Aus dem Institut für Laboratoriumsmedizin

der Ludwig-Maximilians-Universität München

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Massenspektrometrische Methoden in der Laboratoriumsmedizin

Dissertation

zum Erwerb des Doktorgrades der Humanbiologie

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

vorgelegt von

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aus

Tirschenreuth

2014

Mit Genehmigung der Medizinischen Fakultät

der Universität München

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Kumulative Dissertation

Inhaltsverzeichnis

1 EINLEITUNG

1.1 Massenspektrometrische Methoden in der Laboratoriumsmedizin

1.1.1 Laboratoriumsmedizin

Laboruntersuchungen spielen heute eine Schlüsselrolle in fast allen Gebieten der Medizin. Die In-vitro-Diagnostik trägt oft zentral zur Diagnosestellung bei, auch im Sinne einer Früherkennung. Laboruntersuchungen werden zur Prognostik genutzt wie auch zur Beurteilung des Behandlungsverlaufs; dabei sind die Befunde oft essentiell für therapeutische Entscheidungen. Als Fach gliedert sich die Laboratoriumsmedizin insbesondere in die Bereiche Klinische Chemie, Immunchemie, Endokrinologie, Hämatologie, Hämostaseologie, Therapeutisches Drug Monitoring, Mikrobiologie und Transfusionsmedizin.
Als Querschnittsfach ist die Laboratoriumsmedizin mit fast allen klinischen Bereichen verwoben und interdisziplinär in vielfältigste Prozesse eingebunden. Diese reichen von der prä-analytischen Phase (mit Indikationsstellung für Untersuchungen, Probengewinnung und -transport) über die eigentliche Analytik bis zur post-analytischen Phase in der aus Messwerten individuelle Befunde generiert werden, die im klinischen Kontext bewertet werden.

1.1.2 Labormedizinische Untersuchungsverfahren

In der Laboratoriumsmedizin kommt je nach Zielparameter eine Vielzahl von unterschiedlichen Analysetechniken zum Einsatz. Angestrebt wird grundsätzlich ein möglichst hohes Maß an analytischer Zuverlässigkeit und Robustheit bei möglichst geringer Beanspruchung von Ressourcen.

1.1.2.1 Standardtechniken – photometrische und immunometrische Methoden

Bei den photometrischen Methoden handelt es sich um Messverfahren im Wellenlängenbereich des ultravioletten und sichtbaren Lichtes, welche seit den 1960er Jahren angewendet werden. Wurden die Messungen anfänglich mittels eines klassischen Photometers durchgeführt, stehen heute universell einsetzbare Multifunktionsgeräte zur Verfügung (Mehrkanal-Analyzer).

Immunometrische Methoden (bzw. Immunoassays), welche seit den 1980er Jahren in der Laboratoriumsmedizin angewendet werden, beruhen auf einer Antigen-Antikörper-Reaktion. Das Antigen stellt der Zielanalyt dar, welcher eine definierte chemische Struktur besitzt. Für dieses Antigen werden mittels tierbasierten Techniken spezifische Antikörper hergestellt. Bei diesem Messprinzip ist es von großer Bedeutung, dass die Bindung des Antigens an den Antikörper in eine messbare Größe

umgesetzt wird. Grundsätzlich finden Immunoassays Anwendung, wenn der Analyt nicht über stoffspezifische Eigenschaften (Extinktionskoeffizient, enzymatische Aktivität) direkt nachweisbar ist, bzw. wenn der Konzentrationsbereich niedrig ist. Innerhalb der immunometrischen Methoden kommen sowohl homogene Immunoassays (Immunreaktion findet in homogener Phase statt) als auch heterogene Immunoassays (Immunreaktion findet an fester Phase statt) zum Einsatz.

Die Vorteile beider Standardtechniken liegen in der breiten Anwendbarkeit, dem hohen Automatisierungsgrad, sowie dem hohen Probendurchsatz und den relativ geringen Kosten pro Test. Ein weiterer Vorteil der Immunoassays ist deren hohe Sensitivität.
Als Nachteile der photometrischen Methoden sind die teils mangelnde Sensitivität zu nennen. Immunometrische Methoden können hingegen Limitationen hinsichtlich der geringen Spezifität der Antikörper aufweisen, wodurch es zu Kreuzreaktivitäten mit strukturähnlichen Substanzen und somit zu einer Verfälschung des Messsignals kommen kann. Des Weiteren wirken sich potentielle Chargenvarianzen zwischen den verschiedenen Herstellungschargen der Antikörper nachteilig aus. Darüber hinaus sind diese Standardtechniken für viele klinisch interessante Analyte aufgrund von fehlenden speziellen Molekülstrukturen bzw. der mangelnden Epitope methodisch nicht anwendbar (z.B. Methylmalonsäure als Marker eines Vitamin B12-Mangels, freie Metanephrine im Plasma für die Phäochromozytom-Diagnostik).

1.1.2.2 Massenspektrometrische Methoden

Bei der Massenspektrometrie (MS) handelt es sich im Allgemeinen um ein Verfahren zur Messung der Masse von Molekülen. Dieses kann sowohl für die Identifizierung als auch für die Quantifizierung eingesetzt werden. Bei den massenspektrometrischen Methoden in der Laboratoriumsmedizin erfolgt im Allgemeinen zunächst eine Auftrennung des Stoffgemisches entweder mittels Flüssigchromatographie (LC) oder mittels Gaschromatographie (GC). Anschließend findet die Detektion mit einem Massenspektrometer statt. Beim grundsätzlichen Prinzip der MS werden die zu untersuchenden Analyte in die Gasphase überführt. Daraufhin erfolgt die Ionisierung der Substanzen. Die erzeugten Ionen werden durch ein elektrisches Feld beschleunigt und dem Analysator zugeführt. Hier werden die Ionen nach dem Verhältnis Masse zu Ladung (m/z) getrennt. Im Anschluss erfolgt die Detektion der separierten Ionen mittels eines Ionennachweissystems.

Die Geschichte der MS begann Ende des 19. Jahrhunderts mit einer Entdeckung des deutschen Physikers Wilhelm Wien, dass Strahlen von positiv geladenen Teilchen durch ein magnetisches Feld abgelenkt werden können. Weitere Experimente des britischen Physikers Joseph Joe Thomson Anfang des 20. Jahrhunderts zeigten, dass positiv geladene Ionen, welche ein elektromagnetisches Feld passieren, eine Serie von parabolischen Kurvenverläufen zeigen. Hierbei entspricht jeder Kurvenverlauf einem Ion mit einem bestimmten Masse-zu-Ladungsverhältnis und ist des Weiteren abhängig von dessen Geschwindigkeit (1).

[1] Thomson, J.J. Rays of positive electricity; Proc. Royal. Soc. London. A. 1913;89:1-20

Vor ungefähr 50 Jahren fand die MS Einzug in die Laboratoriumsmedizin durch eine Kopplung der GC mit der MS. Bei der GC-MS findet zunächst eine Auftrennung des zu untersuchenden volatilen Stoffgemisches im Gaszustand mittels eines Gaschromatographen statt. Die Trennung erfolgt dabei aufgrund von unterschiedlichen Siedepunkten der Einzelsubstanzen und der unterschiedlichen Wechselwirkungen der Substanzen mit der stationären Phase, welche eine Trennsäule von bis zu 60 m Länge darstellt und durch die die Substanzen mit Hilfe eines inerten Trägergases transportiert werden. Nach der Ionisierung der Substanzen z.B. mittels Elektronenstoß-Ionisation, dient ein Massenspektrometer zur Identifizierung oder Quantifizierung der Zielanalyte. Die GC-MS findet Verwendung für die Analytik von kleinen, flüchtigen Molekülen wie z.B. Fettsäuren und Aminosäuren. Des Weiteren ist diese Technik als Referenzmethode in der externen Qualitätssicherung von großer Bedeutung und spielt eine große Rolle in der Toxikologie. Die Vorteile der GC-MS umfassen eine herausragende Trennleistung, welche durch die vorgeschaltete GC ermöglicht wird und die Generierung von eindeutigen Spektren durch das Massenspektrometer, die mit Spektren-Bibliotheken verglichen werden können. Die GC-MS weist jedoch einige Limitationen auf: begrenzte Anwendbarkeit für die Analytik von Molekülen nichtflüchtiger und polarer Natur, sowie Makromolekülen; umfangreiche Probenvorbereitung im Hinblick auf die Analytextraktion und die Derivatisierung, sowie die langen Analysenzeiten (2).

Die Entwicklung der Elektrospray-Ionisierung (electro spray ionisation, ESI) unter Atmosphärendruck (atmospheric pressure ionisation, API) durch John Fenn in den 1980er Jahren ermöglichte die schonende Ionisierung von großen Biomolekülen. Diese stand im Gegensatz zu den bis dahin vorherrschenden aggressiven Ionisierungstechniken, wie z.B. der Elektronenstoß-Ionisierung. Die ESI erlaubte die Ionisierung von polaren, nicht flüchtigen Molekülen und somit eine Kopplung der Flüssigchromatographie (LC) mit der MS (3).
Bei der LC-MS Analytik in der klinischen Diagnostik ist es unabdingbar, das humane biologische Material (Vollblut, Serum, Plasma, Urin, Speichel) vor dessen Analytik im Rahmen einer Probenvorbereitung von Proteinen und von möglichst vielen Störkomponenten zu befreien und dabei gegebenenfalls den Zielanalyt aufzukonzentrieren. Bei der eigentlichen LC-MS Analytik erfolgt zunächst eine chromatographische Trennung der zu untersuchenden Substanzen. Hierbei liegt der Fokus vor allem auf der Abtrennung der Zielanalyte von Elektrolyten, welche die Ionisierung beeinträchtigen können. Die zu untersuchenden Substanzen werden mittels eines Fließmittels (mobile Phase) über eine analytische Säule transportiert. Diese besteht aus einer stationären Phase mit bestimmten funktionellen Gruppen, welche sehr variable chemische Eigenschaften aufweisen können. Es kommt zu Wechselwirkungen der Zielanalyte mit den funktionellen Gruppen und somit zu einer Auftrennung der Analyte. Anschließend wird das von der analytischen Säule kommende Eluat unter Atmosphärendruck ionisiert wobei hier neben der ESI auch die chemische Ionisierung unter Atmosphärendruck (atmospheric pressure chemical ionisation, APCI) als Ionisierungstechnik eingesetzt werden kann.

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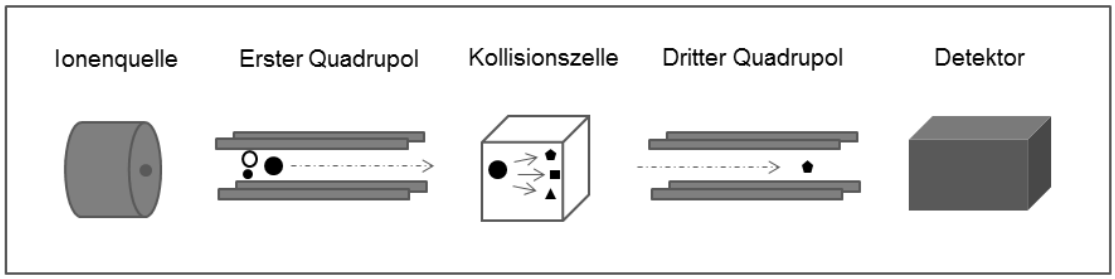
[2] Want, E.J.; Cravatt, B.F.; Siuzdak, G; The expanding role of mass spectrometry in metabolite profiling and characterization; Chembiochem. 2005;6:1941-51
[3] Fenn, J.B.; Mann, M.; Meng, C.K; Wong, S.F.; Whitehouse, C.M.; Electrospray ionization for mass spectrometry of large biomolecules; Science. 1989;246:64-71

Nach der Ionisierung eignet sich für die Quantifizierung der Ionen im Hochvakuum insbesondere die Tandem Massenspektrometrie (MS/MS). In der klinischen Diagnostik sind Triple-Quadrupol Massenspektrometer am weitesten verbreitet. Hierbei wird durch die Kopplung von zwei Analysatoren (Quadrupolen) die Selektivität und Sensitivität einer Quantifizierungsmethode entscheidend verbessert. Zwischen den beiden Quadrupolen befindet sich eine Kollisionszelle, welche in der Begrifflichkeit Triple-Quadrupol Massenspektrometer fälschlicherweise ebenfalls als Quadrupol bezeichnet wird. Zur Quantifizierung eignet sich der Scan Modus selective reaction monitoring (SRM), auch als multi reaction monitoring (MRM) bezeichnet. Hier werden die Ionen mit einem bestimmten Masse zu Ladung-Verhältnis (m/z) im ersten Quadrupol selektiert. Anschließend werden die selektierten Ionen im zweiten Quadrupol – der Kollisionszelle – mit Hilfe eines Kollisionsgases (z.B. Stickstoff oder Argon) fragmentiert wobei sehr spezifische Fragment Ionen entstehen. Im dritten Quadrupol erfolgt die Selektion eines definierten Fragment Ions, welches mittels eines Ionennachweissystems, meist eines Elektronen-Photomultipliers, detektiert wird (Abbildung 1) (2).

