access MS/MS-based analysers offering identical practicability as standard clinical chemistry analysers do [10]. We believe that the implementation of such an instrument concept can be facilitated by the DIDA principle. Our results also suggest the short-term implementation of convenient DIDA functionalities to standard chromatography software. Furthermore, the availability of stable isotope labelled materials specified on the level of reference materials is desirable.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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Quantification of linezolid in serum by LC-MS/MS using semi-automated sample preparation and isotope dilution internal standardization

Abstract

Background: Linezolid serum concentrations have been shown to be highly variable in critically ill patients with often sub-therapeutic drug levels regarding minimal inhibitory concentrations for relevant pathogens. Consequently, therapeutic drug monitoring of linezolid must be considered, requiring a reliable and convenient analytical method. We therefore developed and validated an LC-MS/MS method applying isotope dilution internal standardization and on-line solid phase extraction for serum linezolid quantification.

Methods: Sample preparation was based on protein precipitation and on-line solid phase extraction with two-dimensional liquid chromatography and column switching. Three-fold deuterated linezolid was used as the internal standard. The method was validated involving two separate LC-MS/MS systems covering the concentration range of 0.13–32 mg/L. The run time was 4 min.

Results: Validation revealed good analytical performance, with inaccuracy <6% and imprecision of <7.3% (CV) for six quality control samples (0.38–16.0 mg/L). The method was found to be robust during the validation process and during a pharmacokinetic study so far involving 600 samples. Comparative measurements on two LC-MS/MS systems revealed close agreement.

Conclusions: This LC-MS/MS assay described herein is a convenient, robust and reliable method for linezolid quantification in serum which can be routinely applied using different LC-MS/MS systems. The method can be used for clinical studies and subsequent TDM of linezolid.

Keywords: linezolid; liquid chromatography tandem mass spectrometry (LC-MS/MS); mass spectrometry; on-line solid phase extraction; serum; therapeutic drug monitoring.

Conversion factor of linezolid: [mg/L]×2.96 → [μmol/L]

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Introduction

Severe infections such as sepsis or septic shock remain a major challenge in medicine. The prevalence of sepsis and severe sepsis in ICUs ranges from 20% to 80% with mortality rates of 20%–50% [1–5]. Key elements for the treatment of severe infections include infectious source control, adequate antimicrobial therapy with early treatment, and sufficiently high drug concentration levels [6–9]. In the last few years, linezolid became an important antibiotic for the treatment of infections in critically ill patients [10–12]. It will probably play an even more important role as soon as generic formulations become available after patent expiration of the drug in the near future. The drug has a good in vitro and in vivo activity against Gram-positive organisms including the vast majority of methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococci* (VRE) [13–15]. However, about 10%–30% of linezolid therapies in critically ill patients remain ineffective despite demonstration of sensitive Gram-positive pathogens [16–18]. Moreover, there are reports of various adverse effects such as elevated liver enzymes, gastrointestinal disturbances, or hematological toxicity in about 15%–30% of cases, requiring in some cases discontinuation of the drug [16, 18–20]. The rate of therapy failures and adverse effects might be explained by a high variability of linezolid serum concentrations in critically ill patients. Such high variability has been observed in single-center studies [21–23]. Moreover, particularly low and high linezolid concentrations in critically ill patients have been shown to correlate with therapeutic failure and adverse effects, respectively [17, 20]. These facts are taken into account by different scientific researchers suggesting a therapeutic drug monitoring of linezolid for critically ill patients [20, 23]. Moreover,

several expert panels recommend therapeutic drug monitoring for antimicrobial agents for critically ill patients in general [24, 25]. However, tests for linezolid quantification are not commercially available to date. Several methods using high performance liquid chromatography with UV detection (HPLC-UV) have been published [26–28]. The main disadvantages of these methods are long run-times and rather unspecific UV-detection which may be prone to interferences, especially in critically ill patients with often extended co-medication. Liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for quantification of linezolid have been described [29–31]. The aim of our project was to improve the robustness and reliability of linezolid monitoring using LC-MS/MS by introducing automated on-line solid phase extraction (SPE) and isotope dilution internal standardization.

Materials and methods

Chemicals and reagents

Linezolid and d_3-linezolid pure substances were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Toronto Research Chemicals (Toronto, Ontario, Canada), respectively (Supplemental Data, Figure 1, which accompanies the article at http://www.degruyter.com/view/j/cclm.2014.52.issue-3/issue-files/cclm.2014.52.issue-3.xml). Methanol, acetonitrile and water (each HPLC-grade) were obtained from J.T. Baker (Jackson, TN, USA), and formic acid and zinc sulphate heptahydrate from Merck (Merck, Darmstadt, Germany). All chemicals and solvents were of the highest purity available from commercial sources and used without any further purification.

Instruments

Two different LC-MS/MS systems were used for method development and validation: LC-MS/MS system 1: A Waters Alliance 2795 HPLC-pump (Waters, Milford, USA) together with an additional HPLC-pump (Waters 515, Waters) and a switching valve were coupled splitless with a Quattro Micro tandem mass spectrometry (Waters). LC-MS/MS system 2: A Waters Alliance 2695 HPLC-pump (Waters) together with an additional HPLC pump (CLC300, Chromsystems, Munich, Germany) and a switching valve were coupled splitless with a Micromass Quattro LC tandem mass spectrometry (Waters).

Patient samples for method validation

We used leftover routine patient samples for method validation after anonymization, as approved by the Institutional Review Board. Serum samples from patients on linezolid medication were used to prepare a serum pool for validation procedures and to compare linezolid results obtained from the two different LC-MS/MS systems. Serum samples from patients without linezolid medication were used for evaluation of the specificity of the method and, after spiking with linezolid, for preparation of internal quality control (QC)-samples.

Calibrators and quality control samples

Seven calibrators with linezolid concentrations of 0, 0.13, 0.5, 2.0, 4.0, 8.0, and 32.0 mg/L, and three QC-samples with 0.5, 4.0, and 16.0 mg/L linezolid (QC A-C, all samples on serum basis) were manufactured on individual request by Recipe (Recipe Chemicals & Instruments, Munich, Germany). For preparation of additional internal QC-samples, a linezolid stock solution consisting of 76.2 mg/L linezolid dissolved in methanol/water 30/70 (v/v) was prepared. Drug-free human serum was spiked with this stock solution to yield QC-samples with linezolid concentrations of 0.38 and 6.0 mg/L (Control A and B). Finally, a serum pool from patients on linezolid was used as an additional internal QC-sample. All calibrators and QC-samples were stored at –20°C after aliquoting.

Sample preparation and liquid chromatography

A semi-automated sample preparation protocol with a manual protein precipitation step and automated on-line SPE was used. In 2.0 mL polypropylene cups (Eppendorf, Hamburg, Germany), 50 µL of the serum sample was precipitated with 450 µL of the precipitation solution consisting of an aqueous zinc sulphate heptahydrate solution (50 g/L) and methanol 1/4 (v/v), and d_3-linezolid as the internal standard (0.4 mg/L). After shaking for 5 min at room temperature, specimens were centrifuged at 15 000 *g* for 10 min at 15°C. The clear supernatant was transferred into HPLC vials and placed into an HPLC-autosampler. The automated on-line SPE protocol included three steps: At time zero in position A (Supplemental Data Figure 2), 10 µL of the supernatant was injected and loaded on the on-line SPE column (Waters Oasis HLB column, 2.1×20 mm; 25 µm particle size, Waters) delivered in mobile phase A consisting of water/methanol/formic acid 89.9/10/0.1 (v/v/v) at a flow rate of 4 mL/min. In parallel, the analytical column (Kinetex XB-C18, 100×2.1 mm, 2.6 µm particle size, Phenomenex, Torrance, USA) was equilibrated with water/acetonitrile/formic acid 59.9/40/0.1 (v/v/v) (mobile phase B) at a flow rate of 0.25 mL/min. After 0.7 min, the switching valve was changed to position B and the extraction column was now eluted with mobile phase B in a back-flush mode onto the analytical column. After further 1.3 min, the switching valve was changed back to position A. Whereas the mobile phase for the analytical column did not change, the extraction column was now washed for 1 min with methanol/acetonitrile 80/20 (v/v) (mobile phase C) and then re-equilibrated for 1 min with mobile phase A at a flow rate of 4 mL/min. In total, the run-time was 4 min. The analytical column was kept at 40°C.

Mass spectrometry conditions

Electrospray ionization in the positive mode was used for both instruments. Supplemental Data Table 1 shows the settings applied for both instruments resulting in an optimal ion yield. Several productions were

generated. A collision-induced product ion scan of linezolid is shown in Figure 1. Quantifiers and qualifiers for linezolid and d_3-linezolid were recorded with multiple-reaction monitoring (MRM) transitions, cone voltages, and cone collisions as shown in Table 1. The ratios of quantifier and qualifier were assessed. The dwell time was 0.075 ms for both analyte and internal standard. For quantification, the Waters QuanLynx software module was used with the following settings: Smoothing method, Mean; smoothing width, 2; smoothing iterations, 2; polynome type, linear; origin, excluded; weighting function, 1/x; axis transformation, none. Mass signal width at 50% height was 0.6 Da.

Method validation

The method was validated based on a protocol involving both LC-MS/MS systems. All validation tests with the exception of stability tests were performed on both LC-MS/MS systems. A signal to noise ratio of 10:1 of the lowest calibrator was defined as a system suitability test in each run.

For evaluation of linearity, accuracy, and precision of the analytical method, QC-samples were used. Each sample was analyzed five times in five independent series.

To evaluate the process efficiency, methanol and drug free serum were both spiked with linezolid to a concentration of 6.0 mg/L, aliquoted to five samples each, precipitated and measured at the different LC-MS/MS systems. The process efficiency was calculated according to Matuszewski [32].

For verification of the specificity, we used 10 serum samples of critically ill ICU patients treated with a broad range of different drugs not including linezolid. The internal standard was omitted in this experiment. MRM chromatograms of linezolid were inspected for peaks in the MRM traces of linezolid and d_3-linezolid. Pureness of the internal standard was tested by analyzing drug-free serum only spiked with the internal standard thereby inspecting the MRM traces of linezolid for a peak signal.

To test for carry-over, the highest calibrator (32 mg/L of linezolid) was measured in five injections then followed by analyzis of drug free serum. The MRM traces of the drug free serum were inspected for peaks.

To verify the measuring range of the method, it was tested if the lowest calibrator fulfilled criteria of a lower limit of quantification (LLOQ) (inaccuracy <±20%, imprecision <20% CV, and signal to noise ratio >10:1).

For comparison of both instruments, linezolid serum concentrations in samples of 20 critically ill patients treated with linezolid were determined on both instruments (identical extracts of calibrators and patient samples).

Stability of linezolid as detected by the method was evaluated. Linezolid serum concentrations of three QC-samples (QC A, QC C, and serum pool) were determined after storage at room temperature (RT) for 12 and 24 h and after storage at 4°C, –20°C and –80°C for 24 h, 7 d, 30 d and 60 d. The results were compared with the results found in the initial analytical run. Moreover, the effect of freeze thaw cycles was evaluated. Linezolid concentrations after storage at –20°C for 24 h were compared with linezolid concentrations that were thawed three times to RT in the same time. Linezolid stability in sample extracts was determined (autosampler stability). Extracts of the six QC-samples (QC A-C, control A, B and serum pool) were stored at 4°C and at RT for both 1 day and 1 week. The results found for these samples were compared with the results found in the initial analytical run. All stability tests were performed in triplicate.

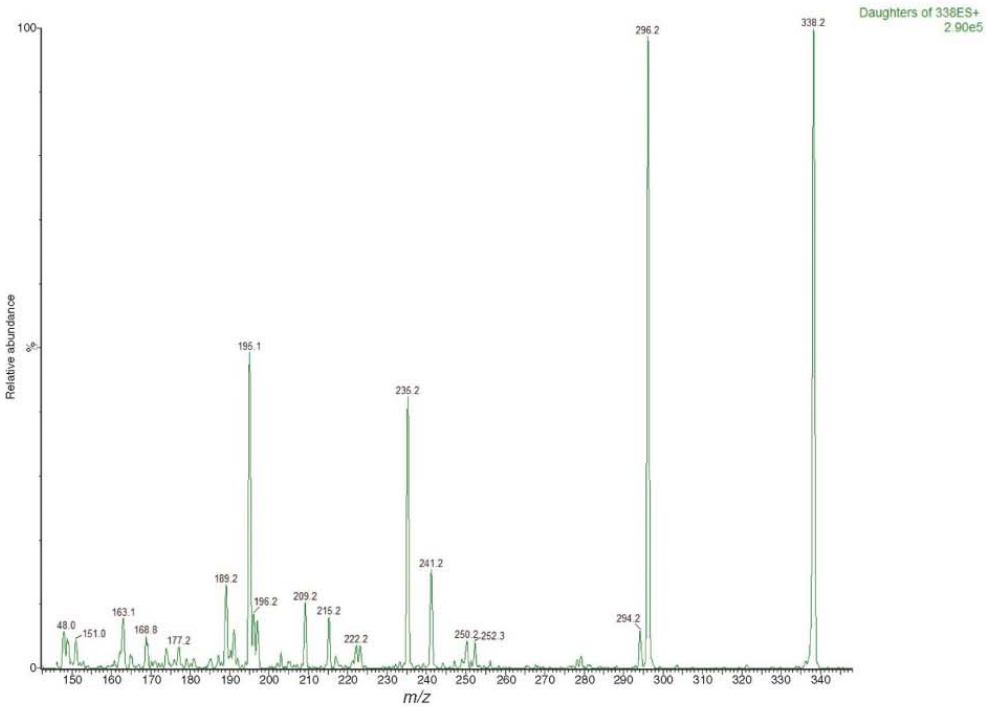

Figure 1 Product ion scan of linezolid.

