Development of mass spectrometric methods for the quantitation of drugs of abuse and therapeutic drugs (DAT/TDM) in clinical samples

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I. Zusammenfassung

Das Ziel der vorliegenden Arbeit war die Entwicklung massenspektrometrischer Verfahren zur Gehaltsbestimmung von Wirkstoffen und Drogen in Körperflüssigkeiten. Im Besonderen wurden dabei neue Technologien für die Aufreinigung und Trennung der Analyten untersucht und Vorteile zu konventionellen Methoden aufgezeigt.

Als Beispiele dienten drei Substanzen und deren Metaboliten sowie strukturähnliche Substanzen: Acetaminophen, ein weit verbreitetes Analgetikum, und zwei Herzglykoside, Digoxin und Digitoxin. Zwei Hochleistungsflüssigkeitschromatographie-Tandem-Massenspektrometrie Methoden basierend auf konventionellen Probenvorbereitungs- und Trennmethoden wurden entwickelt, welche die Quantifizierung aus Serum und Plasma ermöglichten. Nach erfolgreicher Validierung dieser Methoden erfolgte ein Vergleich mit anderen Laboratorien basierend auf Patientenkollektiven.

In einem nächsten Schritt wurde die mögliche Beschleunigung und Automatisierung der Messsysteme untersucht. Eine Aufarbeitung mit magnetischen Mikropartikeln ist eine schnelle und automatisierbare Alternative für die Extraktion von Serumproben, wie am Beispiel des Acetaminophens bewiesen werden konnte. Das Potential der Ionenmobilitätstrennung konnte beispielhaft für Digitoxin und drei seiner Metabolite demonstriert werden. Dazu wurde eine differentielle Mobilitätsspektrometrie Methode ohne chromatographische Trennung entwickelt, die eine zehnfache Beschleunigung der Messung bei gleichbleibender Leistung erlaubte.

II. Abstract

The aim of this dissertation was the development of mass spectrometric assays for the quantitation of therapeutic drugs and drugs of abuse in body fluids. New purification and separation techniques were used and their benefit on the performance of the assay was shown in comparison to established sample preparation and separation methods.

Three representative pharmacologically active substances, including their metabolites and structurally similar compounds were chosen for this study: acetaminophen, a common analgesic and antipyruvic drug, and two cardiac glycosides, namely digoxin and digitoxin. Two LC-MS/MS methods were established for the quantitation of these analytes in serum and plasma. Both assays were validated and results for patient samples were compared to other laboratories for the main target substances.

In a next step, analysis time reductions and automation of the analysis using new purification and separation technologies was investigated. An alternative sample preparation method using magnetic microparticles as extraction support was established for acetaminophen, which allowed specific and automated extraction of the target substances. The potential of differential ion mobility spectrometry (DMS) as an alternative separation method in comparison to high performance liquid chromatography was shown by means of digitoxin and three metabolites. This assay allowed a 10-fold speed increase while maintaining similar performance as compared to the conventional LC-MS/MS assay.

III. Abbreviations

APAP	*N*-acetyl-*p*-aminophenol
CCS	collision cross section
DG	digoxin
DAT	drugs of abuse testing
DMS	differential ion mobility spectrometry
DoE	design of experiment
DRUID	Driving Under the Influence of Drugs
DT	digitoxin
hcPS	hyper-crosslinked polystyrene
HPLC	high performance liquid chromatography
IMS	ion mobility spectrometry
JCTLM	Joint Committee for Traceability in Laboratory Medicine
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LLE	liquid-liquid extraction
ng/mL	nanograms per milliliter
PFP	pentafluorphenyl
pg/mL	picograms per milliliter
ROSITA	Roadside Testing Assessment
SLE	supported liquid extraction
SPE	solid phase extraction
TDM	therapeutic drug monitoring
µg/mL	micrograms per milliliter

IV. Introduction

Drug of abuse testing (DAT) and therapeutic drug monitoring (TDM) have the common requirement of needing fast and accurate methods to identify and quantify pharmacologically active substances and their metabolites in body fluids. [1, 2] Significant progress has been made in the discovery and synthesis of new drugs for therapeutic as well as for abusive use. [3, 4] TDM is needed to ensure correct dosing and to achieve a therapeutic level with minimized unwanted or sometimes even life-threatening side-effects. [5-8] In the DAT field, analytical methods are required to identify quickly what substance the tested person is under the influence of, and at what concentration level. [2, 9] In both cases, analytical tools used to assess these problems have to be able to quickly adapt to the broad panel of new drugs available on the market.

Most methods described for these applications are based on spectrophotometry or immunoassays. Their lack of specificity has led to the rise of mass spectrometric methods, which provide fast, versatile and economic alternatives. Compared to immunoassays, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) offers the additional possibility of analyte multiplexing. [10-12] Many pharmacologically active substances are quickly metabolized after intake and are often not detectable in their native form in body fluids. Known metabolites and degradation products can be included in LC-MS/MS assays, in addition to the native substance. [13] Additionally, LC-MS/MS offers the possibility of metabolite profiling. [14, 15] Before application for clinical use with patient samples, assays are subject to validation including at least the assessment of sensitivity, trueness and precision, to ensure that the reported results will be safe to use for medical decisions. [16] This is particularly important for drugs with narrow therapeutic windows, *e.g.* aminoglycosides, which can cause nephrotoxicity or ototoxicity when dosed in wrong concentrations. [17]

Most DAT and TDM assays use serum, plasma or urine as sample matrix. Because of the growing need for simple, fast and non-invasive sample collection methods for which no medical personnel is required, new methods are developed based on alternative sample matrices (*e.g.* oral fluid, hair, sweat, exhaled breath *etc.*). [9, 18-24] These methods are useful in the context of programs such as DRUID (Driving Under the Influence of Drugs) [25] or ROSITA (RoadSIde Testing Assessment),[26] but they are prone to problems such as low

analyte concentration, inhomogeneity and possible false-positive results by exposure to food, tobacco or cosmetic products. [21] Improvement of these assays is therefore necessary to reach the reliability of results obtained from conventional matrices such as serum or plasma.

LC-MS/MS assays comprise a sample preparation step to purify the target analyte(s) and a separation step via chromatography to separate analytes from residual matrix components before mass spectrometric detection. The goal of this thesis was to investigate the possibility of a generic LC-MS/MS system for analysis of various types of analytes in the DAT/TDM field. For this work, a high pressure liquid chromatography (HPLC) system with a reversed phase chromatography column (pentafluorphenyl (PFP) phase) was used, which allowed the separation of analytes with a broad range of chemical properties. It was hyphenated to a triple quadrupole mass spectrometer, facilitating high sensitive detection. Special attention was paid towards optimization of the sample preparation procedure, speed-up and possible automation of the workflow.

Many sample preparation techniques have been established and allow thorough clean-up of samples in complex matrices such as body fluids. Depending on the nature of the target analyte(s), methods such as protein precipitation, solid phase extraction (SPE) [27-30] and liquid-liquid extraction (LLE) [31-33] are available to specifically extract one or a combination of target substances from interfering matrix components such as high-abundant proteins, phospholipids, salts *etc.* However, optimization of these techniques for specific applications is often time-consuming, frequently involves multiple steps and requires large sample volumes to achieve sensitive analysis. Also, sample throughput is limited by steps which are performed in batch mode and therefore cause long turnaround times, *e.g.* centrifugation. [34] Another drawback complicating the use of LC-MS/MS as a routine analysis tool is the high costs of the assays. Consumables needed for sample extraction are relatively expensive and well-trained scientists have to operate the system and perform tedious data analysis procedures. These limitations underpin the need for automated, fast and cost-efficient sample analysis methods.

Coated magnetic microparticles have shown high potential as extraction media for sample preparation. [35] Particle suspensions can be handled as liquids, allowing full automation and speed-up of the sample preparation process. Smaller sample volumes can be handled compared to conventional SPE methods due to the high specific surface of the beads. This offers the advantage of either a lower consumption of sample volumes or a higher

concentration of the eluate prior to LC-MS/MS analysis. Several applications have been reported, where either analytes are extracted from a complex matrix or where the matrix components were removed from the sample, leaving a clean extract. [36-38] Magnetic beads have low synthesis costs and produce less waste compared to SPE workflows. A broad range of chemical surface modifications are available for magnetic beads, allowing very specific (*e.g.* MIP, antibody-/aptamer-coating) as well as unspecific extraction mechanisms (reversed phase, ion exchange *etc.*). Thus they can be adapted to many applications including not only DAT/TDM analyses but also proteomics, steroids, hormones and waste water analysis.

Another approach to improve sample throughput is to focus on the optimization of analyte separation after sample clean-up. Major progress has been made in the last years for the development of faster chromatographic systems without compromising separation power. [39, 40] New column materials and HPLC systems are available, which considerably reduce separation times for large collectives of target substances. Other techniques are available to further enhance separation times of complex mixtures of analytes, such as ion mobility spectrometry (IMS). [41] In IMS, ions are directed through a cell filled with an inert gas and are separated based on their difference in mobility in the gas phase by applying an external electrical field. Multiple instrument configurations exist for IMS, *e.g.* differential ion mobility spectrometry (DMS). When included in an LC-MS assay, DMS can either act as a filter prior to MS detection reduce background noise or it can be used as a third dimension of separation to improve the specificity of the assay. [42] This approach has shown great potential for the separation of isobaric compounds (metabolites, endogenous substances *etc.*) [43, 44] that can hardly be differentiated by conventional chromatography. DMS separation can be improved by adding ion pairing reagents to analyte mixtures. [45-49] Migration behavior of analyte clusters in the DMS cell can be predicted using adequate modeling software tools. [50, 51]

Conventional methods of drug testing using LC-MS/MS already play an increasingly important role in clinical and forensic laboratories. Further developments in technology are needed to allow generic routine measurement of analytes in clinical laboratories and support the implementation of new therapeutic drugs and detection of narcotics in the future. The objective of this study was to demonstrate the possibility of a generic LC-MS/MS based system for fast, versatile and automatable quantitation of pharmacologically active substances in complex biofluids. The experimental strategy was first to establish conventional

quantication methods for three representative analytes and their metabolites as test systems. Then, detailed investigation of new purification (magnetic bead based sample preparation) and separation technologies (DMS) was planned in terms of reduction of both analysis time and manual handling.

V. Summary and Conclusions

Three representative analytes were exemplary chosen for development of the new instrumental platform described in this work: acetaminophen, a widely used over-the-counter analgetic and antipyruvic drug which is mainly metabolized in the liver and can cause severe liver damage in case of an overdose, and digoxin and digitoxin, two cardiac glycosides with a very narrow therapeutic range. Detailed descriptions of this work can be found in the publications listed in chapter VII.

1. Acetaminophen

In a first part, an LC-MS/MS assay was developed to quantify acetaminophen (*N*-acetyl-*p*-aminophenol, APAP), four of its metabolites (acetaminophen-glucuronide, acetaminophen-sulfate, acetaminophen-mercapturate and acetaminophen-cysteine), its antidote (*N*-acetylcysteine) and four additional compounds (phenacetin, phenetidine, imipramine and amitriptyline) known to cross-react with the immunoassay-based acetaminophen test in human serum and plasma. Because of the high concentration levels expected for these analytes in serum (up to 200 µg/mL), a simple protein reduction and filtration step was sufficient to allow quantitation of all target compounds in the desired concentration range. Chromatographic separation was performed on a PFP separation column. Analytes were detected using a triple quadrupole mass spectrometer. This method was validated and showed good performance in terms of accuracy, precision and linearity. Method comparison was carried out by means of data from a routine laboratory using 77 patient and spiked samples and showed good correlation. Our assay has been accepted and listed as a certified reference method by the Joint Committee for Traceability in Laboratory medicine (JCTLM).[52]

In a second stage of the project, an alternative sample preparation method based on extraction using magnetic microparticles was developed for the analyte combination described above. Because of the large number of parameters suspected to have an influence on the extraction process, experiments were thoroughly planned using design of experiment (DoE). Several bead types were screened and one suitable type was found that allowed simultaneous extraction of the target substances with a very broad range of polarities directly from serum. The resulting assay was validated and compared to the developed reference method using 68 native patient samples. It was shown that the correlation between both methods was excellent, further underpinning the good performance of the assay using magnetic beads in

terms of accuracy. Analysis time was reduced by a factor of 4 while maintaining the same imprecision as the reference method (CV 2-4% for APAP and 2-9% for metabolites); further time reductions can even be achieved by using an automated liquid handling system in the 96 well-plate format. The magnetic bead based method was fast, simple and could be readily applied to simultaneous extraction of analytes with a wide range of chemical polarities. Extension of the method to other target substances should be readily possible.

2. Digoxin/Digitoxin

The next part of the thesis focused on the quantitation of two cardiac glycosides: digoxin (DG) and digitoxin (DT). Contrary to acetaminophen, the concentration levels of these drugs in human serum were much lower, only a few nanograms per milliliter (ng/mL), making the availability of a very sensitive analysis system crucial for quantitation. The developed assay was targeted towards digoxin, digitoxin and eleven of their metabolites (digoxin-bis-digitoxose, digoxin-mono-digitoxose, digitoxin-bis-digitoxose, digitoxin-mono-digitoxose, digoxigenin, digitoxigenin, dihydrodigoxin, acetyl- and methyldigoxin, deslanoside and lanatoside). Because of the different chemical properties of the target substances and their very low concentration levels, extensive sample preparation had to be performed before LC-MS/MS analysis. A combination of protein precipitation and supported liquid extraction (SLE) was necessary to reach the needed sensitivity of the assay. Chromatographic separation and MS detection were performed on the same platform described previously for acetaminophen. The assay was validated; trueness was verified for the two main analytes by analyzing 29 spiked and native samples for DG and 8 native samples for DT and comparing the results to established reference methods for DG and DT.

In a next step, the potential benefit of differential ion mobility spectrometry (DMS) as a potential replacement for chromatographic separation was investigated using DT and three of its metabolites. The influence of chemical modifiers and ionic additives on the separation potential of the DMS cell was investigated and showed that it only improved slightly with gas-phase modifiers. The decisive factor was shown to be cluster formation of the analytes with alkali ions. Separation efficiency increased with increasing size of the counter ion, yielding the optimum results with cesium. It was demonstrated that DMS on its own could be used for separation of analyte mixtures. Using a ballistic chromatographic approach, serum samples could be quantified by DMS-MS/MS in less than 1.5 min, which allowed a speed-up

of the analysis of factor 10 by maintaining the same imprecision of 2-13% compared to the previously described method.