Abbildung 1. Schematischer Aufbau eines Triple-Quadrupol Massenspektrometers



Die LC-MS/MS Technologie ermöglicht die Analytik sowohl von niedermolekularen Analyten, welche endogen im Menschen zirkulieren, als auch von Xenobiotika. Bedingt durch die eingeschränkte Sensitivität der Massenspektrometer der frühen Generation, war die Analytik auf Substanzen begrenzt, welche im Konzentrationsbereich zwischen mmol/L und µmol/L in humanen biologischen Materialien zirkulieren. Inzwischen ist es mit hochempfindlichen Massenspektrometern möglich, selbst Analyte im Konzentrationsbereich von pmol/L zu messen. Die LC-MS/MS findet Anwendung im Bereich der Arzneistoffanalytik (Therapeutisches Drug Monitoring, TDM), im Rahmen des Neugeborenen-Screening auf angeborene Stoffwechselstörungen und auch im Bereich der Endokrinologie z.B. für die Analytik von 25-Hydroxyvitamin D und Testosteron.

In Tabelle 1 sind die Vor- und Nachteile der LC-MS/MS Analytik in der klinischen Routinediagnostik gegenübergestellt. Obwohl einige Nachteile nicht von der Hand zu weisen sind – vor allem bezüglich der Komplexität der Analysetechnik – setzte sich die LC-MS/MS in der Laboratoriumsmedizin in den letzten Jahren immer mehr durch. Vor allem die breite Einsetzbarkeit dieser Technologie, sowie das große Potential für vollautomatische Front-End Module sind für die klinische Diagnostik von enormer Bedeutung.

Tabelle 1. Vor- und Nachteile der LC-MS/MS in der Laboratoriumsmedizin

Vorteile	Nachteile
- Breite Einsetzbarkeit für die Analytik von unterschiedlichsten Substanzen (niederer oder hoher molekularer Masse, unterschiedlicher Polarität, hoher oder niederer Konzentration) aus verschiedensten Matrizes. - Möglichkeit einer sehr spezifischen Analytik, da für den Analyten ein genau definierter Massenübergang herangezogen wird. Auch kann aufgrund dieses Prinzips sehr hohe Sensitivität erreicht werden. - Option der simultanen Quantifizierung von vielen verschiedenen Analyten innerhalb eines Analysenlaufs, das für ein Metabolite-Profiling eingesetzt werden kann – z.B. im Hinblick auf Vitamin D. - Verwendung von stabilisotopen-markierten internen Standards ermöglicht die Kompensation von nahezu allen Matrix-Effekten. - Möglichkeit einer eigenständigen und flexiblen Methodenentwicklung, wobei die Methoden aufgrund verfügbarer Referenzmaterialien und Referenzmethoden ein hohes Maß an Standardisierung aufweisen können. - LC-MS/MS Methodenentwicklung deutlich kostengünstiger, im Vergleich zur Immunoassay-Technologie; diese erfordert kostspielige Entwicklung von analytischen Antikörpern, welche chargenabhängige Varianzen im Hinblick auf die Spezifität aufweisen können. - Im Vergleich zur GC-MS wesentlich weniger aufwändige Probenvorbereitung und deutlich kürzere Analysenlaufzeiten - Bezüglich Automatisierung bietet die LC-MS/MS im Vergleich zur GC-MS großes Potential für vollautomatische Front-End Module; diese sind für eine erleichterte Bedienbarkeit und Robustheit der Technologie von großer Bedeutung.	- Methodenentwicklung und -implementierung sehr analytspezifisch und nur durch LC-MS/MS Applikationsspezialisten durchführbar. - Praktische Routineanwendung der LC-MS/MS Technik erfordert sehr gut ausgebildetes Personal aufgrund komplexer und nicht standardisierter Hard- und Software. - LC-MS/MS Analytik umfasst sehr viele manuelle Arbeitsschritte, welche eine sehr hohe Fehlerquote aufweisen können. - LC-MS/MS Analytik erfordert fast bei allen Methoden eine Probenvorbereitung um Matrixeffekte zu minimieren oder den Analyt aufzukonzentrieren. - Für Wartung und Reparatur der LC-MS/MS Technologie meist zu wenige gut qualifizierte Techniker verfügbar. - Eingeschränkte kommerzielle Verfügbarkeit von internen Standard-Substanzen für verschiedene Zielanalyte mit potentieller Relevanz für die klinische Diagnostik mittels LC-MS/MS. - Wie bei allen anderen zur Verfügung stehenden Untersuchungsverfahren ist die Verfügbarkeit von Referenzmethoden, Referenzmaterialien und externer Ringversuche nicht für alle relevanten Zielanalyte gegeben. - Im Gegensatz zu vollautomatischen Standard-Immunoanalyzern wurde die vollständige Automatisierung bisher nicht realisiert.

Die übergeordnete Aufgabe des Promotionsprojekts war es die Anwendungsmöglichkeiten der LC-MS/MS Analytik zu beleuchten und deren Handhabbarkeit zu verbessern. Hierzu wurde in Teilprojekten zum einen eine LC-MS/MS Methode für die sehr sensitive Messung eines vorgeschlagenen Biomarkers entwickelt. Zum anderen wurde eine LC-MS/MS Methode entwickelt, die auf einer innovativen Probenvorbereitungstechnik basiert. Es wurde überprüft inwieweit diese Technik eine vollautomatische LC-MS/MS Analytik ermöglicht und somit die Handhabbarkeit der LC-MS/MS Analytik verbessert.

1.2 Publikationen der Teilprojekte

1.2.1 Baecher, S.; Kroiss, M.; Fassnacht, M.; Vogeser, M. No endogenous ouabain is detectable in human plasma by ultra-sensitive UPLC-MS/MS Clin. Chim. Acta. 2014;431:87-92

Die aktuelle Generation von Massenspektrometern ermöglicht mit einer herausragenden Sensitivität die Analytik von endogenen Steroidhormonen und Biomarkern, welche in Konzentrationsbereichen von pmol/L – nmol/L im Menschen zirkulieren (z.B. Androstendion, 17-Hydroxyprogesteron, freie Metanephrine im Plasma). Als ein möglicher potentieller Biomarker wird Ouabain (Synonym G-Strophanthin) diskutiert – ein Analyt, der neben Digitoxin und Digoxin zur Gruppe der Herzglykoside gehört. Es ist bekannt, dass an der Natrium-Kalium-ATPase des Menschen eine spezifische Bindungsstelle für Herzglykoside existiert. Dies gab Anfang der 1990er den Anstoß, endogene kardiotone Steroide zu isolieren. In verschiedenen klinischen Studien deuteten immunometrische Ergebnisse darauf hin, dass ein sog. *endogenes Ouabain* im Menschen synthetisiert wird. Die körpereigene Synthese von Ouabain sollte dabei durch körperliche Aktivität und Bluthochdruck hervorgerufen werden und mit der Schwere von bestimmten Erkrankungen wie z.B. dem Nierenversagen und der Herzinsuffizienz korrelieren. Die gemessenen Konzentrationen des vorgeschlagenen *endogenen Ouabains* in gesunden Probanden lagen im Bereich von 60 – 530 pmol/L. Es ist hervorzuheben, dass es sich bei den herangezogenen Immunoassays um nicht standardisierte „in-house assays“ handelte. Ein bis in die frühen 1990er Jahre kommerziell verfügbarer Immunoassay ist seitdem nicht mehr erhältlich.

Das Ziel dieses Teilprojekts der Promotionsarbeit war die Entwicklung einer spezifischen und zuverlässigen LC-MS/MS Methode für die Messung von Ouabain, einem potentiell kardiovaskulären Biomarker, in humanem Plasma. Die entwickelte Ultra Performances Liquid Chromatography (UPLC) Tandem Massenspektrometrie (MS/MS) Methode umfasste einen stabil-isotop markierten Internen

Standard um Matrixeffekte zu kompensieren. Um die Sensitivität der Methode zu erhöhen wurde als Probenvorbereitung eine Festphasenextraktion durchgeführt. Diese spezifische und sensitive UPLC-MS/MS Methode wurde umfassend validiert und wies ein unteres Quantifizierungslimit (lower limit of quantification, LLOQ) von 1.7 pmol/L auf. Trotz dieses extrem niedrigen LLOQ konnte der Analyt in keiner Patientenprobe detektiert werden.

Die Ergebnisse dieser Arbeit weisen eindeutig darauf hin, dass die Immunoassays, welche in der Vergangenheit zur Quantifizierung des vermeintlichen Ouabains herangezogen wurden, Substanzen detektierten, die mit Ouabain strukturell nicht identisch sind. Es liegt nahe, dass diese Diskrepanzen zwischen der immunologischen und der massenspektrometrischen Analytik durch Kreuzreaktivitäten von endogenen, strukturell mit Ouabain verwandten Substanzen verursacht wurden.

Mit unserer Arbeit wurde die bisherige Literatur über das *endogene Ouabain* widerlegt. Dabei ist zu betonen, dass die Isolierung, Identifizierung und Charakterisierung des *endogenen Ouabains* von einer einzigen Forschergruppe durchgeführt wurde. Auch die bisher in der Literatur verwendeten „in-house assays“ zur Quantifizierung des *endogenen Ouabains* basieren auf einem Immunoassay dieser Forschergruppe.

Die hochspezifische Technik hat einen neuen Weg für die Forschung auf diesem Feld gewiesen. Um den hoch interessanten potentiellen Biomarker – eine mögliche endogene kardiotone Substanz – zu identifizieren erfordert es spezielle Technologien. Für das „Non-Targeted Screening“ eignet sich dabei besonders die hochauflösende MS. Auch eine Analyt-Extraktion bzw. -Anreicherung mittels immobilisierter Natrium-Kalium-ATPase im Rahmen der Probenvorbereitung könnte für die Analytik von Nutzen sein.

