Aus dem Institut für Laboratoriumsmedizin
der Ludwig-Maximilians-Universität München

Direktor: Univ. Prof. Dr. med. Daniel Teupser

Untersuchungen zur quantitativen Massenspektrometrie in der Laboratoriumsmedizin

Dissertation

zum Erwerb des Doktorgrades der Humanbiologie

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

vorgelegt von

Katrin König

aus

Freising

2014

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	Prof. Dr. med. Michael Vogeser
Mitberichterstatter:	Prof. Dr. Dr. Thomas Demant
	Prof. Dr. Roland Beckmann
Dekan:	Prof. Dr. med. Dr. h.c. M. Reiser, FACR, FRCR
Tag der mündlichen Prüfung:	20.05.2014

Für Oma…

Kumulative Dissertation

Inhaltsverzeichnis

1 EINLEITUNG

1.1 Massenspektrometrie in der Laboratoriumsmedizin

Ein wesentlicher Anteil klinischer Entscheidungen beruht heute auf qualitativen und quantitativen Laboruntersuchungen von Körperflüssigkeiten, vornehmlich von Blut und Urin. Laboruntersuchungen sind häufig entscheidend für die Diagnosestellung (z.B. Troponin-Messung beim akuten Koronar-Syndrom), für die Beurteilung der Prognose (z.B. Lactat-Messung bei Patienten mit Infektionen), für die Überwachung von Arzneimittelbehandlungen (z.B. Kontrolle von Leberparametern unter Behandlung mit Statinen), für eine Risikoabschätzung (z.B. Cholesterin-Analytik hinsichtlich kardiovaskulärer Erkrankungen) sowie für das Monitoring der Therapie-Effizienz (z.B. CEA-Messung unter Chemotherapie). Durch die heute verfügbaren, voll automatisierten Analysesysteme ist es möglich, entsprechende Messungen schnell und wirtschaftlich auch im wenig spezialisierten Laborumfeld durchzuführen. Technologische Haupt-Säulen dieser Systeme sind die Photometrie und immunometrische Methoden. Diese seit den 1960er- bzw. seit den 1980er-Jahren verfügbaren Methoden haben heute einen sehr hohen technischen Standard erreicht und sind Grundlage von außerordentlich benutzerfreundlichen, geschlossenen Hochdurchsatz-Analysesystemen, die einen Multi-Kanal-„random-access“-Betrieb als Standardgeräte der Labormedizin erlauben. Dennoch weisen diese Basis-Technologien prinzipielle analytische Limitierungen auf, die ihre Anwendbarkeit in der medizinischen Diagnostik einschränken. Dies betrifft vor allem die für viele Fragestellungen unzureichende analytische Spezifität und eine oft ungenügende Sensitivität. Für eine Vielzahl potentiell klinisch wertvoller Analyten ist die Entwicklung von Messverfahren auf Basis der Standardtechniken gar nicht möglich. Beispielsweise kann die Methylmalonsäure als spezifischer Marker eines Vitamin B12-Mangels weder photometrisch noch mit Immunoassay quantifiziert werden. Massenspektrometrische Verfahren können das der Labordiagnostik zugängliche Analyten-Spektrum ganz wesentlich erweitern. Als Detektionsprinzip erfordert die Massenspektrometrie – im Gegensatz zur Photometrie – keine speziellen Molekülstrukturen, und – im Gegensatz zur Immunoassay-Technologie – keine immunogenen molekularen Epitope. In der technischen Umsetzung der Gaschromatographie-Massenspektrometrie (GC-MS) wird das Grundprinzip der Massenspektrometrie seit den 1970er Jahren in der Labormedizin genutzt. Diese Technologie erlaubt die höchst spezifische Detektion auch primär unbekannter Substanzen in diagnostischen Proben und – unter Anwendung der Isotopen-Verdünnungstechnik – matrix-unabhängige quantitative Analysen. Allerdings ist die Anwendung der GC-MS auf thermo-stabile Analyten von Molekulargewichten unter 800 Dalton begrenzt. Vor allem aber wird die Anwendung durch die Notwendigkeit einer sehr aufwändigen Probenvorbereitung mit Derivatisierung und eine sehr komplexe Geräte-Handhabung begrenzt. Dadurch hat die GC-MS – trotz ihrer enormen analytischen Vorzüge – im medizinischen Routinelabor keinen Anwendungsbereich gefunden. Gleichwohl ist diese Technik in hoch-spezialisierten Laboren

eine wichtige flankierende Technik der Labormedizin geworden. Insbesondere stellt sie die zentrale Säule der Toxikologie dar und spielt als Referenzmethode in der externen Qualitätssicherung eine Schlüsselrolle.

In den 1990er Jahren sind bahnbrechende, technologische Fortschritte erzielt worden, die heute prinzipiell eine wesentlich breitere Anwendung von massenspektrometrischen Methoden in der Labormedizin erlauben als auf Basis der GC-MS alleine. Durch die Entwicklung von Verfahren, die eine Ionisierung der Ziel-Analyten direkt und ohne Derivatisierung aus wässriger Probenmatrix bei Atmosphärendruck erlaubt, hat sich das Analyten-Spektrum der Massenspektrometrie im Grunde auf alle biologisch relevanten Stoffklassen erweitert. Entsprechende atmospheric pressure ionisation (API) Verfahren sind schonend und nicht-desintegrierend, so dass prinzipiell auch die Analyse von intakten Proteinen oder Nukleinsäure-Amplifikaten erfolgen kann. Flugzeit-Massenspektrometrie und Tandem-Massenspektrometrie sind innovative Verfahren der Ionen-Manipulation, die durch eine enorm hohe Massenauflösung bzw. durch die Einbeziehung des molekularen Desintegrationsmusters in die Analyten-Detektion ein sehr hohes Maß an analytischer Spezifität eröffnen. Dies wiederum reduziert die Anforderungen an die Fraktionierung des Probengemisches vor einer massenspektrometrischen Analytik entscheidend. Die Verbindung von Flüssigkeitschromatographie (liquid chromatography, LC) mit Tandem-Massenspektrometern (Koppelung zweier Quadrupol-Massenfilter mit zwischengeschalteter Kollisionszelle) als „LC-MS/MS“ hat sich seit Ende der 1990er Jahre als sehr versatiles und leistungsfähiges Gerätekonzept in der Labormedizin erwiesen. Wesentliche Vorzüge dieser Technologie gegenüber den Standardtechniken auf Basis von Photometrie und Immunometrie umfassen:

- Hochspezifische Detektion der Analyten ohne Kreuzreaktionen wie bei Immunoassays
- Kompensation von Matrix-Effekten durch den Einsatz stabilisotopen-markierter interner Standards
- Simultane Quantifizierung einer Vielzahl von Substanzen in *einem* Analysenlauf mit der Möglichkeit eines Profiling zum Beispiel von Steroid-Hormonen
- Hochflexible und kurzfristige Entwicklung von Methoden durch den Systemanwender ohne Abhängigkeit von der Diagnostika-Industrie
- Einfache, kostengünstige Einsatzmaterialien wie Standard-Lösungsmittel (im Gegensatz zu der höchst komplexen und für den Endanwender nicht realisierbaren Entwicklung von analytischen Antikörpern)
- Reagenzien-unabhängige Standardisierung und damit potentiell gute Übereinstimmung von Wertelagen von Labor zu Labor und über die Zeit sowie methoden-unabhängige Referenzwerte
- Keine Lot-zu-Lot-Varianzen wie bei Immunoassay-Reagenzien

Seit nun mehr als einer Dekade hat die LC-MS/MS-Technologie Einzug in die labormedizinische Diagnostik gefunden. Bereits jetzt ist der Umfang der Anwendung hier erheblich größer als dies für die wesentlich länger implementierte GC-MS gilt. Ältestes und global am weitesten verbreitetes

Einsatzgebiet ist das Stoffwechsel-Screening bei Neugeborenen. In zahlreichen Ländern werden in zentralen Screening-Laboratorien getrocknete Blutproben aller Neugeborenen auf über 30 Metaboliten simultan untersucht; hierdurch können neben der Phenylketonurie mehrere behandelbare Stoffwechseldefekte erkannt werden. Die LC-MS/MS hat sich des Weiteren als ideale Technologie für das therapeutische Drug-Monitoring (TDM) erwiesen. TDM zielt darauf ab, durch die Messung der Blut-Konzentrationen von Arzneimitteln bzw. der entsprechenden aktiven Metaboliten im Hinblick auf validierte Zielbereiche eine individualisierte Optimierung von Dosierungsregimen zu ermöglichen. Besondere Stärken der LC-MS/MS liegen hierbei in der schnellen, industrie-unabhängigen Methodenentwicklung für neue Pharmaka sowie in der spezifischen Detektion aktiver Metaboliten ohne Miterfassung nicht relevanter Metaboliten oder verwandter Stoffe. Insbesondere zum TDM von Immunsuppressiva in der Transplantationsmedizin hat die LC-MS/MS weltweit mittlerweile weite Verbreitung gefunden. Aber auch das TDM von Psychopharmaka erfolgt in vielen spezialisierten Laboratorien mittels LC-MS/MS. Im Bereich der endokrinologischen Diagnostik kommt die LC-MS/MS zunehmend zur Quantifizierung von Steroiden zum Einsatz.
Obgleich die technologischen Grundcharakteristika der LC-MS/MS prinzipiell eine wesentlich bessere Praktikabilität ermöglichen als dies für die GC-MS gilt – insbesondere, da keine Derivatisierungsprozesse notwendig sind – ist die Anwendung gegenwärtiger LC-MS/MS-Methoden in klinischen Routinelaboren noch keineswegs umfassend realisiert. Sie bleibt derzeit noch relativ wenigen, spezialisierten Laboratorien vorbehalten. Gründe hierfür sind u.a.:

- Die Handhabung von Software- und Hardware-Komponenten von gegenwärtigen LC-MS/MS-Konfigurationen ist komplex und verlangt sehr gut qualifiziertes technisches Personal.
- Die Entwicklung, Implementierung und Aufrechterhaltung analytischer Methoden muss im Wesentlichen durch den Anwender selbst erfolgen, was Spezialisten mit akademischem Hintergrund vor Ort erfordert.
- Die Handhabung weist eine Vielzahl von Fehlermöglichkeiten auf.
- Geräte-Konfigurationen sind höchst heterogen und nicht standardisiert wie bei Standard-Analysensystemen der Labormedizin.
- Im Gegensatz zu den Standard-Techniken Photometrie und Immunoassay ist die direkte Analyse von Serum in aller Regel nicht möglich, vielmehr muss eine Probenvorbereitung erfolgen, die weit weniger aufwändig ist als bei der GC-MS, jedoch gleichwohl eine hohe Bindung von gut qualifiziertem Personal erfordert.
- Für einige potentielle Zielanalyten sind geeignete interne Standard-Substanzen bislang nicht verfügbar, beispielsweise für biologisch produzierte Antibiotika wie Vancomycin.

Es muss heute festgestellt werden, dass die LC-MS/MS-Technologie zwar technologisch einen wesentlichen Beitrag dazu liefern kann, die labormedizinische Diagnostik zu verbessern, die Umsetzung in die klinische Routineversorgung aber erst ansatzweise für wenige Analyten in einer sehr begrenzten Anzahl von Laboren erfolgt ist. Voraussetzung hierfür ist im Wesentlichen, dass die Praktikabilität von LC-MS/MS-Anwendungen künftig deutlich verbessert wird, und zwar bis zu dem

Niveau, das moderne Photometrie- und Immunoassay-basierte Analysensysteme heute bieten. Eine entsprechend erforderliche komplette Automation des gesamten Analysenprozesses muss demnach einen MS/MS-basierten „random-access“-Multikanal-Analyzer zum Ziel haben, der von normal qualifiziertem Laborpersonal im Routinelabor rund um die Uhr zu bedienen ist. Zweifellos ist ein solches globales Ziel nur im industriellen Rahmen realisierbar; dennoch hat die labormedizinische Forschung die Einzelkomponenten solcher Lösungen zu entwickeln, zu untersuchen sowie Leistungs- und Praktikabilitätsanforderungen aufzuzeigen.
Sollte es gelingen, durch technologische Weiterentwicklungen die LC-MS/MS kompatibel mit den Arbeitsabläufen in modernen medizinischen Großlaboratorien zu gestalten, werden der Labormedizin wichtige Perspektiven eröffnet:

- Höchst spezifisches TDM von potentiell allen verabreichten Pharmaka mit der Möglichkeit einer individualisierten Behandlung
- Profil-Analysen von Hormonen und Metaboliten mit labor-unabhängigen, allgemein gültigen Richtwerten
- Basierend auf der Isotopenverdünnungs-Technik ein analytisches Qualitätsniveau von Referenzmethoden auch im Routinelabor
- Völlig neue Möglichkeiten bei diagnostischen Ansätzen ausgehend vom Konzept „Metabolomics“ (der umfassende Beschreibung niedermolekularer Komponenten von Probenmaterialien)
- Hoch-parallelisierte Messung von Analyten unterschiedlicher chemischer Klassen aus unterschiedlichen Stoffwechselwegen
- Biomathematische Beschreibung von potentiell krankheits-assoziierten Mustern

Eine wesentliche Herausforderung an die labormedizinische Forschung besteht also derzeit darin, zu untersuchen, ob und wie die Praktikabilität von LC-MS/MS-Konfigurationen soweit zu optimieren ist, dass eine Einbindung dieser höchst leistungsfähigen Technologie in die Arbeits- und Betriebsabläufe moderner Hochdurchsatz-Routinelaboratorien möglich wird, sowie die Anwendbarkeit der Technologie für eine Vielzahl von einzelnen, klinisch relevanten Analyten zu überprüfen.
Die übergeordnete Fragestellung der hier dargestellten Promotionsarbeit bezog sich entsprechend auf die Anwendungsperspektiven der LC-MS/MS-Technologie im klinischen Labor. Dazu wurden in Teilprojekten zum einen innovative Methoden der Proben-Vorfraktionierung untersucht, zum anderen die Entwicklung eines Referenzmessverfahrens für einen Analyt von wesentlicher klinischer Bedeutung angegangen.