3. Outlook

The work described in this thesis represents a significant step towards establishment of a generic system for quantitation of various drugs and their metabolites in body fluids using LC-MS/MS. The developed mass spectrometry techniques have proven to be a very useful approach to solving the studied analytical problems due to their versatility, specificity and fast method development time as compared to immunoassay-based tests. Nevertheless, as described before, several limitations were encountered and needed to be assessed before implementation of LC-MS/MS as routine analysis tool.

In our experiments, the evaluated technologies showed promising results and proved to be applicable to a large number of different analytes exhibiting a broad range of polarities in different concentration ranges. A workflow combining sample extraction with magnetic beads and detection by DMS-MS/MS allowed a significant reduction of analysis time, full automation of the process and considerable decrease of cost per result, while maintaining the analytical performance of assays based on established techniques.

These results will undoubtedly support implementation of LC-MS/MS as a routine tool for DAT/TDM applications in clinical laboratories in the future. However, several issues still need to be assessed before this can be fully realized. Firstly, the robustness of the system needs to be further improved to the point where random access analysis of various targets is possible and frequent hardware maintenance is not required anymore. Furthermore, new calibration concepts should be implemented, to enable weekly instead of daily calibration of the system. Finally, user interaction time could be further minimized if adequate software tools were available for automated and reliable data interpretation.

VI. References

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VII. List of publications

The results of this dissertation have been published in the following peer-reviewed journals:

1. C. Bylda, R. Thiele, U. Kobold, D.A. Volmer, Simultaneous quantification of acetaminophen and structurally related compounds in human serum and plasma, *Drug Test. Anal*., **2014**, 6, 451-460.

2. C. Bylda, V. Velichkova, J. Bolle, R. Thiele, U. Kobold, D.A. Volmer, Magnetic beads as an extraction medium for simultaneous quantification of acetaminophen and structurally related compounds in human serum, *Drug Test. Anal*., **2015**, 7, 457-466.

3. C. Bylda, R. Thiele, U. Kobold, D.A. Volmer, Simultaneous quantification of digoxin, digitoxin and their metabolites in serum using high-performance liquid chromatography tandem mass spectrometry, *Drug Test. Anal*., **2015**, in press, DOI: 10.1002/dta.1781.

4. C. Bylda, R. Thiele, U. Kobold, A. Bujotzek, D.A. Volmer, Rapid quantification of digitoxin and its metabolites using differential ion mobility spectrometry-tandem mass spectrometry, *Anal. Chem*., **2015**, 87 (4), 2121-2128.

Additionally, the following review article was included in the dissertation as an introduction to the research field but does not contain any relevant research results:

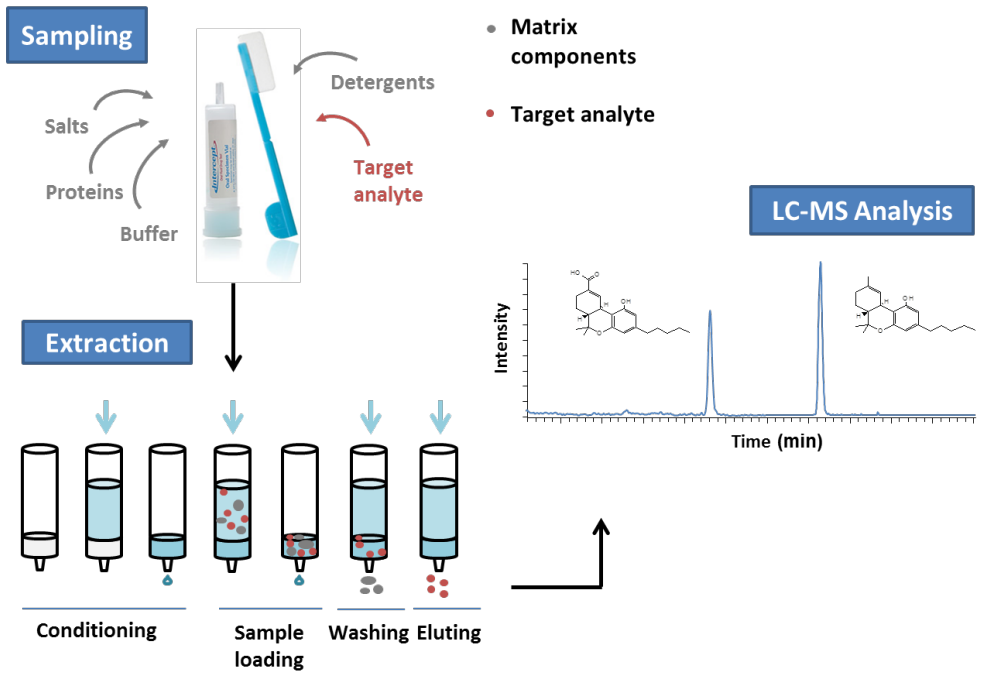
5. C. Bylda., R. Thiele, U. Kobold, D.A. Volmer, Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS, *Analyst*, **2014**, 139, 2265-2276.

Publication 1

Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS

C. Bylda, R. Thiele, U. Kobold, D.A. Volmer

Analyst, **2014**, 139, 2265-2276



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Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS

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Liquid chromatography-mass spectrometry analysis of small molecules from biofluids requires sensitive and robust assays. Because of the very complex nature of many biological samples, efficient sample preparation protocols to remove unwanted components and to selectively extract the compounds of interest are an essential part of almost every bioanalytical workflow. This review describes the most common problems encountered during sample preparation, ways to optimize established sample preparation techniques and important recent developments to reduce or eliminate major interferents from biofluids.

Introduction

The primary goal of sample preparation is to isolate one or several target analytes from the other components of the sample mixture (matrix). Depending on their nature and concentration levels, co-components of the sample matrix can influence the quantitation of target analyte(s) during subsequent liquid chromatography-mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) experiments if not removed prior to analysis. The development of new LC-MS/MS methods for small molecules in biological fluids is becoming increasingly more challenging, because of the need to continuously achieve higher sensitivity and better assay robustness in complex biofluids such as serum, plasma, urine, oral fluid or cerebrospinal fluid (CSF). In addition, because of the very low concentration levels

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of pharmaceutical targets, samples often need to be pre-concentrated before analysis. Unfortunately, this does not only increase the concentration of the desired compound in the sample extract but also often raises the levels of interfering components. As a result, very specific and effective sample clean-up procedures are required for sensitive and selective LC-MS/MS assays today.[1]

This short review presents the main difficulties encountered during sample preparation for analysis of small molecules from biofluids by LC-MS/MS and summarizes several critical factors that particular attention should be paid to, followed by an overview of the latest developments in sample preparation techniques to overcome common difficulties with complex biofluids.

Matrix effects

The general term used today to describe problems encountered during analysis of complex biological samples is "matrix effects". These effects are usually caused by endogenous (*e.g.* metabolites of the target analyte, proteins or lipids) or exogenous (all substances introduced during sample processing and analysis) compounds. Depending on their chemical properties, it may or may not be necessary to remove all of these interferents from the sample before injection into the LC-MS system. Also, only matrix compounds coeluting with target analytes during the chromatographic separation prior to MS analysis can cause a change in the response of the analyte, either positive (ion enhancement effect) or negative (ion suppression effect).[2]

Different methods have been presented to examine matrix effects. A common approach is the post-extraction spike method,[3–5] where the peak area of the target analyte that has been spiked into the biological matrix prior to the sample preparation is compared to the area of the same analyte spiked post-extraction into the biological fluid extract. The ratio between the two values represents the absolute matrix effect. The relative matrix effect is determined by comparing several lots of the biological matrix.[3] Obviously, both absolute and relative matrix effects depend strongly on the target analyte and the ionization technique used for LC-MS/MS.

Another popular method is post-column infusion,[6–8] where possible matrix effects are assessed by continuous post-column infusion of the analyte after injection of a processed blank serum sample onto the chromatography column. Any variation of signal intensity at or near the retention times of the analyte would indicate the presence of substances from the matrix interfering with the analysis.

Matrix effects have been shown to be dependent on the ionization methods used for the LC-MS method,[3] which are usually either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) in most modern LC-MS/MS assays. The chemical structures and the concentration levels of both analyte and co-eluting mixture components determine whether they outcompete each other during the ionization process.[9] For example, ESI is particularly sensitive to co-eluting phospholipids because ESI is strongly biased towards surfactants,[10] which enrich at the surface of the droplets during the liquid/gas-phase ion transfer. That is, phospholipids at the surface of droplets can inhibit ejection of analyte ions trapped inside the droplets. On the other hand, APCI is often less affected by suppression effects, as there is no competition between compounds to enter the gas-phase of the mass spectrometer. Nevertheless, APCI still experiences matrix effects in multicomponent samples. As biofluids contain numerous endogenous molecules, often at high levels, with potentially very high basicities and surface activities, ion suppression effects will almost always be present in any LC-MS/MS assay.

Different strategies are available to eliminate or reduce matrix effects. One approach is to optimize the chromatographic separation to separate the analytes from interfering compounds.[1,11,12] This can, however, result in long chromatographic run times. Another approach is to optimize the sample preparation, to obtain clean extracts of the target analytes. With proper sample preparation and the use of isotopically labeled standards, many matrix effects can be eliminated or strongly reduced. Some cases remain, however, where the high variability of the matrix composition makes the use of standard addition calibration necessary.[13–16]

There are several well-known causes for matrix effects in the analysis of clinically-relevant substances from biological samples. For example, hemolyzed or lipaemic samples have great influence on the analysis of serum and plasma samples.[17,18] Cases also have been reported, where buffers used for solid-phase extraction (SPE) triggered matrix effects in LC-MS/MS.[19] The most important interferents, however, are phospholipids, which not only affect MS response of many analytes greatly, but which are also very difficult to remove from the samples.

Phospholipids

Phospholipids (PPL) are major constituents of cell membranes and are therefore very abundant in serum and plasma.[20] They consist of two functional groups: a hydrophilic head group composed of phosphate and choline units, and a hydrophobic tail, made up of fatty acyl chains. The most abundant phospholipids are glycerophosphocholines (GPChos) (70% of total phospholipids) and lysophosphatidylcholines (10% of total phospholipids) (Fig. 1).[11] These two groups are known to cause serious ion suppression effects in LC-MS analysis, caused by competition for space on the surface of droplets formed during

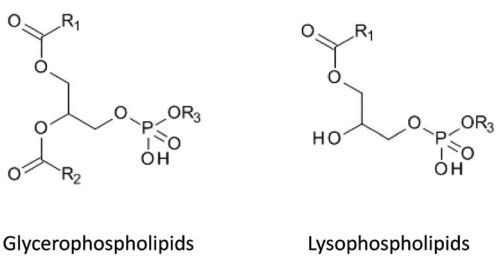

Fig. 1 Chemical structures of the two most important groups of phospholipids.

the ESI process (*vide supra*).[3,10] Phospholipids are present at different concentration levels in serum and plasma samples, depending on the sampling device used.[21] A very simple method to monitor possible ion suppression effects from GPCho was described by Little *et al.* as in-source multiple reaction monitoring (IS-MRM).[22] Using the positive ion mode, a common product ion for the most abundant GPCho is trimethylammonium-ethyl phosphate at *m*/*z* 184, which was monitored during analysis of an analyte-free sample. This class-specific product ion was generated using in-source dissociation of the eluting GPCho during the chromatographic run.[22] Other methods have been described that allow screening for less abundant phospholipids by adding a precursor ion in the negative mode or by using positive ion neutral loss scans.[23]

Studies have shown that the use of methanol as a mobile phase for chromatographic separation provided significant advantages over acetonitrile, because elution of all GPCho occurred in a very narrow time window and their retention behavior on reversed-phase columns could be predicted and decreased by increasing the percentage of the organic phase.[24] The PPL tended to elute at a high content of the organic mobile phase[25] and were completely removed from the system at the end of a run by flushing the analytical column with isopropanol.[26]

The behavior of PPL has also been investigated on hydrophobic interaction liquid chromatography (HILIC) columns:[27] the compounds were focused into 2 groups of peaks (phosphatidyl cholines and lyso-phosphatidyl cholines) and eluted completely from the column in a one gradient cycle. In comparison, on a reversed-phase material, a strong carry-over was observed from one gradient cycle to another.[27]

In some cases, where retention times of target analytes and PPL overlapped, elution of the target substance could be shifted after adding mobile phase modifiers.[27]

Internal standards

The use of isotope-labeled internal standards can help overcome most of the matrix effects during sample preparation and LC-MS/MS analysis. However, in some cases the internal standard cannot completely fulfill its purpose, because of slight differences in the chemical behavior of the target analyte and internal standard. For example, particular attention has to be paid to analytes showing strong protein binding.[28] Generally it is necessary to allow enough time for the internal standard to properly equilibrate and bind to the protein before extraction, to ensure identical behavior of the internal standard and target analyte.[29] A method has been described to determine the extent of protein binding of corticosteroids.[30] In theory, this method could be extended to other substances and be used to compare the protein-affinity of an analyte and its internal standard. It is important that the release of analytes from the protein (*e.g.* by adding organic solvents for protein precipitation, *o*-phosphoric acid for breakdown of non-covalent intermolecular interactions[31] or dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) for reduction of disulfide bonds) has the same impact on the analyte and isotope-labeled standard. A case was reported, where the higher susceptibility of the internal standard for matrix effects than the target analyte led to an underestimation of up to 50% in the presence of specific buffers used for SPE sample preparation (Fig. 2).[19]

Generally, ^{13}C, ^{15}N or ^{18}O-labeled internal standards are preferable to deuterium labeled analogs,[32] because slight differences of physicochemical properties between hydrogen and deuterium can result in small shifts of retention times of the analyte and internal standard. In some cases, this has led to a different degree of ion suppression for the analyte and the internal standard, resulting in changed analyte/internal standard peak area ratios.[33,34] Also, deuterium–hydrogen back-exchange can occur, which has led to false positive results.[35] Unfortunately, in many cases only deuterated compounds are commercially available, which increases the need to carefully investigate the stability of the reference standards and the influence of matrix effects on the method.

Optimization of established sample preparation methods

Even though there has been some recent interest in quantitative analysis of pharmaceutical compounds from biological samples using ambient, direct mass spectrometry techniques such as desorption electrospray ionization (DESI) or direct analysis in real time (DART), with little or no prior sample preparation or chromatography,[36] sample clean-up remains a critical step in most LC-MS analyses of small molecules in biofluids.