Table 1 Precursor and product ions and MS-parameters.

Analyte	Precursor ion, *m/z*	Quantifier			Qualifier		
		Product ion, *m/z*	Cone voltage	Collision energy	Product ion, *m/z*	Cone voltage	Collision energy
Quattro Micro							
Linezolid	338.3	296.2	30	18	235.2	30	20
D_3-linezolid	341.3	297.2	30	18	235.2	30	20
Micromass Quattro LC							
Linezolid	338.2	296.3	35	17	235.2	35	21
D_3-linezolid	341.3	297.3	35	18	235.2	35	18

Linezolid quantification in clinical samples

To test the applicability of the method to clinical samples, the method was used to determine linezolid concentrations in samples of a critically ill patient receiving linezolid as clinically indicated. This study was carried out according to the principles of the Declaration of Helsinki. In a patient enrolled in a clinical study approved by the Institutional Review Board, linezolid concentrations between the second and the fourth linezolid infusion were recorded. A total of 600 mg of linezolid was infused over 30 min at the following time points: −0.5 h to time point zero, and 11.5–12 h. Blood samples were collected at the time points 0.5, 1, 3, 6.25, 8.45 and 11.5 h after the end of each linezolid infusion. Each blood sample was immediately centrifuged and serum was aliquoted and stored at −80°C until analyzis.

Statistics

In addition to determination of the intra-assay coefficient of variation (CV), the inter-assay CV and the accuracy, we determined the total error of the method by calculation of the normalized root mean square error (%RMSE) represented by the formula

$$\%RMSE = \frac{\sqrt{\frac{\sum_{i=1}^{n}(X_i - X_0)^2}{n}}}{X_0}$$

where X_0 is the target value of a QC-sample and X_i the measured value [33–35].

Results

A representative chromatogram of a serum sample of a patient treated with linezolid is shown in Figure 2. Linezolid and d_3-linezolid coeluted at a retention time of about 2.5 min. The method was found to be linear over the calibrator concentration range. In all analytical series the regression coefficient r^2 was >0.999 for calibrators. The slope calibration lines were 0.17%±6.8% and 0.14%±4.9% for systems 1 and 2, respectively. The mean ion ratio of quantifier and qualifier from all analyzes during the validation process was 2.5 (CV 4.9%, range 2.0–2.9) and 1.9 (CV 3.9%, range 1.5–2.2) for systems 1 and 2, respectively. The method was found to be robust on both instruments during the validation process and during measurements of 600 serum samples of critically ill patients treated with linezolid with no malfunction and constant column back-pressure.

Data on analytical accuracy and reproducibility are shown in Table 2. For each of the five QC-samples the inaccuracy was ≤6% on both systems. The imprecision assessed involving both systems was <7.3% CV (inter-assay) and <5.2% CV (intra-assay) for the six QC-samples.

Determination of the efficiency of sample preparation according to Matuszewski [32] (n=5) revealed efficiency of 109% and 103% for systems 1 and 2, respectively.

The analyzis of the 10 serum samples of critically ill ICU patients verified the specificity of the method; no peaks were observed in the MRM traces of linezolid.

Only negligible carry-over was observed. The peak areas found in blank sample compared to the highest calibrator sample repeatedly injected directly before were 0.05% (range 0.03%–0.07%) and 0.04% (range 0.00%–0.07%) for instruments 1 and 2, respectively.

The lowest calibrator showed an inaccuracy of 9.4% and 8.8%, an imprecision of 7.6% CV and 4.9% CV, and a signal to noise ratio range of 114–358:1 and 114–170:1 for instruments 1 and 2, respectively (n=5). In accordance with the criteria described in Material and methods, the lowest calibrator was accepted as the LLOQ.

Close agreement of results for 20 clinical samples was observed by using both LC-MS/MS systems. Figure 3 shows the results from both instruments with a mean value of 6.2 mg/L (range 1.2–13.0 mg/L) and 6.0 mg/L (range 1.2–12.3 mg/L) on systems 1 and 2, respectively (n=5). R^2 was >0.99. Linear regression demonstrated the following equation: $y_{system\ 1} = 0.96x_{system\ 2} + 0.05$ mg/L.

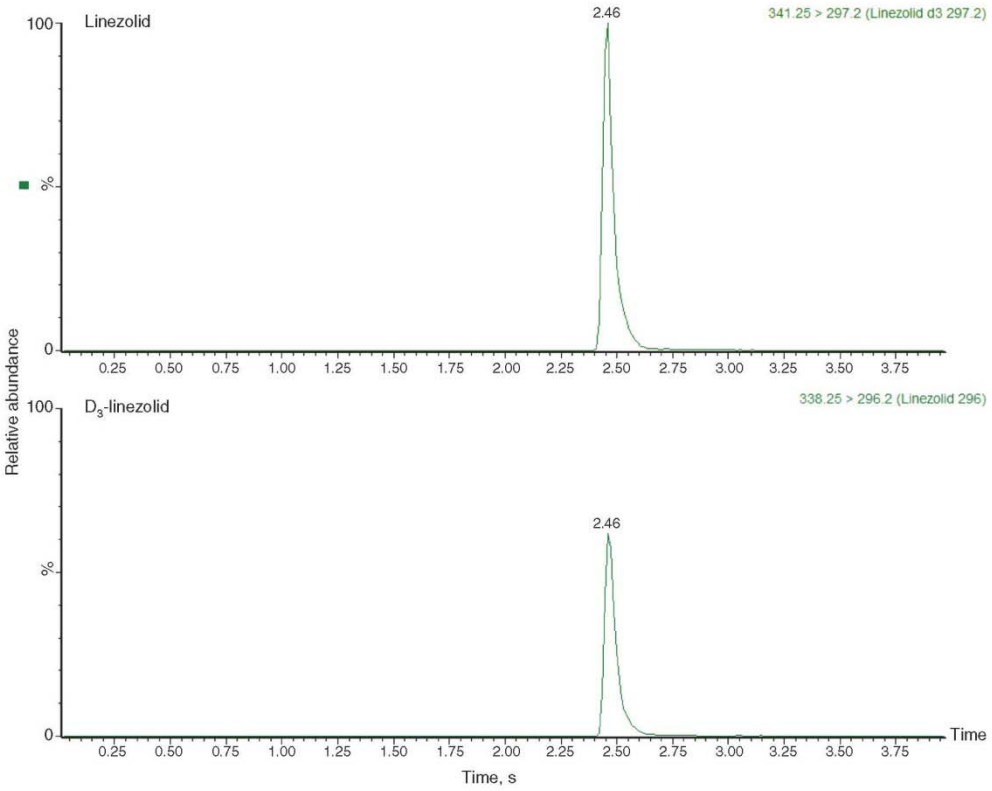

Figure 2 Representative MS/MS chromatogram of linezolid (patient serum sample containing 4.13 mg/L linezolid, with d_3-linezolid as internal standard).

Table 2 Analytical inaccuracy and imprecision of quality control samples.

Sample target concentration	QC A, 0.5 mg/L	QC B, 4.0 mg/L	QC C, 16.0 mg/L	Control A, 0.38 mg/L	Control B, 6.0 mg/L	Serum pool, ~8.6 mg/L
Quattro Micro						
Observed mean concentration, mg/L	0.50	4.12	16.20	0.40	6.30	8.71
Accuracy, % (n=5)	99	103	101	106	105	
CV Intra-assay, % (n=5)	3.72	2.62	5.23	2.49	1.65	2.42
%RMSE, %[a]	4.79	3.13	4.84	2.33	3.08	
CV Inter-assay, % (n=5)	7.34	2.48	2.97	2.64	2.70	5.05
%RMSE, %	6.56	3.81	2.96	6.33	5.61	
Micromass Quattro LC						
Observed mean concentration, mg/L	0.49	4.06	16.16	0.38	6.04	8.41
Accuracy, % (n=5)	98	101	101	102	101	
CV Intra-assay, % (n=5)	2.71	3.22	3.86	1.89	0.96	1.37
%RMSE, %	2.42	3.92	3.48	4.17	4.39	
CV Inter-assay, % (n=5)	4.28	2.01	4.23	2.26	3.57	1.95
%RMSE, %	4.25	2.28	3.96	2.82	3.26	
Both instruments						
Accuracy, % (n=10)	99	102	101	104	103	
CV Inter-assay, % (n=10)	5.72	2.30	3.45	3.05	3.73	4.10
%RMSE, %	5.53	3.14	3.49	4.90	4.59	

[a]%RMSE, normalized root mean square deviation as indicated in Materials and methods.

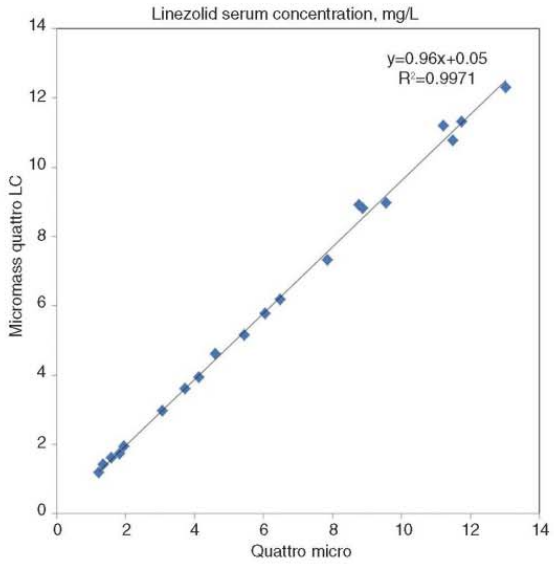

Figure 3 Comparison of patients' serum linezolid results obtained from two different LC-MS/MS systems (n=20); analyzes of identical analytical series including calibrators.

Linezolid was found to be stable in serum samples and in serum extracts according to the evaluation protocol (Table 3, Supplemental Data Tables 2 and 3).

A representative time-concentration course observed in sera of a patient under i.v. linezolid is shown in Figure 4. Trough levels were 2.7, 3.3, and 3.4 mg/L at time points −0.5, +11.5, and +23.5 h, respectively.

Discussion

We hereby describe an isotope dilution LC-MS/MS method for the quantification of the antibacterial drug linezolid which is now widely used for the treatment of life-threatening infections. According to our validation study, the method offers the level of analytical performance which is required for use as a routine method in therapeutic drug monitoring of linezolid. The observed reproducibility (coefficients of variation below 7.3%), accuracy (bias below 6%), and total error (<6.6%) fulfilled the commonly accepted limits.

While previously described LC-MS/MS methods for the quantification of linezolid rely on sample preparation protocols consisting of mere protein precipitation [29–31] or protein precipitation combined with time-consuming manual extraction protocols [18, 36, 37], we implemented an automated solid phase extraction step in the chromatographic work-up of samples, realizing two-dimensional chromatography (LC/LC). This additional pre-fraction aims to reduce the content of matrix constituents (e.g., amino acids, salts) of the samples applied to the analytical column and finally the mass spectrometer ion source. According to our long-term experiences with large-scale therapeutic drug monitoring (TDM) of immunosuppressants applying this approach, on-line SPE indeed minimizes the contamination of LC-MS/MS instruments resulting in a very substantial reduction in maintenance requirements compared to mere protein depletion as the sample preparation. This substantial increase in robustness is in a trade-off with somewhat higher demand of

Table 3 Stability of three serum-based quality control samples (QC A, C, and serum pool).

Sample	Storage time	Recovery, %[a] ± standard deviation at a storage temperature of			
		RT	4°C	−20°C	−80°C
QC A (0.5 mg/L)	12 h	101.6±4.2			
	24 h	102.7±2.0	99.2±2.7	103.2±3.2	99.9±3.4
	7 d		109.3±6.6	101.8±3.4	108.5±5.7
	30 d		106.5±6.2	103.7±3.0	101.3±2.2
	60 d		104.7±3.1	102.1±5.7	109.8±2.7
QC C (16.0 mg/L)	12 h	100.6±4.1			
	24 h	101.1±1.8	99.7±3.0	98.6±1.7	96.9±3.6
	7 d		98.8±4.9	105.7±1.3	103.7±5.8
	30 d		99.1±3.3	102.6±4.6	100.5±3.9
	60 d		97.5±4.4	100.7±5.4	100.7±2.8
Pooled serum (~8.6 mg/L)	12 h	100.9±2.3			
	24 h	101.4±3.3	98.8±1.0	104.3±1.3	97.6±2.3
	7 d		103.1±1.4	99.5±3.4	102.9±4.3
	30 d		102.4±1.8	105.4±4.6	105±4.0
	60 d		102.6±0.7	98.6±2.1	105.1±2.7

[a]Each value represents the recovery based on means of three different measurements.