1.2 Magnet-Partikel basierte Verfahren für die Vorbereitung von Serum- und Plasmaproben für die LC-MS/MS-Analytik

Die Vorbereitung von Serum- und Plasmaproben für die quantitative Analytik kleiner Moleküle mittels LC-MS/MS erfordert im Rahmen einer Probenvorbereitung grundsätzlich die Entfernung der Protein-Matrix. Dies ist unverzichtbar, da die der MS/MS-Analyse vorgeschaltete konventionelle chromatographische Probenfraktionierung mittels Trennsäulen erfolgt, die von Serum-Proteinen bereits nach wenigen Injektionen verstopft werden würden. Die chromatographische Fraktionierung wiederum ist erforderlich, um u.a. zu erreichen, dass die jeweiligen Zielanalyten nicht mit Stoffen co-eluieren, die ihre Ionisierung beeinträchtigen, was vor allem für Serum-Elektrolyte gilt. Die Probenvorbereitung zielt ferner insgesamt darauf ab, Stör-Substanzen abzureichern und den Zielanalyten anzureichern, um hierdurch eine möglichst hohe Signal-Ausbeute zu erzielen. Als konventionelle Probenvorbereitungstechniken werden für die LC-MS/MS heute im Wesentlichen drei Techniken verwendet:

- Die reine Proteinfällung mit Zentrifugation ist kostengünstig, allerdings schwer zu automatisieren und wenig effizient.
- Eine nachgeschaltete automatisierte Festphasen-Extraktion im Sinne einer zweidimensionalen LC (LC/LC oder *on*-line solid phase extraction (SPE) führt zu deutlich robusteren Methoden und ist weit verbreitet. Die Verwendung von Einweg-Festphasen-Extraktionskartuschen (sog. *off*-line SPE) wird ebenfalls häufig verwendet. Sie erlaubt es, Analyten anzureichern und damit die Sensitivität einer Methode zu erhöhen. Nachteilig sind die hohen Materialkosten und ebenfalls die schlechte Automatisierbarkeit.
- Bei der Flüssigphasen-Extraktion kommen organische Lösungsmittel zum Einsatz; es wird eine hohe Extraktions-Effizienz erreicht bei hohem Arbeitsaufwand und ebenfalls schlechter Automatisierbarkeit.

In der Tat ist die komplette Automation der Probenvorbereitung heute die zentrale Herausforderung bei der Entwicklung von künftigen LC-MS/MS-Analyzern für Standard-Routinelaboratorien. Die Verwendung von magnetischen Mikropartikeln mit definierter, extraktiver Oberflächenmodifikation stellt einen innovativen Ansatz dieser Problemstellung dar. Solche Partikel können als Suspension pipettiert werden, verhalten sich aber nach magnetischer Immobilisation wie eine feste Phase. Die Extraktionsschritte Adsorption-Waschen-Elution können auf diese Weise wie bei in Kartuschen gepackten Partikeln sequentiell vorgenommen werden, wobei dieser Prozess hierbei einfach zu automatisieren ist.

Im Rahmen des Promotionsprojektes wurde zunächst die Anwendbarkeit von C18-modifizierten Partikeln zur Extraktion des klinisch relevanten Analyten Mycophenolsäure untersucht. Hierbei konnte eine hohe Extraktionseffizienz festgestellt und eine leistungsfähige analytische Methode entwickelt

werden. Da hierbei kein Zentrifugationsschritt erforderlich ist, kann diese Methode auf Standard-Pipettier-Automaten implementiert werden (siehe 2.1).
In einer zweiten Teilarbeit wurde die Anwendbarkeit von ferromagnetischen Mikropartikeln zur Proteindepletion getestet. Als Zielanalyt wurde Amiodaron ausgewählt. Auch hier konnte durch die reproduzierbare, effektive Probenvorbereitung ein leicht automatisierbares Protokoll entwickelt werden. In Kombination mit einer nachgeschalteten on-line SPE gelang es, eine leistungsfähige analytische Methode zu entwickeln (siehe 2.2).

1.3 Referenzmethode für die Messung von Vancomycin im Serum

Vancomycin ist eines der Arzneimittel, bei denen am häufigsten ein TDM vorgenommen wird. Hierfür finden unterschiedliche, automatisierte Immunoassays Verwendung. Externe Qualitätssicherungsmaßnahmen (Ringversuche) zeigen deutliche Wertelageunterschiede mit Differenzen in den Mittelwerten von bis zu 40% zwischen unterschiedlichen Tests. Es muss angenommen werden, dass – abhängig vom verwendeten Assay – unterschiedliche klinische Entscheidungen aufgrund des TDM von Vancomycin getroffen werden, da keine methodenspezifischen Werte verfügbar sind. Entsprechend ist es von großer klinischer Bedeutung, eine Harmonisierung durch optimierte Standardisierung des Vancomycin-TDM zu erreichen. Insgesamt ist die Standardisierung komplexer Analyseverfahren ein übergeordnetes Ziel der labormedizinischen Forschung – vor allem auch in diesem Bereich gewinnt die LC-MS/MS ständig an Bedeutung. Während der Analyt Vancomycin aufgrund seiner thermischen Labilität nicht mit der traditionellen Referenzmethoden-Technologie der GC-MS adressiert werden kann, ist die Entwicklung von LC-MS/MS-Verfahren möglich. Ein besonderes Problem stellt in diesem Fall jedoch die interne Standardisierung dar: Da Vancomycin ein biologisches Produkt ist und nicht – wie die meisten Pharmaka – voll-synthetisch hergestellt wird, ist die Gewinnung eines einheitlich stabilisotopen-markierten Standards schwer möglich. Bisher beschriebene LC-MS/MS-Methoden für die Messung von Vancomycin im Serum bezogen strukturell kaum ähnliche Substanzen als interne Standards ein, was die Validität dieser Methoden fragwürdig macht. In einem Teilprojekt der hier referierten Promotionsarbeit wurde durch Derivatisierung von Vancomycin ein interner Standard hergestellt, der dem Zielanalyten strukturell sehr ähnlich ist. Auf dieser Basis wurde eine LC-MS/MS-Methode aufgebaut, die vor allem als Referenzmethode für die Standardisierung von Routine-Immunoassays zur Vancomycin-Messung dienen soll. Die Methode wurde umfassend validiert, wobei eine wesentliche Novität in einer bi-zentrischen Untersuchung der Methode lag (siehe 2.3).

In der zusammenschauenden Betrachtung erbringen die publizierten Teilprojekte der Promotionsarbeit relevanten Erkenntnisgewinn hinsichtlich der Entwicklungsperspektiven der LC-MS/MS-Technologie im klinischen Labor. Es konnte gezeigt werden, dass Magnet-Partikel-basierte

Verfahren aussichtsreich für die Entwicklung voll-automatisierter Methoden sind. Des Weiteren wurde gezeigt, dass auf Basis der LC-MS/MS die Entwicklung einer Referenzmethode für den klinisch bedeutsamen Analyt Vancomycin möglich ist.

1.4 Zusammenfassung / Summary

Übergreifendes Ziel der Promotionsarbeit war es, die Perspektiven der Anwendung der LC-MS/MS-Technologie in der labormedizinischen Analytik weiter zu charakterisieren. Dabei wurde in Teilprojekten die Anwendung oberflächen-definierter magnetischer Mikropartikel für die Probenvorbereitung von LC-MS/MS-Methoden untersucht. Hierfür wurden unterschiedliche Ansätze betrachtet: Zum einen die Extraktion von Zielanalyten aus der Probe basierend auf der reversiblen Adsorption des Analyten an Partikeln. Zum anderen die Protein-Depletion der Probenmatrix mit Hilfe von magnetischen Mikro-Partikeln. Für beide Ansätze gelang es, für exemplarische Analyten, valide analytische Methoden zu entwickeln und ihre Leistungscharakteristika zu beschreiben. Beide Verfahren erwiesen sich als hoch effizient. Da ferromagnetische Partikel einerseits als Suspension pipettiert werden, andererseits reversibel zu einer festen Phase immobilisiert werden können, bieten sie ideale Voraussetzungen für die Entwicklung voll automatisierter Probenvorbereitungsverfahren. Solche Systeme wiederum sind erforderlich, um die Anwendung der LC-MS/MS in der Labormedizin zukünftig nicht nur in spezialisierten Laboratorien, sondern auch in Standard-Laboren realisieren zu können und so für die Medizin in der Breite die wesentlichen Innovationen der Massenspektrometrie zugänglich zu machen.

In einem zweiten Teilprojekt konnte gezeigt werden, dass es möglich ist, für den Analyten Vancomycin eine LC-MS/MS-basierte Referenzmethode zu entwickeln, obgleich die Darstellung eines stabilisotopen-markierten internen Standards derzeit nicht realisierbar ist. Dies wurde durch die Synthese eines Vancomycin-Derivates zur internen Standardisierung erreicht. In einer ausführlichen Validierung konnte gezeigt werden, dass dieses Verfahren die analytischen Charakteristika für die Etablierung eines Standardisierung-Projektes zur Bestimmung von Vancomycin im Serum bietet. Ein solches System ist die entscheidende Voraussetzung für eine Harmonisierung des therapeutischen Drug Monitorings von Vancomycin und damit von erheblicher klinischer Relevanz.

Summary

This doctoral thesis was conducted with the aim of further characterising the perspectives of applying the technology of LC-MS/MS-technology within the field of clinical laboratory analytics. In the course of this frame project, two sub-projects addressed the application of surface-defined, magnetic micro-particles for sample-preparation of LC-MS/MS-methods. For this purpose, various approaches were studied: On the one hand, extraction of target-analytes from samples, based on reversible adsorption of the analyte to particles, and – on the other hand – protein-depletion of the sample-matrix using

magnetic micro-particles. For both approaches, it was found possible to develop analytical methods and to describe their characteristics of performance for exemplary analytes. Both approaches were found to be highly efficient. Due to the fact that ferro-magnetic particles can be pipetted as a suspension on the one hand, and can be reversibly immobilised as a solid phase on the other hand, this approach offers ideal conditions for developing fully-automated procedures of sample-preparation. Indeed, such procedures are required in order to realise application of LC-MS/MS in laboratory medicine not only in specialised laboratories but also in regular laboratories in the future. This will contribute to making the fundamental innovations of mass-spectrometry available for medicine in general.

The results of a second sub-project demonstrated that the development of a LC-MS/MS-based reference method for the quantification of vancomycin is possible, despite the fact that a stable-isotope labelled internal standard cannot be realised. This result was achieved by synthesis of a vancomycin-derivative as an internal standard. By thorough validation, it was proven that this procedure does provide the analytical characteristics which are required for establishing a standardisation project for measurement of vancomycin in serum. Such a reference measurement system is the essential prerequisite for harmonisation of therapeutical drug monitoring of vancomycin and is therefore of significant clinical relevance.

2 ORIGINALPUBLIKATIONEN

2.1 König, K.; Vogeser, M. „Sample preparation for measurement of plasma mycophenolic acid concentrations using chromatographically functionalized magnetic micro-particles" Eur. J. Mass Spec. 2012, 18, 413-417.

K. König and M. Vogeser, *Eur. J. Mass Spectrom.* **18,** 413–417 (2012)
Received: 8 May 2012 ■ *Revised: 30 August 2012* ■ *Accepted: 7 September 2012* ■ *Publication: 6 December 2012*

EJMS EUROPEAN JOURNAL OF MASS SPECTROMETRY

ejmsprotocols

Sample preparation for measurement of plasma mycophenolic acid concentrations using chromatographically functionalized magnetic micro-particles

Katrin König* and Michael Vogeser

Institute of Laboratory Medicine, University of Munich, Munich, 81375 München, Germany. E-mail: katrin.koenig@med.uni-muenchen.de

Utilizing chromatographically modified magnetic micro-particles is an innovative principle of sample preparation for quantitative analysis of small molecules in complex biomedical samples by liquid chromatography tandem mass spectrometry. Since no vacuum or pressure has to be applied—in contrast to cartridge based solid phase extraction protocols—the principle's main characteristics are potentially straightforward automation and a high extraction performance (in terms of µg of extraction material per µL of sample). Following first descriptions of the approach, this article reports, the validation of a magnetic particle-based, analytical method for the quantification of the immunosuppressant mycophenolic acid in plasma. This sample preparation technology has shown a good performance for this clinically relevant analyte. As a result, we conclude that further work towards the implementation of this technology in a multi-analyte approach on robotic systems, aiming towards a fully automated process, is justified.

Keywords: chromatography, ferromagnetic micro particles, LC-MS/MS, quantification, sample preparation

Introduction

Previously, the application of C18-functionalized magnetic particles for the extractive sample preparation of small molecule analytes in the context of quantitative bio-analytical liquid chromatography tandem mass spectrometry (LC-MS/MS) methods has been proposed.[1,2] These initial reports demonstrated the feasibility of this approach for exemplary analytes to be quantified in serum or urine. The aim of the study described in the present manuscript was to further validate this technology of sample preparation for an additional analyte of clinical relevance and applying an extended evaluation scheme for biomedical analyses.

Functionalized magnetic particles are commercially available and in use for sample fractionation involved in the work-up of protein analyses following tryptic digest[3,4]—this represents a *qualitative* rather than a quantitative approach in *large* molecule characterisation. In contrast, applying chromatographically modified ferromagnetic micro-particles for *quantitative* analyses of *small* molecules is novel.