1.2.2 Baecher, S.; Geyer, R.; Lehmann, C.; Vogeser, M.

Absorptive chemistry based extraction for LC-MS/MS analysis of small molecule analytes from biological fluids – an application for 25-hydroxyvitamin D

Clin. Chem. Lab. Med. 2014;52:363-71

Wie schon im allgemeinen Teil angesprochen, ist es für die LC-MS/MS Analytik von humanen biologischen Materialien unabdingbar, eine Probenvorbereitung durchzuführen. Hierfür kommen vor allem die Proteinfällung, Flüssig-Flüssig-Extraktion (liquid liquid extraction, LLE), Festphasenextraktion (solid phase extraction, SPE), und die On-line SPE zum Einsatz. Die Wahl der Extraktionstechnik ist abhängig von der Art und dem Konzentrationsbereich des zu messenden Analyten, sowie deren Eignung für einen hohen Probendurchsatz, welcher für die Routineanalytik von großer Bedeutung ist. Diese bisher zur Verfügung stehenden Techniken sind für die Komplettautomatisierung der Probenvorbereitung bzw. der LC-MS/MS Analytik nur bedingt oder gar

nicht geeignet. Um die LC-MS/MS in der klinischen Routinediagnostik weiter zu etablieren, ist eine Komplettautomatisierung von herausragender Bedeutung.
Eine innovative Technik stellt ein gelartiges Polymermaterial dar, welches in Wells einer Mikrotiterplatte (96-well plate) immobilisiert ist und definierte chemische Absorptionseigenschaften besitzt. Diese Technologie wird *Tecan Immobilized Coating Extraction*™ (*TICE*™) genannt und ermöglicht eine einfache Automatisierung auf sog. Liquid-Handling Systemen (Pipettierautomaten). Auf diesem Prinzip beruht die Tecan *AC Extraction Plate*™. Ziel dieses Teilprojekts der Promotionsarbeit war es in Kooperation mit Tecan eine vollständig automatisierte LC-MS/MS Methode zu entwickeln welche die Tecan *AC Extraction Plate*™ zur Analytextraktion heranzieht und des Weiteren deren Performance und Handhabbarkeit zu testen. Hierfür wurde 25-Hydroxyvitamin D als Beispielanalyt herangezogen, da diesem weltweit über die letzten Jahre ein immer größer werdendes Interesse zukommt (4). Ein auf *TICE*™ basierendes Protokoll für die Extraktion von 25-Hydroxyvitamin D aus Serum wurde in Kooperation mit Tecan entwickelt und auf einem Tecan *Freedom EVO*® Pipettierautomaten implementiert. Für die Quantifizierung wurde eine UPLC-MS/MS Methode verwendet, die auf dem Prinzip der stabil-isotop markierten Internen Standardisierung beruht. Die Performance der Methode wurde mittels eines umfassenden Protokolls geprüft und zeigte sehr gute Ergebnisse hinsichtlich Richtigkeit, Präzision, Linearität, Wiederfindung und Matrixeffekt. Des Weiteren wurde ein enorm hoher Probendurchsatz erreicht, indem 96 Proben in weniger als 60 Minuten prozessiert wurden. Außerdem wurde die Methode mit einer zweiten Tandem Massenspektrometrie Methode verglichen, in welcher eine konventionelle Probenvorbereitung verwendet wurde – eine Kombination aus Proteinfällung mit einer zweidimensionalen UPLC. Aus diesem Methodenvergleich resultierte eine sehr gute Übereinstimmung.
In diesem Teilprojekt konnte mittels *TICE*™ eine vollständig automatisierte LC-MS/MS Analytik von 25-Hydroxyvitamin D in Serum umgesetzt werden. Dies ist ein großer Schritt um die LC-MS/MS Analytik in der klinischen Routinediagnostik weiter zu etablieren.

1.3 Zusammenfassung / Conclusion

Im Rahmen der Promotionsarbeit wurden in Teilprojekten die Anwendungsmöglichkeiten der LC-MS/MS Analytik beleuchtet und dessen Handhabbarkeit für die klinische Routinediagnostik verbessert. Hierzu wurde zum einen eine LC-MS/MS Methode für die sehr sensitive Messung eines potentiellen Biomarkers entwickelt. Zum anderen wurde ein Extraktionsprotokoll für eine innovative Probenvorbereitungstechnik entwickelt und diese Technik auf deren Eignung zur Umsetzung einer vollständig automatisierten LC-MS/MS Methode getestet.
Im ersten Teilprojekt konnte gezeigt werden, dass mit den gegenwärtig verfügbaren Hochleistungs-LC-MS/MS Apparaturen eine Quantifizierung von niedermolekularen Substanzen aus Plasma bis zu einer Konzentration im niedrigen pmol/L-Bereich möglich ist. Des Weiteren wurde mit den

[4] Van den Ouweland, J.M.W.; Vogeser, M.; Bächer, S. Vitamin D and metabolites measurement by tandem mass spectrometry. Rev. Endocr. Metab. Disord. 2013;14:159-84

Ergebnissen dieser Arbeit die bisher veröffentlichte Literatur über das *endogene Ouabain* widerlegt. Weitere Forschungsarbeit ist notwendig um den hoch interessanten potentiellen Biomarker – eine mögliche endogene kardiotone Substanz – zu identifizieren.
In einem zweiten Teilprojekt wurde mittels der innovativen Probenvorbereitungstechnologie *TICE*™ eine vollständig automatisierte LC-MS/MS Analytik von 25-Hydroxyvitamin D in Serum ermöglicht. Im Rahmen weiterer Experimente sollte getestet werden, inwiefern sich *TICE*™ für andere unpolare Analyte, wie z.B. Serum-Steroide eignet. Außerdem wäre die Entwicklung eines Extraktionsmaterials, welches sich für die chemische Absorption von polaren Analyten eignet, von großem Interesse. Das hier dargestellte Prinzip birgt großes Potential eine vollautomatisierte LC-MS/MS Analytik in der Routinediagnostik zu etablieren.
Beide Teilprojekte weisen neue Wege für Anwendungen der LC-MS/MS in der medizinischen Diagnostik. Zum einen hinsichtlich der hohen Sensitivität und Spezifität – obwohl diese zum Teil auch zum Umdenken zwingt. Umdenken beispielsweise im Sinne der Nachweisbarkeit von *endogenem Ouabain*, respektive der Fehldeutung einer existierenden strukturähnlichen Substanz. Zum anderen hinsichtlich der Potentiale von Automationskonzepten.

Within the scope of this Ph.D. thesis, the application possibilities of LC-MS/MS analysis were investigated and its handling for clinical routine diagnostic was improved in different projects. For this purpose a LC-MS/MS method for an ultra-sensitive analysis of a potential biomarker was developed in a first project. In a second project, an extraction protocol for an innovative sample preparation technique was developed and the performance of this technique was tested concerning its suitability for implementation of a fully automated LC-MS/MS method.
The first project showed that the currently available high-performance LC-MS/MS equipment allows a quantification of low molecule analytes in plasma up to a low pmol/L concentration range. Moreover, the results of this study refuted the hitherto published literature about the so called *endogenous ouabain*. Further research is necessary to identify the highly interesting potential biomarker – an endogenous cardiotonic steroid.
Within the second project a fully automated LC-MS/MS analysis of 25-hydroxyvitamin D in serum was realized using the innovative sample preparation technique *TICE*™. An ongoing investigation is addressing the applicability of *TICE*™ for further lipophilic analytes such as serum steroids. Furthermore, the development of an extraction material for the chemical absorption of polar analytes would be of great interest. With this project a fully automated LC-MS/MS front-end module for the clinical diagnostic has been accomplished.
Both projects guide new ways for applications of LC-MS/MS in medical diagnostic. On the one hand, concerning the high sensitivity and specificity – although this partly forces rethinking. Rethinking for example in terms of the detectability of *endogenous ouabain*, and of the misinterpretation of an existent structural similar substance, respectively. On the other hand concerning potentials of automation concepts.

2 ORIGINALPUBLIKATIONEN

2.1 Baecher, S.; Kroiss, M.; Fassnacht, M.; Vogeser, M.

No endogenous ouabain is detectable in human plasma by ultra-sensitive UPLC-MS/MS

Clin. Chim. Acta. 2014;431:87-92

Clinica Chimica Acta 431 (2014) 87–92

Contents lists available at ScienceDirect

Clinica Chimica Acta

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

No endogenous ouabain is detectable in human plasma by ultra-sensitive UPLC-MS/MS

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ARTICLE INFO

Article history:
Received 23 October 2013
Received in revised form 18 January 2014
Accepted 23 January 2014
Available online 7 February 2014

Keywords:
Endogenous ouabain
LC-MS/MS
Cardiotonic steroids
Cardiac glycosides
Immunoassay

ABSTRACT

Background: The presence of a binding site for cardiac glycosides, such as digitoxin and digoxin, in the sodium–potassium–ATPase, stimulated attempts to isolate endogenous cardiotonic steroids. Using immunoassays, clinical studies found the cardenolide ouabain to be secreted endogenously in response to exercise and untreated hypertension and to be correlated with severity of clinical conditions such as kidney failure and dilated cardiomyopathy. The assays used were not standardized and the mean concentrations of endogenous ouabain reported for healthy controls ranged from 60 to 530 pmol/l. None of these immunoassays is available any more. Therefore, the aim of this study was to develop a highly specific and reliable method for measurement of ouabain in human plasma based on isotope dilution liquid chromatography tandem-mass spectrometry (ID-LC-MS/MS).
Method: An ultra-sensitive and specific ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method was developed which applied solid phase extraction of plasma for sample preparation.
Results: The method was comprehensively validated and had a lower limit of quantification of 1.7 pmol/l. However, despite this very low detection limit ouabain was not observed in plasma samples from patients with and without heart failure.
Conclusion: Our results suggest that immunoassays previously used to quantify assumed endogenous ouabain detected compounds which are *not* structurally identical with ouabain. Cross reactivity of structurally related compounds of endogenous origin may cause these discrepancies between immunological and mass spectrometric analyses. Conclusive characterization of assumed endogenous counterparts of digoxin in a biomarker discovery approach seems to require distinct analytical techniques.

1. Introduction

Cardiac glycosides comprise a group of compounds which share structural features of a steroid backbone coupled with a lactone (= aglycone) and one or more desoxy-sugar structures. By competitively binding to mammalian Na^+/K^+-ATPases, they convey positive inotropy, negative chronotropy and dromotropy to the heart and are therefore also called cardiotonic steroids. This group of compounds include the classes of cardenolides which are synthesized by plants, such as digoxin, digitoxin and ouabain (synonymous: g-strophanthin, extracted from seed of *Strophanthus gratus* and the root/bark of *Acokanthera ouabaio*) and the group of bufadienolides (bufalin, marinobufagenin, proscillaridin), produced by toads. The description of a specific binding site with high affinity for cardiac glycosides on mammalian Na^+/K^+-ATPases [1,2] prompted speculation about endogenous, physiologically relevant counterparts of exogenous cardiac glycosides. The term *endogenous digitalis like factor* (EDLF) was coined for this putative compound or group of compounds. Attempts to isolate and to characterize respective compounds from various biological sources (e.g. plasma, urine, hypothalamus) started in the 1980ies [3–9]. In 1991, a working group from the University of Maryland and the Upjohn Company suggested from their data on a preparation from large quantities of human plasma that a mammalian endogenous cardiac glycoside might be structurally, biochemically and immunologically indistinguishable from ouabain [10–12]. The chemical structure of the cardenolide ouabain is shown in Fig. 1. Immunometric methods were developed for the quantification of this compound in human blood [13]. While initially the term *ouabaine-like compound* (OLC) was used, in the majority of subsequent clinical investigation the analyte was denominated as ouabain

Abbreviations: EDLF, endogenous digitalis like factor; OLC, ouabain-like compound; EO, endogenous ouabain; ACTH, adrenocorticotropic hormone; LC-MS, liquid chromatography mass spectrometry; API, atmospheric pressure ionization; QC, quality control; IS, internal standard; RT, room temperature; TFA, trifluoroacetic acid; HPLC, high performance liquid chromatography; UPLC-MS/MS, ultra performance liquid chromatography tandem mass spectrometry; ESI, electrospray ionization; MRM, multi reaction monitoring; r^2, coefficient of determination; LLOQ, lower limit of quantification; CV, coefficient of variation; S/N, signal-to-noise ratio; BNP, B-type natriuretic peptide; NYHA, New York Heart Association; GC-MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance; RIA, radioimmunoassay; ELISA, enzyme linked immunosorbent assay.

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http://dx.doi.org/10.1016/j.cca.2014.01.038

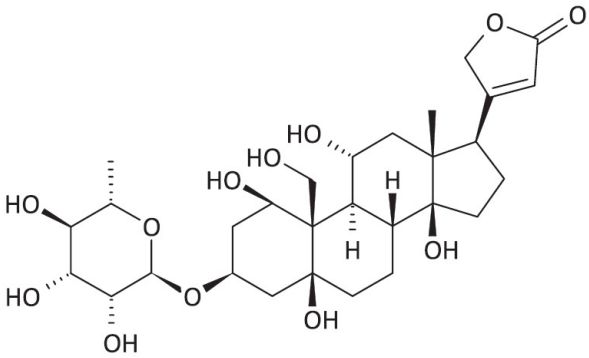

Fig. 1. Chemical structure of ouabain.

or *endogenous ouabain* (EO). Several authors have suggested that the glomerulosa cells of the adrenal cortex synthesize these compounds in an adrenocorticotropic hormone (ACTH) — and angiotensin II-dependent fashion [14–16]. However, the origin of ouabain in the adrenal cortex has not been unequivocally demonstrated and the actual pathway of its biosynthesis remains elusive [17–21].