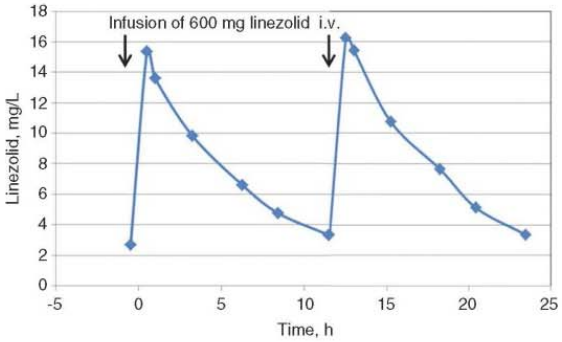

Figure 4 Serum concentration-time profile of linezolid of a critically ill patient after repeated infusion of 600 mg linezolid.

solvents, hardware (additional HPLC-pump, switching valve, extraction column), and run time (approx. 1 min per sample). However, when comparing automated on-line SPE with SPE using separate extraction columns or even with solvent extraction as manual extraction protocols, a very substantial reduction of manual workload is realized in this automated protocol. Besides optimizing the robustness and convenience of LC-MS/MS methods, on-line SPE also reduces the impact of matrix effects on ionization, and consequently maximizes the reliability of methods.

Whereas recent studies used structurally unrelated [29, 30, 36–38] or structurally related compounds [39] of linezolid as internal standards, we applied for the first time the principle of isotope dilution internal standardization for this analyte. For this purpose, we used threefold deuterated linezolid which is commercially available. Since target analyte and internal standard are very similar in their physico-chemical properties, variances of individual samples impacting the ionization are compensated almost completely. Consequently, isotope dilution mass spectrometry realizes the highest attainable level of reliability [40].

Regarding the features of on-line solid phase extraction combined with isotope dilution internal standardization, the data indicate that the method described herein is superior to previously reported methods for the quantification of linezolid. Also, this methodology provides convenience and reliability in the therapeutic drug monitoring of linezolid in the ICU setting. We applied an innovative validation protocol which was based on results obtained from two different LC-MS/MS instruments. This protocol is also feasible in a routine clinical laboratory setting. Here, typically it is required to implement assays on several instruments in order to maintain analytical services also on a back-up instrument during down-times of the standard instrument. Furthermore, from our extended experiences with TDM of immunosuppressants it is necessary to have a systematic comparison of results obtained from different instruments in the evaluation of clinical LC-MS/MS methods. It should be noted that the description of LC-MS/MS is complex since different instruments are highly heterogeneous even in one individual laboratory. While main features such as mode of ionization, selection of precursor and product ions, dwell time or composition of mobile phases can be standardized, parameters such as actual mass resolution or individually optimized settings for source voltages are specific for individual instruments. Indeed validation protocols for clinical LC-MS/MS application should address the instrument-transfer or up-scale potential of the described method. This feature is not included in the FDA protocol for analyzis in pharmacological research [41]. For the method reported herein a very close agreement between results from two instruments was observed.

A previous study revealed that chance for success of linezolid treatment in seriously ill patients with Gram-positive infections appeared to be higher when linezolid concentrations remained above the minimal inhibitory concentration (MIC) for the causative pathogen within the entire dosing interval [17]. Adverse effects may rise when trough levels exceed 10 mg/L, however, data supporting this threshold are very rare [23]. Based on these studies and with respect to MIC distributions of relevant pathogens (mostly being below 2–4 mg/L [42–44]), a preliminary serum trough target concentration range of 2–4 mg/L to 10 mg/L can be assumed for linezolid [23, 44]. This range is covered by the validated working range of our method. In an ongoing investigation we currently correlate serum concentration profiles of linezolid of patients with life threatening infections with outcome. These data will contribute to the evaluation of a potential benefit of linezolid TDM and to the development of sampling strategies and target concentration ranges in this study. Several hundreds of samples have been analyzed for linezolid with the method described here. The method was found to be highly robust and to maintain analytical performance also in a quasi-routine setting.

In conclusion, our data support the use of the isotope dilution, and on-line SPE LC-MS/MS method for the quantification of linezolid in patients' sera for both clinical research and routine TDM of linezolid.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analyzis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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Variability of linezolid concentrations after standard dosing in critically ill patients: a prospective observational study

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Abstract

Introduction: Severe infections in intensive care patients show high morbidity and mortality rates. Linezolid is an antimicrobial drug frequently used in critically ill patients. Recent data indicates that there might be high variability of linezolid serum concentrations in intensive care patients receiving standard doses. This study was aimed to evaluate whether standard dosing of linezolid leads to therapeutic serum concentrations in critically ill patients.

Methods: In this prospective observational study, 30 critically ill adult patients with suspected infections received standard dosing of 600 mg linezolid intravenously twice a day. Over 4 days, multiple serum samples were obtained from each patient, in order to determine the linezolid concentrations by liquid chromatography tandem mass spectrometry.

Results: A high variability of serum linezolid concentrations was observed (range of area under the linezolid concentration time curve over 24 hours (AUC_{24}) 50.1 to 453.9 mg/L, median 143.3 mg*h/L; range of trough concentrations (C_{min}) < 0.13 to 14.49 mg/L, median 2.06 mg/L). Furthermore, potentially subtherapeutic linezolid concentrations over 24 hours and at single time points (defined according to the literature as AUC_{24} < 200 mg*h/L and C_{min} < 2 mg/L) were observed for 63% and 50% of the patients, respectively. Finally, potentially toxic levels (defined as AUC_{24} > 400 mg*h/L and C_{min} > 10 mg/L) were observed for 7 of the patients.

Conclusions: A high variability of linezolid serum concentrations with a substantial percentage of potentially subtherapeutic levels was observed in intensive care patients. The findings suggest that therapeutic drug monitoring of linezolid might be helpful for adequate dosing of linezolid in critically ill patients.

Trial registration: Clinicaltrials.gov NCT01793012. Registered 24 January 2013.

Introduction

Severe infections in ICU patients remain a major challenge in modern medicine. The prevalence of severe infections such as sepsis or septic shock in ICU patients ranges from 20 to 80% with high mortality rates of 20 to 50% [1-5]. Consequently, there is a substantial need for optimizing antimicrobial therapy. Key elements for the treatment of infections include an adequate antimicrobial therapy with an early initiation and with sufficiently high drug concentration levels [6-10]. Furthermore, sufficiently high drug concentrations are required to limit the development of antimicrobial resistance [11].

About 50% of bloodstream infections in critically ill patients are caused by Gram-positive bacteria [12,13]. A major part of these Gram-positive infections are represented by multidrug-resistant strains (for example, methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE)), which are particularly frequent in ICUs [12-17]. Linezolid has good *in vitro* and *in vivo* activity against these organisms and is

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an important antibiotic for the treatment of infections in critically ill patients [9,16,18-22]. The volume of distribution in adults of this hydrophilic antibiotic approximates to the total body water content of 40 to 60 L [23]. The plasma elimination half-life is mostly reported to be between 3.1 and 4.9 h with a clearance rate between 6.4 and 14.8 L/h [23]. Linezolid is metabolized by liver enzymes to two major inactive metabolites, an aminoethoxyacetic acid and a hydroxyethyl glycine, which are excreted predominantly - together with the parent substance - in urine [24,25]. Because of its intrinsic chemico-physical and pharmacokinetic characteristics, it is assumed that adequate serum linezolid concentrations will be achieved most of the time when using the recommended dose of 600 mg every 12 hours and that therapeutic drug monitoring (TDM) might not be necessary [26,27]. This assumption is based on reports showing adequate linezolid concentrations in healthy volunteers or non-critically ill patients [27-29]. According to the manufacturer, no dose adjustment of linezolid is necessary in the case of renal or liver impairment. Consequently, expert panels recommend standardized doses of 600 mg linezolid twice a day also for patients with severe infections such as sepsis or septic shock [30]. However, 10 to 30% of critically ill patients receiving linezolid have treatment failure despite isolation of Gram-positive organisms sensitive to linezolid [16,31,32]. Of these, 15 to 30% of patients suffer furthermore from adverse effects such as elevated liver enzymes, gastrointestinal disturbances or hematological toxicity [32-34]. The rate of therapy failure and adverse effects may be in part explained by a high variability of linezolid serum concentrations in critically ill patients.

Though there are have been few studies evaluating blood levels and pharmacokinetic parameters of linezolid in critically ill patients [26,35-41], it is still very difficult for physicians to decide if therapeutic levels are reached after standard dosing with linezolid when treating critically ill patients. The studies to date have found variable results with regard to linezolid blood levels. A substantial number demonstrated that inadequate levels occur [36,37,39,41] whereas others concluded that standard doses are mostly sufficient [35,38]. Low numbers of study patients, the lack of use of compartment models, and the retrospective design of most studies leave inconclusive information about this topic within the existing literature. Moreover, there are only preliminary data for linezolid blood levels in specific subgroups of ICU patients, such as critically ill patients on continuous renal replacement therapy (CRRT), those on extracorporeal lung assist (ECLA) and patients who have undergone organ transplantation [41,42]. Indeed, most of these studies excluded particular patient groups, therefore, do not represent the full spectrum of different patients in ICUs. We therefore designed a prospective observational study to analyze the variability of linezolid serum concentrations in relation to preliminary target concentration ranges in a heterogeneous group of critically ill patients with suspected infections. The primary aim of the study was to evaluate whether linezolid serum concentrations in different critically ill patients were within the defined therapeutic range.

Materials and methods

Patients

The study population originated from medical-surgical critically ill patients hospitalized in two ICUs within the Department of Anesthesiology, University Hospital of Munich between March and November 2013. Patients were eligible for inclusion if they had a severe infection (confirmed or suspected by clinical assessment) and were treated with linezolid intravenously by short-duration infusions according to the clinic guidelines, and in accordance with the German Paul-Ehrlich-Society and the guidelines of the Infectious Disease Society of America [30,43]. Patients were only excluded if they were under the age of 18 years, if their planned hospitalization was less than 4 days, or if the first linezolid administration was given more than 48 h before study enrollment. Written informed consent was obtained from all patients or their legal representatives.

Study design

The monocentric, prospective observational study was performed at the University Hospital of Munich. The study protocol (ClinicalTrials.gov, NCT01793012) was approved by the Institutional Review Board of the Medical Faculty of the Ludwig-Maximilians-University (registration number 428-12) and carried out according to the principles of the Declaration of Helsinki. Enrolled patients (n = 30) received 600 mg linezolid twice a day by short-duration infusion (15 to 60 minutes). Day 1 of the study was defined as that day on which the first linezolid trough level (C_{min}) was determined (see Additional file 1). This was directly before the second or third linezolid administration in all patients except patients 2 and 27, for whom the study start was directly before the fifth and fourth linezolid administration, respectively. Serum samples from the arterial line for antibiotic determination were collected at multiple time points before (C_{min}), during, and after the two linezolid administrations on day 1; and before, during, and after one of the two linezolid administrations on days 2, 3 and 4 (in total 26 to 43 samples per patient). The exact time of blood sampling was recorded by the medical staff. Samples were immediately sent to the Institute of Laboratory Medicine, University Hospital of Munich, centrifuged (3,000 g, 10 minutes) and aliquoted into 2-ml polypropylene tubes (Eppendorf, Hamburg, Germany). Serum aliquots were stored within one hour after blood sampling at −80°C.

Determination of clinical and laboratory parameters

Clinical patient data and diagnosis in the ICU were recorded. Sepsis was defined according to the Society of Critical Care Medicine/European Society of Intensive Care Medicine (SCCM/ESICM) Consensus Conference Committee [10]. The severity of the patient's clinical condition was characterized using the acute physiology and chronic health evaluation (APACHE) II score. To assess renal function, creatinine concentrations in both serum and 24-h urine samples were determined using an enzymatic photometric test on an automated chemistry analyzer (Model AU5822: Beckmann Coulter, Brea, CA, USA). Creatinine clearance (CL_{crea}) was calculated using the formula:

$$CL_{crea} = (C_{urine} * V_{urine}) : (C_{serum} * time),$$

where C_{urine} is the creatinine concentration in urine, V_{urine} is the urine volume, and C_{serum} is the serum creatinine concentration.

Determination of linezolid concentrations

Serum linezolid concentrations were determined using a previously described liquid chromatography tandem mass spectrometry (LC-MS/MS) method [44]. Briefly, sample preparation was based on protein precipitation and on-line solid phase extraction with two-dimensional liquid chromatography and column switching. Three-fold deuterated linezolid was used as the internal standard. Control samples were used from both a commercial provider and from in-house production. Validation revealed good analytical performance showing inaccuracy <6% and imprecision <7.3% (coefficient of variation) for all quality control samples. The lower limit of quantification was 0.13 mg/L. The method was found to be robust over the course of the study.

Pharmacokinetic analysis

We analyzed linezolid plasma concentrations with a compartmental pharmacokinetic model based on nonlinear mixed-effects modeling. For model estimation we used the NONMEM 7.2® program (Icon Development Solutions, Hanover, MD, USA) with the FOCE-I estimation algorithm. The aim of the pharmacokinetic analysis was to determine individual concentration time courses. We assumed that the population parameters were log-normally distributed. The individual post-hoc concentration predictions obtained from NONMEM were used to predict the time course of linezolid plasma concentrations and to calculate the area under the concentration time curve over 24 h (AUC_{24})-values. Model selection was based on the NONMEM objective function, goodness-of-fit plots, and median absolute performance errors as described by Varvel *et al.* [45]. For graphical analysis we used PLTTools 5.0 PLTsoft, San Francisco, CA USA [46]. Linezolid plasma concentrations were calculated for each patient based on individual pharmacokinetic parameters in 10-minute steps.