Since no vacuum or positive pressure has to be applied in this approach, just as in classical, cartridge-based solid-phase extraction, the potential of straightforward automation can clearly be considered one of the advantages of this procedure of sample preparation (as widely carried out in DNA-purification based on ferromagnetic silica particles[5]). Furthermore, a very high efficacy of extraction was observed in terms of the amount of extraction material required per µL of sample material. This was due to the obviously more intense and permeating interaction between the extraction material and the sample matrix, when compared with packed solid-phase extraction materials.

Although the analytical specificity of LC-MS/MS is potentially very high, medical diagnostic applications in highly complex biological sample materials—such as plasma or urine—require efficient pre-fractionation.[6] This is due to the extensive presence of compounds that potentially interfere with the analyte's ionization and due to compounds that

ISSN: 1469-0667
doi: 10.1255/ejms.1197

potentially share mass transitions with the target analyte—such as isomers or conjugate metabolites, whenever disintegration during ionization may occur (in-source transformation).[7] Should efficient sample preparation be able to avoid such potential analytical interference, the requirement of further chromatographic fractionation by LC could be minimized, reducing over-all analytical run times.

Progressive and comprehensive automation is of eminent importance in the context of clinical laboratory applications of LC-MS/MS, where random access work-flows with short turn-around-times and minimal manual workload are desired.[8] As a matter of fact, laborious sample preparation, up to now, has been a major drawback for the widespread implementation of LC-MS/MS in clinical chemistry,[9] which is why there is an urgent need for novel technologies in this field.

Our methodological study is focused on the immunosuppressant drug, mycophenolic acid, which is used in transplant patients and in the treatment of autoimmune diseases. The drug acts as a reversible, non-competitive inhibitor of inosine-monophosphate dehydrogenase type 2. Preventive measures against intoxication or organ rejection caused by inappropriate dosing is of great importance. Due to the fact that significant inter-patient variability has been demonstrated, therapeutic drug monitoring is widely used for the individualisation of therapeutic regimens.[10–13]

Methods and results

Extraction protocol

C18-functionalized ferromagnetic micro-particles (Dynal Dynabeads RPC18, Invitrogen, Karlsruhe, Germany; 12.5 mg mL^{-1}; mean particle diameter 1 µm) were used for sample extraction. In order to prepare a particle working suspension, 100 µL of the original particle suspension were transferred into a 1.5 mL polypropylene tube which was placed in a magnetic particle separator (Magnamedics MM-Separator M 12+12). Particles are immobilized to the wall of the tube so that the particle-free fluid could be removed with a pipette tip. After removing the tube from the separation device, 100 µL of washing solution (0.1% trifluoroacetic acid) were repeatedly added and dispensed with a pipette tip. Again, the washing solution was removed after immobilisation of the particles. Finally, the particles were re-suspended in 1 mL of water to obtain a working suspension with an amount of 1.25 mg magnetic particle per mL of volume. For plasma sample extraction further on, 100 µL of sample and 50 µL of the 1.25 mg mL^{-1} working magnetic particle solution were applied. This gives a total of 62.5 µg of extraction material added to the sample. The ideal amount of magnetic particle per µL of sample was discovered by serial recovery testing in a range between 10.25 µg and 102.5 µg per 100 µL of sample.

For the purposes of internal standardisation, a solution containing 10 mg L^{-1} of mycophenolic acid carboxybutoxy ether in acetonitrile/water (1/1, vol/vol) was used.

As the first step of sample preparation, 20 µL of this internal standard solution were pipetted into a 1.5 mL polypropylene microcentrifuge tube containing 100 µL of plasma sample. After vortex mixing, 50 µL of the ferromagnetic micro-particle working solution were added, which was mixed by aspirating and dispensing five times with a pipette tip. For analyte adsorption to the extraction material, the samples were left at room temperature for five minutes. Thereafter, the tubes were placed on the magnetic separator and particle free, extracted matrix was discarded. The tubes were then removed from the magnet and 100 µL of washing solution (0.1% trifluoroacetic acid) were added. Again, mixing was done by pipetting up and down five times. After immobilisation of the particles, the washing solution was removed. This washing step was repeated. For analyte elution, the particles were re-suspended in 100 µL of a desorption solution (50% acetonitrile in 0.1% trifluoroacetic acid) and incubated at room temperature for five minutes. The particles were immobilized by the magnet separator and the eluate was transferred into HPLC vials with micro inserts.

The LC-MS/MS system consisted of a Waters Alliance 2975 HPLC coupled to a Waters Quattro Micro triple stage mass spectrometer.

A Sunfire C18 (100 mm × 2.1 mm, 3.5 µm) (Waters, Eschborn, Germany) was used for analysis, equipped with a LiChroCart C18 pre-column (Merck, Darmstadt, Germany). The column oven was set to 30°C, the injection volume was 20 µL. The mobile phase consisted of acetonitrile/0.1% formic acid (1/1 vol/vol).

The MS/MS instrument was run in positive ionization mode with the following settings: capillary voltage was 1.36 kV, cone voltage 30 eV, collision energy 25 eV, source temperature 120°C and desolvation temperature 350°C. For mycophenolic acid, the mass transition *m/z* 321 → 207 was recorded and for the internal standard mycophenolic acid carboxybutoxy ether *m/z* 421 → 207.[14]

Validation

Specificity of the method was investigated by analysis of blank, drug-free serum samples, not adding the internal standard. There were no peak signals detected at the retention time of the analyte and the internal standard (mycophenolic acid: 6.7 min; mycophenolic acid carboxybutoxy ether: 6.0 min). The limit of detection (LOD) was found at 0.3 mg L^{-1}. A representative chromatogram is shown in Figure 1. It was investigated without addition of the internal standard. Signal-to-noise ratio was greater than 10 : 1. The limit of quantitation (LOQ) was found at 0.5 mg L^{-1} (coefficient of variation ≤ 15% for the six-fold measurement of a sample).

In order to test the linearity of the analytical system, an eight-point calibration series of mycophenolic acid spiked plasma (0.5/1/2/4/5/8/10/15 mg L^{-1}) was analysed. A linear calibration function was observed (r^2>0.99). For the following quantification runs, a five point plasma-based calibration curve was used (1/2/5/10/15 mg L^{-1}).

For purposes of testing the recovery of the extraction method, washing solutions obtained during the analyses of spiked samples (3.5 mg L^{-1}, n = 3) were analysed by HPLC/MS-MS for mycophenolic acid and the internal standard

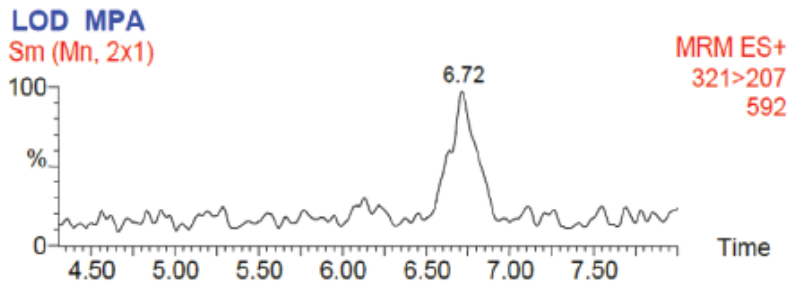

Figure 1. Chromatogram for mycophenolic acid showing the limit of detection at a concentration of 0.3 mg L^{-1}.

compound. No analytes were found and thus a recovery rate of nearly 100% can be supposed.

The extent of ion suppression was tested by applying the post column infusion experiment:[15] A solution of mycophenolic acid and mycophenolic acid carboxybutoxy ether (1 mg L^{-1}) was infused with a syringe pump at a constant rate, using a T-piece into the column effluent prior to continuous transfer to the MS/MS instrument. From this configuration, a background signal was generated. The impact of the injection of matrix samples on this background signal was observed. Upon injection of extract, which was obtained by magnetic particle sample preparation from a pooled blank plasma sample, a drop in the background signal intensity was observed in a typical manner. However, this period of ion suppression clearly preceded the elution of the analyte (mycophenolic acid: 6.7 min; mycophenolic acid carboxybutoxy ether: 6.0 min). As a result, it can be concluded that the method is free from relevant ion suppression.

For validation, three quality control (QC) samples in the typical concentration range were prepared by spiking drug-free human plasma with mycophenolic acid (QC1 = 2.1 mg L^{-1}; QC2 = 3.5 mg L^{-1}; QC3 = 10.2 mg L^{-1}). Independent from the process of sample preparation, the precision of the instrument configuration was tested as a system suitability check by repeatedly injecting the extract of QC 3 ($n = 6$) from one vial. The coefficient of variation was 1.6%.

Reproducibility and accuracy of the entire method was tested over four series ($n = 6$).

A coefficient of variation of less than 10% was observed for the three QC samples (Table 1). Standard deviation was presented as an error bar (Figure 2).

Agreement of the results, obtained using this LC-MS/MS method, with our routine method, based on HPLC-UV and a commercial kit (Chromsystems, München, Germany), was tested using eight residual samples sent for clinically indicated mycophenolic acid monitoring. The range of deviation between these two measurements was ±6.7% (Table 2).

Discussion

We were able to demonstrate that the use of chromatographically modified ferromagnetic micro-particles for the sample preparation of LC-MS/MS methods can realize a level of performance that is required for analyses in clinical laboratories. Up to now, only the mere feasibility of this approach, without clinically relevant validation data, has been shown. With our micro-particle-based protocol, we obtained extracts that did not induce relevant ion suppression, a key goal for sample preparation in biomedical LC-MS/MS analyses.

By analysing mycophenolic acid in the washing solution, we were able to demonstrate that no target analyte was lost during washing and that the sample clean-up was effective. Proteins were removed with the washing solution. The analyte elution was effective with a low background signal. Background signal was proven by the analysis of blank, drug free human serum.

The essential strength of magnetic particle-based sample preparation is the fact that efficient solid-phase extraction can be performed, while this solid phase can be handled as a fluid. This is a huge advantage in comparison to cartridge-based approaches. In such standard solid-phase extraction applications, positive or negative pressure has to be applied in order to pass sample materials through the packed extraction particles, which is technically demanding. Furthermore,

Table 1. Reproducibility of the magnetic particle-based LC-MS/MS method for the quantification of mycophenolic acid in plasma. $n = 24$

Target concentration (mg L^{-1})	Observed mean concentration (mg L^{-1})	Interassay coefficient of variation (%) ($n = 24$)
2.1 (QC 1)	1.9	7.8 %
3.5 (QC 2)	3.2	3.1 %
10.2 (QC 3)	9.6	5.6 %

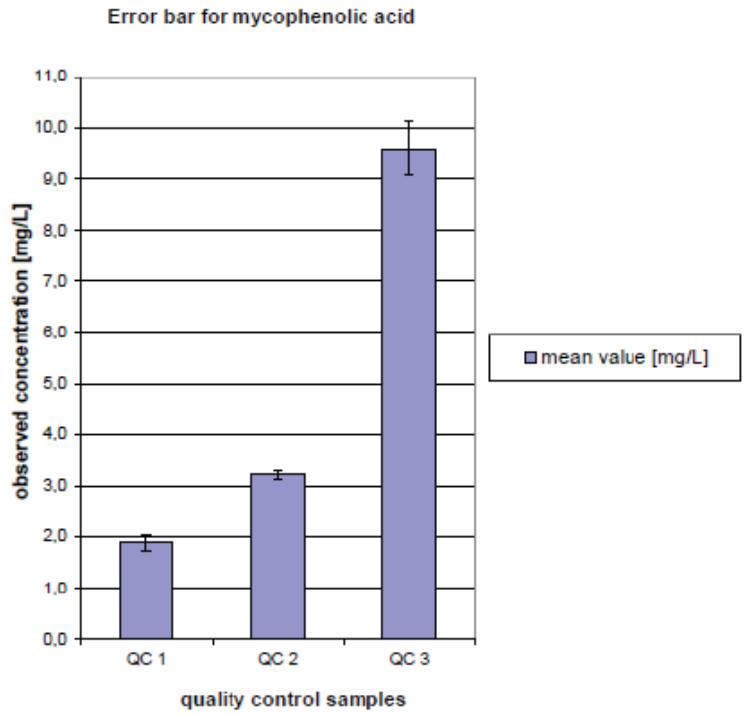

Figure 2. Standard deviation for all three quality control samples presented as error bar.

classical methods generate large volumes of solid waste from infections. Since interaction of the liquid samples with the packed extraction particles is fairly poor, large amounts of rather expensive materials are required. While conventional, solid-phase extraction cartridges contain roughly 100 mg of extraction material, we were able to reduce the volume of particles to less than 100 µg per sample.

Sample clean-up using functionalized ferromagnetic micro-particles may indeed open up new horizons in clinical mass spectrometry applications. Variation of particles with different surface modifications, as well as variation in washing and desorption solutions, offer a wide spectrum of potential analytes amenable to this technology in the future.

The use of coated, ferromagnetic micro-particles has become a key technology of modern immunoassays in clinical laboratories.[16] Our data and experience with a clinically-relevant example analyte further encourages the vision that the same could become true for clinical LC-MS/MS analyses

Table 2. Comparison of mycophenolic acid plasma concentration results, obtained by the clinical routine method (HPLC UV, reagent kit from Chromsystems, Munich, Germany) and the magnetic particle-based LC-MS/MS method.

#	HPLC-UV, Chromsystems ($mg\,L^{-1}$)	Ferromagnetic micro-particles ($mg\,L^{-1}$)	Bias %
1	1.5	1.4	−6.7
2	4.1	3.9	−4.9
3	4.3	4.5	4.7
4	2.4	2.3	−4.2
5	0.8	0.8	0.0
6	3.1	2.9	−6.5
7	3.4	3.3	−2.9
8	2.3	2.4	4.3

and stimulates respective continued work. As a next step towards achieving this goal, the implementation of magnetic particle-based extraction protocols on robotic liquid-handling systems seems warranted now. When using and combining different particle surface modifications, this technology may vastly facilitate the development of generic and convenient front-end modules for clinical mass spectrometry.