Compared to healthy controls, increased plasma ouabain was reported in several pathological conditions such as hypertension [21–24] and heart failure [23,25–28]. Using immunoassays developed in few different laboratories, mean endogenous ouabain concentrations ranging from 60 to as high as 530 pmol/l were described in healthy individuals (Table 1). Despite these interesting findings, immunoassays for the quantification of ouabain in biological samples are not available any more. There are a few attempts reported to analyze plasma ouabain by using liquid chromatography mass spectrometry (LC-MS) instead of and as an alternative to immunoassays [27,29,30]. The methodologies used, however, did not approach quantification and did not involve internal standardization. Regarding on the one hand the potential usefulness of plasma ouabain measurement as a cardiovascular biomarker, and on the other hand the lack of commercially available immunoassays for further research in this field, we decided to develop a reliable and convenient, antibody-free method for the quantification of ouabain in human plasma, based on atmospheric pressure ionization (API) tandem-mass spectrometry. This technology allows the analysis of small molecules on a very high level of metrological reliability: detection of analytes is based on their molecular mass and their molecular disintegration patterns; matrix effects are compensated for by application of stable isotope labeled compounds for internal analytical standardization [31]. We envisioned to establish conclusive reference ranges for endogenous ouabain based on this technology.

2. Materials and methods

2.1. Materials and chemicals

Ouabain octahydrat (purity 99%) and lithium chloride were from Sigma-Aldrich (Schnelldorf, Germany). Ouabain-d3 (purity 97%) was obtained from Toronto research chemicals (Toronto, Canada).

Table 1
Studies reporting mean (range) endogenous ouabain plasma levels; [a] healthy volunteers, [b] patients with congestive heart failure, [c] patients with endstage renal disease, [d] patients with essential hypertension, [e] patients with idiopathic hyperaldosteronism, [f] critically ill patients, [g] normotensive individuals, [h] patients with low-renin hypertension, [i] patients with mild hypertension; FAM, families with positive history of hypertension.

Author	Reference	Assay type	Analyte description	Mean (range) endogenous ouabain (EO) plasma level [pmol/l]
Gottlieb et al., 1992	[25]	ELISA	Endogenous ouabain	[a] 440 (160–770) (n = 19) [b] 1520 (20–8270) (n = 16)
Lewis et al., 1994	[26]	ELISA (in-house assay)	Ouabain	[a] 320 (130–560) (n = 9) (without HPLC) [b] 650 (250–1600) (n = 6) (without HPLC) [c] 440 (240–620) (n = 6) (without HPLC) [a,b,c] no evidence of EO ([a]n = 9, [b]n = 6, [c]n = 6) (with HPLC)
		ELISA kit (Du Pont-NEN)		[a] 60 (50–70) (n = 5) (without HPLC) [a] no evidence of EO (with HPLC)
Rossi et al., 1995	[21]	ELISA	Endogenous ouabain	[a] 530 ± 100 (±SEM) (n = 19) [d] 3390 ± 570 (±SEM) (n = 64) [e] 4090 ± 1120 (±SEM) (n = 24)
Gonick et al., 1998	[42]	DELFIA	Ouabain	[a] 93 ± 15 (±SEM) (n = 38) [b] 102 ± 25 (±SEM) (n = 7) [c] 144 ± 23 (±SEM) (n = 24) [d] 128 ± 51 (±SEM) (n = 27) [e] 226 ± 140 (±SEM) (n = 5)
Berendes, 2003	[43]	ELISA	Endogenous ouabain	[a] 380 ± 310 (±SD) (n = 62) [f] 1340 ± 900 (±SD) (n = 343)
Wang et al., 2003	[44]	RIA	Ouabain	[a] 144 ± 45 (±SD) (women) (n = 197) [a] 158 ± 48 (±SD) (men) (n = 182)
Manunta et al., 2005	[22]	RIA	Endogenous ouabain	[g] 221.5 ± 10.95 (±SD) (FAM+ subjects) (n = 41) [g] 179.6 ± 9.58 (±SD) (FAM- subjects) (n = 45)
Balzan et al., 2005	[24]	RIA	Endogenous ouabain	[a] 366 ± 40 (±SEM) (n = 13) [h] 939 ± 219 (±SEM) (n = 13)
Pitzalis et al., 2006	[27]	RIA	Endogenous ouabain	[a] 230.36 (53–409) (young subjects) (n = 151) [a] 222.69 (66–726) (old subjects) (n = 52) [b] 235.80 (105–589) NYHA 1 (n = 41) [b] 247.96 (72–740) NYHA 2 (n = 74) [b] 426.52 (98–956) NYHA 3 (n = 25)
Manunta et al., 2006	[45]	RIA	Endogenous ouabain	[a] 430 ± 80 (±SE) (normal diet) (n = 13) [a] 5800 ± 2200 (±SE) (high salt diet) (n = 13)
Stella et al., 2007	[30]	RIA	Endogenous ouabain	[c] 930 ± 290 (±SD) (n = 156)
Manunta et al., 2008	[46]	RIA	Endogenous ouabain	[i] EO quartile <157: 132.5 ± 3.0 (±SEM) (n = 39) [i] EO quartile 157–236: 193.0 ± 3.8 (±SEM) (n = 40) [i] EO quartile 236–337: 279.9 ± 4.5 (±SEM) (n = 37) [i] EO quartile > 337: 547.2 ± 27.5 (±SEM) (n = 39)
Kolmakova et al., 2011	[47]	DELFIA	Endogenous ouabain	[a] Mean similar to [c] (n = 19) [c] 280 ± 20 (±SEM) (n = 25)

Table 2
Target concentrations of ouabain in QC materials and validation results. [a)] n = 5 determinations; [b)] n = 4 series x 5 determinations.

	Target conc. [pmol/l]	Intra-day precision CV [a] [%]	Inter-day precision CV [b] [%]	Mean accuracy [a] [%]	Recovery in stability study [%]						
					RT 24 h	8 °C 24 h	RT 48 h	8 °C 48 h	RT 120 h	8 °C 120 h	freeze and thaw
QC Level low	6.9	1.3	5.0	99.2	99.5	107.2	105.5	108.4	101.9	97.6	107.7
QC Level medium	70.0	1.6	2.5	101.8	100.6	102.2	106.3	105.6	91.4	104.5	103.7
QC Level high	138.0	1.8	3.1	97.5	100.9	103.8	105.7	102.8	94.7	99.1	98.9

Acetonitrile, methanol, water and trifluoroacetic acid were from Biosolve (Valkenswaard, the Netherlands).

2.2. Calibrator samples and quality control (QC) material

Calibrator samples were prepared by spiking a pool of EDTA plasma from leftover samples with a solution of ouabain in methanol 20% in order to obtain seven concentration levels: 1.7, 3.5, 8.6, 17.3, 43.1, 86.3 and 172.5 pmol/l. Calibrator samples were aliquoted à 500 µl and stored at −20 °C. In the same way QC samples were prepared in three levels: QC Level low 6.9 pmol/l, QC Level medium 70.0 pmol/l, QC Level high 138.0 pmol/l (Table 2). The QC materials were aliquoted (à 500 µl) and stored at −20 °C. An internal standard (IS) ouabain-d3 stock solution (c = 82.7 µmol/l) was prepared in methanol and an IS working solution was prepared in methanol 20% (c = 827 pmol/l) which were stored at −20 °C and 8 °C, respectively.

2.3. Sample preparation

25 µl IS working solution was added to 500 µl calibrator samples, QC materials or plasma samples and mixed thoroughly for 5 min at room temperature (RT). Samples were diluted with 500 µl 0.1% trifluoroacetic acid (TFA) in water, mixed thoroughly for 5 min at RT and centrifuged at 3000 rcf for 30 min at 4 °C. Samples were extracted applying solid phase extraction using Oasis MAX cartridges (30 mg, 1 cc, 1 ml; Waters, Eschborn, Germany) which were conditioned with 1 ml acetonitrile and equilibrated with 1 ml water. Supernatants were loaded onto the columns which were subsequently washed with 1 ml water. Analytes were eluted with 2 × 600 µl 25% acetonitrile. The combined eluate was evaporated to dryness using a BACHOFER Speed Vac Concentrator® (BACHOFER GmbH, Reutlingen, Germany). Residues were reconstituted using 200 µl 5% methanol, mixed thoroughly for 5 min and the resulting solutions were centrifuged at 10000 rcf for 3 min at RT. Supernatants were transferred into high performance liquid chromatography (HPLC) vials with small volume insert and analyzed using ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS).

2.4. UPLC-MS/MS

UPLC-MS/MS analysis was performed using a Waters ACQUITY UPLC system and Waters XEVO TQ-S tandem quadrupole mass spectrometer (Waters, Milford, USA). After injection of 20 µl of extracted sample (full loop injection), separation was achieved on an ACQUITY UPLC HSS-T3 column (2.1 mm x 100 mm, 1.8 µm; Waters, Milford, USA) equipped with a ACQUITY UPLC HSS-T3 VanGuard pre-column (2.1 mm x 5 mm, 1.8 µm; Waters, Milford, USA) (column temperature 40 °C) with the following linear gradient of mobile phase A (water) and B (methanol): initial: 5% B; 0–0.5 min 5% B; 0.5–3.5 min 5–35% B; 3.5–5.5 min 35% B; 5.5–9.0 min 35–90% B; ouabain and IS eluted at 5.3 min. After analyte separation the analytical column was washed and equilibrated. The total run time was 12.5 min with a constant flow rate of 0.35 ml/min. The effluent was combined with 0.25 mM LiCl (flow rate 2.5 µl/min) using a T-piece and post-column-infusion. The UPLC was interfaced with a Waters XEVO TQ-S operating in atmospheric pressure electrospray ionization (ESI) positive mode. Ion source settings were: capillary voltage, 4.25 kV; desolvation temperatures, 650 °C; source temperature, 150 °C; nitrogen was used as desolvation gas, cone gas and nebulizer gas at a flow rate of 1000 l/h, 150 l/h and 7 bar; argon was used as collision gas at a flow rate of 0.15 ml/min. Lithium-adduct fragments of ouabain and IS were detected using multi reaction monitoring (MRM) technique. An overview of the MRM-parameters is shown in Table 3. System operation, data acquisition and data processing were controlled using MassLynx V4.1 software (Waters, Milford, USA).

2.5. Method validation

2.5.1. Linearity

Linearity was determined by assessing four replicates of the seven-point calibration curves. The response was considered to be linear if in all assays the %-deviation of recalculated results of each calibrator from target value was <10% and if the coefficient of determination (r^2) was >0.99, calculated using least-squares linear regression.

2.5.2. Lower limit of quantification (LLOQ)

LLOQ was evaluated by analyzing the calibrator sample with the lowest concentration (1.7 pmol/l) five times. The acceptance criterion for LLOQ was defined as a %-deviation from target value <10%, a total coefficient of variation (CV) <20%, and a signal-to-noise ratio (S/N) >10 for analyte peaks.

2.5.3. Intra-day precision (repeatability) and inter-day precision (intermediate precision)

Intra-day precision (repeatability) and inter-day precision (intermediate precision) were determined using the QC materials given in Table 2. For assessment of the intra-day precision the CVs were calculated for five-fold determination in one series and for assessment of the inter-day precision the CVs were calculated for five-fold determination in four series (n = 4 × 5).

2.5.4. Accuracy

To assess the accuracy of the method, mean concentrations found for the QC samples in imprecision study were compared with the target concentrations (Table 2).

2.5.5. Stability

Analyte stability was investigated in QC materials. For this purpose, short-term temperature stabilities (24 h, 48 h and 120 h) at RT and at 8 °C were assessed. Furthermore, freeze and thaw stability was evaluated (one freeze-thaw cycle). The concentrations found for QC samples treated in this way were compared with the respective target concentrations (Table 2)

Table 3
MRM-parameters.