Assessment of target concentration ranges

The thresholds for potential therapeutic efficacy were defined as C_{min} >2 mg/L and/or AUC_{24} > 200 mg*h/L. The rationale behind these two thresholds was the findings of Rayner *et al.*, showing a higher therapeutic success in seriously ill patients when linezolid exceeds the minimum inhibitory concentration (MIC) over the entire dosing interval or when AUC_{24}/MIC-values are higher than 80 to 120 [31]. We defined the MIC as the concentration that inhibits the growth of 90% of important relevant infectious pathogens (MIC_{90}) (particularly *S. aureus* and Enterococcus species) [47,48] and therefore, we set the threshold of potential therapeutic efficacy of C_{min} at 2 mg/L. As in Rayner *et al.*, we set the AUC_{24}/MIC_{90} value at 100, corresponding to a threshold for potential therapeutic efficacy of AUC_{24}-values of 200 mg*h/L. The threshold for potential therapeutic toxicity was defined as trough levels >10 mg/L or AUC_{24} values >400 mg*h/L according to the literature [23,26,34].

Statistics

The AUC_{24} was calculated by means of the trapezoidal rule using concentration values as predicted by the pharmacokinetic model (individual post-hoc concentration time course). Patients were divided into three groups in relation to the defined target concentration ranges based on their C_{min}- and AUC_{24}-values. Non-continuous parameters were expressed as percentages and numbers, and compared by means of the Chi-square test. Continuous parameters were expressed as median values and ranges, and compared by the Jonckheere-Terpstra test. A *P*-value below 0.05 was considered statistically significant. All calculations were performed using SAS (version 9.3, SAS Institute Inc., Cary, NC, USA).

Results

Twenty male and ten female patients with a median age of 57 years (range, 28 to 84 years) and a median body mass index (BMI) of 25.5 kg/m^2 (range, 16 to 35 kg/m^2) were included. The most frequent causes of sepsis were pneumonia and peritonitis (Table 1). Ten patients were lung-transplant, and five were liver-transplant recipients. Patients had high variability in APACHE II scores (range 9 to 37, median 27.5). Of the 25 patients who were not on CRRT, 5 had a reduced creatinine clearance of <50 ml/minute. Five patients were being treated with CRRT and seven patients were treated with ECLA. Detailed parameters of the corresponding CRRT and ECLA systems are shown in Additional files 2 and 3.

Linezolid plasma concentrations were best described by a two-compartment model with an individual (post-hoc)

Table 1 Characteristics of the study population

Patient number	APACHE II score	Mean creatinine clearance (ml/minute)[a]	CRRT (+/–)	ECLA (+/–)	Organ transplantation[b]	Clinical condition
1	20	89	-	+	Lung	Septic pneumonia
2	12	129	-	-	-	ARDS
3	20	21	-	-	-	Septic peritonitis
4	28	114	-	-	-	Septic pleural empyema
5	23	97	-	+	-	ARDS
6	33	72	-	+	-	Septic pneumonia
7	9	117	-	-	Lung	Septic pneumonia
8	28	119	-	-	Lung	Septic pneumonia
9	28	-	+	-	Liver	Septic peritonitis
10	29	94	-	-	-	Septic pneumonia
11	33	35	-	-	Lung	Septic pneumonia
12	31	102	-	+	-	Septic pneumonia
13	14	127	-	-	-	Septic pneumonia
14	14	69	-	-	Lung	Septic pneumonia
15	32	42	-	-	-	Septic endocarditis
16	19	76	-	+	-	Septic pneumonia
17	35	-	+	-	Liver	Septic peritonitis
18	21	33	-	-	Lung	Septic pneumonia
19	27	55	-	-	Lung	Septic pneumonia
20	17	64	-	-	Lung	Septic pleural empyema
21	30	68	-	-	-	Septic pneumonia
22	23	-	+	-	Liver	Septic peritonitis
23	14	85	-	-	Liver	Septic peritonitis
24	24	-	+	-	Liver	Septic peritonitis
25	37	74	-	-	Lung	Septic pneumonia
26	25	83	-	+	Lung	Septic pneumonia
27	28	-	+	-	-	Septic peritonitis
28	34	163	-	+	-	Septic pneumonia
29	32	126	-	-	-	ARDS
30	29	37	-	-	-	Septic peritonitis
Median	27.5	83				
(range)	(9 to 37)	(21 to 163)				

[a]Mean value of the 4 study days; [b]within the last 28 days. APACHE II, acute physiology and chronic health evaluation II score; CRRT, continuous renal replacement therapy; ECLA, extracorporeal lung assist; ARDS, acute respiratory distress syndrome.

median prediction error of 1% and a median absolute prediction error of 13%. The parameter estimates (standard error) of the population model were: volume of distribution of the first compartment = 19.3 (3.9) L, volume of distribution of the second compartment = 26.4 (3.8) L, elimination clearance = 8.3 (0.9) L/h and inter-compartmental clearance = 56.0 (19.3) L/h.

Figure 1 shows the concentration time curves of serum linezolid for each patient. A high inter-patient variability was observed. The high inter-patient variability could be quantified when AUC_{24}-values ranged from 50.1 to 453.9 mg*h/L (median 143.3 mg*h/L) (Table 2). The high inter-patient variability was also observed for single C_{min}-values (range >100-fold, from <0.13 to 14.49 mg/L, median 2.06 mg/L) (Table 2). To obtain further information about the usefulness of c_{min}-values for TDM, C_{min} values were correlated with corresponding AUC_{24} values giving an r^2 value of 0.79 (Additional file 4).

In addition to the inter-patient variability, high intra-patient variability of C_{min} values was also observed. Nine of thirty patients had maximum C_{min} values, more than 5-fold higher than the minimum C_{min} values (range of

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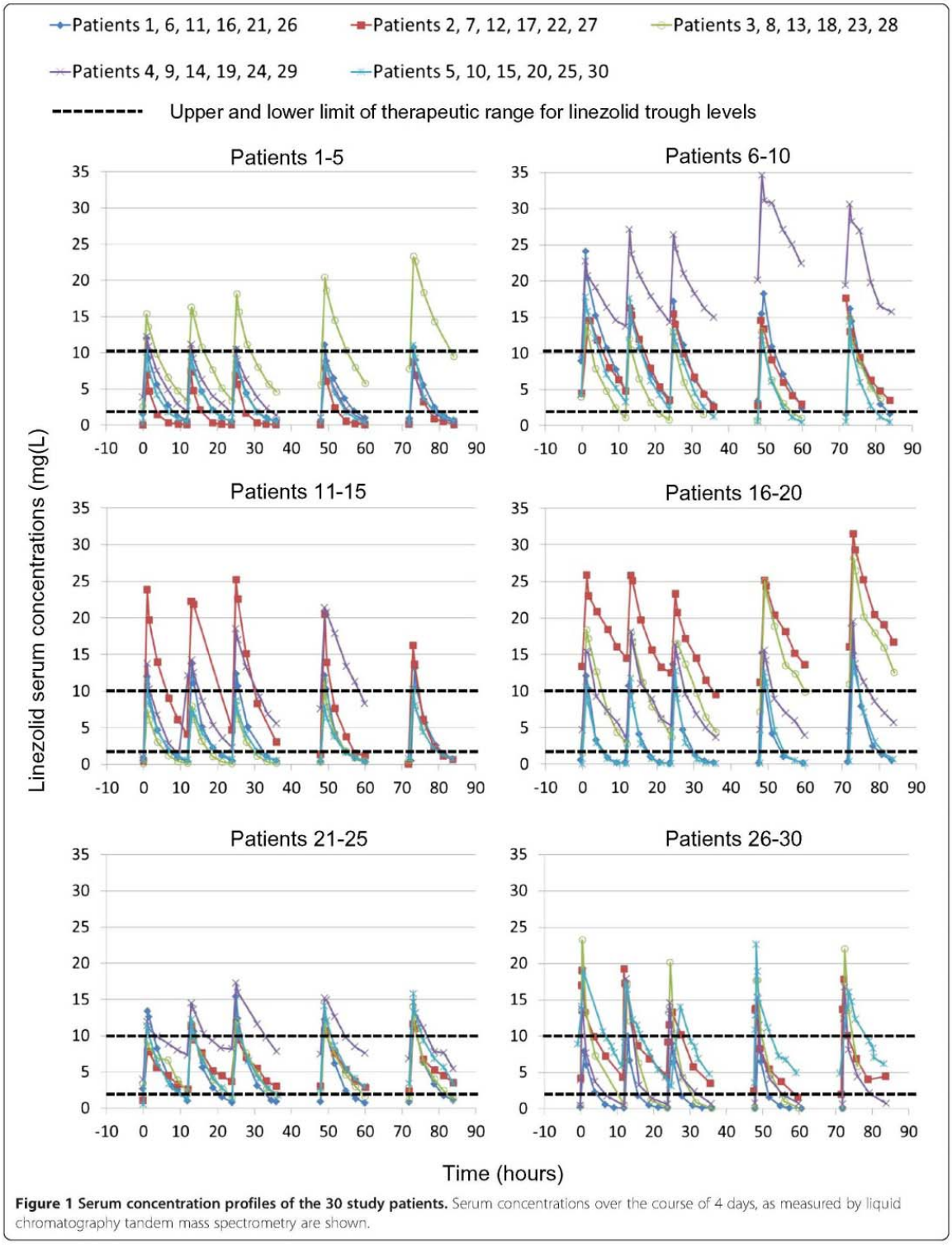

Figure 1 Serum concentration profiles of the 30 study patients. Serum concentrations over the course of 4 days, as measured by liquid chromatography tandem mass spectrometry are shown.

Table 2 Linezolid pharmacokinetic parameters following intravenous administration of 600 mg twice daily in critically ill ICU patients

Patient number	AUC_{24} (mg*h/L)[a]	C_{min} (mg/L)[b]
1	99.1	0.67
2	50.1	<0.13[c]
3	258.2	3.35
4	118.3	1.89
5	82.3	0.46
6	213.7	3.15
7	206.3	3.59
8	138.9	1.17
9	453.9	13.75
10	165.3	3.26
11	105.3	0.43
12	224.7	4.13
13	67.6	0.14
14	266.9	2.31
15	80.8	0.70
16	101.7	0.18
17	442.0	14.49
18	325.6	2.75
19	217.8	3.48
20	94.8	<0.13
21	122.0	1.03
22	144.6	3.70
23	141.8	2.22
24	250.8	8.18
25	157.1	1.45
26	50.8	<0.13
27	165.1	4.22
28	106.2	0.17
29	86.5	0.45
30	244.1	5.86
Median	143.3	2.06
Range	50.1 to 453.9	<0.13 to 14.49

[a]As determined by the NONMEM system, values from the start of the third administration of linezolid over 24 h; [b]as analyzed by liquid chromatography tandem mass spectrometry, values obtained directly before the fourth administration of linezolid if not indicated otherwise;
[c]value obtained directly before the fifth administration of linezolid. AUC_{24}, concentration time curve over 24 h; C_{min}, linezolid trough level.

maximum/minimum C_{min} values 1 to 36) (Figure 1; see also Additional file 5). The C_{min} of most patients did not change in a consistent pattern over the 4 days of the study; only in patients 3, 14, 18 and 25 did we observe an increase, and in patient 4 a decrease of C_{min} values over time (Figure 1).

Optimal pharmacodynamic exposure over 24 h with AUC_{24} values between 200 and 400 mg*h/L and at a single time point with C_{min} values between 2 and 10 mg/L was observed in 30% and 43% of the patients, respectively (Table 3, Figure 1). Regarding these AUC_{24} and C_{min} values, 63% and 50% of the study patients had linezolid concentrations below the lower limit of the corresponding target concentration range, respectively, and 7% had linezolid concentrations above the target concentration range. Moreover, only 17% of the patients continuously attained optimal C_{min} values between 2 and 10 mg/L over 4 days (see Additional file 5).

Patients on CRRT had significantly higher C_{min} values and AUC_{24} values than patients without CRRT ($P = 0.005$ for AUC_{24} and $P = 0.001$ for C_{min}) (Table 3). Similarly, patients who had undergone liver transplantation had significantly higher AUC_{24} and C_{min} values than in non- (liver and lung) transplant patients ($P = 0.036$ for AUC_{24} and $P = 0.012$ for C_{min}). Other characteristics such as gender, lung transplantation and therapy with ECLA did not have any significant influence on AUC_{24} or C_{min} values. Variability of linezolid levels was high in patient groups on CRRT and ECLA, and in the liver and lung transplantation groups, with each group showing a substantial proportion (≥40%) outside the target concentration range. The distributions of continuous parameters in relation to linezolid target ranges are shown in Table 4. A trend towards higher linezolid serum levels was observed in patients with reduced creatinine clearance, although these changes were not significant ($P = 0.102$ for C_{min} and $P = 0.051$ for AUC_{24}) (Table 4, Additional file 6). Other continuous parameters such as age, BMI, and APACHE-II score did not have any significant influence on AUC_{24} or C_{min} values.