Acknowledgement

This study was supported by the Joint German Association of Clinical Chemistry and Laboratory Medicine (DGKL; Stiftung Pathophysiologie und Molekulare Diagnostik).

References

1. M. Vogeser, A. Geiger, R. Herrmann and U. Kobold, "Sample preparation for liquid chromatography-tandem mass spectrometry using functionalized ferromagnetic micro-particles", *Clin. Biochem.* **41,** 1417 (2008). doi: 10.1016/j.clinbiochem.2008.08.001
2. I.I. Sukhanova, M.A. Dikunets, E.D. Viryus and G.M. Rodchenkov, "Magnetic separation as a new method for the extraction of small molecules from biological fluids of humans", *J. Anal. Chem.* **66,** 9:807-814 (2011). doi: 10.1134/S1061934811090164
3. G.M. Fiedler, A.B. Leichtle, J. Kase, S. Baumann, U. Ceglarek, K. Felix, T. Conrad, H. Witzigmann, A. Weimann, C. Schutte, J. Hauss, M. Buchler and J. Thiery, "Serum peptidome profiling revealed platelet factor 4 as a potential discriminating peptide associated with pancreatic cancer", *Clin. Cancer Res.* **15,** 3812 (2009). doi: 10.1158/1078-0432.CCR-08-2701
4. G.M. Fiedler, S. Baumann, A. Leichtle, A. Oltmann, J. Kase, J. Thiery and U. Ceglarek, "Standardized peptidome profiling of human urine by magnetic bead separation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry", *Clin. Chem.* **53,** 421 (2007).doi: 10.1373/clinchem.2006.077834
5. J. Verheyen, R. Kaiser, M. Bozic, M. Timmen-Wego, B.K. Maier and H.H. Kessler, "Extraction of viral nucleic acids: comparison of five automated nucleic acid extraction platforms", *J. Clin. Virol.* **54,** 255 (2012). doi: 10.1016/j.jcv.2012.03.008
6. P.L. Kole, G. Venkatesh, J. Kotecha and R. Sheshala, "Recent advances in sample preparation techniques for effective bioanalytical methods", *Biomed. Chromatogr.* **25,** 199 (2011). doi: 10.1002/bmc.156
7. M. Vogeser and U. Spohrer, "Pitfall in the high-throughput quantification of whole blood cyclosporin A using liquid chromatography-tandem mass spectrometry", *Clin. Chem. Lab. Med.* **43,** 400 (2005). doi: 10.1515/CCLM.2005.072
8. M. Vogeser and C. Seger, "A decade of HPLC-MS/MS in the routine clinical laboratory—goals for further developments", *Clin. Biochem.* **41,** 649 (2008). doi: 10.1016/j.clinbiochem.2008.02.017
9. J.M. van den Ouweland and I.P. Kema, "The role of liquid chromatography-tandem mass spectrometry in the clinical laboratory", *J. Chromatogr. B* **883/884,**1 (2012). doi: 10.1016/j.jchromb.2011.11.044
10. C.G. Patel, A.E. Mendonza, F. Akhlaghi, O. Majid, A.K. Trull, T. Lee and D.W. Holt, "Determination of total mycophenolic acid and its glucuronide metabolite using liquid chromatography with ultraviolet detection and unbound mycophenolic acid using tandem mass spectrometry", *J. Chromatogr. B* **813,** 1-2:287-294 (2004). doi: 10.1016/j.jchromb.2004.10.004
11. T.M. Annesley and L.T. Clayton, "Quantification of mycophenolic acid and glucuronide metabolite in human serum by HPLC-tandem mass spectrometry", *Clin. Chem.* **51,** 872 (2005). doi: 10.1373/clinchem.2004.047357
12. A. Buchwald, K. Winkler and T. Epting, "Validation of an LC-MS/MS method to determine five immunosuppressants with deuterated internal standards including MPA", *BMC Clin. Pharmacol.* **12,** (2012), doi: 10.1186/1472-6904-12-2
13. I.S. Westley, B.C. Sallustio and R.G. Morris, "Validation of a high-performance liquid chromatography method for the measurement of mycophenolic acid and its glucuronide metabolites in plasma", *Clin. Biochem.* **38,** 824 (2005). doi: 10.1016/j.clinbiochem.2005.05.008
14. M. Vogeser, R. Zachoval, U. Spohrer and K. Jacob, "Potential lack of specificity using electrospray tandem-mass spectrometry for the analysis of mycophenolic acid in serum", *Ther. Drug Monit.* **23,** 722 (2012).
15. T.M. Annesley, "Ion suppression in mass spectrometry", *Clin. Chem.* **49,** 1041 (2003).
16. H.T. Kuo, J.Z. Yeh, C.M. Jiang and M.C. Wu, "Magnetic particle-linked anti hCG beta antibody for immunoassay of human chorionic gonadotropin (hCG), potential application to early pregnancy diagnosis", *J. Immunol. Methods* **381,** 32 (2012). doi: 10.1016/j.jim.2012.04.006

2.2 König, K.; Goethel, S.; Rusu, V.; Vogeser, M. „Deproteination of serum samples for LC-MS/MS analyses by applying magnetic micro-particles“ Clin. Biochem. 2013, 46, 652-655.

Clinical Biochemistry 46 (2013) 652–655

Contents lists available at SciVerse ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Deproteination of serum samples for LC–MS/MS analyses by applying magnetic micro-particles

Katrin König [a,*], Sven F. Goethel [b], Viorel M. Rusu [b], Michael Vogeser [a]

[a] Institute of Laboratory Medicine, Hospital of the University of Munich, Munich, Germany
[b] MagnaMedics Diagnostics BV, Geleen, The Netherlands

ARTICLE INFO

Article history:
Received 4 October 2012
Received in revised form 20 December 2012
Accepted 10 January 2013
Available online 18 January 2013

Keywords:
Magnetic micro particles
LC–MS/MS
Quantification
Sample preparation
Protein depletion
Automation

ABSTRACT

Objectives: Investigation of the practicability and performance of a magnetic micro-particle based method for protein depletion of serum samples, preceding the quantitative analysis of small molecules by LC–MS/MS.

Design and methods: A commercially available kit including a protein denaturation reagent and functionalized magnetic particles together with a magnetic separator device was tested by addressing the quantification of amiodarone in serum as an exemplary analyte by LC–MS/MS with on-line SPE. A standard method validation protocol was applied.

Results: The sample preparation protocol was found to be convenient, straightforward and robust. Validation data characterized the entire analytical method – combining particle-based protein depletion and two-dimensional chromatography – as compatible with the analytical needs regarding selectivity, accuracy (102–106%), linearity ($r^2 \geq 0.99$), reproducibility (CV <7%), and control of ion suppression.

Conclusions: Since this novel approach of sample preparation does neither require centrifugation nor the technically demanding application of positive or negative pressure, as in conventional solid phase extraction protocols, it seems highly attractive for developing fully automatized preparation systems for LC–MS/MS analyzers.

1. Introduction

For more than a decade, the technology of LC–MS/MS has been demonstrating its usefulness and applicability in laboratory medicine [1]. Essential strengths of this technology include the straightforward implementation of new methods without the need for antibody development, its capability of performing multi-analyte quantification, and high analytical reliability, in particular when applying the principle of stable isotope dilution internal standardization. However, still the application of this powerful technology is limited to rather specialized laboratories and cannot be considered a standard technology in clinical laboratories. Reasons for this can be found in the fact that there is still no commitment of big companies in the diagnostic industry towards this technology, and the expensive instrumentation required, as well as the need for laborious protocols for sample preparation. Consequently, concepts to streamline sample preparation are of utmost importance for further development in clinical mass spectrometry; this particularly includes the goal of full automation of the entire analytical process in LC–MS/MS [1,2].

Thanks to the high analytical specificity of LC–MS/MS, the requirements of sample preparation are rather limited for this technology, for instance, when being compared to conventional HPLC, LC–MS/MS applies far less analyte-specific UV-detection. However, a main requirement for quantification of small molecules using LC–MS/MS is the removal of proteins from serum or plasma samples. This is due to the fact that the latter interfere with conventional chromatographic separation and electrospray ionization. Protein precipitation by organic solvents, salts or acids – followed by centrifugation – is the most widely applied technique for protein removal from blood derived materials. This approach is rather cost-effective and straightforward; on the other hand, it is difficult to include centrifugation into robotic automation solutions, especially when random access instead of batching is desired. As an alternative to centrifugation, deproteination following protein denaturation can be achieved by ultrafiltration. Solutions for this aim are commercially available, based on 96 well filtration plates [3–5]. However, this process cannot be easily automated since positive pressure or vacuum has to be applied. Besides this, the required consumables are expensive and batching is required.

Surface-functionalized magnetic micro-particles have been suggested for sample preparation protocols in different fields of mass-spectrometric analyses, addressing large and small molecules alike [6–9]. Magnetic-particle based protocols are ideally suited for automation since no pressure or vacuum has to be applied. As a matter of fact, this basic principle of sample manipulation experienced a high acceptance and widespread use in immunoanalyzers, as well as in fully automated DNA-extraction.

For the first time, the application of a kit for deproteination of serum samples, based on protein-denaturation by using a proprietary reagent which is supported by magnetic micro-particles (MagnaMedics

* Corresponding author at: Institute of Laboratory Medicine, Hospital of the University of Munich, Munich, Germany, Marchioninistr. 15, 81375 München, Germany.
E-mail address: katrin.koenig@med.uni-muenchen.de (K. König).

0009-9120/$ – see front matter © 2013 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.clinbiochem.2013.01.003

Diagnostics BV, Geleen, The Netherlands) is herewith reported. The aim of our study was to investigate the practicability and performance of this technology within the context of a LC–MS/MS with on-line SPE method for quantification of an exemplary, clinically relevant analyte.

2. Methods and results

2.1. Chemicals and reagents

Stock solutions of amiodarone hydrochloride (Sigma-Aldrich, Schnelldorf, Germany) and amiodarone-D4 (Toronto Research Chemicals, North York, Canada) with a concentration of 100 mg/L were separately prepared in methanol (J.T. Baker, Griesheim, Germany) and stored at −20 °C. Seven calibrators (0.2, 0.5, 1.1, 1.6, 2.2, 3.3, and 5.4 mg/L) and four quality control samples (QCs) (0.3, 1.1, 1.6 and 2.7 mg/L) were prepared by spiking drug-free human serum.

2.2. HPLC–MS/MS conditions

The LC–MS/MS system consisted of a Waters Alliance 2975 HPLC-module, coupled via a switching-valve with a Waters Quattro Micro MS/MS-instrument (Waters, Milford, USA). Waters XBridge C8 (100 mm × 3.5 mm, 2.1 µm), equipped with a LiChroCart C8 pre-column (Merck, Darmstadt, Germany), was used as the analytical column. As described previously [10], on-line solid phase extraction was applied, using Waters OASIS HLB (20 mm × 2.1 mm, 25 µm) as the extraction column and an additional isocratic HPLC pump (Waters 600). The mobile phase for the analytical column was 90% methanol/10% water (J.T. Baker, Griesheim, Germany) containing 0.1% formic acid (Merck, Darmstadt, Germany) delivered with a flow rate of 0.4 mL/min (kept at 40 °C). The HPLC pump flow for the extraction column was set at 4.0 mL/min with a mobile phase of 90% water and 10% methanol for sample clean-up. The injection volume was 20 µL. The total analytical run-time was 6.5 min.

The MS/MS instrument was run in positive ionization mode with the following settings: capillary voltage of 2.0 kV, cone voltage of 40.0 V, collision energy of 30 eV, source temperature set at 120 °C, and desolvation temperature set at 350 °C. For amiodarone, the mass transition m/z 646 → 201 was recorded, and for deuterated amiodarone the mass transition was m/z 650 → 201 [11].

2.3. Sample preparation

For preparation of serum samples, a pre-commercial MagSi kit was used (MagnaMedics Diagnostics BV, Geleen, The Netherlands); this includes a suspension of proprietary surface-modified MagSi magnetic micro particles and a proprietary protein denaturation reagent. For extraction, 100 µL of serum was added into a 1.5 mL polypropylene microcentrifuge tubes. Subsequently, 25 µL of amiodarone internal standard solution and 25 µL of magnetic particle suspension were added. After adding 200 µL of precipitation organic-solvent mix from the kit, the tubes were vortex mixed and incubated at room temperature for 2 min. In addition, the samples were sonicated for about 5 s, as suggested by the manufacturer. Then, the tubes were placed in a magnetic particle separator (MagnaMedics MM-Separator M 12 + 12) in order to immobilize the micro-particles towards the wall of the tube. After a few seconds, the particle-free fluid was transferred with a pipette directly from the tubes into HPLC-vials for LC–MS/MS analysis with on-line SPE.

3. Results

3.1. Validation

A full validation protocol, according to FDA Guidelines, was conducted, addressing specificity, accuracy, intra- and inter-day imprecision, limit of detection, limit of quantitation, linearity, recovery, stability of processed samples and defrosting stability [12].

Linear response of the calibration series ranging, from 0.2 to 5.4 mg/L, was observed in all validation series. A linear calibration function, weighted 1/x, with a regression coefficient of (r^2) ≥ 0.99 was observed.

In order to assess the specificity/selectivity of the assay, a serum-pool taken from intensive-care patients who did not receive amiodarone was analyzed six times without adding an internal standard. There were no interferences at the retention time of amiodarone (3.7 min).