Analyte	Parent ion [m/z]	Product ion [m/z]	Cone voltage [V]	CE [V]	dwell time [sec]	Rt [min]
Ouabain	591.3	445.25	110	48	0.015	5.3
Ouabain-d3	594.3	448.25	110	48	0.015	5.3

2.5.6. Recovery and matrix effect

For evaluation of recovery and matrix effect according to Matuszewski et al. [32] the following samples were analyzed:

(A) Spiked sample: A defined amount of ouabain was spiked to a plasma sample and then submitted to sample preparation
(B) Spiked eluate: a defined amount of ouabain was spiked to reconstituted eluat obtained after sample preparation
(C) Spiked elution solvent: an identical amount of ouabain was spiked to elution solvent

The percentage of recovery and matrix effect, respectively, was calculated using the peak areas of the MRM trace of ouabain according to the following equations:

$$\text{Recovery } [\%] = (A \div B) \text{ x } 100$$

$$\text{Matrix effect } [\%] = (B \div C) \text{ x } 100$$

2.5.7. Post column-infusion experiment

Ion suppression/enhancement was further studied according to Bonfiglio et al. [33] by a post-column-infusion experiment: A solution of 17 nmol/l of ouabain and IS, respectively, was infused post-column into the mobile phase of the analytical set-up with a constant flow rate of 5 μl/min using a T-piece. By this setting a continuous background signal was generated in the MRM-traces of the analytes. The ion count of these traces was monitored upon injection of elution solvent and extracted plasma samples, respectively. MRM chromatograms were visually inspected for a variation in the ion yield (i.e., background signal) at the retention time of the target analytes following the injection of the specified samples.

2.6. Patients

While it was the initial aim of our work to characterize ouabain concentration in a large and well defined population in a second stage of our project, we decided to first apply our analytical method to a pilot-set of clinical samples before recruiting prospectively. For this aim 30 residual human plasma samples from a clinical laboratory were analyzed using the UPLC-MS/MS method. 25 samples were from patients with severe heart failure and plasma B-type natriuretic peptide (BNP) values >524 pg/ml. Five samples were from individuals with BNP values <30 pg/ml. The BNP values were obtained using an automated immunoassay system (ADVIA Centaur XP, Siemens Healthcare Diagnostics GmbH, Erlangen, Germany). According to the manual of the assay a BNP value >524 pg/ml is typically found in New York Heart Association (NYHA) Class III. De-identified leftover sample materials were used for this study as approved by the institutional review board. These EDTA-anti coagulated samples (centrifuged at 3000 rcf for 15 min) were sent to the Institute in order to measure BNP. The leftover material was preserved at −20 °C not longer than 6 h after sampling. The samples were stored at −20 °C for maximum one month prior UPLC-MS/MS analysis.

3. Results

3.1. UPLC-MS/MS method

With the UPLC-MS/MS method, ouabain and ouabain-d3 eluted at 5.3 min. In Fig. 2 a chromatogram of the calibrator sample with the lowest analyte concentration (1.7 pmol/l) in EDTA plasma is shown (signal-to-noise ratio = 38).

3.2. Method validation

Linear regression analysis showed linearity for ouabain over its calibration range (1.7 pmol/l–172.5 pmol/l) with a %-deviation of each calibrator from target value <10% and r^2 >0.99.

A LLOQ (%-deviation from target value <10%; CV <20% and S/N >10) was verified as 1.7 pmol/l based on analysis of the lowest calibrator.

CV's observed for intra-day precision (repeatability) and inter-day precision (intermediate precision) for all QC samples were ≤1.8% and ≤5.0%, respectively. Accuracy related to QC-samples ranged from 97.5% to 101.8%. Ouabain in plasma showed short term stability (24 h, 48 h, 120 h) at RT and 8 °C as well as freeze–thaw stability (one freeze–thaw cycle). Results of the validation series in their entirety are given in Table 2.

Recovery and matrix effect was 94% and 40%, respectively. Ion suppression testing showed a base line signal suppression of about 10–20% over the whole chromatographic run time and no further signal suppression at the typical retention time of the analyte and the IS.

3.3. Analyzing human plasma samples

Analyzing the pilot-set of clinical samples in none of the 30 human plasma samples ouabain could be detected within the calibrator range. This was true for the 5 patients with normal BNP (presumably from patients without heart failure), but also for all patients with high BNP (median BNP 1705 pg/ml, mean BNP 2278 pg/ml, range 858–7175 pg/ml). Considering those strikingly unexpected but analytically sound and valid results, we decided not to proceed with recruiting reference population samples in our project.

4. Discussion

In this article we describe an extremely sensitive, highly specific and comprehensively validated UPLC-MS/MS method for the detection and quantification of ouabain in human plasma. Using this reliable method in a pilot-set of clinical samples from patients with and without heart failure, ouabain was not detected in any sample. This is in striking contrast to previously reported immunoassay-based results, which describe detectable ouabain in *all* samples. Regarding those findings we did not find it reasonable to proceed in our project with recruiting well defined populations for measurement of ouabain.

While gas chromatography–mass spectrometry (GC-MS) has been recognized for several decades as a platform for reference methods, LC-MS/MS has become available in the late 1990ies [34]. In contrast to GC-MS this technology is applicable for highly polar and thermo-labile compounds such as cardiac glycosides as well. After ionization of target analytes from the extracts of biological samples, detection is based on the molecular mass of the target analyte and in addition to its disintegration pattern induced by collision of target analyte ions with an inert gas. This "multiple-reaction-monitoring" principle realizes on the one hand very high specificity for the respective target analyte based on a "mass transition", and on the other hand background signal is almost completely eliminated, enabling very sensitive assays with high signal-to-noise ratio. Addition of stable isotope labeled target analyte molecules for internal standardization allows the compensation of all matrix effects. The method as described in the material and method section was the result of an extended process of optimization focussed on the lower level of quantification. It involved a highly efficient solid phase extraction protocol and post column infusion of LiCl in order to increase ionization yield by forming Li-adducts of the target analyte, finally enabling reliable quantification of ouabain in concentrations as low as 1.7 pmol/l in plasma-based spiked calibrator samples. The method was extensively validated according the Guidelines of the FDA for use in pharmacological research (http://catalogue.nla.gov.au/Record/4834734) [35], also addressing sample stability. The method

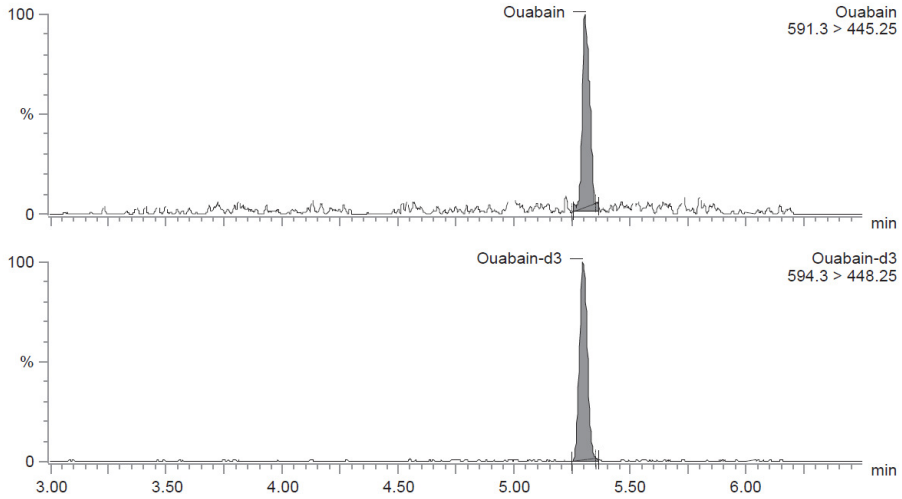

Fig. 2. Chromatogram recorded for the calibrator sample with the lowest analyte concentration (1.7 pmol/l) in EDTA plasma. Upper trace: target analyte ouabain, S/N = 38; lower trace: threefold deuterated IS.

showed excellent performance concerning sensitivity, precision, accuracy and recovery. A relevant degree of matrix associated signal suppression was observed, however, this did not compromise trueness of results, since isotope dilution internal standardization was applied. Ouabain revealed stability in EDTA plasma in context of the evaluated time and conditions, respectively. Since patients' samples were processed identically to the calibrator samples which were plasma-based, we can also rule out adsorption of the analyte to tube or vial surfaces or plasma proteins. In summary, there is no reasonable doubt in the reliability of our method with respect to its intended use.

Given the lower level of quantification of 1.7 pmol/l, our result indicates that endogenous ouabain – if actually existing in the human circulation – would be present in only femtomolar concentrations. In contrast, using different immunoassays mean concentrations of ouabain in plasma of healthy persons ranging from 60 to 530 pmol/l have been reported (Table 1). Notably, in *all* samples of these studies ouabain was found to be present. Direct comparison between the two analytical approaches was not possible in the context of our investigation since no immunoassay for the quantification of ouabain is available at present.

The most likely explanation for these discrepant results – ouabain is detected in human plasma by immunoassays but not by the highly specific LC-MS/MS method described herein – is the limited specificity of so far used immunoassays for the quantification of (seeming) ouabain secreted from endogenous sources. It appears reasonable to assume that these immunoassays suffer from cross-reaction with structurally related compounds which may indeed have between-individual dynamics in cardiovascular diseases. Limited specificity is a widely recognized limitation of immunoassays, in particular in a single-antibody, competitive set-up. Our LC-MS/MS method, however, strictly detects an analyte with the molecular mass (584.5 Da) and molecular disintegration behavior assigned for ouabain — if present within the calibration range.

Indeed, the investigations performed to characterize the molecular structure of endogenous targets of ouabain immunoassay appear questionable [12]. In the most relevant publication of Mathews et al. the daughter ion spectra of endogenous digitalis like factor (EDLF) and commercially available ouabain were compared and exhibited only uncertain agreement [12]. In a review of Hamlyn et al., these discrepancies were addressed and an "isomer of ouabain" in plasma was suggested [36]. The molecular structure of ouabain in human plasma has never been characterized using nuclear magnetic resonance (NMR) technique which is crucial for reliable structure elucidation.

In two studies, "endogenous ouabain" was measured by radioimmunoassay (RIA) and LC-MS/MS was used to 'prove the presence of endogenous ouabain' by analyzing it in four plasma samples [27,30]. In both studies it was stated that the results determined by LC-MS/MS and by RIA were "correlated in linear regression analysis". However, exact ouabain concentrations were not given. In one of the studies, the correlation between plasma ouabain concentrations determined by these respective methods is shown in a figure; the approximate concentrations measured by LC-MS/MS were about 2–20% (~3–50 pmol/l) compared to the approximate concentrations measured by RIA (~140–300 pmol/l). Notably, the LC-MS/MS method used in these studies was not adequately described nor validated. Our results are also in contrast to a report by Komiyama et al. [29] addressing an *ouabainlike factor* in an extract of 80 ml plasma using LC-MS. However, when reviewing primary data displayed in this article we cannot agree that a conclusive identification of the assumed analyte is given. The detected peaks, which are shown for various potential daughter ions, exhibit 2–3 data points each which is not sufficient for reliable analyte identification. Notably, also digoxin is claimed to be observed in this plasma extract from untreated individuals in this article. No method validation was performed.

Notably, various studies were published previously which questioned the existence of ouabain from endogenous sources in human plasma [17,26,37–41]. Lewis et al. reported discrepant ouabain concentrations in human plasma using different assay conditions [26]. The authors were unable to detect ouabain with various immunometric methods (enzyme linked immunosorbent assay (ELISA) techniques) after precedent HPLC fractionation, while without HPLC fractionation ouabain was reported in concentrations between 50 and 560 pmol/l in healthy volunteers.

We conclude from our results that it does not seem useful to address the compound ouabain in plasma by using LC-MS/MS in the further research on proposed endogenous modulators of myocardial Na^+/K^+-ATPases. Structurally slightly different compounds may account for the measurement results of previous studies relying on respective immunoassays for this compound in plasma. In this context it may be recognized as a certain drawback of LC-MS/MS that already very minor molecular modification of intended target analytes makes compounds undetectable for this technology, while immunoassay may function as a "group test" due to some inherent degree of poly-specificity. Future research in the exciting field of endogenous cardio-vascular regulating compounds should therefore address

biomarker identification with appropriate technologies including immobilized Na^+/K^+-ATPases for analyte extraction and high-resolution mass spectrometry for unknown screening.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgment

None.

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2.2 Baecher, S.; Geyer, R.; Lehmann, C.; Vogeser, M.