Discussion

This study shows that the recommended standard dosing of linezolid leads to subtherapeutic linezolid plasma concentrations in about every second critically ill patient. Furthermore, a high variability of linezolid levels was observed in the study population with a majority (57 to 70%) detected outside the target ranges. Although there were insufficient levels in the majority of patients, inappropriate high levels occurred in a small number of patients. Finally, this variability of linezolid levels was not only observed between the different patients, but also within individual patients over the course of 4 days.

Our findings are in line with other studies also observing very low AUC_{24} or C_{min} values with the majority being insufficient [37,39,41]. In contrast to some other studies [37,39], we used two different approaches to define the lower threshold of the target range. First, we used AUC_{24}/MIC in concordance with other studies [26,35,36,38,40,41], which was shown to be the best

Table 3 Distribution of patients in relation to the target range of linezolid

Patient groups, number of patients	Number (percentage) of linezolid patients					
	AUC_{24}, mg*h/L[a]			C_{min}, mg/L[b]		
	<200	200 to 400	>400	<2	2 to 10	>10
Total patients, n =30	19 (63)	9 (30)	2 (7)	15 (50)	13 (43)	2 (7)
Male patients, n = 20[c]	13 (65)	5 (25)	2 (10)	11 (55)	7 (35)	2 (10)
Female patients, n = 10	6 (60)	4 (40)		4 (40)	6 (60)	
On CRRT, n = 5[d]	2 (40)	1 (20)	2 (40)		3 (60)	2 (40)
Not on CRRT, n = 25	17 (68)	8 (32)		15 (60)	10 (40)	
On ECLA, n = 7[c]	5 (71)	2 (29)		5 (71)	2 (29)	
Not on ECLA, n = 23	14 (61)	7 (30)	2 (9)	10 (43)	11 (48)	2 (9)
After liver transplantation, n = 5[f]	2 (40)	1 (20)	2 (40)		3 (60)	2 (40)
After lung transplantation, n = 10[c]	6 (60)	4 (40)		6 (60)	4 (40)	
No transplantation, n = 15	11 (73)	4 (27)		9 (60)	6 (40)	

[a]As determined by the NONMEM system, values from the start of the third administration of linezolid; [b]as determined by liquid chromatography tandem mass spectrometry, values obtained directly before the fourth administration of linezolid; [c]no significantly different values in comparison to the corresponding patient group; [d]significantly higher values in patients on CRRT than in those not on CRRT ($P < 0.01$); [f]significantly higher values in patients with liver transplantation than in non-liver-transplant patients ($P < 0.05$). AUC_{24}, concentration time curve over 24 h; C_{min}, linezolid trough level; CRRT, continuous renal replacement therapy; ECLA, extracorporeal lung assist.

parameter to predict efficacy [31]. When we used this approach 63% of our study patients had insufficient linezolid serum levels. Second, we used C_{min} values as a further lower threshold of the target concentration range, as time above the MIC over the entire dosing interval also correlates with efficacy [31]. This value was set at 2 mg/L in accordance with MIC_{90} values of relevant causative pathogens [47,48] as had been done in other studies [26,37,39]. Different efficacy thresholds might be used in environments where MIC_{90} values of relevant pathogens differ from 2 mg/L. Indeed, linezolid serum concentrations during infection should

Table 4 Distributions of continuous parameters in relation to linezolid target ranges

Patient groups	Median and range of patients					
	AUC_{24} (mg*h/L)[a]			C_{min} (mg/L)[b]		
	<200	200 to 400	>400	<2	2 to 10	>10
Mean creatinine clearance (mL/minute)[c]						
median	89	62		89	70	
range	35 to 163	21 to 117		35 to 163	21 to 117	
(number of patients)	(17)	(8)		(15)	(10)	
APACHE II score						
median	28	24	32	28	24	32
range	12 to 37	9 to 33	28 to 35	12 to 37	9 to 33	28 to 35
(number of patients)	(19)	(9)	(2)	(15)	(13)	(2)
Age (years)						
median	53	59	56	57	57	56
range	28 to 77	34 to 84	50 to 61	28 to 77	29 to 84	50 to 61
(number of patients)	(19)	(9)	(2)	(15)	(13)	(2)
Body mass index (kg/m^2)						
median	24	19	21	22	23	21
range	13 to 32	17 to 26	19 to 23	13 to 32	17 to 28	19 to 23
(number of patients)	(19)	(9)	(2)	(15)	(13)	(2)

[a]As determined by the NONMEM system, values from the start of the third administration of linezolid; [b]as determined by liquid chromatography tandem mass spectrometry, values obtained directly before the fourth administration of linezolid; [c]mean value of the 4 study days. AUC_{24}, concentration time curve over 24 h; C_{min}, linezolid trough level; APACHE II, acute physiology and chronic health evaluation II.

reach sufficient levels for most causative pathogens to ensure efficacy. This might be particularly important, as the identity of most causative pathogens is unknown in the early course of severe infection.

Furthermore, we showed that there is a high variability of linezolid AUC_{24} and C_{min} values with C_{min} values differing more than 100-fold between the different study patients and more than 30-fold within individual patients. This is in line with some recent studies also describing high variability of C_{min} values differing more than 50-fold between different patients [26,34,41]. In fact, the majority of linezolid concentrations in our study was outside the defined linezolid target concentration ranges, supporting the concept of TDM. We set the upper threshold of the target concentration range for C_{min} values at 10 mg/L. This was done in accordance with other studies, because higher concentrations have been shown to be associated with drug-related toxicity [23,26,34], whereas Pea *at al.* showed that in long-term treatment with linezolid an upper threshold of the target concentration range of 7 mg/L should be favored [49]. Indeed, in the study of Pea *et al.*, all patients with C_{min} values >10 mg/L of linezolid had substantial platelet reduction (>30%) during long-term linezolid treatment, whereas no patient had these adverse effects with C_{min} values <4 mg/L. Despite the rather high upper threshold used in our study, 7% of the patients had linezolid concentrations above the target range. The fact that for critically ill patients, the two parameters AUC_{24}/MIC >80 to 120 and time above MIC over the entire dosing interval strongly correlated with treatment efficacy [31], and that elevated linezolid concentrations correlated with adverse effects [23,34], show that both AUC_{24} and C_{min} values correlate with efficacy and toxicity. This strongly supports the concept of linezolid target concentration ranges in terms of TDM. The good linear relationship between C_{min} and AUC_{24} values described by Pea *et al.* [26] was confirmed in our study ($r^2 = 0.79$). C_{min} might therefore be a useful parameter for TDM of linezolid in clinical practice. As a high variation of C_{min} values within individual patients was observed in this study, we would recommend repetitive determinations of linezolid C_{min} values during infection treatment.

Finally, the linezolid serum concentrations in different critically ill patients, such as those on CRRT or ECLA, and patients who had undergone liver or lung transplantation, were evaluated. Only a few studies have evaluated the pharmacokinetics of linezolid in critically ill patients on CRRT [50-53], thereby using different CRRT systems such as continuous venovenous hemodiafiltration (CVVHDF) and continuous venovenous hemofiltration (CVVH). Linezolid concentrations were partly subtherapeutic and partly within the potential therapeutic range, however, a comparison of linezolid levels in patients not on CRRT was not performed. In our study, linezolid concentrations were tested in patients with CVVHDF and CVVHD. In comparison to the other study patients, significantly higher linezolid levels were observed in patients on CRRT. However, it should be noted that four of the five patients on CRRT were liver transplant recipients and that higher levels of linezolid have been reported in patients after liver transplantation [41]. About 50% of the parent substance linezolid is metabolized by liver enzymes to two major inactive metabolites and are excreted predominantly - together with linezolid - in urine [25]. Higher levels of linezolid in patients after liver transplantations might therefore be due to alterations in the activity of liver enzymes after ischemia/reperfusion [41]. Higher numbers of patients on CRRT and those after liver transplantations with simultaneous evaluation of the liver function, as well as of linezolid and its inactive metabolites in urine will be necessary to definitely understand the impact of CRRT and liver transplantations on linezolid concentrations in critically ill patients. Furthermore, we evaluated linezolid levels in patients after lung transplantation and patients on ECLA, and no significant differences (in the setting used in our study) in comparison to the whole study population were observed. Admittedly, the majority of the linezolid levels were also insufficient in these patients. This is in line with the reported low linezolid levels in three critically ill patients on extracorporeal membrane oxygenation-systems (specific ECLA system) and three critically ill patients after lung transplantation [42,50], which are the only available data on these patients.

The results of this study suggest that the limited availability of linezolid quantification methods in clinical laboratories might pose a serious problem for the antimicrobial therapy of ICU patients. The reason for the limited availability of such methods is in particular the lack of cost-efficient commercially available linezolid quantification tests. Only a few laboratories use custom-made linezolid quantification methods such as high performance liquid chromatography with UV-detection (HPLC-UV) or LC-MS/MS. Development and routine use of these custom-made methods require a high level of human resources, professional specialization and high-technology equipment, which can often only be provided by large or specialized laboratories. The availability of commercial quantification tests (for example, based on HPLC-UV or immunoassay methods) of a therapeutic substance applied in life-threatening conditions might therefore be of particular impact.

The present study considered a number of aspects which have only partially been covered in previous work: (a) we investigated numerous critically ill patients from the whole intensive care spectrum, including patients who had undergone lung and liver transplantation and during CRRT and ECLA; (b) we analyzed a large number of

linezolid plasma samples. This allowed description of individual concentration time courses by pharmacokinetic modeling with a small median prediction error of 1% and a small median absolute prediction error of 13%; (c) finally, this study used a highly accurate method for linezolid quantification [44]. All other studies measuring linezolid concentrations in critically ill patients used HPLC-UV [26,35-41], which may be prone to interference, especially in critically ill patients with extended co-medication. In contrast, we used an LC-MS/MS method, thereby, for the first time, using isotope dilution internal standardization. As target analytes and internal standards are very similar in their physico-chemical properties, variances of individual samples impacting the ionization are compensated almost completely, realizing the highest attainable level of reliability [54]. Furthermore, the use of control samples from both a commercial provider and from in-house production additionally ensures the accuracy of the method. Indeed, there were only minimal undulations in the concentration-time curves of linezolid in individual patients (Figure 1), showing that the study-protocol, including blood sampling and analytical method, was accurately performed.

The high variability of linezolid levels found in our study, with a substantial proportion at insufficient low levels, might contribute to the observed high mortality rate and severity of infection in ICU patients. Furthermore, high variability of linezolid levels may also lead to the development of resistance and drug-related toxicity. As early and effective antimicrobial therapy has a substantial effect on bacterial eradication and patient survival [6,31], optimal individual dosing of antibiotics is of particular importance. Given the fact that the most common cause of death in the ICU in medically advanced nations is severe infection [55] and because of worldwide intentions to reduce morbidity and mortality from sepsis [10], we believe that there is great importance in optimizing individual antimicrobial dosing with the aid of TDM.

Conclusions

We found high variability in linezolid serum concentrations with mostly insufficient low levels in critically ill patients. We therefore suggest general TDM of linezolid in critically ill patients during linezolid therapy. However, future studies will have to investigate whether application of TDM can definitely improve linezolid-dosing protocols and infection-related patient outcome.

Key messages

- High variability of linezolid serum concentrations after standard linezolid dosing in 30 different critically ill patients with suspected infections were observed.
- We observed potentially subtherapeutic levels in the majority of different patients.
- Potentially toxic levels were observed in a minority of different patients.
- Our data suggests that therapeutic drug monitoring might be helpful for adequate dosing of linezolid in critically ill patients.

Additional files

Additional file 1: Figure showing study protocol of blood sampling for linezolid determination. [a]Two to three linezolid infusions before study start with the exception of patients 2 and 27, for whom the study start was directly before the fifth and fourth linezolid administration, respectively: [b]26 to 43 samples per patient.

Additional file 2: Table showing parameters of the continuous renal replacement therapy systems used for each patient in this study.

Additional file 3: Table showing parameters of the extracorporeal lung-assist systems used for each patient in this study.

Additional file 4: Figure showing correlation of linezolid trough level (C_{min}) values and concentration time curve over 24 h (AUC_{24}) values of linezolid. Shown are values for AUC_{24} as determined by the NONMEM system from the beginning of the third administration of linezolid, and C_{min} as determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) directly before the fourth administration of linezolid.

Additional file 5: Figure demonstrating the high variability (inter-patient and intrapatient) of linezolid trough levels (C_{min}) over the course of the study for each patient.

Additional file 6: Table showing concentration time curve over 24 h (AUC_{24}) and linezolid trough level (C_{min}) values of patients from the lowest and highest quartile of covariates.