Limit of detection was defined as the lowest concentration, which could be measured with a signal to noise ratio ≥ 3/1. A serum sample spiked to a concentration of amiodarone of 0.03 mg/L was analyzed in three replicates. The achieved signal to noise ratio was greater than 10/1. In order to assess the limit of quantitation, six samples at a concentration of 0.1 mg/L were prepared. The results were reproducible, at an accuracy of 90–110%.

Recovery of the extraction method was tested by comparing the results of extracted samples at four concentrations (QCs) with unextracted standard solutions spiked to the same concentrations representing 100%. The mean recovery for amiodarone was rated as 98%. Peak areas for amiodarone and internal standard were similar.

In order to test the accuracy and imprecision of the entire method, four quality control samples in typical concentration levels were prepared by spiking serum (target concentrations: QC 1, 0.3 mg/L; QC 2, 1.1 mg/L; QC 3, 1.6 mg/L; and QC 4, 2.7 mg/L). Accuracy was assessed by performing five determinations per QC. The following results were observed: QC 1, 101%; QC 2, 106%; QC 3, 102%; and QC 4, 102%. Accuracy and imprecision was tested in four independent series; in these series, each of the four QC samples were analyzed six times, resulting in 24 results per sample. The results for intra- and interday precision are presented in Table 1. Coefficients of variation of less than 7% were observed for all samples.

Following an initial analysis, the stability of the analyte in the processed samples was determined by storing the HPLC vials containing the extract for 24 h at room temperature. A visual inspection showed no clouding. Repetition of the analytical run after 24 h delivered the following results, in relation to the initial ones (n = 6 each): QC 1, 98%; QC 2, 101%; QC 3, 101%; and QC 4, 99% (Table 2). Results and peak areas remained at the same level.

For investigation of ion suppression effects at the elution time of amiodarone, post column infusion was applied [13]. By infusing a solution of amiodarone (0.5 mg/L) with a syringe pump and a T-piece into the column effluent, a background signal was generated. The injection of an extract from the pooled serum samples without amiodarone resulted in a decrease of the background signal for several seconds; however, at the retention time of amiodarone baseline ionization, efficacy was regained, thus excluding substantial suppression of the ionization of amiodarone by sample extracts. Matrix effects were further investigated by post-extraction spiking experiments in three individual serum samples. The response of spiked extracted samples was analyzed in comparison to spiked neat extraction solvent. For calculation of the

Table 1
Validation results for the quantification of amiodarone by isotope dilution MS/MS with a sample preparation protocol based on micro-particle supported deproteination and two-dimensional chromatography.

Target concentration	QC 1 (0.3 mg/L)	QC 2 (1.1 mg/L)	QC 3 (1.6 mg/L)	QC 4 (2.7 mg/L)
Intra assay (n = 6)				
Observed mean conc.	0.3	1.2	1.7	2.8
CV %	6.6	4.4	3.7	2.7
Inter assay (n = 24, 4 series)				
Observed mean conc.	0.3	1.1	1.6	2.8
CV %	6.1	6.8	6.2	3.0

Table 2
Stability of processed samples, stored for 24 h at room temperature. Recovery was determined in relation to the first measurement.

	QC 1 (0.3 mg/L)	QC 2 (1.1 mg/L)	QC 3 (1.6 mg/L)	QC 4 (2.7 mg/L)
24 h at RT (n=6)				
CV %	0.3	3.4	4.4	1.9
Recovery %	98	101	101	99

matrix effect, the formula described by Matuszewski et al. was used [14,15]. A mean value of −9% was observed, which indicated a low degree of ion suppression.

The robustness of the HPLC system was monitored by documenting column back-pressure and on-line extraction column back-pressure (Fig. 1). During the process of method development and validation, nearly 600 serum samples were measured, while the back-pressure of both columns remained constant.

We used residual 10 serum samples from patients on amiodarone for a comparison between the MagSi kit and the application of conventional deproteination by precipitation with organic solvents. For this, we used 200 µL of acetonitrile/methanol (9/1) added to 100 µL of serum, vortexed for 2 min and centrifuged for 10 min at 16,000 g. The serum amiodarone concentrations ranged from 0.6 to 3.3 mg/L. Very close agreement of the results was observed ($r^2=0.99$; no statistically significant difference according to the paired *t*-test ($p=0.40$); differences of less than 5% for individual sample).

With the same set of samples we tested a potential effect of omitting the sonication step which is recommended for the MagSi kit. Excellent agreement was also found for this experiment ($r^2=0.99$; differences of less than 5% for individual samples).

4. Discussion

With this paper, we report for the first time about a micro-particle based technology for deproteination of serum samples, coupled with two-dimensional liquid chromatography-fractionation of the extracts, as a sample-preparation strategy for quantitative MS/MS analysis in clinical diagnostics. This novel concept was tested and validated for amiodarone as an exemplary analyte of clinical relevance, addressed by isotope dilution internal standardization. Our validation results were fully compliant with the requirements of clinical laboratory testing and we experienced the handling as very convenient and straightforward. In particular very good control of matrix effects was observed, and a high degree of robustness and close agreement of results were obtained using conventional protein precipitation by organic solvents and centrifugation.

The evaluation study was performed in a manual protocol format; as a goal for the near future, however, our work aims to fully automate application, and implement it on robotic liquid handling systems. Hardware components for such manipulation of magnetic micro-particles on respective platforms are available, but may require further optimization.

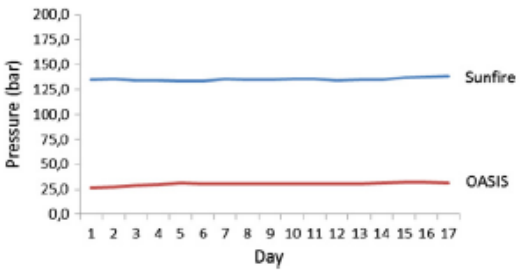

Fig. 1. Back-pressure of analytical column (Sunfire) and on-line extraction column (OASIS) during method development and validation involving nearly 600 sample injections.

The sonication step recommended by the manufacturer of the kit was found to be unnecessary, which facilitates automation. When using manual application of the MagSi kit, relevant time-saving compared to conventional protein precipitation is achieved since no centrifugation is required.

Protein removal is essential for the chromatographic separation of small molecules using LC–MS/MS, since proteins cause clogging of standard HPLC columns after few injections. Conventional approaches towards sample deproteination include protein denaturation using organic solvents, salts or acids, followed by centrifugation for precipitation or filtration of the denatured proteins by using filtration devices which have become available in 96-well format during recent years [3–5]. This latter approach, however, requires the application of vacuum or positive pressure, which is rather complex in pipetting systems. In contrast, the micro-particle supported principle of protein removal, which is described in this paper, can, by far, be more easily automated and cause no infectious solid waste. Neither centrifugation nor application of pressure is required, but merely a time-controlled application of a magnetic field, just like what has been realized in a number of immunoanalyzer systems today.

We have previously demonstrated the applicability of C18-surface modified magnetic micro-particles [6,7] for sample preparation, involved in the quantification of itraconazole, as a representative analyte. In contrast to this generic, multi-step application, the specific compositions of the kit's components used for our present study, are proprietary and have not been disclosed by the manufacturer. However, compared to the earlier reports on protocols for the preparation of biological samples for quantitative small-molecule MS/MS-analytics, the approach tested in our present study is substantially more straightforward: the particles need to be immobilized only once during the whole process, since no separate step for washing or elution is required. Clean-up of the samples beyond this one-step protein-removal – in particular addressing salts, amino acids, and phospholipids, as well as removal of protein-denaturating compounds from the kit and remaining matrix constituents – and concentration of the analyte is achieved by automated solid phase extraction (SPE) in our protocol. Finally, fractionation of the extracted compounds, according to the individual requirements of an analyte-specific MS/MS-assay, is realized by HPLC. Thus, a combined and potentially fully automated, three-step sample processing work-flow has been developed (particle supported protein removal > on-line solid phase extraction > reversed phase chromatography). Given the wide range of materials available for on-line SPE and chromatographic separation, it can be assumed that a very wide spectrum of analytes (regarding polarity and molecular mass) can be addressed by this basic protocol. This, however, will require analyte specific optimization and validation.

On-line solid phase extraction, based on constantly used extraction columns and column-switching – which represents a part of our suggested three-step approach – has been recognized as a very powerful and convenient technology for sample clean-up in clinical tandem mass-spectrometry. By now, this has been applied for a variety of analytes (e.g., immunosuppressants) [16] in a large number of routine laboratories for more than a decade now. The principle of turbo flow chromatography [17] claims the potential for direct analysis of serum samples, however, at the price of very high flow-rates and substantial consumption of expensive chromatography solvents. Nevertheless, efficient protein removal is found to be necessary for most extraction columns, in order to realize the cycle numbers of several thousands of samples with on-line SPE.

Still, the pre-dominant obstacle for a widespread application of isotope dilution MS/MS in the setting of clinical routine laboratories is the evident lack of comprehensive automation [1,2]. Our results and experiences suggest to further address the concept of particle supported protein precipitation with subsequent on-line SPE as a promising and versatile technology for realizing fully-automated, random-access LC–MS/MS analyzers for clinical laboratories in the near future.

Acknowledgment

This study was supported by the Joint German Association of Clinical Chemistry and Laboratory Medicine (DGKL; Stiftung Pathophysiologie und Molekulare Diagnostik).

MagnaMedics Diagnostics BV (Geleen, The Netherlands) supported the study by providing the MagSi kit free of charge; no further funding was obtained from MagnaMedics.

References

[1] Vogeser M, Seger C. A decade of HPLC–MS/MS in the routine clinical laboratory—goals for further developments. Clin Biochem 2008;41:649–62.
[2] Vogeser M, Kirchhoff F. Progress in automation of LC–MS in laboratory medicine. Clin Biochem 2011;44:4–13.
[3] Kole PL, Venkatesh G, Kotecha J, Sheshala R. Recent advances in sample preparation techniques for effective bioanalytical methods. Biomed Chromatogr 2011;25:199–217.
[4] Williams MG, Palandra J, Shobe EM. Rapid determination of rat plasma uridine levels by HPLC–ESI-MS utilizing the Captiva plates for sample preparation. Biomed Chromatogr 2003;17:215–8.
[5] Smalley J, Xin B, Olah TV. Increasing high-throughput discovery bioanalysis using automated selected reaction monitoring compound optimization, ultra-high-pressure liquid chromatography, and single-step sample preparation workflows. Rapid Commun Mass Spectrom 2009;23:3457–64.
[6] Vogeser M, Geiger A, Herrmann R, Kobold U. Preparation of plasma samples for chromatographic analyses using functionalized ferromagnetic micro-particles manipulated in a high pressure liquid system. Clin Biochem 2009;42:915–8.
[7] Vogeser M, Geiger A, Herrmann R, Kobold U. Sample preparation for liquid chromatography–tandem mass spectrometry using functionalized ferromagnetic micro-particles. Clin Biochem 2008;41:1417–9.
[8] Fiedler GM, Baumann S, Leichtle A, Oltmann A, Kase J, Thiery J, et al. Standardized peptidome profiling of human urine by magnetic bead separation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Chem 2007;53:421–8.
[9] Fiedler GM, Leichtle AB, Kase J, Baumann S, Ceglarek U, Felix K, et al. Serum peptidome profiling revealed platelet factor 4 as a potential discriminating peptide associated with pancreatic cancer. Clin Cancer Res 2009;15:3812–9.
[10] Vogeser M, Schiel X, Spohrer U. Quantification of voriconazole in plasma by liquid chromatography–tandem mass spectrometry. Clin Chem Lab Med 2005;43:730–4.
[11] Kuhn J, Gotting C, Kleesiek K. Simultaneous measurement of amiodarone and desethylamiodarone in human plasma and serum by stable isotope dilution liquid chromatography–tandem mass spectrometry assay. J Pharm Biomed Anal 2010;51:210–6.
[12] Guidance for industry-bioanalytical method validation. U.S. Department of Health and Human Services—Food and Drug Administration; 2001 [http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf].
[13] Annesley TM. Ion suppression in mass spectrometry. Clin Chem 2003;49:1041–4.
[14] Chambers E, Wagrowski-Diehl DM, Lu Z, Mazzeo JR. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. J Chromatogr B Analyt Technol Biomed Life Sci 2007;852:22–34.
[15] Matuszewski BK, Constanzer ML, Chavez-Eng CM. Matrix effect in quantitative LC/MS/MS analyses of biological fluids: a method for determination of finasteride in human plasma at picogram per milliliter concentrations. Anal Chem 1998;70:882–9.
[16] Seger C, Tentschert K, Stoggl W, Griesmacher A, Ramsay SL. A rapid HPLC–MS/MS method for the simultaneous quantification of cyclosporine a, tacrolimus, sirolimus and everolimus in human blood samples. Nat Protoc 2009;4:526–34.
[17] Kozak M, Ruzicka R, Bodepudi V, Zonderman J. Quantitative analysis of immunosuppressant drugs in whole blood using high throughput LC–MS/MS. San Jose, CA, USA; Fremont, CA, USA; Franklin, MA, USA: Thermo Fisher Scientific; 2010 [Application Note: 487b].

2.3 König et al. „Quantification of vancomycin in human serum by LC-MS/MS“ Clin.Chem. Lab. Med. 2013, 51(9), 1761–1769.

DE GRUYTER DOI 10.1515/cclm-2013-0142 — Clin Chem Lab Med 2013; aop

Katrin König*, Uwe Kobold, Gerhard Fink, Andreas Leinenbach, Thomas Dülffer, Roland Thiele, Johannes Zander and Michael Vogeser

Quantification of vancomycin in human serum by LC-MS/MS

Abstract

Background: The aim of our work was to develop and validate a reliable LC-MS/MS-based measurement procedure for the quantification of vancomycin in serum, to be applied in the context of efforts to standardize and harmonize therapeutic drug monitoring of this compound using routine assays.