Protein precipitation

The simplest sample preparation approach for biofluids is protein removal. Proteins can be denatured using acids or heat, or removed by using ultrafiltration cut-off membranes.[37] Another possibility is to use organic solvents for protein precipitation (PPT). PPT removes a part of the phospholipid content present in serum and plasma samples, depending on the organic solvent used. Studies have shown that methanol extracts contain 40% more phospholipids compared to acetonitrile,[11] and are also less clean than tetrahydrofuran or ethanol extracts.[38]

Solid-phase extraction (SPE)

Silica-based sorbents in SPE cartridges have excellent retention capacity for PPL when eluted with 100% acetonitrile.[39] Clean extracts were also obtained by including a washing step with up to 50% methanol, but this strongly affected the recovery of polar analytes.[39] Large amounts of methanol eluted significant amounts of phospholipids from silica-based reversed-phase SPE cartridges. Methanol contents of 60, 70 and 80% for elution of samples on phenyl, C8 and C18 phases resulted in a high concentration of phospholipids in the extracts. Acetonitrile appeared to be a stronger eluent for phospholipids on reversed-phase materials when present at levels up to 50%. The same study showed that the recovery of lysophosphatidylcholines decreased with the increasing content of acetonitrile (>50%), reaching its minimum at a 100% organic phase.[40] The retention

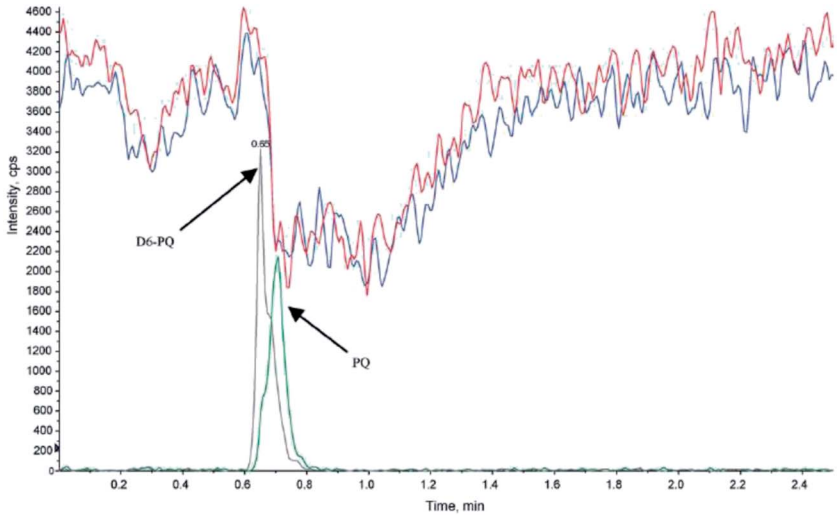

Fig. 2 Injection of extracted blank human plasma (+0.2 μL triethylamine, blue and red traces) with an overlay of the control sample (20 ng mL^{-1}, grey and green traces) containing piperaquine (PQ) and internal standard (d_6-PQ) during post-column infusion at 10 μL min^{-1} of PQ and d_6-PQ (1.2 ng mL^{-1}). Electrospray ionization of the analytes was performed in positive ion mode; the MRM transitions were *m/z* 535 → 288 and *m/z* 541 → 294 for PQ and D6-PQ, respectively (reprinted with permission from ref. 19).

of phospholipids on the sorbent increased by interactions with residual silanol groups, as was shown by comparison of end-capped and non-endcapped materials. Silica-based sorbents were compared to polymeric phases regarding extraction of phospholipids, and the tested materials showed comparable efficiency.[40]

Studies comparing different sample preparation methods in terms of matrix effects and analyte recovery demonstrated that mixed-mode strong anion exchange SPE was more effective than PPT and LLE for polar and non-polar analytes in plasma (Fig. 3).[11,41]

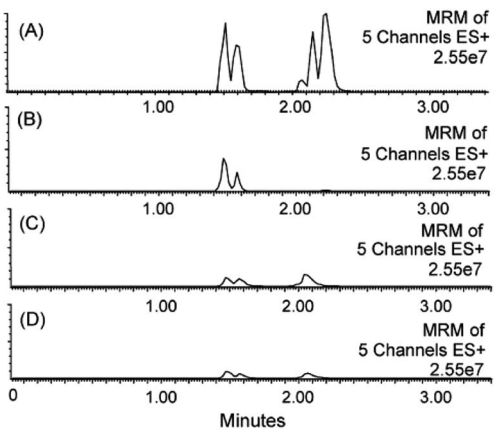

Fig. 3 MRM traces for five residual phospholipids in rat plasma extracts after sample preparation by (A) acetonitrile PPT, (B) reversed-phase polymeric SPE, (C) silica-based pure cation exchange, and (D) mixed-mode cation exchange SPE. The phospholipids monitored were 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (*m/z* 496.35), 1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (*m/z* 524.37), 1-hexadecanoyl-2-(9*Z*,12*Z*-octadecadienoyl)-*sn*-glycero-3-phosphocholine (*m/z* 758.57), 1-(9*Z*,12*Z*-octadecadienoyl)-2-(5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoyl)-*sn*-glycero-3-phosphocholine (*m/z* 806.57) and a fifth glycerophosphocholine lipid of molecular weight 703.57 Da. MRM analysis was performed on an ESI triple quadrupole LC-MS/MS system using a methanol–water gradient at pH 10 (reprinted with permission from ref. 11).

HILIC-SPE was evaluated as an effective method to remove phospholipids from serum and plasma samples.[26] The retention of phospholipids was shown to increase when samples were diluted with acetone. For some applications to urine samples, HILIC materials were more effective than reversed-phase materials.[42] The polar metabolites in urine had to be separated from the salts and other polar components present in urine. Orthogonal separation using both HILIC and reversed-phase materials for sample preparation and chromatography improved the effectiveness of sample clean-up.[42]

Overall, SPE has a very broad range of applications in the LC-MS/MS quantification of small molecules in biofluids.[43–47]

Liquid–liquid extraction (LLE)

Liquid–liquid extraction has found numerous applications for analysis of pharmaceuticals and their metabolites. The concentration of residual phospholipids in the extract is usually lower compared to other techniques such as mixed-mode SPE; on the other hand, the extraction efficiency for highly polar analytes is also lower.[29] The choice of extraction solvent is very important to reduce unspecific extraction of matrix components.[41] Halogenated solvents such as chloroform or dichloromethane[48–50] are commonly used in combination with hydrophilic solvents (*e.g.* alcohols) for extraction of polar compounds; they also have high affinity for lipids.[38]

As non-ionized analytes are more efficiently extracted by organic solvents than charged species, particular attention has to be paid to the pH of the sample prior to LLE. As a general rule, the pH should be between pK_a and (pK_a − 2) for acidic analytes and between pK_a and (pK_a + 2) for basic analytes,[51] to increase the extraction recovery. This obviously applies only if

the stability of the main analyte and its potentially labile metabolites is given in this pH range.[29]

Extraction using methyl-*tert*-butylether (MTBE) has shown good results,[52] but significantly lower analyte recoveries were seen compared to mixed mode SPE and PPT, especially for polar analytes.[11] Only traces of phospholipids were found in MTBE and *n*-butylchloride extracts of serum and plasma samples.[53] However, particular attention has to be paid to the process, when several sample preparation steps are combined. The clean extracts obtained with MTBE for untreated serum or plasma can show a high recovery for phospholipids if the samples contain a high percentage of acetonitrile, *e.g.* after protein precipitation (Fig. 4).[53]

Extraction time also plays an important role for the specific extraction of target analytes compared to matrix components. A study showed that a 5 min extraction time yielded a cleaner extract and better recovery for the target compound than 20 min, indicating that matrix compounds diffuse slower into the extraction solvent.[52]

To improve low recovery rates of LLE for strongly hydrophilic compounds, extraction procedures using water miscible solvents have been considered. Complex methods were reported in the past that use temperatures below 0 °C to achieve phase separation of serum samples and extraction solvent.[54] A more convenient way to achieve phase separation between an aqueous sample and a water-miscible solvent is salt-assisted liquid–liquid extraction (SALLE), where the polarity of the aqueous phase is increased by adding high concentration of salt, leading to phase separation.[55] This approach has been used for quantitation of pharmaceutical compounds from biofluids using LC[56] or LC-MS/MS.[57–59]

Novel sample preparation methods

Many common interferents can be removed with conventional sample preparation methods (*e.g.* protein precipitation, SPE, and LLE), but optimization of these techniques for specific applications is often complex, time-consuming and frequently involves multiple steps. Many common interferents can be removed with conventional sample preparation methods (*e.g.* protein precipitation, SPE, and LLE), but optimization of these techniques for specific applications is often complex, time-consuming and frequently involves multiple steps. Moreover, some challenges involving very small sample volumes and low abundant analytes remain. If repeated analyses are required from the same sample and if no further sampling is possible, sample preparation sometimes has to be performed using a sample volume as low as a few microliters. Similar difficulties apply to assays for metabolites or biomarkers that are present at very low concentration levels in human samples. Here, the method must be able to pre-concentrate the target substance(s), additionally to removing all other components of the matrix. New developments for sample preparation methods are therefore often directed towards simplification and possible automation, miniaturization and specificity enhancements of the clean-up process. New developments for sample preparation methods are therefore often directed towards simplification and possible automation, miniaturization and specificity enhancements of the clean-up process. In the following, the most promising recent developments are briefly summarized.

Supported liquid extraction (SLE)

Even though LLE is mostly a very effective sample preparation method, it has limitations, in particular low sample throughput. Several extractions are often required to improve analyte recovery, sample handling is labor-intensive and time-consuming, and emulsions can form at the interface between liquid layers. These limitations can be overcome by using supported liquid extraction (SLE), where aqueous samples are adsorbed on a porous solid support material, *e.g.* diatomaceous earth. Some studies have shown analyte recovery from SLE that was comparable or higher than LLE.[60]

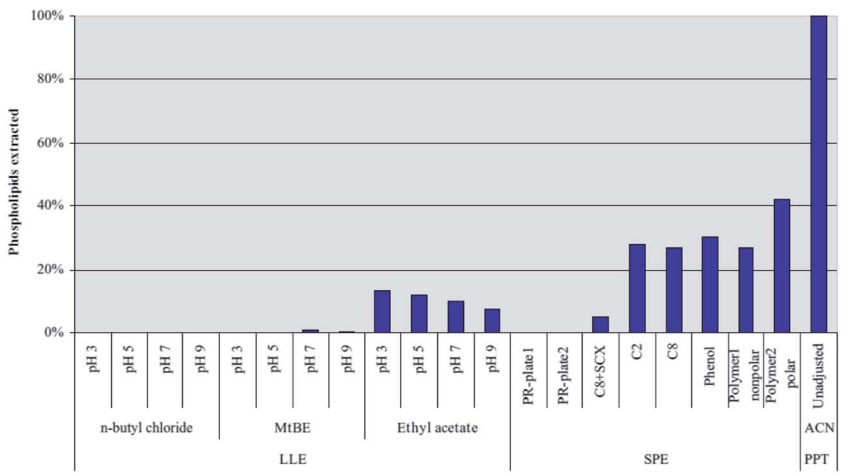

Fig. 4 Extraction of C16:0 lysophosphatidylcholine (C16:0 lyso-PC) from human plasma using liquid–liquid extraction with three different solvents at different pH values. Comparison to solid-phase extraction and two commercial phospholipid removal sorbents (PR-plate 1 and PR-plate 2). Lyso-PC was monitored using the following MRM transition: *m/z* 496 → 184 (reprinted with permission from ref. 53).

SLE has been shown to effectively remove the majority of phospholipids when the extraction conditions were carefully optimized.[61] The efficiency of several extraction solvents was also compared for SLE:[7] ethyl acetate removed about 85%, MTBE removed more than 99% of total phospholipids. Dichloromethane removed 99.5% of the phospholipids when used alone; its removal efficiency decreased to 95% when isopropanol was added. However, addition of water-soluble solvents to the samples (*e.g.*, acetonitrile or methanol) prior to SLE extraction led to higher matrix effects for some analytes.[7] Isopropanol combined with dichloromethane also yielded low concentrations of phospholipids in the extract.[7]

The SLE technique has been implemented in various LC-MS/MS methods recently.[62–67] It was particularly powerful for normal phase separation systems, since the high percentage of organic solvent in the eluate did not need to be evaporated prior to injection into the LC-MS/MS system.[68]

Phospholipid removal plates

The use of hybrid precipitation/SPE plates for selective removal of phospholipids and precipitated proteins has been increasing over the past few years.[4,38,69,70] Several types of these plates are now commercially available, *e.g.* Hybrid SPE™ (Sigma Aldrich), Ostro™ (Waters), Captiva™ ND (Agilent) and Phree™ (Phenomenex). These plates have shown very effective extractions of phospholipids compared to PPT.[71] For example, the Hybrid SPE plate specifically retains phospholipids by Lewis acid–base interactions between zirconia ions – which are bonded to the stationary phase – and the phosphate group of the phospholipids. Acetonitrile with 1% formic acid is used as the precipitation agent; formic acid has important influence on the recovery of the analytes.[72] Hybrid SPE extracts have shown to contain significantly lower phospholipid concentrations as compared to PPT.[4] Ostro uses a combination of protein precipitation and extraction on a C18 sorbent. Several applications using these products have been reported.[73–75]

Other approaches are also possible for removal of phospholipids. A study showed that addition of a colloidal silica suspension together with lanthanum chloride to plasma samples resulted in a reproducible sample clean-up without loss of the analyte of interest.[76]

Magnetic beads

Magnetic particles and nanoparticles (MNPs) are becoming increasingly interesting for sample preparation. They have been used for extraction and pre-concentration of drugs in complex biological fluids.[77–79] They consist of a magnetic core (*e.g.* Fe_3O_4) coated with a polymer material, to which specific functionalities can be added (Fig. 5).[80] Sample preparation steps are similar to SPE (loading, washing and elution). The magnetic particles suspended in solution can be handled as a liquid. Obviously, the big advantage of magnetic beads is that after sample extraction, the beads are pulled to the tube wall, the supernatant is removed and the wall-bound beads washed with an appropriate solvent. The loaded beads are then re-suspended. The entire procedure is fast and simple, and complete automation is readily possible.