Absorptive chemistry **based extraction for LC-MS/MS analysis of small molecule analytes from biological fluids – an application for 25-hydroxyvitamin D**

Clin. Chem. Lab. Med. 2014;52:363-71

DE GRUYTER DOI 10.1515/cclm-2013-0534 — Clin Chem Lab Med 2014; 52(3): 363–371

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Absorptive chemistry based extraction for LC-MS/MS analysis of small molecule analytes from biological fluids – an application for 25-hydroxyvitamin D

Abstract

Background: Already available sample preparation technologies for liquid chromatography-tandem mass spectrometry have substantial shortcomings with respect to automation. A novel approach is based on gel-like polymeric material with defined absorption chemistry, which is immobilized in micro-plate wells. It is referred to as *Tecan Immobilized Coating Extraction*™ (*TICE*™) technology and it enables easy automation on liquid handling systems. We aimed to study the performance of Tecan *AC Extraction Plate*™ based on this principle by addressing 25-hydroxyvitamin D (25OHD) as an exemplary analyte.

Methods: A protocol for extraction of 25OHD from serum samples based on *TICE*™ technology was implemented on a robotic liquid handling system *Freedom EVO*® (Tecan). An isotope-dilution ultra-performance liquid chromatography-tandem mass spectrometry method was used for quantification. Performance was tested according to a comprehensive protocol.

Results: Linearity was found over a range from 4.3 to 65.8 ng/mL for $25OHD_3$. The coefficients of variation for the intra-day and inter-day precision were <6% and accuracy ranged between 96.9% and 99.8% for $25OHD_3$. Recovery was 84% and efficient control of matrix effects was verified. High sample throughput could be observed with 96 samples prepared in <60 min. Close agreement of results was found for clinical samples analyzed with a second tandem mass spectrometry method based on protein precipitation and two-dimensional ultra-performance liquid chromatography for sample preparation ($r=0.988$, $n=73$).

Conclusions: The new *TICE*™ technology was found to be a useful process for sample preparation in clinical mass spectrometry. Full automation suited for routine analysis was achieved.

Keywords: 25-hydroxyvitamin D (25OHD); clinical mass spectrometry; immobilized liquid extraction (ILE); liquid chromatography-tandem mass spectrometry (LC-MS/MS); Tecan *AC Extraction Plate*™; *Tecan Immobilized Coating Extraction*™ (*TICE*™).

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Introduction

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a highly selective technology for analyte detection; however, for application in laboratory medicine, efficient sample prefractionation is crucial for reliability and robustness of respective methods. For small molecule analytes, depletion of proteins from blood-based samples is essential to avoid clogging of packed chromatography columns. Further, matrix components – such as salts – are aimed to be eliminated in order to obtain efficient and constant ion yield, and to minimize contamination of analytical columns and MS analyzer. Concentrating analytes during sample preparation can furthermore improve the analytical sensitivity. These general requirements of sample preparation should ideally be realized by cost-efficient and technically simple, high-throughput protocols.

Several different approaches, in particular protein precipitation (PPT), protein filtration, solvent extraction, solid phase extraction (SPE), and on-line SPE, have been applied so far for sample preparation in clinical LC-MS/MS methods (Table 1).

This spectrum of sample preparation techniques allows the development of appropriate protocols for most analytes; however, all these technologies have substantial shortcomings with respect to full automation (Table 1) [1]. As an alternative approach, *Tecan Immobilized Coating*

Table 1 Extraction methods for sample preparation in clinical LC-MS/MS methods and their advantages, challenges, and automation aspects.

Extraction method	Principle	Advantages	Challenges	Automation aspects
Protein precipitation (PPT)	Addition of e.g., methanol, acetonitrile, or zinc sulfate solution. Subsequent centrifugation, formation of a protein pellet.	Straightforward Inexpensive	High residual content of matrix components. Dilution of samples. Automation challenging.	Full automation requires integration of a centrifuge to a sample preparation robot.
Protein filtration	Addition of denaturation reagents. Subsequent filtration by application of vacuum.	Automation possible.	Vacuum device required. Even vacuum in each well necessary. High content of residual matrix components.	Convenient automation using micro-plate format, liquid handling robots and vacuum device.
Solvent (syn. liquid-liquid) extraction	Addition of immiscible organic solvents. Differential distribution of the analyte in organic solvent and aqueous phase.	Very clean extracts. Potential to concentrate the target analyte. Inexpensive	Solvent evaporation and sample reconstitution required. Potential health hazard by organic solvent.	Automation for higher throughput can hardly be achieved.
Solid phase extraction (SPE)	Prechromatography with disposable chromatographic cartridges or well plates. Technique based on analyte adsorption.	Highly efficient extraction. Automation possible. Potential to concentrate target analytes.	Risk of overload or breakthrough. Expensive consumables.	Automation can be realized requiring technologically demanding application of positive or negative pressure.
On-line SPE	Two-dimensional (2D) chromatography applying a permanently used extraction column after PPT.	Convenient and widely used second clean-up step after PPT. Minimized workload. Extraction column repeatedly used and re-activated during analytical cycle.	Expensive extraction columns. Protein precipitation required. Application of crude serum limited due to rather short life of respective extraction columns (e.g., in turbulent-flow chromatography). Increased run-time per sample.	Requires additional HPLC pump and switching valve, software support. Automation of preceding PPT demanding (see above).
TICE™	ILE-based principle. Extraction plate with AC.	Only pipetting and shaking steps; no application of positive or negative pressure. No conditioning or equilibration. Easy to automate.	Risk of contamination if not kept sealed until usage.	Only shaking device and liquid handling system required. Automated application does not require technically demanding use of vacuum, positive pressure or centrifugation.

Extraction (*TICE™*) technology, an immobilized liquid extraction (ILE) based principle will soon be commercially available (Table 1). In *TICE™* technology, a thin film of a gel-like polymeric extraction material is coated onto micro-plate wells. According to the specific *absorptive chemistry* (AC) of this material, target analytes are *ab*sorbed into the medium – in contrast to SPE where *ad*sorption to a surface takes place. Absorption of target analytes from serum or plasma samples is achieved under well-defined chemical conditions because of a pre-dispensed reagent which aids in pH adjustment and in overcoming protein binding of analytes. After the removal of residual sample matrix and the washing step, analytes are eluted by a small volume of solvent. In contrast to SPE or protein filtration, no positive or negative pressure has to be applied with *TICE™* technology and time-consuming preconditioning of the plates is not required. Consequently, the *TICE™* technology seems to be ideally suited for the development of fully automated sample preparation protocols for clinical mass spectrometry. The *TICE™* technology will be commercialized by Tecan (Männedorf, Switzerland) in a 96-well micro-plate format as *AC Extraction Plate™* (Figure 1).

The aim of our study was to test the performance characteristics of the *AC Extraction Plate™* by addressing

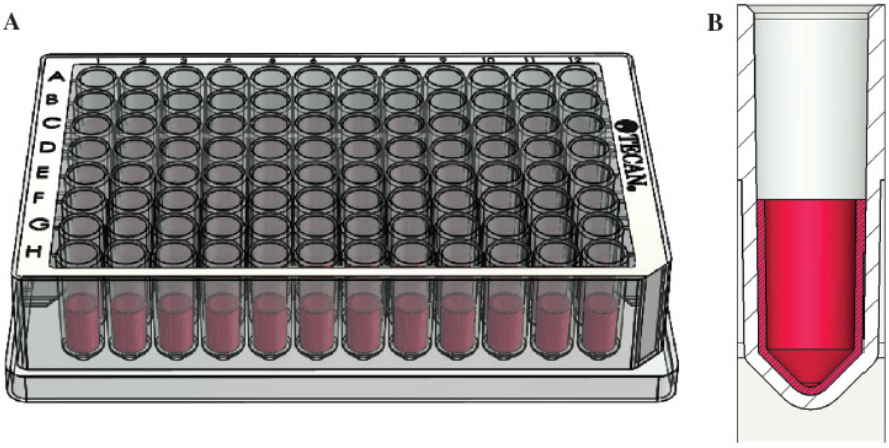

Figure 1 Graphical designs of the *AC Extraction Plate*™ (Tecan) (A) and of a well of the *AC Extraction Plate*™ (B) which is coated from the bottom to about half height with a thin film (the coating itself is transparent, but can be made visible with for example a Nile red solution).

25-hydroxyvitamin D (25OHD) as an exemplary analyte in a fully automated protocol implemented on a robotic liquid handling system (*Freedom EVO*®, Tecan, Switzerland).

Materials and methods

Materials and chemicals

Tecan *AC Extraction Plates*™ and *LiHa Disposable Tips* with filter (200 and 1000 µL) were from Tecan (Männedorf, Switzerland). D_6-25-hydroxyvitamin D_3 (D_6-25OHD_3) was from Cerilliant (Round Rock, USA). Uncoated 96 half-deep well plates (500 µL) and the corresponding pierceable sealing mats were from Eppendorf (Wesseling-Berzdorf, Germany). Acetonitrile, methanol, and water were from Biosolve (Valkenswaard, the Netherlands). Formic acid (99%) was from Merck (Darmstadt, Germany). Sodium carbonate and sodium bicarbonate were obtained from Sigma-Aldrich (Schnelldorf, Germany).

Calibration standards and quality control materials

Commercially available four-level calibrators *3PLUS1® Multilevel Serum calibrator Set 25-OH-Vitamin D3/D2* were from Chromsystems (Munich, Germany) with concentration levels ranging from 4.3 to 65.8 ng/mL for 25OHD_3 and from 14.1 to 54.7 ng/mL for 25OHD_2. Commercially available two-level quality control (QC) material *MassCheck® 25-OH-Vitamin D3/D2 Serum Control* was from Chromsystems (Munich, Germany). According to the manufacturer's declaration these calibrators and controls are traceable to the *NIST standard reference material (SRM) 972*. Further commercial QC material was the two-level QC material *ClinCheck® – Control for 25-OH-Vitamin D2/D3* from Recipe (Munich, Germany). All calibration and QC materials were handled according to the instructions from the manufacturers. Table 2 shows the target concentrations of both analytes in QC materials. Additionally, QC pools were prepared from leftover human serum sample materials in a low and normal 25OHD_3 concentration range to study the reproducibility of the method.

Table 2 Quantification of 25OHD using the automated *TICE*™ UPLC-MS/MS method: target concentrations of 25OHD_3 and 25OHD_2 in QC materials given by the manufacturers and validation results.

QC material	25OHD_3				25OHD_2			
	Target concentration, ng/mL	Intra-day precision[a] CV, %	Inter-day precision[b] CV, %	Accuracy (mean)[a], %	Target concentration, ng/mL	Intra-day precision[a] CV, %	Inter-day precision[b)] CV, %	Accuracy (mean)[a)], %
Chromsystems QC Level I	16.7	3.7	4.2	99.4	17.2	4.9	5.8	101.8
Chromsystems QC Level II	37.7	3.7	5.5	96.9	37.8	3.5	5.8	95.4
Recipe QC Level I	20.5	1.1	5.7	99.2	16.3	2.2	6.9	112.7
Recipe QC Level II	44.3	1.4	4.0	99.8	36.6	4.3	4.3	107.3
QC pool low		2.7	5.2			n.a.	n.a.	
QC pool normal		4.1	5.0			n.a.	n.a.	

n.a. not applicable because no analyte was detected. [a]n=5 determinations; [b]n=4 series×5 determinations.

An internal standard (IS) (D_6-25OHD$_3$) working solution was prepared by diluting the stock solution (concentration 5 µg/mL) with acetonitrile resulting in a concentration of 50 ng/mL.

All calibrator and QC materials as well as IS working solution were stored at -20°C.

Automated *Tecan Immobilized Coating Extraction (TICE™)*

Instrument

AC Extraction Plates™ were handled in a fully automated protocol implemented on a *Freedom EVO*® robotic pipetting system (Tecan, Männedorf, Switzerland). This instrument is an open liquid handling platform on which the components can be modularly implemented for each individual application. The system used in this study was equipped with one robotic arm (liquid handling arm) with four channels for disposable pipetting tips, six 16-position sample tube carriers (for up to 96 samples in total), a reagent carrier with three troughs for the extraction mix, wash solution and elution solvent, a washing station, a plate carrier on a horizontal shaker (*Te-Shake*™) for the *AC Extraction Plate*™, an additional plate carrier for the uncoated injection plate, a barcode reader, disposable pipetting tips in trays, and a dropping station for used pipette tips (Figure 2). The pipetting system was controlled by *Freedom EVOware*® software.

TICE™ solutions for 25OHD

The daily freshly prepared extraction mix (room temperature) is a 2:1 (v/v) mixture of the modifier buffer (0.2 M carbonate buffer stock solution, which is prepared with sodium carbonate and sodium bicarbonate in water:acetonitrile, 95:5, v/v) and the IS working solution (IS in acetonitrile). The wash solution consists of 10% methanol in water and the elution solvent consists of 10% water in methanol.