Methods: Sample preparation was based on protein precipitation followed by ultrafiltration. In order to minimize differential modulation of ionization by matrix constituents extended chromatographic separation was applied leading to a retention time of 9.8 min for the analyte. Measurement was done by HPLC-ESI-MS/MS. For internal standardization the derivative vancomycin-glycin (ISTD) prepared by chemical synthesis was used, HPLC conditions ensured coelution of ISTD with the analyte.

Results: In a bi-center validation total CVs of <4% were observed for quality control material ranging from 5.3 mg/L to 79.4 mg/L; accuracy was ±4%. No relevant ion suppression was observed. Comparative measurement of aliquots from 70 samples at the two validation sites demonstrated close agreement.

Conclusions: Employing a closely related homologue molecule for internal standardization and the use of MS/MS following highly efficient sample pre-fractionation by HPLC, the method described here can be considered to offer the highest level of analytical reliability realized so far for the quantification of vancomycin in human serum. Thus, the method is suitable to be used in a comprehensive reference measurement system for vancomycin.

Keywords: LC-MS/MS; liquid chromatography; mass spectrometry; serum; vancomycin.

***Corresponding author: Katrin König,** Institute of Laboratory Medicine, Hospital of the University of Munich, Marchioninistrasse 15, 81375 Munich, Germany, Phone: +49 89 70953221, E-mail: katrin.koenig@med.uni-muenchen.de

Uwe Kobold, Gerhard Fink, Andreas Leinenbach, Thomas Dülffer and Roland Thiele: Roche Diagnostics GmbH, Penzberg, Germany

Michael Vogeser and Johannes Zander: Institute of Laboratory Medicine, Hospital of the University of Munich, Munich, Germany

Introduction

The glycopeptide compound vancomycin is one of the most widely used antimicrobial agents for the treatment of serious gram-positive infections including methicillin-resistant *Staphylococcus aureus* (MRSA) [1]. Vancomycin is also among the highest volume target analytes in therapeutic drug monitoring (TDM) for many years now [1–3]. TDM of vancomycin mainly aims to balance therapeutic efficacy against the risk of nephrotoxicity. It has been shown in several studies – applying various analytical methods – that high vancomycin trough levels are associated with the incidence and extend of nephrotoxicity [4, 5]. However, low trough levels of vancomycin may lead to increased occurrence of resistant strains of *S. aureus* and failure of treatment in complicated infections. Based on these issues the American Society of Health-System Pharmacists, the Infectious Diseases Society of America, and the Society of Infectious Diseases Pharmacists have published expert panel recommendations for vancomycin TDM, recommending trough serum concentrations of vancomycin of 15–20 mg/L in complicated infections [1].

These recommendations, however, do not take into consideration that routine serum vancomycin quantification by commercial immunoassays is still lacking between-method standardization and substantial method bias can be found [6]. In the proficiency testing scheme of the German Association of Clinical Chemistry and Laboratory Medicine [DGKL; Referenzinstitut für Bioanalytik (RfB), Bonn, Germany] at present 14 tests are monitored. The bias between the lowest reading vancomycin test and the highest reading test is continuously found in the range of 40% in this external quality assessment program. Considering the clear-cut vancomycin target concentration ranges, it is likely that different clinical dosing decisions are made today in a substantial number of patients depending on the assay which is used in an individual institution. Notably, these consensus target concentrations ranges cannot be traced back conclusively to a defined analytical method.

From these considerations it has to be concluded that improved harmonization and standardization of serum

vancomycin measurement is warranted. A future comprehensive reference measurement system for vancomycin measurement has to include, on the one hand, reliable reference materials, but on the other hand, a robust and reliable method for the specification of working calibration materials, and proficiency testing materials, as well as for the evaluation of routine immunometric tests referring to large reference serum panels. The aim of our work was to develop and to validate such a candidate reference method.

Due to the rather high molecular weight and the limited thermal stability of vancomycin, LC-MS/MS was the most promising technology for this aim. Indeed, several LC-MS/MS methods for the quantification of serum vancomycin concentrations have been described previously [7–9]. These methods, however, rely on internal standard compounds with are structurally not related to the target analyze (teicoplanin, atenolol).

LC-MS/MS-based reference methods [10] usually involve stable isotope labeled compounds for internal standardization. Since vancomycin is a bio-product, production of a respective material is hardly possible. Consequently we designed a suited internal standard compound by stable chemical derivatization of vancomycin which showed almost identical behavior during sample preparation and HPLC-MS/MS separation. The molecule was modified only marginal by introduction of a small functional group not changing the polarity of the molecule leading to a different molecular mass.

The intended use of this method protocol described herein is the specification of serum-based samples within the calibration range of the method in both an industrial and research setting. Consequently, high sample throughput did not have major priority in the development of this method, while robustness and reproducibility in different instrument settings was an essential goal. Thus, we decided to apply highly efficient chromatographic fractionation in order to minimize matrix effects, and to apply an innovative bi-centric validation protocol.

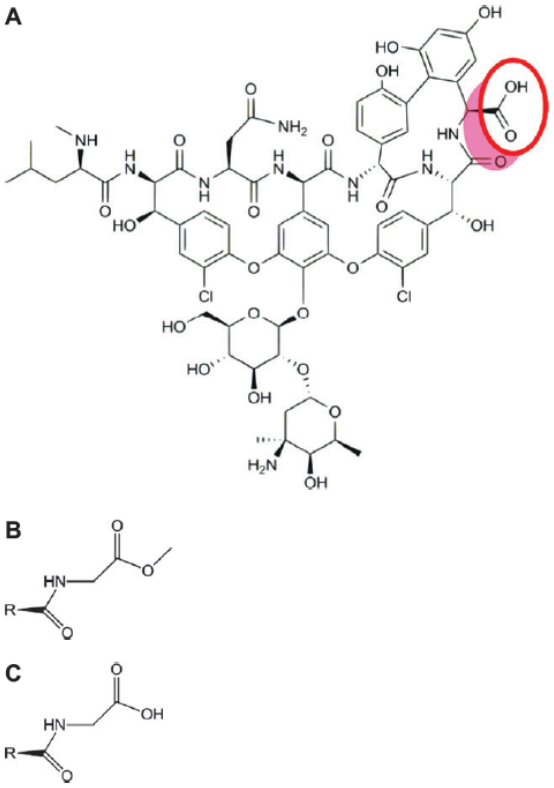

Figure 1 Synthesis of vancomycin-glycine.
(A) Vancomycin (molecular weight: 1449.25; molecular formula: $C_{66}H_{75}Cl_2N_9O_{24}$). The only carbon acid function accessible for formation of an ester derivative is indicated. (B) Peptide coupling of vancomycin to vancomycin-glycine-methyl ester. (C) Saponification of vancomycin-glycine-methyl ester to vancomycin-glycine (molecular weight: 1506.34; molecular formula: $C_{68}H_{78}Cl_2N_{10}O_{25}$).

Materials and methods

Chemicals and reagents

Vancomycin-hydrochloride pure substance was purchased as a USP reference standard from U.S. Pharmacopeia (USP Rockville MD, USA; LOT M0H006; CAS 1404–93–9; molecular weight 1485.71; molecular formula $C_{66}H_{75}Cl_2N_9O_{24}$.HCl; vials containing 99,300 µg vancomycin activity; Figure 1A). USP-based routine human serum calibrators for immunoassays (Cal A, 5 mg/L; Cal B, 40 mg/L; Cal C, 80 mg/L) were purchased from Roche Diagnostics (Mannheim, Germany). The latter samples were used for investigation of accuracy and reproducibility.

Methanol and water (both HPLC-grade) for chromatography were from J.T. Baker (Griesheim, Germany). Formic acid and trichloroacetic acid were from Merck (Darmstadt, Germany).

For synthesis of a derivative of vancomycin to be used as an internal standard, vancomycin-hydrochloride and glycine methyl ester hydrochloride were purchased from Sigma-Aldrich (Steinheim, Germany). O-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU), Dimethyl sulfoxide (DMSO), *N,N*-Dimethylformamide (DMF), diisopropylethylamine, acetic acid, sodium carbonate, water and acetonitrile were from J.T. Baker.

Synthesis of vancomycin-glycine

Peptide coupling

Vancomycin-hydrochloride (200 mg; 135 µmol), glycine methyl ester hydrochloride (33.8 mg; 269 µmol) and HBTU (76.5 mg; 202 µmol) were dissolved in DMSO (2 mL) and DMF (0.66 mL). The resulting

solution was cooled to 0°C, and diisopropylethylamine (117 µL; 673 µmol) was added. After stirring at room temperature for 2.5 h, the mixture was diluted by acetic acid (0.5 mL), water (2 mL) and acetonitrile (0.5 mL). Using preparative reverse phase HPLC (C18; Vydac 218TP152050; 5×25 cm) the reaction product vancomycin-glycine-methyl ester (Vancomycin-Gly-OMe; Figure 1B) was purified and subsequently dried to a white powder (approx. 150 mg).

Saponification of vancomycin-glycine-methyl ester

Vancomycin-Gly-OMe (75 mg; 49.3 µmol) was added to sodium carbonate buffer (2%; 5 mL; pH 10.0) at room temperature. The reaction mixture was stirred for 6 h and then acidified with acetic acid (0.5 mL). The main reaction product vancomycin-glycine (Figure 1C) was again purified by preparative reverse phase HPLC. After drying a white powder was obtained.

For preparative HPLC separation, a Dionex P580P HPG HPLC Binary High-pressure Gradient Pump (Thermo Scientific, Sunnyvale, USA), a Rheodyne switching valve (IDEX, Rohnert Park, USA), a Gynkotek SP-6 detector (Thermo Scientific) and a LKB 2211 Superrac fraction collector (AIE, Haverhill, USA) were used. As column a Vydac C 18 (218TP152050; 5×25 cm) (Grace, Worms, Germany) was used. The mobile phase consisted of Eluent A [aqueous trifluoroacetic acid (0.1% v/v)] and Eluent B [80% acetonitrile/20% water containing trifluoroacetic acid (0.1% v/v)], delivered with a flow rate of 35 mL/min during a run time of 120 min. The gradient elution program was as follows: 100% eluent A for 100 min; increase to 25% eluent B over 3 min, afterwards an increase up to 100% eluent B hold for 7 min. Separation was monitored at 226 nm. The uniform peak fraction eluting around 70 min was collected (Figure 2). The solvent was removed under low pressure and the residue was brought to dryness under vacuum to obtain approximately 40 mg of a white solid.

The final product of this procedure was analyzed by ESI-TOF-MS in the positive mode; this offered the following fragmentation patterns: m/z 1507 (M+H); 1364 [(Vanc-143)$^+$]; 754 [(M+2H)$^{2+}$], which is evidentiary for Vancomycin-Gly; (Vanc-143)$^+$ corresponds to the loss of the 4-amino-4-methyl-5-hydroxy-6-methyl-glucose residue (Figure 1A). Since the molecular structure of the vancomycin molecule offers only one carboxylic acid function which is available for reaction to an ester (Figure 1A), such uniform molecular structure of vancomycin-glycine can be expected.

Preparation of stock solutions, calibrators and quality control samples

The dry pure substance of vancomycin weighted into vials by USP was dissolved in 100 mL of HPLC-grade water according USP handling instructions, leading to a stock solution containing 993 mg/L vancomycin based on USP certificate. An internal standard working solution (5 mg/L) was prepared by dissolving vancomycin-glycine in HPLC-grade water.

Drug-free human serum was spiked with vancomycin stock solution to yield the following five calibrator concentrations: 1.06 mg/L; 21.1 mg/L; 42.2 mg/L; 63.3 mg/L; and 84.4 mg/L. Calibrator 0 was drug free human serum. Calibrator samples were stored at –20°C, the internal standard solution at +4°C during the study.

Quality control samples (QC) in five concentration levels were prepared. QC 1–3 were mixtures of left-over patients' samples found in different concentration ranges in routine analyses (for QC 1, samples between 1 and 9 mg/L; for QC 2, samples between 9 and 13 mg/L; and for QC 3 samples >13 mg/L). QCs 1–3 were used to assess the reproducibility of the method. QC 4 and QC 5 were prepared by spiking drug-free human serum with a vancomycin working solution to a concentration of 40 mg/L, and 80 mg/L, respectively. This

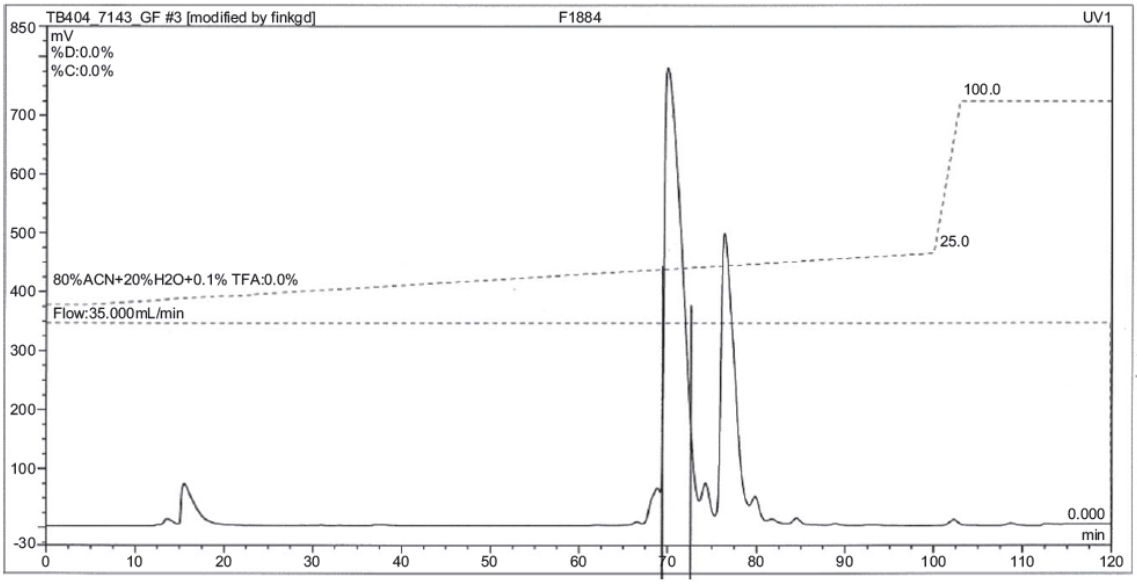

Figure 2 Preparative chromatography applied to isolate vancomycin-glycine eluting after approximately 70 min.

vancomycin solution was prepared separately from the solution used for the preparation of calibrator samples. All QC samples were aliquoted and stored at –20°C until analysis on either validation site.