Several applications have been reported, where either analytes are selectively extracted from a complex matrix[81–83] or where the matrix components were removed from the sample, leaving a clean extract behind that can be directly injected into the LC-MS system.[84] Using matrix-assisted laser desorption/ionization (MALDI), the analytes can also be analyzed without having to be eluted from the magnetic beads first.[85] The possible modifications on the surface of the magnetic beads are similar to conventional SPE and involve hydrophobic coatings, ion exchange functionalities, molecular imprinted polymers (MIP),[86] restricted access[87] or affinity materials.[88] Magnetic particles have also been coated with carbon nanotubes and used to extract aromatic compounds.[89]

Turboflow

Turboflow extraction is usually carried out online before chromatographic separation and uses columns with large particle sizes in conjunction with high flow rates.[90] Samples can be directly injected after dilution; sometimes a protein precipitation step is required before injection. The target analytes are retained in the pores of the column, whereas matrix components are flushed through and discarded directly to waste. The analytes are then eluted from the trapping column using organic solvents. This method has the advantage of fast and generic method development but unfortunately it can also show high carry-over effects.[91] A study reported that this technique had no significant impact on phospholipid removal from serum and plasma samples, and still needed extensive chromatographic separation after clean-up to avoid matrix effects.[92] Other groups reported successful applications for quantification of various substances (drugs, steroids, phenolic compounds, *etc.*)[93–102] in human serum, urine and dried blood spots using reversed-phase, ion exchange or mixed-mode materials.

Monolithic spin column extraction

Monolithic spin column extraction is a fast sample preparation method that uses a spin column packed with octadecyl silane-bonded monolithic silica as the extraction device.[103] The sample is loaded onto the sorbent by centrifugation; the same procedure is performed for washing and elution steps.[104] This technique is fast and easy, requires only small amounts of solvents and allows high sample throughput. Unfortunately, the method can only be applied over a limited pH range because of possible degradation of the monolithic silica phase.[104] Several applications have been reported for quantification of various analytes from human samples, using underivatized,[105] C18,[106–108] ion exchange[109] or mixed-mode phases (C–C18, TiO–C18, C18-ion exchange).[110–112]

Microextraction by packed sorbent (MEPS)

This recent sample preparation technique is based on the miniaturization of conventional SPE, using a gas-tight syringe as extraction device. The method is designed for sample volumes from 10 to 1000 μL and can be connected online to

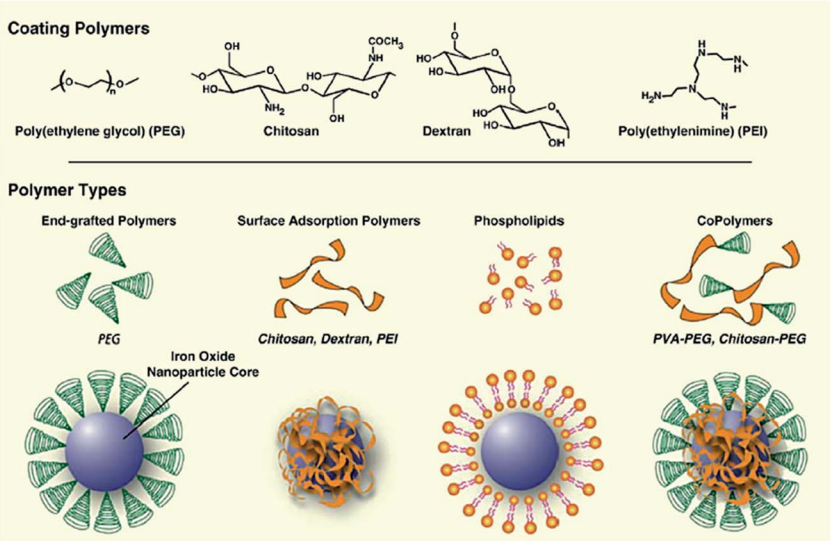

Fig. 5 Assembly of polymers onto the surface of magnetic nanoparticle cores (reprinted with permission from ref. 168).

LC-MS or GC-MS. Compared to conventional SPE, MEPS is easy to use, faster and needs significantly lower amounts of organic solvents. Additionally, MEPS sorbents can be used for up to 100 extractions.[113]

Packing materials for MEPS are similar to sorbents used for SPE. Essentially, any sorbent material and functionalization can be applied. For example, silica-based materials (C2, C8, C18),[114–118] with additional ion exchange functionality[119] or even as mixed-mode materials,[120] restricted access materials (RAM), HILIC, carbon, polystyrene–divinylbenzene copolymers (PS–DVB) or molecular imprinted polymers[113,121] have been utilized for MEPS.

The method has been implemented in several recent reports for quantification of pharmaceutical compounds from human biological samples (urine, plasma, oral fluid and whole blood), including antipsychotic drugs,[119] cardiac drugs,[114] local anesthetics,[115,121] phenolic acid,[116] immunosuppressants,[117] opioids[120] and antidepressants.[118] Recent studies have also reported the successful extraction of trazodone from plasma with polymer nano-fibers as the extraction sorbent.[122]

Carbon nanotubes

Carbon surfaces have the ability to retain substances by strong hydrophobic interactions. These materials are therefore interesting for reversed-phase extractions of hydrophilic substances. Carbon nanotubes (CNTs) are hollow cylinders that consist of one (single-wall carbon nanotubes, SWCNTs) or several (multi-walled carbon nanotubes, MWCNTs) graphene layers.[123] Because of their large surface areas, CNTs have a high adsorption capacity. They show high affinity towards aromatic compounds that can be adsorbed *via* π–π interactions.[124] CNTs can be packed into SPE cartridges or used for dispersive solid phase extraction.[125–127] Common target analytes are small, hydrophobic molecules extracted from water samples. Very few applications to biofluids have been reported so far. A method for quantitation of diuretics from urine[128] has been published as well as plasma peptide analysis.[129] The specificity of the extraction can be enhanced by derivatizing the surface of CNTs with functional groups. A method was recently shown for the determination of anti-inflammatory drugs from urine using carboxylated CNTs for sample clean-up.[130] To further improve both specificity and handling of the sample clean-up, magnetic CNTs coated with molecular imprinted polymers have been synthetized and used for extraction of BSA from serum samples.[131]

Restricted access materials (RAM)

Restricted access materials allow extractive clean-up of biofluids by utilizing physical and chemical diffusion barriers. RAM consist of a porous material with a restrictive and hydrophilic outer surface that prevents retention of large interfering molecules such as proteins and phospholipids, combined with smaller inner pores with hydrophobic surfaces that only molecules with low molecular weight can reach.[132] This technique is commonly used for online sample clean-up, with the advantage that samples dissolved in almost any solvent can be loaded, even MS incompatible solvents, before elution with the mobile phase used for chromatographic separation. There are two types of RAM phases:[133] internal surface phase (ISP) materials use size exclusion to prevent the matrix components from reaching the inner layer; semi-permeable surface (SPS) materials chemically exclude matrix components by polymeric- or protein coating of the outer layer. In both cases, the inner layer can be functionalized to enhance the specificity of the method.[134] Molecular imprinted polymers are a special form of restricted access materials; they are discussed below.

Application of sample clean-up using RAM includes quantification of antimicrobial agents, immunosuppressants *etc.* from human biological samples prior to LC-MS/MS analysis.[135,136] RAM have also been used in combination with magnetic

particles to quantify therapeutic drugs and steroids from biofluids.[87,137–140] An application was published that reported the synthesis of chiral RAM materials for extraction of enantiomeric drugs from plasma samples.[141]

Immunosorbents

Immunosorbents use the principle of antigen–antibody affinity for highly specific retention of target substances. The desired antibody is bound to a solid support or gel, which can be used as SPE or micro-SPE sorbent, MEPS or in columns.[123] The target analytes can be specifically extracted from complex matrices, which allows thorough sample clean-up prior to instrumental analysis. A study has shown that the capacity of monoclonal antibodies was significantly higher than that of polyclonal antibodies.[142] This technique has been used as in-tube SPME to quantify interferon α from plasma samples[143] as well as SPE extraction of ProGRP[144] and ochratoxin[145] from serum. Sample preparation techniques with high specificity towards the target analyte are required if the target analyte is present at very low concentration levels or in cases where structurally similar interferents (*e.g.* isobars) influence the analysis.[146] The immunosorbent extraction usually involves high costs, however, and also requires host animals to grow the required antibodies. Sometimes, the antibodies can be replaced by synthetic alternatives of comparable specificity, such as molecular imprinted polymers or aptamers (see below).

Molecular imprinted polymers (MIPs)

MIPs use the principle of affinity chromatography to maximize the specificity for the analyte(s) of interest. The target analyte or a structurally-related compound is used as a template for the synthesis of the MIP by copolymerization of a complex formed by the template and a functional monomer. The template molecule is then removed, leaving a rigid three-dimensional cavity that is complementary to the target analyte.[147]

The synthesis of these adsorbents is often inexpensive and has shown to be fast and reproducible; the materials also have high capacity and can be regenerated and used several times.[148] The MIP principle enables highly specific extraction of the target and structurally similar compounds (*e.g.* a drug and its metabolites) from complex matrices, and pre-concentration of the sample. The specificity of this technique has been shown in several applications. For example, a MIP sorbent developed for tylosin was able to differentiate between tylosin and the closely related narbomycin as well as the remotely similar tylactone. (Fig. 6). Both the target analyte and structurally similar compound were quantitatively extracted, whereas the interfering substance did not show any affinity for the sorbent.[149]

MIP can be used in various forms, for online or off-line processes such as molecular imprinted solid phase extraction (MISPE),[150] magnetic MIP,[151,152] solid-phase micro-extraction (SPME), needle/micropipette tip, dynamic liquid–liquid–solid micro-extraction (DLLSME) or molecular imprinted stir-bar sorptive extraction (MI-SBSE).[153,154] This concept has been applied to samples with complex matrices, for example, for benzodiazepines in plasma,[155] nucleoside reverse transcriptase

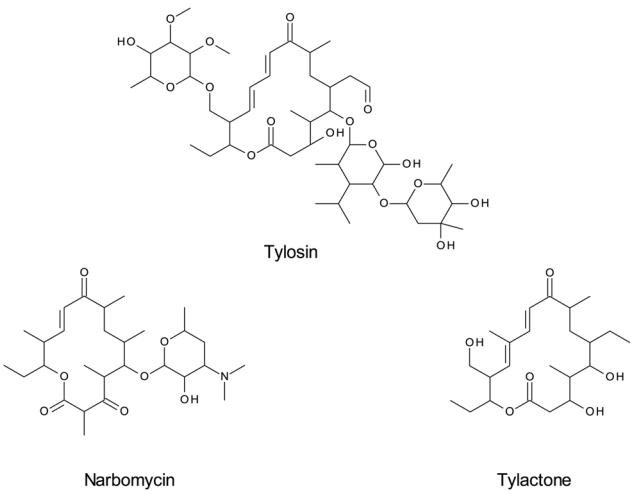

Fig. 6 Structures of tylosin and two structurally-related compounds, narbomycin and tylactone.

inhibitors in serum,[156] cocaine[157] or ketamine[158] from hair extract, testosterone[159] and tobacco-specific cancer biomarkers[160] from urine. MIP-coated fibers for solid phase microextraction (SPME) have also been used for extraction of linezolid from human biofluids.[161] This technique has shown to provide much cleaner extracts than other sample preparation methods such as LLE.[155] However, this technology still needs some improvement and has several drawbacks, including possible template bleeding, sometimes tedious synthesis procedures, and problematic application to aqueous samples.[147,150]

Aptamers

Another possibility to increase specificity for the target analyte is the use of aptamers immobilized on a solid sorbent for sample preparation. Aptamers are synthetic single-stranded oligonucleotides capable of binding specific analytes with a high affinity through hydrogen bonding, van der Waals forces and dipole interactions.[123,162] They are specifically prepared for each target molecule; that is, several nucleic acids have to be tested *in vitro* for each target. Selected nucleic acids with high affinity for the analytes are isolated and amplified using a process called systematic evolution of ligands by exponential enrichment (SELEX).[163] The major advantage compared to antibodies is that aptamers can be synthetized directly, without the need for laboratory animals. They can be regenerated within minutes and reused several times. The technique has been used for the selective extraction of cocaine from plasma[164,165] and for extraction of tetracyclines from biological fluids in combination with ion mobility spectrometry.[166] The high affinity of a target substance to an extraction sorbent is clearly shown in these applications as well as the importance of the sequence of the oligonucleotides. The sequence is specific for a particular compound and will become inactive if the oligonucleotides are grafted in a randomized order.[163] Recoveries of up to 90% confirm the high specificity of this technique, even in complex

samples such as plasma.[163] Aptamers have also been immobilized on polymeric nano-fibers and extraction of thrombin from serum was shown.[167]

Conclusion

Common problems encountered during development of an LC-MS/MS assay for the quantification of small molecules from biological samples include loss of sensitivity and specificity due to matrix effects. Sample preparation is therefore an indispensable part of the analytical workflow. The possible influence of matrix effects on LC-MS/MS assays has been extensively studied and several methods have been published to identify and avoid these effects. Considerable progress has been made in the improvement of sample preparation routines in the last few years. New trends are directed towards either increasing the specificity of the extraction for the target analyte or removing as much of the matrix components as possible. Miniaturization and automation of these techniques are on-going efforts, leading to cheaper, more robust and fully automated LC-MS/MS assays that will significantly impact pharmaceutical analyses of biofluids in the future.