Abbreviations

APACHE: acute physiology and chronic health evaluation; AUC_{24}: concentration time curve over 24 h; BMI: body mass index; C_{min}: linezolid trough level; CRRT: continuous renal replacement therapy; CVVH: continuous venovenous hemofiltration; CVVHD: Continuous venovenous hemodialysis; CVVHDF: continuous venovenous hemodiafiltration; ECLA: extracorporeal lung assist; HPLC-UV: high performance liquid chromatography with UV-detection; LC-MS/MS: liquid chromatography tandem mass spectrometry; MIC: minimal inhibitory concentration; MRSA: methicillin-resistant *Staphylococcus aureus*; SCCM/ESICM: Society of Critical Care Medicine/European Society of Intensive Care Medicine; TDM: therapeutic drug monitoring; VRE: vancomycin-resistant enterococci.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MZ and JZ designed the study and wrote the manuscript; BM measured antibiotic concentrations by LC-MS/MS; CN and GD were responsible for acquisition of data; DN and CH performed the statistical analyses; LMH, MB, TW, BG, LF, DT and MV made substantial contributions to the conception and design of the study including interpretation of results. All authors critically revised the manuscript for important intellectual content and approved the final manuscript. All authors meet key authorship requirements and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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2.4 Zander J, Maier B, Suhr A, Zoller M, Frey L, Teupser D, Vogeser M.

Quantification of piperacillin, tazobactam, cefepime, meropenem, ciprofloxacin and linezolid in serum using an isotope dilution UHPLC-MS/MS method with semi-automated sample preparation.

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Abstract

Background: Recent studies have demonstrated highly variable blood concentrations of piperacillin, tazobactam, cefepime, meropenem, ciprofloxacin and linezolid in critically ill patients with a high incidence of sub-therapeutic levels. Consequently, therapeutic drug monitoring (TDM) of these antibiotics has to be considered, requiring robust and reliable routine analytical methods. The aim of the present work was to develop and validate a multi-analyte ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) method for the simultaneous quantification of the above mentioned antibiotics.

Methods: Sample preparation included a manual protein precipitation step followed by two-dimensional ultra high performance liquid chromatography (2D-UHPLC). Corresponding stable isotope-labeled substances were used as internal standards for all of the analytes, with the exception of tazobactam. The injected sample volume was 7 µL. The run time was 5.0 min.

Results: Inaccuracy was ≤8% and imprecision coefficient of variation (CV) was <9% for all analytes. Only minor matrix effects and negligible carry-over was observed. The method was found to be robust during the validation period.

Conclusions: We were able to develop a reliable 2D-UHPLC-MS/MS method addressing analytes with highly heterogeneous physico-chemical properties. The novel assay may be an efficient tool for an optimized process workflow in clinical laboratories for important antibiotics in regards to TDM.

Keywords: antibiotics; 2D-UHPLC; serum concentration; therapeutic drug monitoring; ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).

Introduction

Severe bacterial infections are a leading cause of morbidity and mortality in intensive care unit (ICU) patients, with mortality rates ranging from 20% to 50% [1–3]. A key element for successful treatment of critically ill patients with life-threatening infections is an adequate antimicrobial therapy, including an early administration of effective antibiotic substances in appropriate doses [4–7]. However, therapeutic drug monitoring (TDM) for the verification of antibiotic serum concentrations is well established for only aminoglycosides and glycopeptides [8]. Few studies have addressed the blood concentrations and pharmacokinetics of other antibiotics, including β-lactam antibiotics, quinolones and oxazolidinones, in critically ill patients with severe infections [9–13]. Those studies found a high variability of key pharmacokinetic parameters in critically ill patients, including insufficient therapeutic levels with the danger of insufficient therapeutic success. The few studies that correlated the serum concentrations of such antibiotics with outcome data confirmed that insufficient levels led to decreased clinical or bacteriologic cures [10, 12, 14–17]. Nevertheless, some antibiotics of these classes

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are important in ICUs because of their broad antimicrobial spectrum, bactericidal activity or low resistance rates. This strongly suggests that the introduction of TDM for several antibiotics in intensive care medicine could potentially lower the risks of inappropriate dosing with potentially sub-optimal therapeutic effects. Individualization of dosing regimens based on TDM could finally lead to decreased mortality and decreased antibiotic resistance [8]. Actually several expert panels therefore recommend the use of TDM for different antibiotics in intensive care patients [8, 18].

However, there are currently no commercially available quantification tests for these antibiotics, resulting in a lack of broadly usable routine techniques for clinical laboratories. Several high performance liquid chromatography methods with UV-detection (HPLC-UV) for the quantification of β-lactam antibiotics, quinolones or oxazolidinones in human blood have been published. The main disadvantage of HPLC-UV include the long run-times and the rather unspecific UV-detection, which might be a problem regarding the various co-medications in critically ill patients. Different single- and multi-analyte liquid chromatography tandem mass spectrometry (LC-MS/MS) methods are described as well [e.g., 19–28]. However, only few studies have described methods for quantifying structurally unrelated antibiotics of these classes with a single analytical method [19, 22, 23, 25, 27]. The development of such methods may be more difficult for analytes of different antibiotic classes because they present a high level of heterogeneity in their physico-chemical properties; however, the use of multi-analyte methods covering relevant antibiotics of typical antimicrobial combination therapies would be time and cost efficient in the routine setting. Therefore, the aim of the present study was to establish a fast and reliable multi-analyte LC-MS/MS method for the simultaneous quantification of antibiotics of different antibiotic classes chosen as promising candidates for a potential TDM in the ICU-setting. We also wanted to implement a two-dimensional ultra-high performance liquid chromatography (2D-UHPLC) method to make high throughput possible.

Materials and methods

Chemicals and reagents

Piperacillin sodium salt, tazobactam sodium salt, meropenem trihydrate, ciprofloxacin and linezolid were provided by Sigma-Aldrich (St. Louis, MO, USA); cefepime dihydrochloride monohydrate and linezolid-D_3 were provided by Toronto Research Chemicals (St. Louis, MO, USA); and cefepime-$D_2{}^{13}C_1$-sulfate, ciprofloxacine-D_8, piperacillin-D_5 sodium salt, and meropenem-D_6 were provided by Alsachim. Water, methanol and acetonitrile (each HPLC-grade) were obtained from J.T. Baker (Jackson, TN, USA), and formic acid was purchased from Merck (Darmstadt, Germany). All chemicals were of the highest purity available from commercial providers.

Patient samples for the validation process

For method validation, leftover routine serum samples were used from anonymized patients as approved by the Institutional Review Board. Serum samples of patients without prior treatment with antibiotics were used to evaluate the specificity of the method and, after spiking with the different antibiotics, for the preparation of the different calibrators and quality control (QC) samples.

Calibrators and quality control (QC) samples

Standard stock solutions for piperacillin, tazobactam, cefepime, meropenem and linezolid were prepared in a methanol-water solution [30/70 (v/v)]. A standard stock solution for ciprofloxacin was prepared in methanol/water/0.1 M hydrochloric acid 30/69.995/0.005 (v/v/v). Eight calibrators and three QC-samples were prepared by spiking drug free serum with the antibiotics (exact concentrations: see Table 1). For QC-samples and calibrators, separate stock solutions were prepared. All calibrators and QC-samples were aliquoted and immediately stored at −80 °C after preparation.

Table 1 Concentrations (mg/L) of calibrators and controls for the different antibiotics.

Antibiotic	Cal[a] 0	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6	Cal 7	QC A	QC B	QC C	Pool
Piperacillin	0	0.50	1.0	2.5	5.0	10.0	30.0	60.0	1.50	16.0	45.2	~34.8
Tazobactam	0	0.25	0.50	1.0	2.5	5.0	10.0	20.0	0.75	6.0	15.1	~7.1
Cefepime	0	0.13	0.25	0.50	2.0	8.0	25.0	50.0	0.38	6.0	39.6	~16.6
Meropenem	0	0.25	0.50	1.0	4.0	10.0	25.0	50.0	0.75	8.0	40.0	~13.0
Ciprofloxacin	0	0.05	0.10	0.20	0.80	2.0	4.0	8.0	0.15	1.0	6.0	~1.1
Linezolid	0	0.10	0.20	0.50	2.0	4.0	10.0	32.0	0.30	5.6	20.1	~3.7

[a]Calibrator.

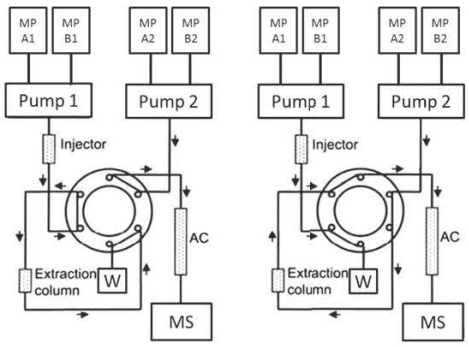
Sample preparation and UHPLC-MS/MS conditions

A semi-automated sample preparation with a manual protein precipitation step was used, followed by fractionation of the analytes by a 2D-UHPLC. Then, 50 µL of human serum and 50 µL of a methanol/water solution [10/90 (v/v)] containing the internal standards (2 mg/L of ciprofloxacin-D_8 and 4 mg/L of piperacillin-D_5, meropenem-D_6, cefepime-$D_3{}^{13}C_1$ and linezolid-D_3) were precipitated in 2 mL polypropylene cups (Eppendorf, Hamburg, Germany) with 100 µL of acetonitrile. After 10 min of shaking at room temperature (RT) and subsequent centrifugation at 15,000 *g* for 10 min at 15 °C, 10 µL of the supernatant was further diluted with 500 µL of a methanol/water solution 10/90 (v/v). A volume of 200 µL of that solution was transferred into a glass vial and placed into the autosampler for ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis. Analyses were performed on a 2D-Waters UHPLC system consisting of an autosampler, a column manager, a switching valve and two Waters Acquity UPLC pumps (pumps 1 and 2) coupled to a Xevo TQ-S instrument (Waters, Milford, MA, USA). The 2D-UHPLC protocol included three steps: on-line sample clean up, transferring the extracted sample to the analytical column including chromatographic separation and re-equilibration. At time zero in position A (Figure 1A), 7 µL of the sample was injected and loaded onto the on-line solid phase extraction column (Oasis HLB Direct Connect HP column, 2.1×30 mm, 20 µm, Waters) with mobile phase A1 [water/formic acid 99.9/0.1 (v/v)] delivered at a flow rate of 2.0 mL/min by pump 1. After 0.6 min, the switching valve entered position B and the extract was eluted by pump 2 in a backflush-mode onto the analytical column (Acquity UPLC BEH Phenyl 1.7 µm, 2.1×100 mm, Waters), which was kept at 50 °C. For chromatographic separation, gradient elution with mobile phase A2 [water/formic acid 99.9/0.1 (v/v)] and mobile phase B2 [methanol/acetonitrile 75/25 (v/v)] was performed according to Figure 1C. After 3.1 min, the switching valve changed back to position A and the extraction column was washed and re-equilibrated with mobile phase A1 and B1 [methanol/acetonitrile 80/20 (v/v)] as described in detail in Figure 1B. Instruments were controlled by the Mass Lynx V4.1 software. The total run time was 5.0 min.

Mass spectrometry conditions

Electrospray ionization in the positive mode was used for all analytes. The following settings were applied: source temperature, 150 °C; desolvation temperature, 500 °C; and desolvation gas flow, 1000 L/h. The capillary voltage was 1.0 kV. For identification of the best mass transitions, collision-induced product scans of the different antibiotics were performed (Supplemental Data, Figure 1) and parameters, such as cone voltage and collision energy, were optimized for each substance. The multiple reaction monitoring (MRM) transitions for antibiotics (quantifier and qualifier) and internal standards with the corresponding cone voltage, collision energy and dwell time are shown in Table 2. For quantification, the Waters

A



B

Solvents delivered by pump 1

Time, min	Solvent flow, mL/min	Rate of mobile phase A1, %	Rate of mobile phase B1, %
0-0.5	2.0	100	0
0.5-0.6	2.0-0.1	100	0
0.6-3.2	0.1	100	0
3.2-3.3	0.1-2.0	100-40	0-60
3.3-3.7	2.0	40	60
3.7-3.8	2.0	40-5	60-95
3.8-4.2	2.0	5	95
4.2-4.3	2.0	5-100	95-0
4.5-5.0	2.0	100	0

C

Solvents delivered by pump 2

Time, min	Solvent flow, mL/min	Rate of mobile phase A2, %	Rate of mobile phase B2, %
0-0.8	0.3	90	10
0.8-1.0	0.3	90-55	10-45
1.0-2.0	0.3	55	45
2.0-2.05	0.3	55-30	45-70
2.05-2.3	0.3	30	70
2.3-2.4	0.3	30-10	70-90
2.4-4.2	0.3	10	90
4.2-4.5	0.3	10-90	90-10
4.5-5.0	0.3	90	10

Figure 1 On-line solid phase extraction system with corresponding solvent flows.
(A) On-line solid phase extraction system at the two different positions; AC, analytical column; MP, mobile phase; MS, mass spectrometry; W, waste. (B) Solvents delivered by pump 1 with corresponding gradient elution. (C) Solvents delivered by pump 2 with corresponding gradient elution.

Table 2 Precursor and product ions and MS-parameters.