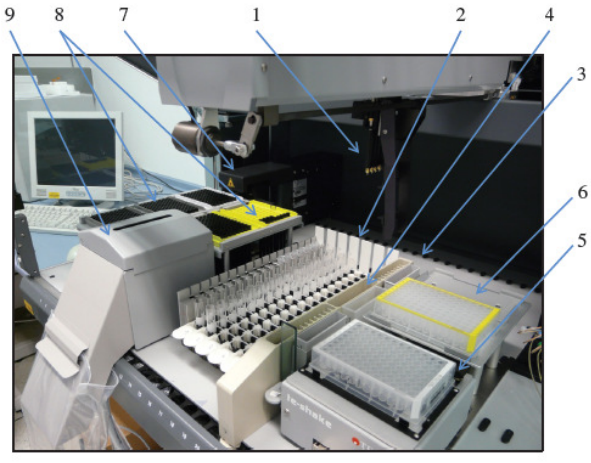

Figure 2 Tecan *Freedom EVO*® pipetting system worktable.
1) Liquid handling arm with four channels for disposable pipetting tips; 2) six 16-position sample tube carriers; 3) reagent carrier; 4) wash station; 5) horizontal shaker for *AC Extraction Plate*™ (Tecan); 6) plate carrier for injection plate (uncoated 96 half-deep well plate, Eppendorf); 7) barcode reader; 8) disposable pipetting tips; and 9) dropping station for used pipette tips.

TICE™ workflow

For automation of 25OHD extraction from serum samples, a simple three-step pipetting and shaking workflow was developed, which includes a loading (extraction) step, a wash step, and an elution step. Few manual working steps were necessary only in the initial set-up and after the finalization of this automated process. The extraction process is displayed in detail in Figure 3.

Ultra-performance liquid chromatography-tandem mass spectrometry

Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) quantification of 25OHD$_3$ and 25OHD$_2$ in extracts was performed using a Waters ACQUITY Ultra Performance LC (UPLC) system and a Waters XEVO TQ-S tandem quadrupole mass spectrometer (Waters, Milford, USA). After injection of 20 µL of extracted sample (full loop injection), separation was achieved on an ACQUITY UPLC BEH C18 column (2.1 mm×50 mm, 1.7 µm, Waters) (column temperature 45°C) with the following linear gradient of mobile phase A [water:methanol (90:10, v/v) with 0.1% formic acid] and mobile phase B [methanol:acetonitrile (80:20, v/v) with 0.1% formic acid]: initial: 70% B; 0.1–1.1 min: 70% B–98% B; 1.1–2.6 min: 98% B; 2.7–5.0 min 70% B. Total run time was 5 min with a constant flow rate of 0.4 mL/min. 25OHD$_3$ and the IS eluted at 1.40 min and 25OHD$_2$ eluted at 1.44 min. UPLC was interfaced with a Waters XEVO TQ-S operating in the atmospheric pressure chemical ionization positive mode. Ion source settings were: corona discharge current, 2.0 µA; probe temperature, 550°C; nitrogen was used as a desolvation gas, cone gas, and nebulizer gas at a flow rate of 900 L/h, 150 L/h, and 2.5 bar; source temperature, 100°C; argon as the collision gas at a flow rate of 0.15 mL/min. Analytes were detected using multi-reaction monitoring (MRM) technique. An overview of the MRM parameters is shown in Table 3. System operation, data acquisition, and data processing were controlled using MassLynx V4.1 software (Waters, Milford, USA).

Method evaluation

The automated *TICE*™ UPLC-MS/MS method was studied to assess linearity, sensitivity, intra-day precision, inter-day precision, and accuracy. Furthermore, recovery and matrix effects were studied.

Linearity

Linearity was determined by assessing four replicates of four-point calibration curves. The response was considered to be linear if in

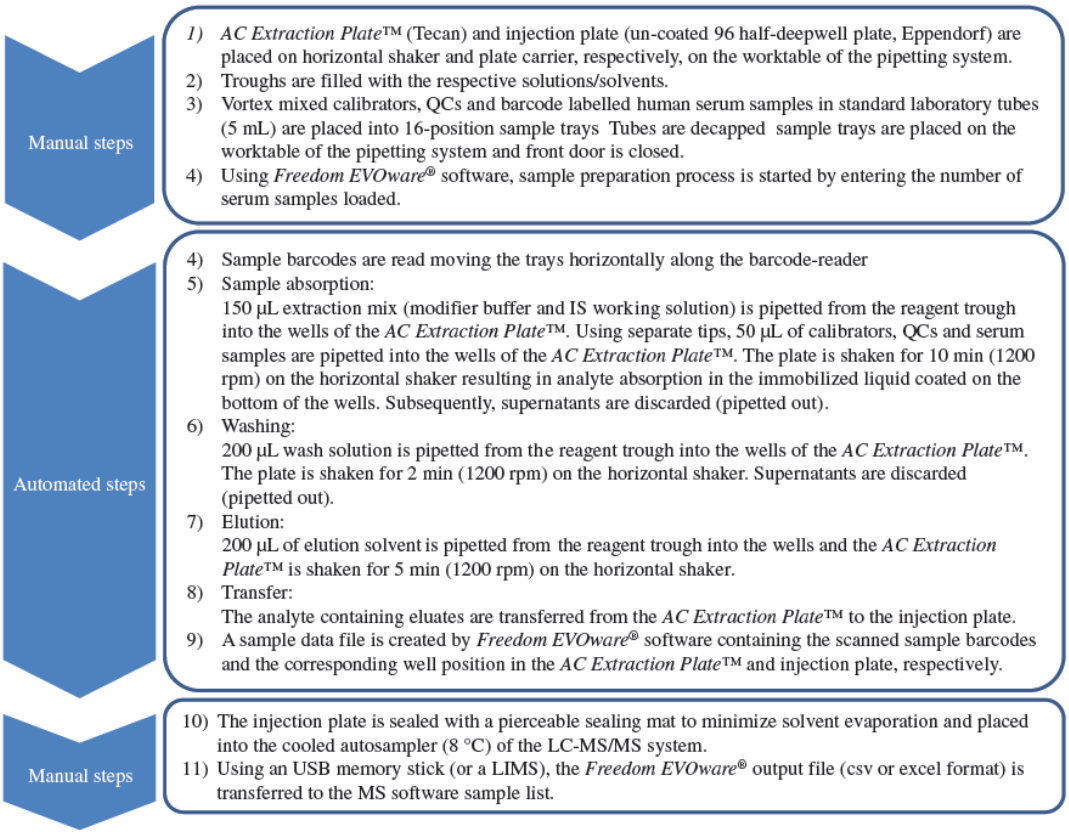

Figure 3 Detailed automated *TICE*™ process.

Table 3 MRM parameters of the automated *TICE*™ UPLC-MS/MS method and the PPT 2D UPLC-MS/MS method.

Analyte	Parention, *m/z*	Production, *m/z*	Cone voltage, V	CE, eV	Dwell time, s	Rt, min
$25OHD_3$ (quantifier ion)	383.3	257.2	25	15	0.025	1.40
$25OHD_3$ (qualifier ion)	383.3	229.2	25	18	0.025	1.40
$25OHD_2$ (quantifier ion)	395.3	269.2	25	15	0.025	1.44
$25OHD_2$ (qualifier ion)	395.3	251.2	25	18	0.025	1.44
D_6-$25OHD_3$ (quantifier ion)	389.3	263.2	25	15	0.025	1.40
D_6-$25OHD_3$ (qualifier ion)	389.3	229.2	25	18	0.025	1.40

all series the percentage deviation of each calibrator sample from target values was <10% and if the coefficient of determination (r^2) was >0.99, calculated using least-squares linear regression.

Sensitivity – lower limit of quantification

Lower limit of quantification (LLOQ) was evaluated analyzing the calibration standards with the lowest analyte concentration. These samples were quantified on four different days. The acceptance criteria for the LLOQ were defined as a percentage deviation from target value <10%, a total coefficient of variation (CV) <20%, and a signal-to-noise ratio (S/N) >10 for analyte peaks.

Intra-day precision (repeatability) and inter-day precision (intermediate precision)

Intra-day precision (repeatability) and inter-day precision (intermediate precision) were determined using the QC materials given in Table 2. For assessment of the intra-day precision the CVs were calculated for five-fold determination in one series and for assessment of

the inter-day precision the CVs were calculated for five-fold determination in four series (n=4×5).

Accuracy

To assess the accuracy of the method, mean concentrations found for the QC samples in the imprecision study were compared with the target concentrations given by the manufacturers (Table 2).

Furthermore, 10 samples from the DEQAS October 2012 (421–425) and January 2013 (426–430) distribution were analyzed. DEQAS is the most acknowledged international proficiency testing scheme for 25OHD measurement (http://www.deqas.org/; [2]). For five of those proficiency testing samples, concentrations found with the *NIST* reference measurement procedure were reported [3].

Recovery and matrix effect

For evaluation of recovery (extraction efficiency) and matrix effect, according to Matuszewski et al. [4] the following samples were analyzed:

(A) Spiked sample: a defined amount of $25OHD_3$ and $25OHD_2$ was spiked to a serum sample and then submitted to sample preparation
(B) Spiked eluate: a defined amount of $25OHD_3$ and $25OHD_2$ was spiked to the eluate obtained after sample preparation
(C) Spiked elution solvent: an identical amount of $25OHD_3$ and $25OHD_2$ was spiked to elution solvent.

For samples (A) and (B) the recorded analyte peak areas of $25OHD_3$ were corrected for their intrinsic content as no analyte-free matrix was available for this experiment (leftover sample pool with $25OHD_3$ concentration approx. 2 ng/mL). The percentage of recovery (extraction efficiency) and matrix effect, respectively, was calculated using the peak areas according to the following equations:

$$\textit{Recovery}\ [\%]=(A/B)\times 100$$

$$\textit{Matrix effect}\ [\%]=(B/C)\times 100$$

Process efficiency (total recovery) results from the combination of recovery (extraction efficiency) and matrix effect.

Postcolumn-infusion experiment

Ion suppression/enhancement was further studied according to Bonfiglio et al. (1999) [5] by a postcolumn-infusion experiment: a solution of 100 ng/mL $25OHD_3$, $25OHD_2$ and D_6-$25OHD_3$, respectively, was infused postcolumn into the mobile phase of the analytical set-up with a constant flow rate of 10 µL/min. By this setting, a continuous background signal was generated in the MRM traces of the analytes. The ion count of these traces was monitored upon injection of elution solvent and extracted serum samples. The MRM chromatograms were visually inspected for a variation in the ion yield (i.e., background signal) at the retention time of the target analytes after injection of the specified samples.

Method comparison

Results obtained with the automated *TICE*™ UPLC-MS/MS method were compared with those obtained by a 2D UPLC-MS/MS method that involved manual PPT (for details of the method see online Supplemental Data, which accompanies the article at http://www.degruyter.com/view/j/cclm.2014.52.issue-3/issue-files/cclm.2014.52.issue-3.xml). Leftover serum samples (n=77) from routine measurement of 25OHD were used after de-identification as approved by the local institutional review board. These samples, covering the clinically relevant concentration range, were divided and analyzed using both methods. Passing-Bablok regression and Bland-Altman analysis were applied for method comparison. The calculations were performed using MedCalc Version 12.7.2 (Mariakerke, Belgium).

Results

Linear regression analysis showed linearity for $25OHD_3$ and $25OHD_2$ over their calibration range of 4.3–65.8 ng/mL and 14.1–54.7 ng/mL, respectively with a percentage deviation of each calibrator from target value <10% and r^2 >0.99.

An LLOQ (percentage deviation from target value <10%; CV <20%; S/N >10) was verified as 4.3 ng/mL for $25OHD_3$ and 14.1 ng/mL for $25OHD_2$ based on the analysis of the lowest calibrator sample.

The CVs observed for intra-day precision (repeatability) and inter-day precision (intermediate precision) for all QC samples were below 7%. Accuracy ranged from 96.9% to 99.8% for $25OHD_3$ and from 95.4% to 112.7% for $25OHD_2$. Results of the validation series in their entirety are given in Table 2.