List of abbreviations

APCI	Atmospheric pressure chemical ionization
BSA	Bovine serum albumin
CNT	Carbon nanotubes
CSF	Cerebrospinal fluid
DART	Direct analysis in real time
DESI	Desorption electrospray ionization
DLLSME	Dynamic liquid–liquid–solid microextraction
DTT	Dithiothreitol
ESI	Electrospray ionization
GC–MS	Gas chromatography-mass spectrometry
GPCho	Glycerophosphocholines
HILIC	Hydrophobic interaction liquid chromatography
IS-MRM	In-source multiple reaction monitoring
ISP	Internal surface phase
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLE	Liquid–liquid extraction
MALDI	Matrix-assisted laser desorption/ionization
MEPS	Microextraction by packed sorbent
MIP	Molecular imprinted polymers
MI-SBSE	Molecular imprinted stir-bar sorptive extraction
MISPE	Molecular imprinted solid phase extraction
MNP	Magnetic nanoparticles
MRM	Multiple reaction monitoring
MTBE	Methyl-*tert*-butylether
MWCNT	Multi-walled carbon nanotubes
PPL	Phospholipids
PPT	Protein precipitation
PQ	Piperaquine
PS–DVB	Polystyrene–divinylbenzene
RAM	Restricted access materials
SALLE	Salt-assisted liquid–liquid extraction
SELEX	Systematic evolution of ligands by exponential enrichment
SLE	Supported liquid extraction
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
SPS	Semi-permeable surface
SWCNT	Single-wall carbon nanotubes
TCEP	Tris(2-carboxyethyl)phosphine

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Publication 2

Simultaneous quantification of acetaminophen and structurally related compounds in human serum and plasma

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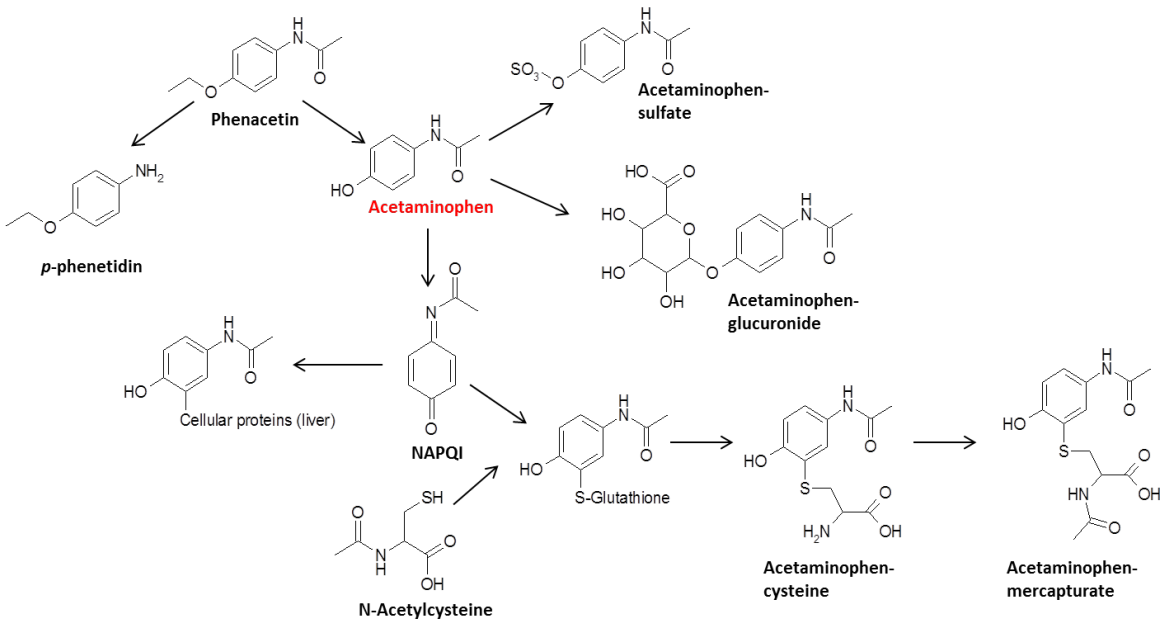

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Simultaneous quantification of acetaminophen and structurally related compounds in human serum and plasma

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The method described in this study allows the simultaneous quantification of acetaminophen (APAP) and nine structurally related compounds, namely acetaminophen metabolites and structurally similar analogs (acetaminophen-glucuronide [APG], -sulfate [APS], mercapturate [APM], -cysteine [APC], *p*-phenetidine, phenacetin), antidote (N-acetylcysteine, NAC), and two tricyclic antidepressants (imipramine and amitryptiline). Due to the relatively high serum concentration levels in the μg/ml range, matrix effects were simply minimized by dilution. The samples were diluted with water and disulfide bonds between serum proteins and analytes reduced using tris(2-carboxyethyl)phosphine. Chromatographic separation of the analytes was achieved by gradient elution using a pentafluorphenyl (PFP) column with subsequent detection by electrospray ionization (ESI) triple quadrupole mass spectrometry in positive and negative ionization multiple reaction monitoring (MRM) modes. Quantification was performed by means of deuterated analogues of the analytes as internal standards. Total run time of the assay was 19 min. The method was fully validated and allowed quantification of the analytes with lower limits of quantification between 50 and 0.5 ng/ml. The calibration curves were linear over the range 0.1–100 μg/ml for APAP, APG, NAC, *p*-phenetidine and phenacetin, 0.03-50 μg/ml for APS, and 0.01-10 μg/ml for APM, APC, imipramine and amitriptyline with correlation coefficients $r^2 > 0.99$. The intra-assay precision was ≤5% for all analytes except NAC (CV < 10%). The inter-day precision was ≤10% for all analytes except NAC (inter-assay precision <11%). This method was used to analyze 77 patient and spiked samples and results were consistent with expected values from a round robin test. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: acetaminophen; LC-MS/MS; immunoassay; metabolites; serum; plasma

Introduction

Acetaminophen (APAP, N-acetyl-*p*-aminophenol), also known as paracetamol, is a widely used over-the-counter drug with analgesic and anti-pyruvic properties. Severe toxic effects in case of an overdose or long-term abuse have been reported.[1] In Europe and the United States, the daily limit is 4 g[2]; in Japan it is set at 1 g.[3] The lethal dose strongly varies between individuals, depending on health condition, drinking habits, and age.[4,5] The threshold for liver damage is known to be approximately 250 mg/kg.[6] APAP poisoning is accompanied by unspecific symptoms such as nausea, vomiting, pallor, and lethargy in the first 24 h. It may take up to 48 h before signs of hepatic failure become apparent.[7]

APAP undergoes several metabolic pathways and exhibits a number of metabolites. The abbreviated metabolism of acetaminophen in the human body is summarized in Figure 1.[8–10] Briefly, APAP is primarily metabolized to stable glucuronic acid (APG) and sulfate (APS) metabolites. A small proportion of APAP is transformed by cytochromes P450 2E1 to the highly reactive metabolite N-acetyl-*p*-benzoquinoneimine (NAPQI),[11] which is normally detoxified by conjugation with glutathione. This complex further reacts to acetaminophen-cysteine (APC) and then to acetaminophen-mercapturate (APM).[12] Excessive dosage of APAP causes overproduction of this metabolite and depletion of free glutathione. In this case, NAPQI binds to liver cell proteins, causing hepatic necrosis. In aqueous medium, NAPQI undergoes redox reactions and hydrolysis and decomposes to further products, which makes quantification in serum very difficult.[13,14]

The damaging action of NAPQI can be stopped by administration of N-acetylcysteine (NAC). The antidote inhibits binding of NAPQI to liver proteins by creating a new source of free glutathione, and also has the ability to directly bind to NAPQI and thus detoxify it.[15,16] However, the second mode of action was shown to have little relevance for the detoxification process of APAP.[17]

Phenacetin was commonly used as an analgesic at the end of the twentieth century. Because of dangerous side-effects, it has been taken off the market in a number of countries. The main metabolite of phenacetin is APAP, but *p*-phenetidine is also formed by deacylation.[18]

Imipramine and amitriptyline are tricyclic antidepressants, which in certain instances are co-administered with APAP. Preliminary tests at Roche Diagnostics during the development of an immunologic APAP assay showed that these substances have the potential to cross-react with immunologic assays.

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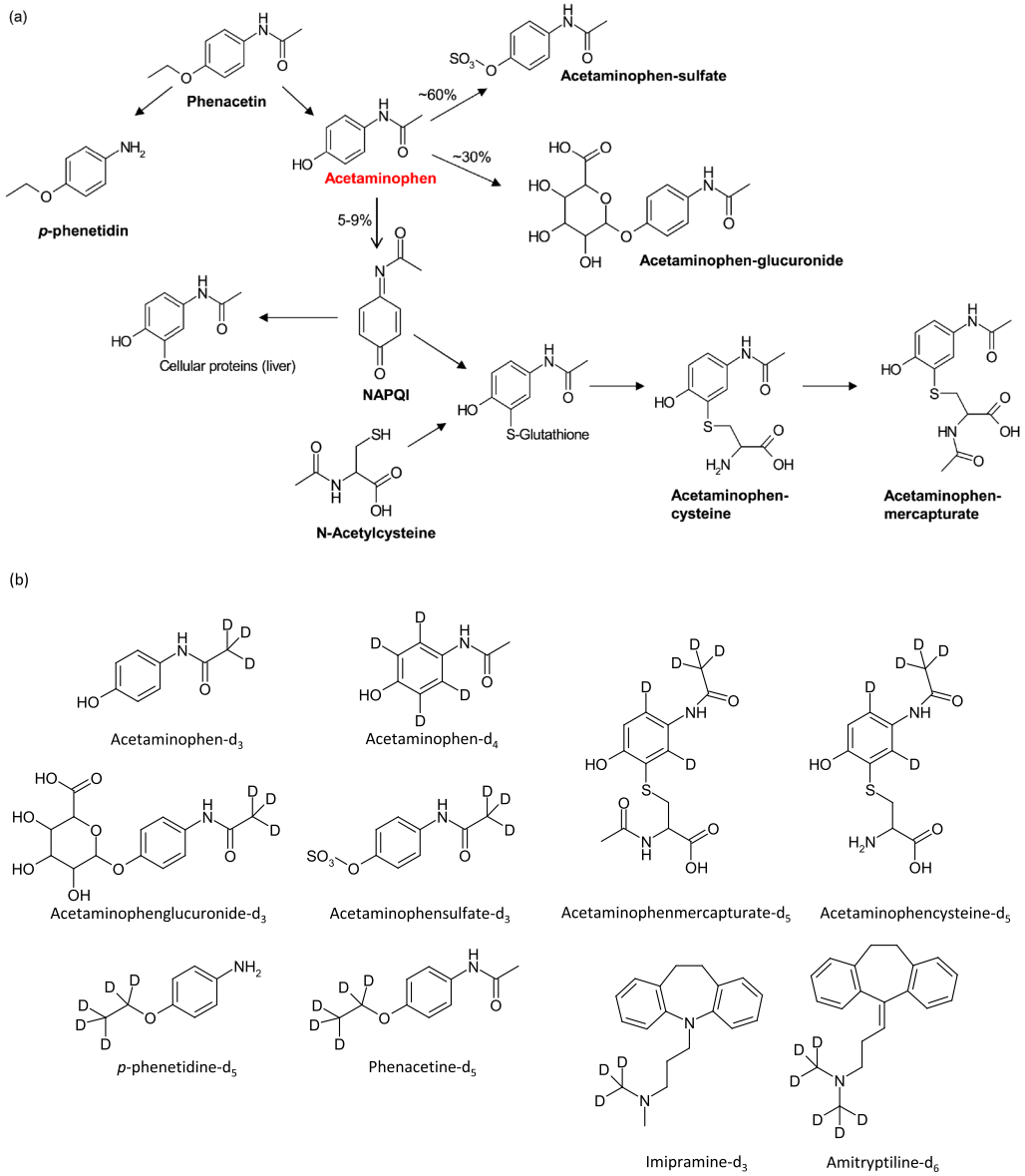

Figure 1. Abbreviated metabolic pathway of acetaminophen and chemical structures of the investigated analytes (a); chemical structures of the isotopically labelled standards (b).

Usually APAP is quantified by immunoassay techniques, either as part of a general screen or as specific target.[19–25] Various other analytical methods have also been applied to detect APAP and its metabolites, such as high performance liquid chromatography (HPLC) in combination with accelerator mass spectrometry (AMS),[26] diode-array detection (DAD),[8] chemical reaction interface for mass spectrometry (CRIMS),[27] UV,[9] as well as capillary electrophoresis (CE) with UV detection.[28] In particular, hyphenated HPLC-mass spectrometry (LC-MS) has shown great potential, because of its specificity and its ability to identify structurally-related substances that could bias the result of immunoassay analysis. Several LC-MS methods have been reported for quantification of APAP[29] or APAP in combination with other analytes, for example other therapeutic drugs,[30–33] or several metabolites in various matrices.[25,34–39] An assay for APAP and all major metabolites was reported for rat plasma.[25] It is essential that methods developed for clinical diagnostics are carefully validated for potential matrix interferences. The sample material usually exhibits a strong influence on the method performance; therefore, methods developed for sample matrices other than human serum cannot be readily transferred without proper evaluation and modifications. To our knowledge, there is no literature LC-MS method for quantifying APAP simultaneously with its major metabolites in human serum and plasma.

Mass spectrometric quantification of NAC has been described.[33,40] Phenacetin and *p*-phenetidine have been included in LC-MS assays for APAP and of its metabolites.[18,41] One study has also reported the simultaneous LC-MS analysis of APAP, imipramine and amitriptyline (amongst others),[42] but no method is available to simultaneously quantify the combination of substances presented in this work in human serum and plasma.

The scope of this work was the development and optimization of a simple, specific and sensitive LC-MS/MS method for quantification of APAP, its main metabolites and several structurally

related compounds in serum as a reference method for an APAP-targeted immunoassay.

Experimental

Reagents and chemicals

The reference material for APAP was purchased from LGC Standards (Wesel, Germany). Imipramine was from Cerilliant (Wesel, Germany) at 1 mg/ml in methanol. Amitriptyline was from Sigma-Aldrich (Steinheim, Germany) and all other analytes (4-acetamidophenyl β-D-glucuronide sodium salt, 4-acetaminophen sulfate potassium salt , 3-(N-acetyl-L-cystein-S-yl) acetami nophen sodium salt , 3-cysteinylacetaminophen trifluoroacetic acid salt , N-acetyl-L-cysteine , *p*-phenetidine, phenacetin) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Deuterated analogues of the analytes were used as internal standards. Imipramine-d3 was ordered from Cerilliant and amitriptyline:HCl (N,N-dimethyl-d_6) from Cambridge Isotope Laboratories (Saarbrücken, Germany) at 100 µg/ml in methanol. The deuterated substances APAP-d_3, APAP-d_4, 4-ace tamidophenyl β-D-glucuronide-d_3 sodium salt (APG-d_3), 4-acetaminophen-d_3 sulfate (APS-d_3), 3-(N-acetyl-L-cystein-S-yl) acetami nophen sodium salt-d_5 (APM-d_5), 3-cysteinylacetaminophen-d_5 trifluoroacetic acid salt (APC-d_3), *p*-phenetidine-d_5 hydrochloride, phenacetin-d_5 were from Toronto Research Chemicals. LC-MS grade solvents were from Biosolve (Valkenvaard, the Netherlands). Formic acid (FA) (98-100%) was purchased from Merck (Darmstadt, Germany) and tris(2-carboxyethyl)phosphine (TCEP) from Sigma-Aldrich. Human serum for therapeutic drug monitoring provided by Roche Diagnostics GmbH (Mannheim, Germany) was used as negative calibrator matrix. Water was generated using a Waters Millipore Milli-Q-Plus purification system (Eschborn, Germany)

Sample preparation

A serum volume of 25 µl was used for each analysis. Each sample was mixed with 25 µl internal standard solution and 400 µl MilliQ water in an Eppendorf vial and equilibrated at room temperature while shaking on a thermomixer for 15 min. Fifty microliters of a 350 mmol/L TCEP solution were then added to the vial and the sample mixed again for 30 min at 37 °C. After this reduction step, the solution was transferred to an ultracentrifugation tube using a regenerated cellulose 30 kDa cutoff membrane. The acidic filtrate (pH between 3 and 4) was then injected into the LC-MS/MS system.