High-performance liquid chromatography conditions

At laboratory site 1 (Munich) a Waters Alliance 2795, at site 2 (Penzberg) a Waters Acquity LC system was used. As analytical column a Fortis C8 (100 mm×2.1 mm, 3 µm) (dichrom GmbH, Marl, Germany) was used. The column temperature was 40°C. The injection volume was 20 µL. The mobile phase consisted of two solvents; Eluent A: aqueous formic acid (0.1% v/v); Eluent B: methanol containing 0.1% formic acid (0.1% v/v). Run time was 21 min with a flow rate of 0.3 mL/min and an gradient elution program as follows: 100% A for 3 min; linear increase to 30% B over 7 min, hold for 2 min; afterwards a linear increase up to 80% B within 1 min, hold for 2 min; return to the initial condition within 1 min and re-equilibration for 5 min. Via a post-column switching valve, the HPLC eluate was directed into the mass spectrometer between 4.0 and 10.5 min after injection; during the residual run time the eluent was diverged into waste.

Mass spectrometric conditions

Mass spectrometric analysis was performed using Waters Quattro Micro instruments in the positive ionization mode on both laboratory sites. The following settings were applied: Capillary voltage, 3.2 kV; cone voltage, 20.0 V; collision energy, 12 eV; source temperature, 120°C; and desolvation temperature 480°C. The dwell time was 0.2 ms; inter-channel delay, 0.02 ms; and inter-scan delay 0.02 ms. Mass spectrometric data were acquired from 4.5 to 12.0 min after injection.

A collision-induced product ion scan of vancomycin is shown in Figure 3. For vancomycin the mass transition 725>1306 was recorded, and for vancomycin-glycine 753>1362. This corresponds to the doubly charged protonated molecules as the precursor ions, and singly charged fragments as the product ions – leading to higher m/z values of the product ions compared to the precursor ions. Mass resolution was tuned to obtain a mass signal width of 1 Da at 50% height of the product ion signal.

Sample preparation

Seventy-five µL of internal standard working solution and 75 µL of the calibrator sample or of unknown serum sample were pipetted into 1.5 mL polypropylene microcentrifuge tubes. For equilibration the solution was shaken at room temperature for 30 min. Subsequently, 300 µL of trichloroacetic acid (15%) were added. Protein precipitation was achieved by shaking at room temperature for 5 min. The tubes were centrifuged at 14,000 *g* for 10 min. The clear supernatant was pipetted into Amicon Ultra-0.5 mL 10k ultra-filtration devices – (Millipore, Billerica, USA; 10,000 nominal molecular weight cut-off). The devices were placed into a centrifuge and filtration was achieved by centrifugal force for 45 min at 7000 *g*. The filtrate (approx. 450 µL) was transferred into HPLC vials with small-volume inserts.

Five-point calibration was applied in a concentration range from 1.06 to 84.4 mg/L. For quantification the Waters QuanLynx soft-

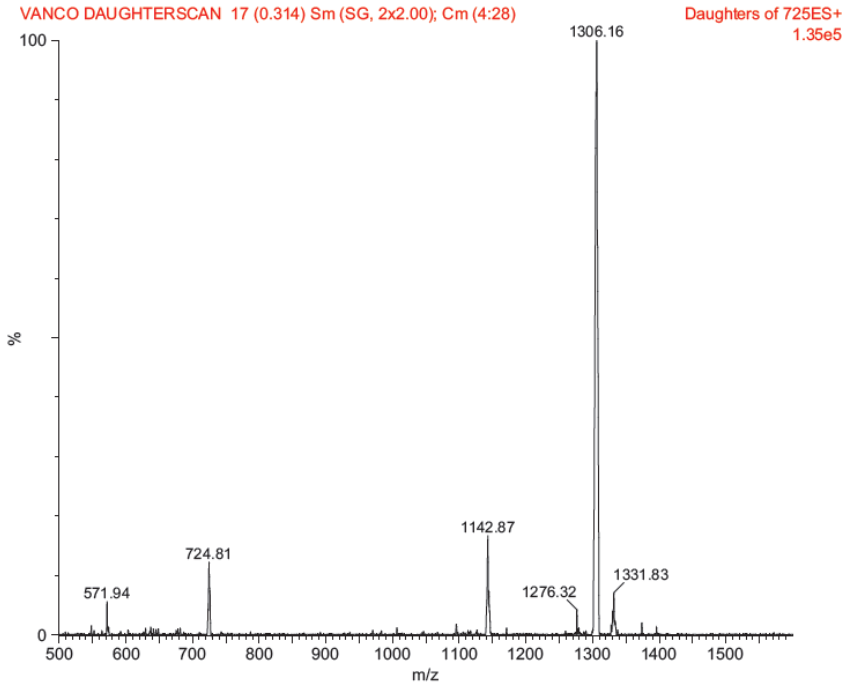

Figure 3 Product ion scan of two-fold charged vancomycin.

ware module was used with the following setting: Polynome type, linear; origin, include; weighting function, 1/x; axis transformation, none; smoothing method, mean; smoothing width, 1; smoothing iterations, 1.

Method validation

The method was validated in a bi-centric protocol: Institute of Laboratory Medicine, Hospital of the University of Munich, Germany, site 1; and Roche Diagnostics, Penzberg, Germany, site 2.

Prior to each analytical run, a system suitability screening test was performed; for this the preparation of calibrator 1 (1 µg/mL) was injected. A signal-to-noise ratio of >10:1 for the MRM-trace of vancomycin was defined as mandatory for a subsequent analytical run.

In order to test the specificity of the method, 10 leftover clinical serum samples from intensive care patients not treated with vancomycin were used. These samples were processed and analyzed without addition of the internal standard solution in order to verify the absence of peaks within the retention time window of the analyte or the internal standard, respectively.

In order to characterize the potential impact of residues of the sample matrix on the ionization of analyte and internal standard, two investigations were performed. In the post-column-infusion setting [11], a pure solution of vancomycin (1 mg/L) was infused with a syringe pump at constant rate using a T-piece into the column effluent, in order to generate a MS/MS background signal. Upon injection of processed serum samples, potential modulation of the background signal was monitored. In a second experiment, extracted serum from three patients not treated with vancomycin was spiked with a solution of vancomycin to a concentration of 21 mg/L each. For comparison, a neat sample in water was spiked in the same way in triplicate. Both sets of samples were analyzed with the MS/MS method and peak areas were recorded in order to estimate potential effects of the serum-derived sample matrix on ionization of vancomycin, as matrix effect (%) according to Matuszewski et al. [12].

Accuracy of the method was tested by analyzing three external reference calibrator samples (USP Calibrators, Roche) and the quality control samples QC 4 and QC 5 which were prepared on validation site 2. Each sample was analyzed in triplicate on both validation sites.

Imprecision of the method was studied by analyzing aliquots of the QC samples 1–5 in two series of six-fold determination on both study sites, leading to a total of 24 results for each sample.

To roughly characterize a lower limit of detection and the performance of the method implementation in a concentration range below the lowest calibrator sample, a spiked sample with a vancomycin concentration of 0.1 µg/mL was injected in triplicate on both study sites and the signal-to-noise ratio was assessed.

In order to test the stability of processed samples, extracts of three patients' samples were stored at +8°C and –20°C. These samples were re-quantified in analytical series after 1 day, 2 days, and 1 week. These results were compared with the results found in the initial analytical run.

Left-over serum from 70 patients' samples send to the Institute of Laboratory Medicine for clinically indicated vancomycin measurement were used to study the agreement between analyses performed with the described LC-MS/MS method on both validation sites. The samples were recruited consecutively without any selection, thus reflecting the typical distribution of concentrations found in a tertiary care hospital. After anonymization, two aliquots of these samples were prepared and stored at –20°C until analysis on either study site within 4 weeks. This procedure was approved by the Institutional Review Board.

Results

A representative LC-MS/MS chromatogram is shown in Figure 4. Analyte and the internal standard compound vancomycin-glycine co-eluted as requested. Fifty MS/MS-data points were acquired over both chromatographic peaks. Minor signals from isobaric compounds were observed eluting slightly beforehand and following the peaks. Their pattern was found constant in both calibrator samples and patients' samples on both study sites, and peak integration was performed consistently. The pattern did not change with the storage time of samples.

In all analytical series the regression coefficient r^2 was ≥0.99 for all calibration runs over the concentration range from 1.06 to 84.41 mg/L. The slope of the calibration line was 0.25 (±5%).

The signal-to-noise ratio observed in the system suitability screening test (injection of the lowest calibrator sample) was >400:1 in all analytical series on study site 1, and >100:1 on site 2.

The handling of the method was found convenient, including the ultrafiltration step. The entire instrumental setting was robust throughout the study period on both sites, in particular regarding HPLC back-pressure and ionization yield.

The analysis of 10 serum samples from patients which were not treated with vancomycin proved the specificity of the method; no peak signals in retention time windows of vancomycin or vancomycin-glycin were observed.

Figure 5 displays the results of the post-column infusion experiment which was performed to characterize the impact of serum-derived sample materials on the ionization yield of vancomycin. The figure shows an overlay of the signal pattern generated by injection of extract from a vancomycin-free sample, and from a patient's sample. During the time period in which the chromatographic eluate was transferred to the MS/MS system a constant signal of the continuously infused analyte can be observed. There was no obvious drop or increase in ion yield at the retention time of vancomycin. In the spiking experiment, a matrix effect of –9% according to Matuszewski et al. [12] was observed.

Table 1 displays the analytical accuracy realized for the analysis of standard and quality control materials;

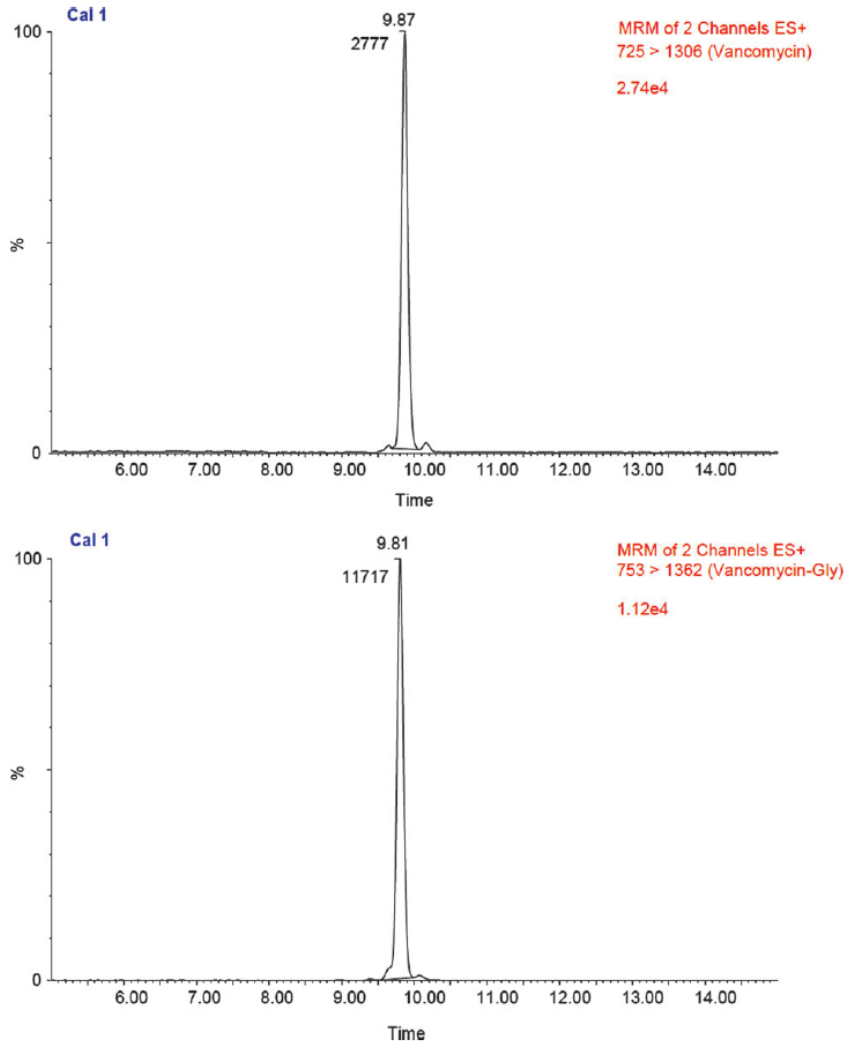

Figure 4 Representative MS/MS chromatogram of vancomycin (calibrator sample 1, with vancomycin-glycin as the internal standard).

for each single determination a bias of ≤±4% was observed for the five materials investigated on both study sites which is within the ±3 SD range of the method.

The data of the imprecision study are given in Table 2. Intra-assay imprecision observed for the five samples ranged from 1.1% to 3.9% CV. The total CV observed for aliquots of these samples analyzed on both study sites (4 series, 24 determinations) ranged from 1.9% to 3.8%.