Analyte	Corresponding internal standard	Quantifier				Qualifier				Dwell time, s
		Precursorion, m/z	Production, m/z	CV[a], V	CE[b], eV	Precursorion, m/z	Production, m/z	CV[a], V	CE[b], eV	
Piperacillin	Piperacillin-D_5	518	160	25	10	518	143	25	26	0.037
Tazobactam	Meropenem-D_6	301	168	26	12	–	–	–	–	0.015
Cefepime	Cefepime-$D_3{}^{13}C_1$	241	227	25	10	–	–	–	–	0.026
Meropenem	Meropenem-D_6	384	141	30	16	384	114	30	24	0.012
Ciprofloxacin	Ciprofloxacin-D_8	332	231	25	36	332	245	25	22	0.020
Linezolid	Linezolid-D_3	338	296	25	21	338	195	25	21	0.037
Piperacillin-D_5	–	523	160	25	9	–	–	–	–	0.037
Cefepime-$D_3{}^{13}C_1$	–	243	227	25	10	–	–	–	–	0.026
Meropenem-D_6	–	390	147	30	16	–	–	–	–	0.012
Ciprofloxacin-D_8	–	340	235	25	36	–	–	–	–	0.020
Linezolid-D_3	–	341	297	25	21	–	–	–	–	0.037

[a]Cone voltage; [b]collision energy.

TargetLynx software module was used with the following settings: polynome type, linear; origin, excluded; weighting function, 1/x; axis transformation, none.

Method validation

The linearity of the method was tested by analyzing the calibrators in five independent series. Inaccuracy and imprecision (both intra- and inter-assay) were examined by replicate analyses (n=5) of the three different QC-samples (and additionally the serum pool for imprecision). Imprecision was expressed as the coefficient of variation (CV). Furthermore, we assessed the total error in the form of the relative root mean square error (%RMSE) described by the equation

$$\%RMSE = \frac{\sqrt{\frac{\sum_{i=1}^{n}(X_i - X_0)^2}{n}}}{X_0}$$

where X_0 is the target value of a QC-sample and X_i is the measured value [28–30]. The performance goals for these quality criteria were defined as values <15% for inaccuracy, imprecision and %RSME.

The mean ion ratios of quantifiers and qualifiers of the standards used to construct the calibration curve were assessed each day according to the recommendations of the Clinical and Laboratory Standards Institute [31]. The second MRM transition was accepted as a qualifier if the ion ratios of the analytical series were similar between the different series and if the ion ratios of all samples of the same series did not differ more than 20% from that of the mean ion ratio of the corresponding standards.

The specificity of the method was verified by analysis of 10 serum samples from different critically ill patients treated with a broad range of substances but not with the antibiotics measured by this UHPLC-MS/MS method. The internal standard was omitted in these experiments. The resulting chromatograms were examined for unspecific peaks that could possibly disturb the quantification of the antibiotics.

In addition to the calibrators used for calculation of the calibration curve calibrator 1 was measured five times to determine whether the lowest calibrator fulfils the criteria of a lower limit of quantification (LLOQ): inaccuracy <20%, imprecision <20% CV and a signal to noise ratio >10:1.

Matrix effect and process efficiency were examined and calculated according to the guideline on bioanalytical method validation of the European Medicines Agency and Matuszewski et al. [32, 33] at two different concentrations (low and high). However, it should be noted that the sample preparation consists of the manual protein precipitation and the online-SPE and that this approach only takes the manual step into account. Sample preparation was performed in three different ways (set 1–3) as described as follows. The final concentration of the antibiotics and the internal standards were identical in all sets. Every set consisted of five independent samples. For set 2 and set 3, hemolyzed, hyperlipidemic and icteric serum samples (each n=5) were additionally evaluated. In the neat solution (set 1), antibiotics and internal standards were spiked into a solution of water/methanol 90/10 (v/v), except for ciprofloxacin and its internal standard, which were spiked into a solution of water/methanol/formic acid 89.99/10/0.01 (v/v/v) to guarantee solubility. For set 2 (spiked eluate), 100 µL acetonitrile were added to 50 µL of antibiotic-free serum and 50 µL of a water/methanol solution 90/10 (v/v). After centrifugation, 10 µL of the supernatant were diluted with 500 µL working solution containing antibiotics and internal standard with the appropriate concentrations. For set 3 (spiked serum), serum was spiked with the antibiotics to the defined concentration and subsequently processed according to the extraction protocol. The mean value of the peak area for every antibiotic and internal standard was calculated for set 1 (A), set 2 (B) and set 3 (C). Matrix effect and process efficiency were calculated as follows:

$$\text{Matrix effect [\%]} = B/A \times 100$$
$$\text{Process efficiency [\%]} = C/A \times 100$$

The internal standard normalized matrix factor was calculated by dividing the matrix effect of the analyte by the matrix effect of the internal standard as described in the guideline on bioanalytical method validation of the European Medicines Agency [32]. The

internal standard normalized process efficiency factor was calculated similarly.

Carry-over was tested five times by injecting a blank after each injection of the highest calibrator. The chromatogram of the blank was inspected for peaks.

To test the stability of the different antibiotics, samples at three different concentrations (QC1, QC3 and serum pool) were stored for 2 h at RT, for 12 h at 4 °C and for 30 and 90 days at −80 °C. The stability was tested for each storage condition by measuring QC1, QC3 and the serum pool three times after the different storage conditions. The mean values were compared to those of the samples, which were quantified immediately after preparation.

Results

A representative UHPLC-MS/MS chromatogram of all antibiotics at the lowest calibrator is shown in Figure 2. Representative UHPLC-MS/MS chromatograms of three samples from patients treated with different combinations of antibiotics are shown in Supplemental Data, Figure 2. The retention times of piperacillin, tazobactam, cefepime, meropenem, ciprofloxacin and linezolid were 3.38 min, 2.41 min, 2.21 min, 2.22 min, 2.32 min, and 2.94 min, respectively.

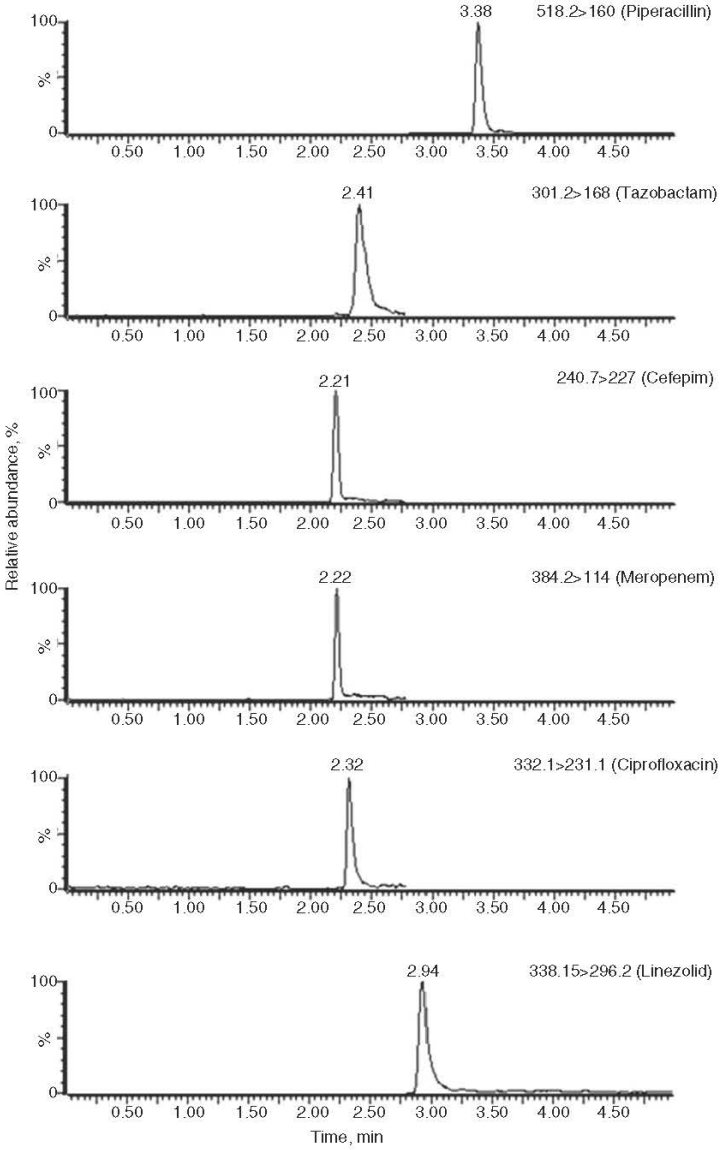


Figure 2 Representative UHPLC-MS/MS chromatogram of the different antibiotics at the lowest calibrator with the following concentrations: piperacillin 0.50 mg/L, tazobactam 0.25 mg/L, cefepime 0.13 mg/L, meropenem 0.25 mg/L, ciprofloxacin 0.05 mg/L and linezolid 0.10 mg/L.

The method was linear over the whole concentration range (r^2>0.99) for all antibiotics. The slopes of the calibration curves were 0.49±0.02 for piperacillin, 0.34±0.01 for tazobactam, 0.55±0.04 for cefepime, 0.61±0.02 for meropenem, 1.74±0.06 for ciprofloxacin, 0.25±0.01 for linezolid. No second MRM transition was found for tazobactam and cefepime, which could be used as a qualifier. Exemplarily, the mean ion ratios of quantifier to qualifier of the standards on the first validation day were 0.31±0.01 for piperacillin, 1.38±0.06 for meropenem, 1.19±0.05 for ciprofloxacin and 1.83±0.05 for linezolid. The mean ion ratios between the different days were similar (CV<15%). No ion ratio of a single run deviated by more than 20% of the mean ion ratios from the corresponding standards (data not shown).

Imprecision and inaccuracy of the method are shown in detail in Table 3. The imprecisions of the three QC-samples and the serum pool of all substances were <9% (intra- and inter-assay). For each of the three QC-samples, the inaccuracies and %RSME of all substances were ≤8% and <9%, respectively, meeting the quality criteria goals defined in Materials and methods.

The specificity of the method was verified by the analysis of 10 different serum samples from critically ill patients without the addition of internal standards. No peaks were observed in the MRM traces of the different antibiotics and internal standards.

Each of the lowest calibrators of the different antibiotics fulfilled the criteria of an LLOQ as described in Materials and methods: for all of the lowest calibrators, inaccuracy was between 0.2% and 11.8%, imprecision was between 5.0% and 9.4% CV and the signal to noise ratio was >100.

Evaluation of the matrix effect and the process efficiency for all antibiotics revealed that matrix effects were between 92% and 118% and that process efficiency was between 85% and 99% (Table 4). The internal standard normalized matrix factors and process efficiency factors were between 95% and 105% and between 92% and 105%, respectively. CVs of the normalized values were <9% for all analytes. Hemolytic, icteric or hyperlipidemic sera did not lead to results that were substantially different from the results of the normal serum (deviations of the normalized matrix effect and process efficiency ≤5% for piperacillin, ≤8% for tazobactam, ≤3% for cefepime, ≤8% for meropenem, ≤11% for ciprofloxacin and ≤2% for linezolid from values determined in normal serum).

Only negligible carry-over was observed for all antibiotics. The ratio between human drug-free serum and the highest calibrator was 0.13% for piperacillin, 0.17% for cefepime, 0.08% for meropenem, 0.18% for ciprofloxacin and 0.06% for linezolid. No carry-over was observed for tazobactam.

The method was found to be robust over the validation process with no malfunction of the system and constant column pressure.

Antibiotics were shown to be stable when stored for 2 h at RT, for 12 h at 4 °C and for 30 and 90 days at −80 °C. All samples were within 90%–110% of the comparative concentrations.

Discussion

We developed a fast and reliable 2D-UHPLC-MS/MS multimethod for the quantification of piperacillin, tazobactam, cefepime, meropenem, ciprofloxacin and linezolid, which are widely used for treatment of severe infections in critically ill patients. The method showed inaccuracy and CV of <10% for the different control samples, including all analytical substances, thereby meeting the quality standards that are widely accepted for the introduction of TDM.

While diverse previous LC-MS/MS methods have employed mere protein precipitation, manual solid phase extraction or protein precipitation combined with time-consuming manual extraction protocols [20, 22–24, 26, 27, 34, 35], we implemented a semi-automated sample preparation with a manual protein precipitation step and a subsequent 2D-UHPLC. A similar approach was employed by recent studies for ciprofloxacin, daptomycin, oxacillin, levofloxacin, rifampicin and other non-antibiotic substances [25]. The use of a 2D-UHPLC not only reduces the hands-on-time in sample preparation but also minimizes matrix effects and protects the analytical column and the mass spectrometer by washing potentially interfering matrix compounds remaining after protein precipitation (mainly salts and protein residues) into the waste. This can finally lead to a reduction of maintenance requirements, and may be particular important in daily routine use.

Only few studies [19, 22, 23, 25, 27] have described multi-analyte LC-MS/MS methods for the quantification of various antibiotics from different antibiotic classes. One challenge in the development of a multi-analyte method covering antibiotics of different antibiotic classes is the high variability of the antibiotics' physico-chemical properties, such as polarity. This presents difficulty regarding the choice of the column as well as the ideal mobile phases and the protocol of the gradient elution program. Nevertheless, a time- and cost-efficient multi-analyte method covering different promising candidates

Table 3 Analytical inaccuracy and imprecision of quality control samples.