Quantification

Calibration samples were prepared by spiking analyte-free human serum with a stock solution containing all analytes. Depending on the expected concentration levels of the substances in human serum, different amounts for each substance were spiked into the calibrator samples. APAP, APG, NAC, *p*-phenetidine and phenacetin were spiked in concentrations between 0.1 and 100 µg/ml, APS between 0.03 and 50 µg/ml and APM, APC, imipramine and amitriptyline between 0.01 and 10 µg/ml. The concentrations of all calibrators for each analyte are shown in Table 1. Deuterated internal standards were added to each sample at different concentration levels. APAP-d_3 and APG-d_3 at 50 µg/ml, APS-d_3 at 25 µg/ml, APAP-d_4 at 10 µg/ml, APM-d_5, APC-d_5, *p*-phenetidine-d_5 and phenacetin-d_5 at 5 µg/ml and imipramine-d_3 and amitriptyline-d_6 at 2.5 µg/ml.

The compounds were quantified using the multiple reaction monitoring (MRM) mode of the mass spectrometer. Calibration curves were constructed using analyte/internal standard peak area ratios for all analytes. Unknown samples were quantified by comparing the ratio of the integrated area of the analyte and the corresponding internal standard to the corrected calibration curve.

Chromatography and mass spectrometry

The chromatographic system consisted of Dionex (Germering, Germany) U3000 binary pump, column oven and autosampler. The analytes were separated on a Phenomenex (Aschaffenburg, Germany) Kinetex PFP column (2.6 µm, 150 x 3.0 mm, 100 Å). The mobile phase was acetonitrile-water (5:95 *v/v*) + 0.1% formic acid (eluent A), acetonitrile:water (50:50 *v/v*) + 0.1% formic acid (eluent B) and acetonitrile + 0.1% formic acid (eluent C). Gradient elution at a flow rate of 300 µl/min was performed (Table 2). The total runtime of analysis was 19 min. The column temperature was maintained at 40 °C during separation.

The mass spectrometer was a Thermo Scientific (Bremen, Germany) TSQ Vantage triple quadrupole equipped with a heated electrospray ionization source (HESI). The sprayer voltage was set to +4500 V in positive and -3250 V in negative ionization mode, capillary temperature was kept constant at 250 °C, nitrogen as sheath gas was set to 60 psi, the HF amplitude of the S-Lens was 87 V, and the argon pressure in the collision cell 1 mTorr. The multiple reaction monitoring (MRM) transitions and optimized collision energies (CE) for each transition are summarized in Table 3.

Table 1. Concentrations of all investigated analytes in the calibrators [µg/ml]

Cal #	APAP	APG	APS	APC	APM	NAC	Phene-tidin	Phena-cetin	Imipra-min	Amitrip-tylin
0	*200.0*	*200.0*	*100.0*	*20.0*	*20.0*	*200.0*	*200.0*	*200.0*	*20.0*	*20.0*
1	100.0	100.0	50.0	10.0	10.0	100.0	100,0	100.0	10.0	10.0
2	50.0	50.0	25.0	5.00	5.00	50.0	50.0	50.0	5.00	5.00
3	30.0	30.0	15.0	3.00	3.00	30.0	30.0	30.0	3.00	3.00
4	10.0	10.0	5.00	1.00	1.00	10.0	10.0	10.0	1.00	1.00
5	5.00	5.00	2.50	0.50	0.50	5.00	5.00	5.00	0.50	0.50
6	1.00	1.00	0.50	0.10	0.10	1.00	1.00	1.00	0.10	0.10
7	0.75	0.75	0.38	0.08	0.07	0.75	0.75	0.75	0.07	0.07
8	0.05	0.05	0.03	0.01	0.01	0.05	0.05	0.05	0.01	0.01

Table 2. Eluent gradient composition of the gradient

Time [min]	%B	%C
0	0	0
6	50	0
13	0	100
16	0	100
16.1	0	0
19	0	0

The total acquisition time was divided into five segments, to increase the number of data points per peak. The first segment was further divided in two scan events, where the polarity was constantly switched between positive and the negative ionization mode, to record transitions of APC, APG and their deuterated standards (positive mode) as well as transitions of NAC (negative mode). During the following 0.9 min, APAP, APS, *p*-phenetidine and their internal standards were recorded as well as APAP-d_4 used as internal standard for NAC. In the third segment (5.8–7.8 min), *p*-phenetidine and APM plus deuterated standards were monitored. The fourth segment (7.8–11.6 min) was for phenacetin and phenacetin-d_5. During the remainder of the chromatographic run, imipramine, amitriptyline and their internal standards were monitored.r

Method validation

System suitability test

At the beginning of each sequence, the following system suitability test was carried out: an aqueous solution of all analytes at the concentration level of Cal 8 (Table 1) was injected into the system. Signal-to-noise ratios for all analytes were required to be 10:1. Additionally, it was checked whether all peaks appeared inside their intended time segments.

Accuracy, precision

The accuracy was determined for all analytes with three replicates at three concentration levels, by comparing calculated concentrations with the theoretical values. For this procedure, different concentrations of analytes were spiked into analyte-free matrix. These concentrations are given in Table 4. For the main analyte APAP, the purity of the reference material was investigated by several analytical methods. The APAP content of the materials was determined by quantitative nuclear magnetic resonance (qNMR), the percentage of inorganic impurities was investigated using inductively coupled plasma mass spectrometry (ICP-MS) and the slopes of the calibration curves were compared by HPLC-MS/MS. The quality of the used reference material was compared to other materials, namely Sigma Aldrich BioXtra and Sigma Aldrich 'meets USP qualifications'. Recovery was calculated in terms of bias as percent deviation of the measured mean from the corresponding theoretical concentration. Precision was evaluated by performing six determinations at three concentration levels (low, mid, and high). Each sample was analyzed in duplicate. The intra-assay precision was calculated for the mean of all six preparations. For the inter-assay precision, the same experiment was repeated by a different operator on a different day. The coefficient of variation (CV) was determined using all 12 measurements for each concentration level.

Table 4. Concentrations of all analytes in samples used for the validation of the method

Substances	High level [µg/ml]	Middle level [µg/ml]	Low level [µg/ml]
APAP	50	10	1
APG	50	10	1
APS	25	5	0.5
APM	5	1	0.1
APC	5	1	0.1
NAC	50	10	1
p-Phenetidine	50	10	1
Phenacetin	50	10	1
Imipramine	5	1	0.1
Amitryptiline	5	1	0.1

Specificity, ion suppression[43,44]

Possible matrix-dependent ion suppression effects were assessed. The possible transfer of this method to different matrices such as plasma collected in Lithium-heparin or EDTA-tubes was also investigated. The specificity of the method was also verified by analyzing analyte-free serum and plasma samples. Ion suppression from glycerophosphocholines (GPCho) was measured in selected ion monitoring (SIM) mode over the entire run by monitoring the ion at *m/z* 184 and 104 formed by in-source collision-induced dissociation (CID) using a high declustering voltage of the mass spectrometer (40 V). All GPCho compounds yield the common product

Table 3. MRM transitions and applied collision energies (CE [V], in parentheses) for all investigated substances and their corresponding internal standards

Compound	MRM transition	Internal standard	MRM transitions
APAP	*m/z* 152.1 ➔ 65.0 (25), 110.0 (15)	APAP-d_3	*m/z* 155.1 ➔ 65.0, 111.1 (25)
APG	*m/z* 328.1 ➔ 151.9 (18)	APG-d_3	*m/z* 331.0 ➔ 154.8 (18)
APS	*m/z* 231.9 ➔ 109.9, 151.9 (18)	APS-d_3	m/z 234.9 ➔ 110.9, 154.9 (18)
APM	*m/z* 313.1 ➔ 165.8, 207.9 (20)	APM-d_5	*m/z* 318.1 ➔ 167.8, 212.0 (20)
APC	*m/z* 271.0 ➔ 140.0, 182.0 (18)	APC-d_5	*m/z* 276.1 ➔ 142.9, 186.7 (18)
NAC	*m/z* 161.6 ➔ 28.1, 81.3, 161.6 (10)	APAP-d_4	*m/z* 156.1 ➔ 69.1, 114.1 (23)
p-Phenetidine	*m/z* 138.1 ➔ 93.0, 110.0 (20)	*p*-Phenetidine-d_5	*m/z* 143.1 ➔ 93.0, 111.0 (18)
Phenacetin	*m/z* 180.0 ➔ 110.0, 138.1 (22)	Phenacetin-d_5	*m/z* 185.1 ➔ 111.0, 143.1 (18)
Imipramine	*m/z* 281.2 ➔ 58.0, 86.0 (25)	Imipramine-d_3	*m/z* 284.2 ➔ 61.1, 89.1 (25)
Amitriptyline	*m/z* 278.2 ➔ 90.9, 233.1 (20)	Amitriptyline-d_6	*m/z* 284.3 ➔ 91.0, 233.1 (25)

ion (trimethylammonium-ethylphosphate, *m/z* 184) that sometimes further dissociates to trimethylammoniumethylate (*m/z* 104).[44]

Linearity, sensitivity

The linearity of the method was assessed by extending the 8-point calibration range from 80% of the lowest concentration to 120% of the highest calibrator. The lower limit of quantification (LLOQ) in serum was determined using the isotopically labelled substances, because of small endogenous amounts of analyte already contained in the sample matrix. The LLOQ was defined as the lowest concentration measured with a CV $< 20\%$ over six measurements.

Stability

Stability of the samples was determined by storing serum calibrators containing all 10 analytes at 4 °C and -20 °C for seven days and comparing the recovery before and after storage. Additionally, serum samples containing only APAP were stressed at temperatures ranging from -20 °C to 35 °C for up to 12 weeks. The APAP amount was determined before the beginning of the experiment. Sample aliquots were then analyzed after one day and after one, three, six and twelve weeks, and recoveries calculated compared to the original value.

Results and discussion

The aim of this study was the development of LC-MS/MS reference method for quantification of the very common analgesic drug, APAP, its antidote and several structurally related compounds (including metabolites) known to cross-react with APAP-specific immunoassays. The main requirement for this method was the ability to quantify the analytes in serum samples as well as the possibility to transfer the technique to plasma samples (heparin and EDTA tubes). The calibration range of APAP was 0.1–100 μg/ml; calibration ranges for the other analytes were adapted to the expected concentration levels in human serum based on the metabolism of the parent drug, and on therapeutic ranges for the other quantified substances. The very broad polarity range of the target substances put limitations on the sample preparation protocol. After a reduction step to cleave disulfide bonds between target molecules and matrix proteins, the samples were diluted and filtered before injection into the LC-MS/MS system.

Optimization of sample preparation

The samples were first incubated by rolling the tubes for 15 min with the internal standard solution. This step allowed equilibration of the analytes with the deuterated standards and improved the reproducibility of the method. After initial experiments, it became clear that N-acetylcysteine was not detectable in serum without pretreatment. Therefore, a reduction agent was used to cleave the disulfide bonds between N-acetylcysteine and proteins from the sample matrix. Two reducing agents were tested, DTT and TCEP. The test results showed that DTT needed longer incubation times than TCEP and had an unpleasant odor. Therefore, TCEP was chosen as reducing agent. Because of the wide spectrum of polarities of the target analytes, a simple sample preparation approach (dilution/filtration) was initially investigated before more complex and time-consuming methods such as solid-phase extraction (SPE) or liquid/liquid extractions (LLE). Fortunately, the detection sensitivity of the LC-MS/MS far exceeded the required sensitivity from the analytes in the samples. Therefore, matrix effects were simply 'diluted out'.

Optimization of LC-MS conditions

Several reversed-phase materials (Waters XTerra C18, Phenomenex Kinetex C18, Agilent Zorbax C8 and SB-CN, Thermo Fisher Scientific Hypercarb, Phenomenex Kinetex PFP) were tested for the chromatographic separation of the analytes. Optimum results were achieved with the pentafluorphenyl-phase (PFP) column, which allowed resolving the very polar APAP metabolites and separation of this group of compounds from other, less polar analytes (Figure 2).

Ionization for LC-MS was performed via electrospray ionization (ESI). Optimization of ionization and fragmentation conditions was performed using standard solutions of each analyte. All analytes except for NAC exhibited high sensitivity in positive ionization mode. NAC showed better results in negative ionization mode; polarity of ionization was therefore switched during each run. A compromise for interface conditions had to be used to detect all analytes with sufficiently high sensitivity. Since APAP was the primary target, the optimum settings for ionization of APAP were applied to all other target molecules (i.e. settings for capillary temperature, sheath gas pressure, sprayer voltage and S-Lens potential). Fragmentation conditions were optimized as well; in these experiments, the collision gas pressure was kept constant for all analytes and the collision energy was optimized for each MRM transition. For MRM, the two most intense and selective product ions were chosen for each target substance. Unspecific transitions such as H_2O or CO_2 losses were avoided. The collision energy was set to the value for which the sum of the intensities of both product ions was the highest.

Ion suppression

Ion suppression effects from the matrix were assessed by continuous post-column infusion of the analyte after injection of a processed blank serum sample. Any variation of signal intensity near the retention times of the analytes would indicate the presence of substances from the matrix interfering with the analysis.[43] For this experiment, 10 serum blank samples were processed in identical manner to the regular samples. The filtrates were pooled and equally distributed over 10 vials. The experiment was carried a single time for each analyte. No sudden increases or decreases of signal intensities were noticed close to the retention time of the target analytes. The observed chromatogram for APAP is shown in Figure 3. The matrix does not seem to contain any substance that interferes with quantification of the analytes.

An additional experiment was carried out to assess the specific influence of glycerophosphocholines (GPCho), which are known to cause matrix ionization effects in LC-MS/MS methods[44] (see Experimental for experimental details). Analyte-free serum and plasma samples were processed and injected into the LC-MS-system. In all tested matrices, no signal was recorded for the fragments of GPCho compounds at the retention time of the analytes. This experiment confirmed that the sample matrix does not interfere with the quantification of the analytes.