Close agreement of total 25OHD concentrations found with the *TICE*™ UPLC-MS/MS method with results reported for the DEQAS proficiency testing was observed (Figure 4).

Recovery (extraction efficiency) of the automated *TICE*™ method was 84% for $25OHD_3$ and 80% for $25OHD_2$.

Negligible matrix effect was observed with minimal ionization enhancement for both analytes (105% for $25OHD_3$ and 109% for $25OHD_2$) which was also found in the postcolumn-infusion experiment (Figure 5). Therefore, process efficiency (total recovery) and recovery calculated as extraction efficiency was almost equal for this method.

Results of the method comparison between the *TICE*™-based method and a second 2D-UPLC-based method with PPT are shown as Passing-Bablok regression analysis and Bland-Altman plot in Figure 6. Four samples were excluded from the analysis of the method comparison study because results were below the LLOQ of both methods. For the remaining 73 samples, Passing-Bablok regression revealed the following equation:

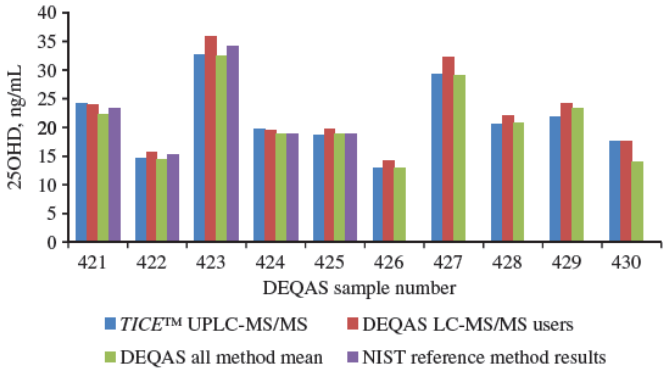

Figure 4 Comparison of total 25OHD concentrations of DEQAS samples found by the automated *TICE*™ UPLC-MS/MS method together with reported results from DEQAS survey.

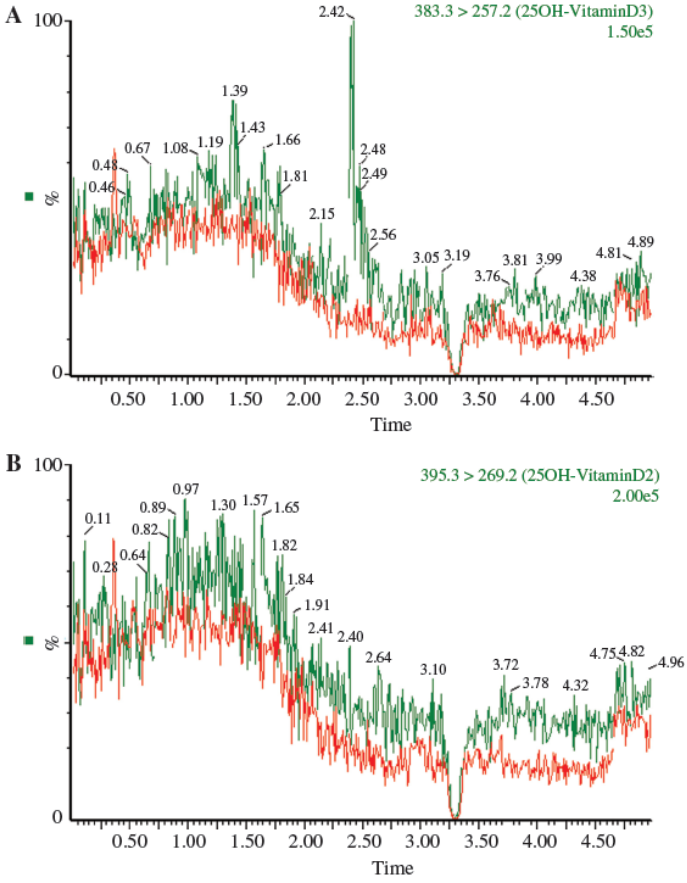

Figure 5 Postcolumn infusion experiment.
Overlay of the monitored ion count after injection of elution solvent (red) and extracted serum sample (green) of (A) $25OHD_3$ MRM-trace (retention time=1.40 min) and (B) $25OHD_2$ MRM-trace (retention time=1.44 min).

[Automated *TICE*™ UPLC-MS/MS]=0.96×[PPT 2D UPLC-MS/MS]+0.28 ng/mL

95% confidence interval (CI) for slope: 0.93–0.99

95% CI for intercept: -0.44–0.69

Correlation coefficient (r)=0.988

The mean 25OHD concentration found was 22.3 ng/mL (range 4.7–55.0 ng/mL) for the automated *TICE*™ UPLC-MS/MS method and 22.8 ng/mL (range 4.5–56.9 ng/mL) for the

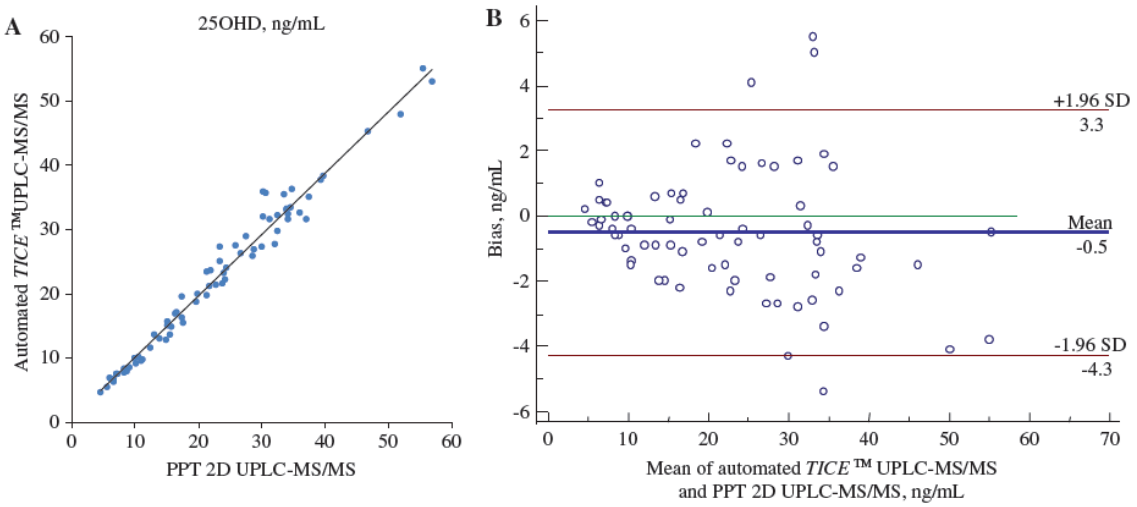

Figure 6 Comparison of 25OHD serum concentrations found in 73 clinical samples using the automated *TICE*™ UPLC-MS/MS method and the PPT 2D UPLC-MS/MS method shown as (A) Passing-Bablok regression analysis and (B) Bland-Altman plot.

PPT 2D UPLC-MS/MS method, corresponding to an overall mean bias of -0.5 ng/mL and -2.1%, respectively, between the two methods (Figure 6B). In one of the 77 samples significant amount of $25OHD_2$ was found with both UPLC-MS/MS methods (*TICE*™ UPLC-MS/MS method: 24.9 ng/mL; PPT 2D UPLC-MS/MS method: 24.7 ng/mL).

No malfunctions of the pipetting system occurred during the study and the handling was found highly convenient. Preparation of 96 samples was achieved within <60 min applying the fully automated protocol.

Discussion

In this article, we report for the first time about a comprehensive evaluation of *Tecan Immobilized Coating Extraction*™ (*TICE*™) as an innovative principle of sample preparation for clinical mass spectrometry. Quantification of serum 25OHD – as an exemplary analyte – was performed using UPLC-MS/MS, after extraction of the analytes using a *TICE*™-product which will be commercially available soon. Extraction of large sample batches was fully automated using a robotic liquid handling system. The *TICE*™ UPLC-MS/MS analytical system was found to fulfill the requirements for use in diagnostic testing of the study analytes regarding reproducibility and accuracy. Good analytical recovery (extraction efficiency) and efficient control of matrix effects on ionization was verified. Close agreement of results with those found with an independent sample preparation technique was observed. High throughput was realized with preparation of 96 samples in <60 min. The obtained samples were of high purity and do not have to be purified subsequently using on-line SPE. In comparison, about 4 h have to be scheduled for manually performed PPT sample preparation of 96 samples for the PPT 2D UPLC-MS/MS method. Such protein-precipitated samples have a high content of residual matrix components and have to be further purified applying on-line SPE prior to LC-MS/MS analysis. Notably, on-line SPE requires an additional HPLC pump and switching valve as well as software support.

In general, *TICE*™ can be applied involving automation but also manually using multi-channel pipettes. While *TICE*™ without automation may be considered for smaller batches (<50 samples) the full benefit of *TICE*™ can be observed for fully automated handling of batches ≥50 samples.

An essential advantage of automated *TICE*™ over already available automated technologies – such as 96-well plate-based SPE or protein filtration – is that automated application does not require the technically demanding use of vacuum, positive pressure, or centrifugation. Conditioning and equilibration of the extraction material is not necessary, further contributing to fast, automated extraction protocols (Table 1).

As *TICE*™ is not surface-*ad*sorption related as in SPE, but is based on *ab*sorption and distribution of the target analyte within a volume of extraction material, highly efficient extractive interaction seems to be realized within very small amounts of extraction material coated as a thin film onto micro-plate wells. Additionally, with the workflow depending only on pipetting and shaking capabilities

any number of samples between 1 and 96 can be chosen to be processed at start of a batch.

With the instrument configuration used in our study, extracts are collected batch-wise in a separate uncoated 96-well micro-plate which is transferred manually to the chromatography autosampler. By implementation of an HPLC injection port on the worktable of the robotic liquid handling system, we intend to achieve direct coupling of the sample preparation system with the chromatographic/mass spectrometric system in a subsequent development project. By integrating the software systems of robotic system and MS system, this would realize a fully integrated MS/MS-based clinical analyzer. Indeed such complete integration of sample preparation and mass spectrometry analysis seems to be essential to develop LC-MS/MS to a convenient standard technology for the clinical laboratory. An ongoing investigation is addressing the applicability of *TICE*™ for further lipophilic analytes such as serum steroids. For routine analysis of 25OHD in serum, a *TICE*™-based UPLC-MS/MS method was found to be fit for this purpose. Indeed, as 25OHD continues to be a high-volume test in many countries, there is a substantial interest in convenient and reliable methods for the quantification of this analyte [6]. In our opinion, the *TICE*™ technology will be an important contribution in this context.

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 played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Research funding: The consumables were provided by Tecan.

Employment or leadership: CL is an employee of Tecan Schweiz AG.

Honorarium: None declared.

Received July 12, 2013; accepted September 10, 2013; previously published online October 9, 2013

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DANKSAGUNG

Die vorliegende Arbeit wurde in der Zeit von Oktober 2011 bis Dezember 2013 am Institut für Laboratoriumsmedizin des Klinikum der Universität München in der Arbeitsgruppe von Herrn Prof. Dr. Michael Vogeser angefertigt.

Herrn Prof. Michael Vogeser gilt mein besonderer Dank für die sehr interessante Themenstellung dieser Arbeit, die weitgehende Freiheit bei der Bearbeitung, sowie die fachliche und moralische Unterstützung.

Ganz besonderer Dank gebührt Roche Diagnostics, die sowohl für die Finanzierung dieser Stelle verantwortlich waren, als auch in wissenschaftlichen Belangen zur Seite standen. Dabei gebührt mein Dank insbesondere Herrn Dr. Uwe Kobold.

Vielen Dank an die Co-Autoren der wissenschaftlichen Publikationen, Herrn Dr. Roland Geyer, Frau Dr. Christine Lehmann, Herrn Dr. Matthias Kroiss und Herrn Prof. Dr. Michael Fassnacht, die mich bei der Planung, Durchführung, Interpretation und beim Verfassen der Fachartikel unterstützt haben.

Den Mitarbeitern des Instituts für Laboratoriumsmedizin danke ich für die gute Zusammenarbeit.

Meinen Eltern Brigitte und Alfred Bächer, sowie meinen Lebensgefährten Martin Riedel, danke ich besonders herzlich dafür, dass sie mich ohne Unterlass nach besten Kräften während der Durchführung dieser Arbeit unterstützten.