Sample	QC A[a]	QC B[a]	QC C[a]	Serum pool[a]
Piperacillin				
Target concentration of QCs, mg/L	1.50	16.0	45.2	(~34.8)
Observed mean concentration, mg/L	1.44	15.2	42.9	34.8
Inaccuracy, % (n=5)	−4	−5	−5	
CV Intra-assay, % (n=5)	3.18	2.04	1.05	1.72
%RMSE, %	3.28	5.93	7.34	
CV Inter-assay, % (n=5)	0.86	5.22	4.33	3.43
%RMSE, %	3.90	6.67	6.29	
Tazobactam				
Target concentration of QCs, mg/L	0.75	6.0	15.1	(~7.1)
Observed mean concentration, mg/L	0.70	5.62	14.6	7.14
Inaccuracy, % (n=5)	−6	−6	−3	
CV Intra-assay, % (n=5)	6.76	3.57	2.28	4.34
%RMSE, %	7.42	7.32	7.11	
CV Inter-assay, % (n=5)	6.75	5.84	8.25	3.98
%RMSE, %	8.61	8.04	7.81	
Cefepime				
Target concentration of QCs, mg/L	0.38	6.0	39.6	(~16.6)
Observed mean concentration, mg/L	0.37	5.53	38.2	16.6
Inaccuracy, % (n=5)	−1	−8	−4	
CV Intra-assay, % (n=5)	8.53	4.46	3.47	4.57
%RMSE,%	7.61	8.77	5.75	
CV Inter-assay, % (n=5)	6.04	5.74	4.07	3.71
%RMSE, %	5.47	9.18	5.01	
Meropenem				
Target concentration of QCs, mg/L	0.75	8.0	40.0	(~13.0)
Observed mean concentration, mg/L	0.73	8.28	40.2	13.0
Inaccuracy, % (n=5)	−3	+4	+1	
CV Intra-assay, % (n=5)	3.72	2.65	1.72	3.80
%RMSE, %	4.34	5.20	3.93	
CV Inter-assay, % (n=5)	4.80	5.30	5.51	6.64
%RMSE, %	5.12	6.04	4.98	
Ciprofloxacin				
Target concentration of QC, mg/L	0.15	1.0	6.0	(~1.1)
Observed mean concentration, mg/L	0.15	1.00	5.77	1.14
Inaccuracy, % (n=5)	0	0	−4	
CV Intra-assay, % (n=5)	3.20	4.02	1.08	1.42
%RMSE, %	5.63	4.44	6.39	
CV Inter-assay, % (n=5)	7.25	4.72	5.60	4.29
%RMSE, %	6.50	4.21	6.41	
Linezolid				
Target concentration of QCs, mg/L	0.30	5.6	20.1	(~3.7)
Observed mean concentration, mg/L	0.29	5.45	19.5	3.70
Inaccuracy, % (n=5)	−3	−3	−3	
CV Intra-assay, % (n=5)	5.02	1.24	1.89	1.45
%RMSE, %	4.97	2.92	5.94	
CV Inter-assay, % (n=5)	3.59	4.71	5.04	3.06
%RMSE, %	4.28	4.95	5.23	

[a]Of the appropriate antibiotic substance. %RMSE, normalized root mean square deviation as indicated in Materials and methods.

for a routine TDM is of particular interest for the laboratory workflow because critically ill patients are primarily treated with a combination of antibiotics from different antibiotic classes, and these patients are one of the most important patient groups for the introduction of TDM [8]. In contrast, measuring each antibiotic in a mono-analyte method is time consuming because for each analyte a separate calibration series has to be performed. Furthermore,

Table 4 Matrix effect and process efficiency of the different antibiotic substances.

Antibiotic substance	Concentration, mg/L	Matrix effect, % (n=5)			Process efficiency, % (n=5)		
		ME[a]	MF_n[b]	CV_{ME}[c]	PE[d]	PEF_n[e]	CV_{PE}[f]
Piperacillin	1.50	97	97	0.69	95	97	5.06
	45.2	92	104	1.14	97	104	2.92
Tazobactam	0.75	99	102	8.94	88	102	3.25
	15.1	99	98	2.44	95	105	7.45
Cefepime	0.38	99	99	1.92	90	97	8.00
	39.6	101	105	3.24	94	103	3.48
Meropenem	0.75	96	95	4.23	85	92	5.58
	40.0	102	105	3.30	94	104	3.93
Ciprofloxacin	0.15	112	95	5.15	98	93	5.32
	6.0	118	105	1.75	93	96	1.98
Linezolid	0.30	98	98	2.58	95	98	4.97
	20.1	104	105	1.03	99	105	3.02

[a]Absolute matrix effect; [b]internal standard normalized matrix factor as described in Materials and methods; [c]coefficient of variation of internal standard normalized matrix factor; [d]absolute process efficiency; [e]internal standard normalized process efficiency factor as described in Materials and methods; [f]coefficient of variation of internal standard normalized process efficiency factor.

time-consuming and error prone exchanges of solvents and columns is required if several distinct mono-analyte methods are utilized in a specific clinical laboratory. The application of multi-analyte methods substantially facilitates the implementation of an extended antibiotic TDM service in a hospital. Indeed, analyses of antibiotic samples on site without the need to ship samples to centralized laboratories – as might be appropriate for steroid hormone testing, e.g., – is of utmost importance to enable potential individualization of antibiotic regimens with the need to rapidly adapt antibiotic doses for optimal outcome. Being able to measure six analytes in 5 min is therefore a huge advantage of this method in comparison to single-analyte methods. Indeed, it might be a general goal in a clinical mass spectrometry facility to minimize the number of mono-analyte methods to reduce the amount of conversion work. However, this is a challenge for analytes differing in their polarity properties. In contrast to other studies, we chose only antibiotics that are widely used in ICU-settings and for which no quantification tests are commercially available. Indeed, it has been shown that the most frequent antibiotic classes used in ICUs are penicillins (often combined with β-lactamase inhibitors), cephalosporins, carbapenems and chinolons [36], with meropenem, piperacillin-tazobactam, and ciprofloxacin being the most frequent used antibiotics of their respective antibiotic classes in ICUs in 2012 [37]. Furthermore, the chosen antibiotics showed a high variability of serum concentrations with a major part of potentially insufficient levels in critically ill patients in recent studies [9–11, 13, 16, 38]. Therefore, we believe that these antibiotics are particular promising candidates for a routine TDM for critically ill patients. To the best of our knowledge, this study shows for the first time that antibiotics of those four classes most frequently used in ICUs can be measured using a single semi-automated LC-MS/MS method. As this method already covers antibiotics with a high heterogeneity of physico-chemical characteristics, further promising antibiotic candidates, especially of the antibiotic classes already covered in this study, might be easily implemented in this multi-analyte method.

Using a method in a routine setting requires the compliance of different quality criteria. The inaccuracy and imprecision of the method were within the performance goals described in Material and methods and meet the limits that are generally accepted for the introduction of TDM. In addition to the determination of inaccuracy and imprecision, we determined the total error in the form of the %RMSE, which is a quality criterion required for routine use implementation according to the Guidelines of the German Federal Medical Council (RiliBÄK) [30]. The values of %RSME for all antibiotics were within the limits allowed by the RiliBÄK for most analytes. Furthermore, matrix effects and process efficiencies were evaluated. For all analytes used in this study, only minor matrix effects and good process efficiencies were observed. Moreover, matrix effect and loss of analyte due to sample preparation for the analytes could be almost fully compensated by the use of isotope-labeled internal standards (normalized values, Table 4). Also the CVs of the normalized values were <9% for all analytes and

consequently within the limits suggested by the European Medicines Agency [32]. For tazobactam, no corresponding isotope-labeled internal standard was available, but the internal standard normalized values for tazobactam indicate that meropenem-D_6 is a suitable internal standard for tazobactam. The use of this structurally unrelated internal standard might also be acceptable because tazobactam itself has no antibacterial effect and is only necessary to prevent degradation of piperacillin. Furthermore, it is only used in fixed combinations with piperacillin, and recent studies indicate that their pharmacokinetics may be similar [39]. Further studies may be necessary to investigate whether measuring piperacillin concentrations is sufficient for the appropriate dosing of the fixed combination of piperacillin-tazobactam. With the exception of tazobactam, the corresponding isotope-labeled internal standards were used for all of the analytes. This presents a clear advantage in contrast to most other studies where either no [23, 26] or only a few [24, 25] corresponding isotope-labeled internal standards were used for the quantification of antibiotics. The use of such internal standards might improve the method as isotope dilution mass spectrometry confers the highest attainable level of reliability [40].

Using this method in routine TDM leads to the problem that only preliminary target ranges are described for all of the antibiotics in this study. Only very few data concerning correlation between clinical or microbiological outcome data and minimal inhibitory concentrations of causative bacteria in critically ill patients are available [10, 12, 16]. One study has shown that the success of cefepime treatment in seriously ill patients with infections appeared to be higher when cefepime concentrations remained above the minimal inhibitory concentration (MIC) for the causative pathogen within the entire dosing interval [16]. Another study showed that the outcome of febrile neutropenic patients was significantly better if the meropenem concentrations were above the median MIC-values (MIC_{50}-values) of causative pathogens more than 75% of the time. However, the exact MIC-values of the different causative bacteria were not determined [14]. It has been recommended that β-lactam antibiotics should exceed the MIC of the respective organism for 100% of the dosing interval [41, 42]. This allows defining preliminary trough values of β-lactam serum concentrations on the basis of clinical breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [43]. As in the early course of infections the causative strains are mostly unknown and as there is a large variance of clinical breakpoint values of different bacterial species, the clinical breakpoints of *Pseudomonas aeruginosa* which is a problematic strain with an elevated clinical breakpoint commonly isolated in ICU-patients may serve as the empiric target threshold [13, 44]. Based on this, preliminary trough values for β-lactam antibiotics may be 16 mg/L for piperacillin, 8 mg/L for cefepime and 2 mg/L for meropenem. For the oxazolidinone linezolid, a preliminary trough value may be 2 mg/L, as discussed previously [38]. However, it should be noted that concentrations required for maximal efficacy against causative bacteria are still controversially discussed for the different antibiotics [13, 45, 46] and that there are no guidelines for how to adapt antibiotic concentrations after receipt of quantitative results.

It is even more difficult to determine efficacy thresholds for ciprofloxacin. In the study of Forrest et al., it was shown that the concentration time curve (AUC) over 24 h/MIC-ratio should be higher than 250 for optimal efficacy of ciprofloxacin in critically ill patients [10]. However, it is difficult to determine an AUC in a routine TDM setting because of the required multiple blood sampling. Due to this, the use of antibiotic peak concentration/MIC as a suitable alternative parameter should be evaluated, as has been shown for the related chinolone levofloxacin [17]. This question will be investigated in an ongoing study where we compare AUC/MIC values with maximum concentrations/MIC values of ciprofloxacin in critically ill patients using the method described here to define convenient preliminary efficacy thresholds for a routine TDM for this patient group.

In conclusion, we developed a fast and reliable multianalyte 2D-UHPLC-MS/MS method covering important antibiotics from different antibiotic classes. This method can be recommended for routine use after performance verification on individual systems. Furthermore, it can be used for future studies, which are needed to define evidence-based target concentration ranges and to definitively elucidate the impact of the TDM of these antibiotics for specific patient groups.

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3 Anhänge

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3.2 Abkürzungsverzeichnis

Sollte die Abkürzung für einen englischen Begriff stehen, so ist die deutsche Übersetzung in Klammern aufgeführt.

$Area_A$	Fläche des Analyten
$Area_{IS}$	Fläche des internen Standards
AUC	Area under the curve (Fläche unter der Konzentrations-Zeit-Kurve)
AUC_{24}	AUC über 24 Stunden
c_A	Konzentration des Analyten
c_{DIDA}	Konzentration, die mittels DIDA ermittelt wurde
c_{IS}	Konzentration des internen Standards
c_{Kon}	Konzentration, die über 6-Punkt-Kalibration ermittelt wurde
c_{min}	Talspiegel
DIDA	Direct Isotope Dilution Analysis (Direkte Isotopenverdünnungsmethode)
HPLC	High Performance Liquid Chromatography (Hochleistungsflüssigkeitschromatographie)
IS	Interner Standard
LC	Liquid Chromatography (Flüssigkeitschromatographie)
MHK	Minimale Hemmkonzentration
MHK_{90}	Konzentration, die das Wachstum von 90% der getesteten Stämme hemmt
MRSA	Methicillin resistenter Staphylococcus aureus
MS	Massenspektrometrie
MS/MS	Tandem-Massenspektrometrie
SPE	Solid Phase Extraction (Festphasenextraktion)
TDM	Therapeutisches Drug Monitoring
UHPLC	Ultra High Performance Liquid Chromatography (Ultrahochleistungsflüssigkeitschromatographie)
VRE	Vancomycin-resistente Enterokokken

3.3 Eidesstattliche Versicherung

Maier Barbara Elisabeth

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

„Untersuchungen zur Anwendung der Flüssigkeitschromatographie-Massenspektrometrie in der labormedizinischen Routinediagnostik“

selbstständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 30.04.2015

3.4 Veröffentlichungen

Publikationen

Kirchhoff F, **Maier B**, Rieger C, Ostermann H, Spöhrer U, Vogeser M. An on-line solid phase extraction procedure for the routine quantification of caspofungin by liquid chromatography-tandem mass spectrometry. Clin Chem Lab Med 2011; 50(3):521-4.

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3.5 Lebenslauf

3.6 Danksagung