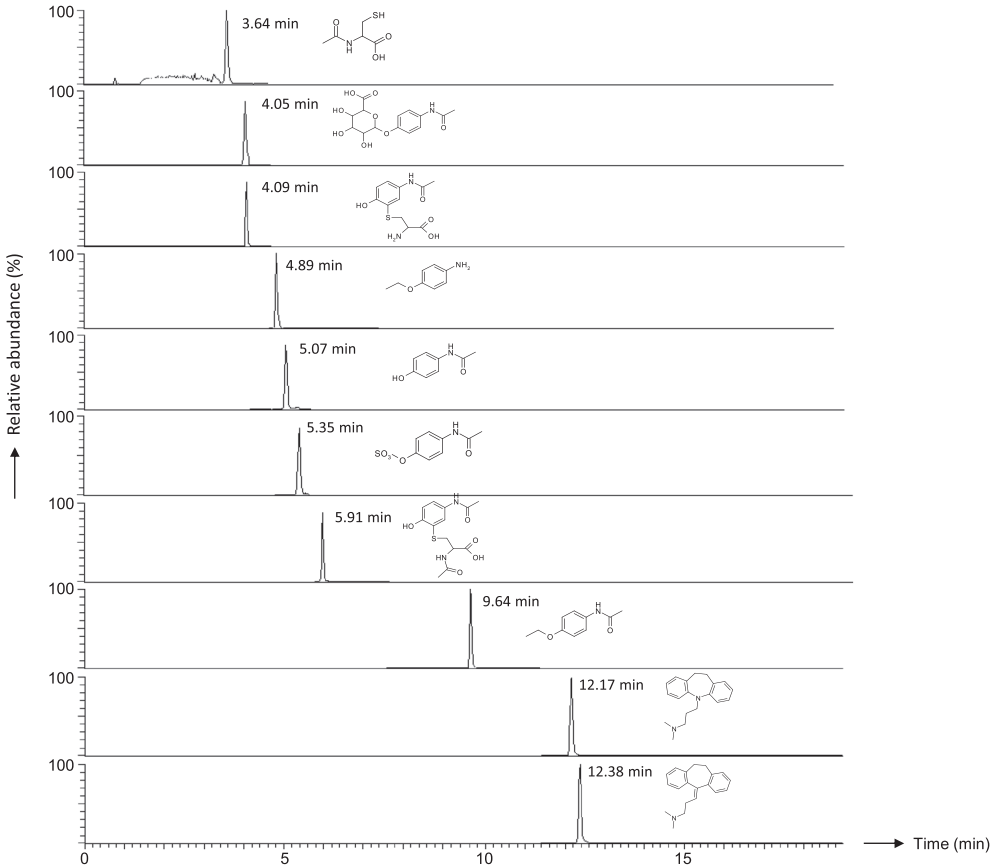

Figure 2. MRM chromatographic traces of the first (A) and second (B) group of substances eluting from the HPLC column. Sample: human serum, analyte concentration range, 1.0–10 μg/ml.

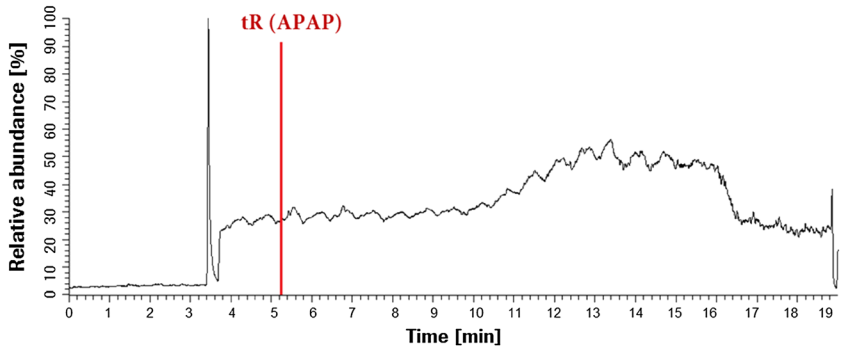

Figure 3. Post-column infusion of APAP in analysis of processed matrix blank.

Traceability of the reference material

The purity of our reference material was examined to ensure the traceability of the method. Several techniques of analysis were employed to characterize the reference substance for APAP (USP reference standard, [A]) and two similar materials (Sigma 'meets USP specifications' [B] and Sigma 'BioXtra quality' [C]). Analysis by qNMR showed that absolute APAP content of these materials was between 98.8% and 98.9%. The percentage of inorganic impurities was determined by ICP-MS. Substance A exhibited the highest purity containing only 11 mg/kg silicon. Both Sigma materials also showed traces of inorganic impurities: B contained 15 mg/kg silicon and 18 mg/kg potassium, whereas C contained 1 mg/kg silicon and 14 mg/kg calcium. Analysis by LC-MS/MS showed a variation of 3% in the slopes of calibration curves determined from these materials, the slope of the USP standard being slightly lower than the Sigma materials.

Transferability to plasma samples

The assay was originally developed for quantification of acetaminophen and the other analytes from serum. To determine whether this method could also be adapted to plasma samples, two sets of calibrators were prepared, one in EDTA and a second in a heparin plasma pool of nine donors. These samples were processed as usual and compared to the reference calibration

Table 5. Precision of the assay

Substances	Precision high level (%)		Precision middle level (%)		Precision low level (%)	
	Intra-assay (n = 6)	Inter-assay (n = 12)	Intra-assay (n = 6)	Inter-assay (n = 12)	Intra-assay (n = 6)	Inter-assay (n = 12)
APAP	2	4	3	4	5	5
APG	2	3	3	4	5	5
APS	1	3	2	3	4	4
APM	2	3	2	3	4	3
APC	2	3	3	4	5	4
NAC	3	4	3	5	10	11
p-Phenetidine	2	7	4	6	5	4
Phenacetin	2	2	2	3	5	7
Imipramine	1	3	2	3	2	3
Amitryptiline	1	3		3		4

curve in human serum for therapeutic drug monitoring. The regression equations of all three calibration curves were compared: the variation of the slopes was 2%. The slopes of the calibration curves in plasma were both smaller than the calibration curve in serum. These values are well within with the specifications of the method, thus it is readily possible to quantify the panel of analytes in plasma with this assay.

Validation

The investigated concentration levels of the target analytes in the samples 'low', 'mid' and 'high' are given in Table 4. These levels were used to assess precision and accuracy of the method.

The accuracy of the method was determined by comparing the average measured concentration of a spiked sample ($n = 3$ preparations) to the originally spiked amount. The values were between 95% and 101% for the high level, 92% and 102% for the mid level and 92% and 105% for the low level.

The calculated CVs for intra- and inter-assay precision are summarized in Table 5. All intra- and inter-assay coefficients were lower than 5% and 7%, respectively, showing overall good precision of the method. NAC was considered separately as precision values for this substance were higher than for other analytes because no isotopically labelled standard was available for this analyte and the chosen internal standard did not have the same binding affinity to proteins than NAC. The reduction step with TCEP, where cleavage of disulfide bonds between NAC and serum proteins occurs, was therefore not compensated by an internal standard, ultimately resulting in higher imprecision.

The linearity of the method was investigated for each analyte by adding two points to the calibration curve and measuring them three-fold. The coefficients of regression were determined for these new calibration curves; they were always > 0.992, thus demonstrating good linearity for all analytes in the desired calibration ranges.

The detection sensitivity was assessed by spiking several concentrations of target analytes below the smallest calibrator level into therapeutic drug-free-serum. These samples were measured six times; the lower limits of quantification (LLOQ) with corresponding CV values are summarized in Table 6 for each target molecule. This experiment was not carried out for NAC, because the LLOQ for this analyte corresponded to the lowest concentration of the calibration range.

The investigated calibration ranges were different for all target analytes; they were chosen according to expected concentrations found in the human body. The main focus of this method was not to achieve high detection sensitivity for all substances, but rather to be able to detect signs of possible poisoning with APAP or to show the presence of other therapeutic drugs capable of falsifying the results of the immunoassay screening. Therefore, it was only required to achieve low LLOQ values for APC and APM as they represent only 5-10% of the metabolic pathway of APAP, and for imipramine and amitriptyline, because these drugs are not prescribed in high doses. For the other substances, the main focus was to achieve a robust method with linearity over a broad concentration range.

The stability of the calibrators was investigated by determining the recovery of the target substances after 2 and 7 days storage at different temperatures. The calibrators were prepared and analyzed on day 1 and aliquots were stored at 4 °C and -20 °C. Aliquots were taken out and measured again after 2 and 7 days storage time. The regression coefficients and slopes of the calibration curves were compared to analysis of calibrators on day 1. All coefficients of regression were 0.991 and the variation of the slopes was ≤ 6%, regardless of the storage temperature. The mixed calibrators in serum can be stored at 4 °C or -20 °C for at least one week. Additionally, the shelf-life of the samples was simulated with an accelerated testing model based on the Arrhenius equation. Using this model, the long-term stability can be estimated by observing

Table 6. Lower limits of quantification (LLOQ) and coefficients of variation (CV)

Substance	LLOQ [ng/ml]	CV [%]
APAP-d_3	20 ng/ml	17
APG-d_3	40 ng/ml	15
APS-d_3	24 ng/ml	13
APM-d_5	4 ng/ml	17
APC-d_5	0.5 ng/ml	11
NAC	50 ng/ml	n.a.
p-Phenetidine-d_5	40 ng/ml	15
Phenacetin-d_5	10 ng/ml	14
Imipramine-d_3	1 ng/ml	12
Amitryptiline-d_6	1 ng/ml	11

Table 7. Long-term stability of serum samples spiked with acetaminophen

Sample #	Conc. at $t = 0$	Recoveries after simulation of long-term storage at -20 °C (%)	
	[µg/ml]	5 years (12 weeks 25 °C)	5 years (6 weeks 35 °C)
A		Blank	
B	10.2	96	100
C	73.6	96	96
D	100.8	97	103
E	293.8	95	103
F	501.6	96	103

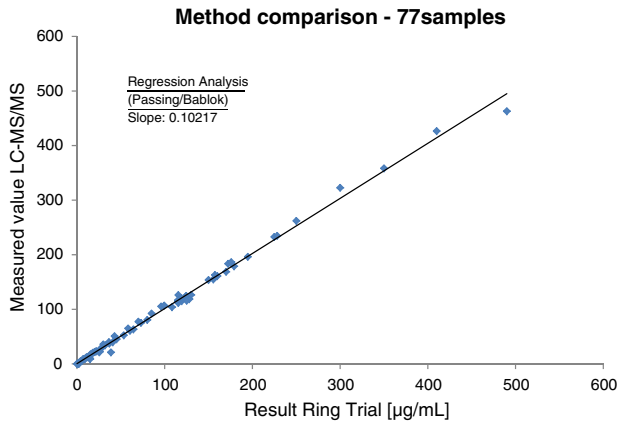

Figure 4. Comparison of measured LC-MS/MS concentration values for 77 samples (spiked and patient samples) with previously measured immunoassay values from Round Robin test.

the degradation rates at high temperatures.[45] For this experiment, blank serum samples were spiked with 5 APAP concentrations and were stressed at -20 °C, 2–8 °C, 25 °C and 35 °C for a total time of 12 weeks, simulating a storage of up to 5 years at -20 °C. Aliquots were regularly taken and analyzed, and the obtained values compared to the measured concentrations on the day of the spiking. The recoveries for the aliquots measured after 12 weeks are shown in Table 7. These results demonstrate that serum samples containing APAP are stable up to five years when stored at -20 °C.

Application to analysis of patient and spiked samples

After validation, the method was used to analyze a total of 77 samples. Nine of these samples were anonymized patient samples from an unrelated study, while other samples were obtained by spiking analyte-free matrix. For these samples, serum of healthy volunteers was collected and treated in agreement with the local ethics guidelines (Ethik Kommission der Bayerischen Landesärztekammer – # 11101, January 2012).

The APAP content of these samples was previously measured by immunoassay in the context of a round robin trial. The values obtained with our method were compared to the overall result of the round robin trial as shown in Figure 4. Regression analysis with Passing/Bablok showed the following coefficients: slope, 1.0217 and intercept, 0.1350. This data shows good correlation of the result for APAP with both methods. The content of metabolites and structure-related compounds was also determined in the patient samples with LC-MS/MS. No APAP structure-related substance capable of biasing the result of the immunoassay was detected. However, high concentrations of metabolites were found in some patient samples (Table 8). The high NAC concentrations found in these samples indicate that the patients had already been treated with the antidote. These results also illustrate that APAP is quickly metabolized and that the ability to quantify the metabolites is potentially of much higher importance than simply measuring the parent drug in cases of drug overdose.

Conclusions

An LC-MS/MS assay for quantifying APAP in human serum was developed in this study. The metabolites of the parent drug, a commonly used antidote and four other substances structurally related to the target analyte were also quantified with the same method. The samples were reduced using TCEP and filtered by ultracentrifugation with a cut-off membrane before chromatographic separation and quantification by LC-MS/MS using deuterated standards. The method was fully validated and exhibited good precision with intra- and inter-day precisions between 1% and 7% (exception: NAC with values between 3% and 11%). The linearity of the assay was demonstrated over the calibration range of all analytes, with coefficients of regression > 0.99. LLOQ of APAP was 20 ng/ml. For all other substances, LLOQ varied between 0.5 ng/ml and 50 ng/ml.

Table 8. Concentrations of all investigated analytes in patient serum samples

Conc. [µg/ml]	#1	#2	#3	#4	#5	#6	#7	#8	#9
APAP	37	23	23	40	63	45	12	75	17
APG	n.d.	76	> 100	> 100	> 100	28	47	> 100	66
APS	47	23	41	50	36	20	11	90	37
APM	0.4	0.3	0.4	0.3	0.2	0.2	0.9	0.3	1.2
APC	1.6	1.4	2.0	1.6	0.8	1.2	13	1.8	3.2
NAC	>100	82	100	>100	>100	n.d.	n.d.	>100	>100
p-Phenetidine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenacetin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Imipramin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Amitriptylin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not detected

Although the calibrators were serum-based, this method also allowed quantification of the analytes in plasma samples (collected with EDTA or lithium heparin tubes).

To our knowledge, no other method has been reported that allows this combination of substances to be determined simultaneously. The method is a useful tool to help diagnose and confirm APAP overdoses. Its potential has been demonstrated during the analysis of 77 samples. It showed good correlation to the average results of a round robin trial based on immunoassay-analysis.