In a sample spiked to a vancomycin concentration of 0.1 mg/L, a mean signal-to-noise ratio from three injections of 52:1 (site 1) and 14:1 (site 2) was observed.

Stability of processed samples (autosampler on board stability) is shown in Table 3; sample extracts were found to be stable for at least 1 week at +8°C with bias of ≤±3% compared to initial measurement.

For 70 unselected left-over serum samples which were analyzed to study the between-laboratory agreement of the method, mean concentrations of 9.4 mg/L and 9.9 mg/L were observed on site 1 and site 2, respectively, with median concentrations of 8.8 mg/L and 9.6 mg/L (Figure 6). Coefficient of variation observed for the internal standard peak area was 14% on site 1 and 10% on site 2. Pearson's r was 0.99. Linear regression demonstrated the following equation:

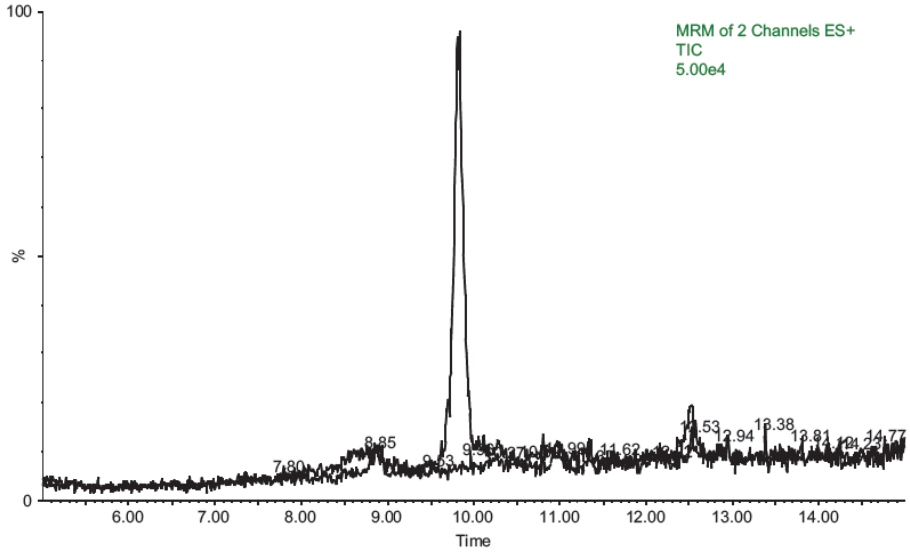

Figure 5 Post-column infusion experiment to assess matrix effects on the ionization of vancomycin. Overlay of a run with extract from drug-free serum and of a clinical sample.

site 2=1.04 * site 1+0.03 (95% confidence interval for slope: 1.00–1.09; 95% confidence interval for intercept: −0.42 mg/L to 0.49 mg/L)

Discussion

We here report a HPLC-MS/MS method for the quantification of the antibiotic compound vancomycin in serum, which is intended to be used in the context of standardization and harmonization of routine assays for this analyte. An essential novelty of the method protocol is the fact that an internal standard compound was prepared as a homologue by derivatization of vancomycin to vancomycin-glycin. Compared to previously reported LC-MS/MS methods for vancomycin quantification [7–9], the molecular structure of this internal standard compound is much closer related to that of the target analyte and ensures identical chromatographic behavior in the chosen setup. In general, matrix effect on the ionization of analytes is best compensated for by the use of stable isotope labeled internal standard compounds; however, since vancomycin is a biological product such labeled compounds are not available. We conclude that the method for the quantification of vancomycin described here has the highest metrological level realized so far, suggesting this protocol as a candidate reference method.

A bi-centric method validation characterized the method as specific, accurate and precise. The between-laboratory agreement was found acceptable with <4% bias for the analysis of a large series of authentic clinical

Table 1 Analytical accuracy observed for external calibration material (Cal A-C) and quality control material manufactured on study site 2 (both materials based on USP vancomycin standard).

Target concentration	Cal A, 5.0 mg/L	Cal B, 40.0 mg/L	Cal C, 80.0 mg/L	QC 4, 39.7 mg/L	QC 5, 79.4 mg/L
Penzberg (n=3)					
Observed mean conc.	5.1	40.4	79.6	40.1	78.0
Recovery %	104/100/102	101/100/102	101/99/99	100/101/99	96/98/99
Großhadern (n=3)					
Observed mean conc.	5.1	40.6	80.4	40.4	81.8
Recovery %	104/100/104	103/102/100	103/99/100	99/102/103	103/101/103

Table 2 Imprecision (coefficient of variation) observed for quality control materials.

	QC 1, 5.3 mg/L	QC 2, 12.4 mg/L	QC 3, 13.9 mg/L	QC 4, 39.7 mg/L	QC 5, 79.4 mg/L
Penzberg					
Intra-assay (n=6)					
Day A					
CV %	3.5	1.4	2.6	1.3	1.4
Intra-assay (n=6)					
Day B					
CV %	3.9	1.5	1.1	1.6	2.7
Großhadern					
Intra-assay (n=6)					
Day A					
CV %	1.5	1.5	1.3	2.1	0.8
Intra-assay (n=6)					
Day B					
CV %	1.4	1.1	3.1	2.0	1.6
Pooled data from both study sites					
Inter-assay (n=24)					
CV %	2.8	2.2	3.8	1.9	2.2

sample materials on two study sites. Thus, the performance of this method – which is based on a homologue for internal standardization – is almost equal to that of typical isotope dilution LC-MS/MS methods. Control of matrix effects was verified by post-column-infusion and spiking experiments.

Minor isobaric interference was observed in the MS/MS chromatograms of analyte and internal standard (Figure 4); the nature of the respective compounds has not been disclosed. Efficient chromatographic separation and consistent peak integration is important for optimum reproducibility of analytical series.

The method described here was *not* optimized regarding a routine use in the clinical laboratory. Extended chromatography was applied in order to minimize matrix effects on the ionization of the analyte and the internal standard, thereby optimizing the analytical reliability. This, however, resulted in a retention time of 9.8 min and a total run-time above 20 min. The sample preparation protocol is straightforward and based on protein precipitation. Subsequent ultrafiltration was performed in order to maximize the stability and robustness of the method.

To date no generally accepted validation protocol for quantitative bio-medical analytical methods using LC-MS/MS is available. The widely used FDA protocol [13] has important limitations and is mainly designed for the

Table 3 Stability of sample extracts (autosampler stability).

Recovery in %	Serum 1		Serum 2		Serum 3	
Storage	+8°C	–20°C	+8°C	–20°C	+8°C	–20°C
Penzberg						
1 day	102	102	104	101	102	101
2 days	104	109	103	104	104	104
1 week	99	99	98	97	100	98
Großhadern						
1 day	96	99	99	95	100	101
2 days	98	97	101	97	99	101
1 week	100	96	103	96	101	101

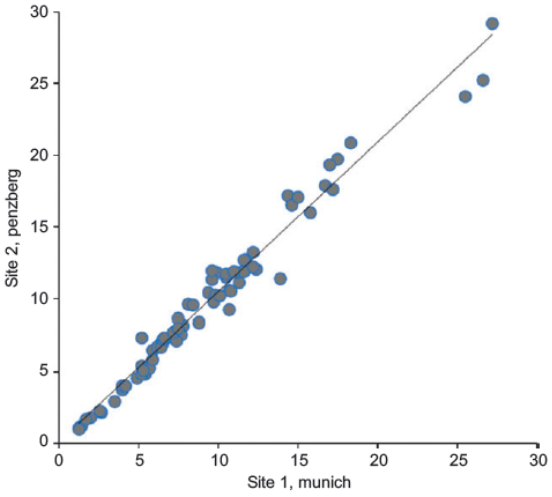

Figure 6 Between-laboratory comparison of serum vancomycin measurement by LC-MS/MS (n=70; vancomycin in mg/L).

validation of pharmacokinetic batch studies in individual laboratories. However, a prerequisite for a reference method is robustness over time and space. Our approach of a bi-centric validation can give an impression on the general robustness of the method realized in different instrument installations and laboratories. We suggest such an assessment of between-instrument or between-laboratory agreement for validation protocols applied for clinical mass spectrometry methods, just as it is standard in the validation of commercial immunoassays.

We defined a signal-to-noise ratio for the lowest concentration calibrator sample which is required as the acceptance criterion in a system suitability test. The concentration range below the lowest calibrator sample was not addressed systematically in the validation study, since this would be outside the intended use of the method.

Besides a robust analytical method, the purity of standard compounds used is crucial for the implementation of a reference measurement system for vancomycin in the future. Detailed characterization of the primary vancomycin standard has not been addressed in this work. All concentrations are based on the USP Pharmacopeia certificates delivered with the purchased material.

In summary, we were able to describe a robust and reliable LC-MS/MS-based measurement procedure for the quantification of vancomycin in human serum which may be used in the context of a reference measurement system. Such a system may contribute to increase the added clinical value of routine vancomycin therapeutic drug monitoring which is so far limited by rather poor agreement between routine methods.

Acknowledgments: We thank the Referenzinstitut für Bioanalytik (RfB) for the permission to report the results of a vancomycin proficiency testing program.

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 funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.

Received February 22, 2013; accepted March 21, 2013

References

1. Rybak M, Lomaestro B, Rotschafer JC, Moellering R Jr., Craig W, Billeter M, et al. Therapeutic monitoring of vancomycin in adult patients: a consensus review of the American Society of Health-System Pharmacists, the Infectious Diseases Society of America, and the Society of Infectious Diseases Pharmacists. Am J Health-Syst Pharm 2009;66:82–98.
2. Roberts JA, Norris R, Paterson DL, Martin JH. Therapeutic drug monitoring of antimicrobials. Br J Clin Pharmacol 2012;73:27–36.
3. Moise-Broder PA, Forrest A, Birmingham MC, Schentag JJ. Pharmacodynamics of vancomycin and other antimicrobials in patients with Staphylococcus aureus lower respiratory tract infections. Clin Pharmacokinet 2004;43:925–42.
4. Cano EL, Haque NZ, Welch VL, Cely CM, Peyrani P, Scerpella EG, et al. Incidence of nephrotoxicity and association with vancomycin use in intensive care unit patients with pneumonia: retrospective analysis of the IMPACT-HAP Database. Clin Ther 2012;34:149–57.
5. Wong-Beringer A, Joo J, Tse E, Beringer P. Vancomycin-associated nephrotoxicity: a critical appraisal of risk with high-dose therapy. Int J Antimicrob Agents 2011;37:95–101.
6. Wilson JF, Davis AC, Tobin CM. Evaluation of commercial assays for vancomycin and aminoglycosides in serum: a comparison of accuracy and precision based on external quality assessment. J Antimicrob Chemother 2003;52:78–82.
7. Cass RT, Villa JS, Karr DE, Schmidt DE Jr. Rapid bioanalysis of vancomycin in serum and urine by high-performance liquid chromatography tandem mass spectrometry using on-line sample extraction and parallel analytical columns. Rapid Commun Mass Spectrom 2001;15:406–12.
8. Zhang T, Watson DG, Azike C, Tettey JN, Stearns AT, Binning AR, et al. Determination of vancomycin in serum by liquid chromatography-high resolution full scan mass spectrometry. J Chromatogr B 2007;857:352–6.
9. Shibata N, Ishida M, Prasad YV, Gao W, Yoshikawa Y, Takada K. Highly sensitive quantification of vancomycin in plasma samples using liquid chromatography-tandem mass spectrometry and oral bioavailability in rats. J Chromatogr B 2003;789:211–8.
10. Stepman HC, Vanderroost A, Van Uytfanghe K, Thienpont LM. Candidate reference measurement procedures for serum 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 by using isotope-dilution liquid chromatography-tandem mass spectrometry. Clin Chem 2011;57:441–8.
11. Annesley TM. Ion suppression in mass spectrometry. Clin Chem 2003;49:1041–4.
12. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem 2003;75:3019–30.
13. Guidance for Industry-Bioanalytical Method Validation. U.S. Department of Health and Human Services-Food and Drug Administration; 2001. Available from: http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070107.pdf. Accessed on February, 2012.

3 ANHÄNGE

3.1 Eidesstattliche Versicherung

König Katrin

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

„Untersuchungen zur quantitativen Massenspektrometrie in der Laboratoriumsmedizin“

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, 06.05.2013

3.2 Danksagung

Mein Dank gilt vor allem Herrn Prof. Dr. M. Vogeser für die hervorragende fachliche und menschliche Betreuung dieser Arbeit.

Der DGKL-Stiftung für Pathobiochemie und Molekulare Diagnostik danke ich für die Förderung des dargestellten Forschungsprojektes, sowie meinen Kooperationspartnern Roche Diagnostics GmbH und MagnaMedics Diagnostics BV für die gute und produktive Zusammenarbeit.

Danke auch an meine Mitdoktorandinnen Silvia Bächer und vor allem Barbara Maier für eine stets angenehme Atmosphäre und die schöne Zeit am Institut.

Vielen Dank an meine Familie, an meinen Papa, Arnold, der mich besonders während meines Studiums unterstützt hat. An meine Mama, Ingrid, für die emotionale Unterstützung in schönen, aber auch in schwierigen Momenten und an Ihren Mann Ulli, für die zahlreichen Gespräche und wertvollen Ratschläge. Nicht zu vergessen meinen Bruder Gregor, der gerade in den letzten Jahren ein unglaublich guter Freund geworden ist!

Ganz besonders möchte ich mich an dieser Stelle bei meinem Mann Mathias bedanken, der mit seinem Vertrauen in mich eine unglaublich große Stütze ist. Danke, dass Du immer für mich da bist!