The main purpose of this method is the future use as reference method for an immunoassay-based APAP-test, which can be biased by metabolites of the target analytes or other therapeutic drugs with similar structures and therefore generate false-positive results.

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Publication 3

Magnetic beads as an extraction medium for simultaneous quantification of acetaminophen and structurally related compounds in human serum

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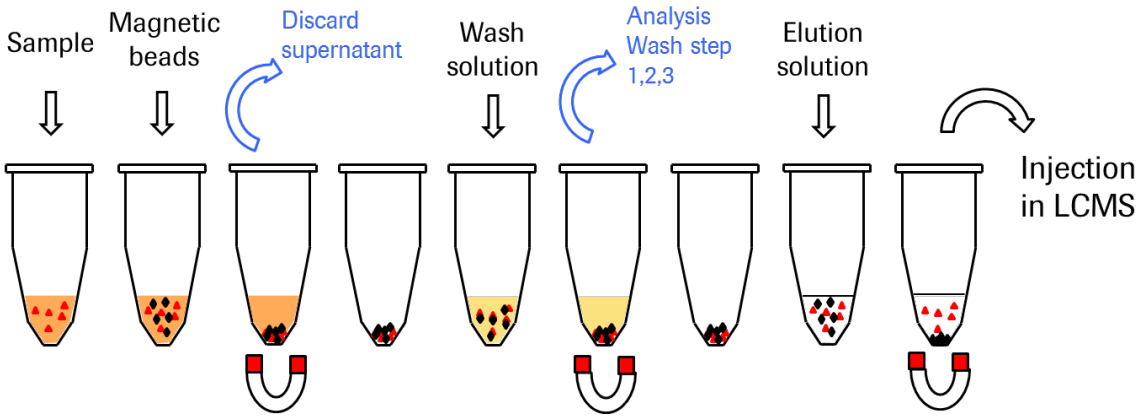

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Magnetic beads as an extraction medium for simultaneous quantification of acetaminophen and structurally related compounds in human serum

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This paper describes a sample preparation method that complements a previously published liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for acetaminophen and eight structurally-related compounds in human serum (C. Bylda, R. Thiele, U. Kobold, D.A. Volmer. *Drug Test. Anal.* 2014, *6*, 451). The analytes (acetaminophen [APAP] + metabolites acetaminophen-glucuronide [APG], -cysteine [APC], -mercapturate [APM] and -cysteine [APC], structurally similar analogues phenacetin and p-phenetidine, as well as tricyclic antidepressants imipramine and amitryptiline) were extracted from serum using magnetized hyper-crosslinked polystyrene particles. The sample preparation protocol was developed by means of a design of experiments (DoE) statistical approach. Using three representative compounds from the analyte panel with different polarities (high, medium, and low), two screening designs were used to identify factors that exhibited significant impact on recovery of the analytes. These parameters were then optimized to permit extraction of the complete target panel exhibiting a broad range of chemical polarities. Liquid chromatographic separations were achieved by gradient elution using a pentafluorphenyl column with subsequent detection by electrospray ionization-triple quadrupole mass spectrometry in multiple reaction monitoring (MRM) mode. The method was linear over the range 0.1–100 μg/mL for APAP, APG, p-phenetidine and phenacetin, 0.03–50 μg/mL for APS, and 0.01–10 μg/mL for APM, APC, imipramine and amitriptyline, with $R^2 > 0.99$. The assay exhibited good precision with CVs ranging from 2 to 9% for all analytes; the accuracy was assessed by comparing two LC-MS/MS methods using a set of 68 patient samples. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords: acetaminophen; magnetic beads; LC-MS/MS; design of experiments

Introduction

The pharmaceutical industry is continuously seeking to improve its analytical assays, generally to perform analyses that are faster, cheaper, and more reliable. Efforts at automating liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays are often aimed at improving the sample preparation step. Magnetic particles have recently attracted considerable interest from analytical scientists, because they offer the unique ability of rapid and specific sample extraction and clean-up that can be readily automated.[1,2] These particles consist of a magnetic core, which is coated with a polymer material that can be derivatized using a wide range of functional groups. Several types have been described in the literature; some with porous structure to specifically retain target analytes, for example restricted access materials (RAM)[3,4] or molecular imprinted polymers (MIP)[5,6]; others with very specific functional groups (e.g. immunosorbents),[7] or unspecific materials such as non-polar coatings (C8, C18,…),[8–11] ion exchange groups,[12–14] or moieties that provide aromatic interactions. The extraction procedure is similar to solid-phase extraction (SPE); i.e., loading, washing, and elution, with the added advantage that the suspended magnetic beads can be handled as a liquid. These materials also provide very high extraction efficiencies because of their large specific surface area per weight compared to packed SPE materials. Possible applications of these sample preparation approaches include drugs of abuse testing (DAT) or therapeutic drug monitoring (TDM). Magnetic particles have frequently been used for extraction and pre-concentration of drugs in complex biological fluids,[2,9,11,12,15,16] mostly for extraction of highly hydrophobic analytes using reversed-phase materials.[2,9,12,17–19] Only a few studies have reported on the extraction of strongly polar analytes from biological fluids. Extraction of polar analytes is generally more challenging than extraction of non-polar substances. Wang *et al.* proposed the use of restricted access materials (RAM) for extraction of polar therapeutic drugs from urine.[3] Efficient extraction was shown but the approach required long incubation and elution times because of slow diffusion of analytes in the pores of the particles. Other groups have applied functionalized magnetic beads to precipitate and remove the matrix components (proteins, lipids, etc.) from the sample, leaving a clean extract.[17] This approach is

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faster because only a single pipetting step is needed. It has shown good results for non-polar targets and should also be efficient for hydrophilic targets. One major disadvantage is the required dilution of samples during the workflow to support protein precipitation in serum. Because of the organic solvent in the resulting extract, samples usually have to be further diluted prior to LC-MS analysis. Strong hydrophilic compounds can also be captured by implementing extraction materials of high specificity; for example MIP[5,20–23] or immunospecific sorbents.[7] While these materials give assays with high sensitivity, the materials are difficult to synthesize and can give template bleeding (MIP).

In this study, magnetic beads were applied to the quantitative analysis of acetaminophen (APAP), which is a widely used over-the-counter drug that can have severe toxic side effects in case of an overdose or long-term abuse.[24] We have recently presented a LC-MS/MS assay for simultaneous quantification of APAP and other pharmaceutically relevant substances in human serum and plasma.[25] That assay was developed for a group of analytes with broad range of polarities, namely APAP, its metabolites acetaminophen-glucuronide (APG), -sulfate (APS), -cysteine (APC) and -mercapturate (APM) as well as phenacetin, *p*-phenetidine, imipramine and amitriptyline. The present study extends the previous work; it describes the application of magnetic hyper-crosslinked polystyrene (PS) particles to simultaneous extraction of the above target substances, with the future potential for full automation of the process. For the method development work, all instrumental parameters that had the potential to influence the extraction process were carefully optimized. Conventional optimization procedures investigate parameters such as temperature, pH, buffer *etc.* one by one; that is, one parameter is systematically varied, while all others are kept constant. That procedure is time-consuming and, importantly, second order interactions are sometimes overlooked and the optimum extraction efficiency not always found. By using a software-controlled statistical approach in this work instead, *viz.*, design of experiments (DoE),[26] the influence of all parameters (including their second order interactions) were simultaneously investigated, resulting in a considerably reduced number of experiments and the possibility to identify significant outliers of the measurements.

Experimental

Reagents and chemicals

The USP reference standard for APAP was purchased from LGC Standards (Wesel, Germany). The metabolites 4-acetamidophenyl β-D-glucuronide sodium salt (APG), 4-acetaminophen sulfate potassium salt (APS), 3-(N-acetyl-L-cystein-S-yl) acetaminophen sodium salt (APM) and 3-cysteinylacetaminophen trifluoroacetic acid salt (APC) as well as N-acetyl-L-cysteine, p-phenetidine and phenacetin were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Imipramine (1 mg/mL in methanol) was from Cerilliant (Wesel, Germany). Amitriptyline was from Sigma-Aldrich (Steinheim, Germany). Deuterated analogues of the analytes were used as internal standards. Imipramine-d3 was obtained from Cerilliant and amitriptyline:HCl (N,N-dimethyl-d6) from Cambridge Isotope Laboratories (Saarbrücken, Germany) at 100 µg/mL in methanol. The deuterated substances APAP-d3, 4-acetamidophenyl β-D-glucuronide-d3 sodium salt (APG-d3), 4-acetaminophen-d3 sulfate (APS-d3), 3-(N-acetyl-L-cystein-S-yl) acetaminophen sodium salt-d5 (APM-d5), 3-cysteinylacetaminophen-d5 trifluoroacetic acid salt (APC-d5), p-phenetidine-d5 hydrochloride, phenacetin-d5 were from Toronto Research Chemicals. LC-MS grade solvents were from Biosolve (Valkenvaard, the Netherlands). Formic acid (FA) (98 100%) and Sodium phosphate dihydrate were purchased from Merck (Darmstadt, Germany) and tris(2-carboxyethyl)phosphine (TCEP) from Sigma-Aldrich. Human serum pools from Tennessee Blood Services (Memphis, TN, USA) were used as negative calibrator matrix. Water was generated using a Waters Millipore Milli-Q-Plus purification system (Eschborn, Germany).

Extraction beads

Melamine resin particles (MF, diameter 2.15 µm) were purchased from Microparticles GmbH (Berlin, Germany) and native carboxylic acid coated particles (Dynabeads M-270, diameter 2.8 µm) were from Life Technologies (Thermo Fischer, Bremen, Germany); the derivatization of the surface with alkylchains was performed at Roche Diagnostics GmbH (Penzberg, Germany). Polystyrene micro-particles (diameter ~4 µm) were synthetized by Roche Diagnostics GmbH. C_8/C_{18} coated magnetic microparticles were from Agilent Technologies (Böblingen, Germany). The smaller hyper-crosslinked polystyrene (hcPS) beads exhibited a diameter range from 25 to 40 µm, the larger beads were Dowex Optipore SD2 from Dow Chemical Company (Amsterdam, the Netherlands) with a diameter distribution from 160 µm to 1 mm.

Sample preparation

A graphic scheme of the sample preparation steps is shown in Figure 1. For each sample, 25 µL of serum were mixed with 25 µL of internal standard solution and 400 µL of water. After mixing the samples for at least 20 min using a thermomixer, 50 µL of TCEP were added to the mixture and samples mixed for another 30 min at 37°C. Then, 25 mg of dry magnetic beads were added to each sample. The beads were allowed to interact with the samples for 15 min under gentle rolling conditions, so that the analytes could access the entire surface of the particles. The supernatants were then discarded and the magnetic beads washed with 500 µL of water. The washing solutions were also discarded before elution with 120 µL of acetonitrile/water 70:30 *v/v*. From the eluate, 100 µL were then withdrawn from the vials and diluted 1:3 with water, before injection into the LC-MS/MS system.

Instrumentation

The chromatographic system consisted of Dionex (Germering, Germany) U3000 binary pump, column oven and autosampler. Chromatographic separation was achieved on a Phenomenex (Aschaffenburg, Germany) Kinetex PFP column (2.6 µm, 150 x 3.0 mm, 100 Å). Gradient elution at a flow rate of 300 µL/min was performed (Table 1). The mobile phase was water+0.1% formic acid (eluent A), water/ACN 50:50 v/v +0.1% FA (eluent B) and acetonitrile +0.1% formic acid (eluent C). The total run time of analysis was 19 min. The separation column was heated to 40°C during separation. Two mass spectrometers were used for the described work. Preliminary experiments were carried out on a Thermo Fisher Scientific (Bremen, Germany) LTQ ion trap equipped with an electrospray ionization source (ESI). The final quantification method was then transferred to a TSQ Vantage triple quadrupole mass spectrometer (QqQ) equipped with a heated electrospray ionization source (HESI). Analyses were performed in positive ionization mode. The ion source parameters were set to the following values: sprayer

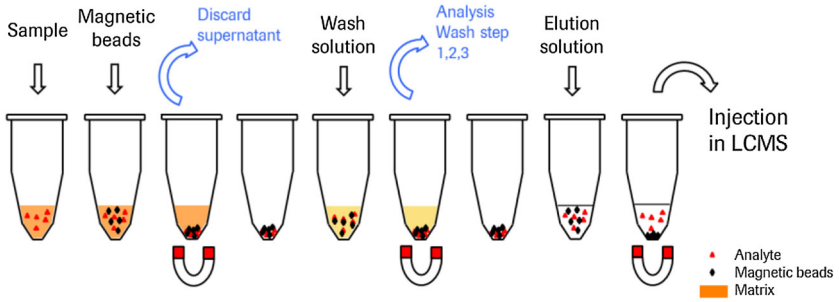

Figure 1. Sample preparation steps using magnetic particles.

Table 1. Eluent gradient composition

Time [min]	% Eluent B	% Eluent C
0	0	0
6	50	0
13	0	100
16	0	100
16.1	0	0
19	0	0

voltage: 4500 V (TSQ)/4000 V (LTQ), capillary temperature: 250°C (TSQ)/350°C (LTQ), vaporizer temperature: 350°C (TSQ)/source heater temperature: 150°C (LTQ), auxiliary gas: 10 psi (TSQ)/8 psi (LTQ), sheath gas 30 psi (TSQ)/28 psi (LTQ). Nitrogen was used as sheath and auxiliary gas. The argon pressure in the collision cell was 1.0 mTorr. MRM transitions and optimized collision energies (CE) for each transition are summarized in Table 2.

Performance data

The analytical performance of the method was investigated by determining precision and accuracy, as well as linearity, recovery and specificity. The regression model used was linear with $1/X$ weighting. Linearity was ensured for the analytes with a regression parameter $R^2 > 0.99$. Precision was determined using three spiked serum samples with concentrations ranging from 1.5 to 25 µg/mL, depending on the analyte. Each sample was processed and analyzed six times; the coefficient of variation (CV) was calculated for the mean of all six preparations. To assess the accuracy of the method, a set of patient samples was measured and compared to the results obtained with a previously described and fully validated LC-MS method (see below). Recovery for all analytes was determined by comparison with peak areas of corresponding deuterated internal standards in duplicate from eight calibrator samples. The mean over the 16 values was calculated for each fraction of the workflow: wash steps 1, 2 and 3, and eluate. The recovery was calculated by comparing the amount of substance found in each fraction to the sum of all fractions. Specificity was verified by analyzing two different pools of analyte-free serum samples purchased from Tennessee Blood Services. Additionally, ion suppression from glycerophosphocholines (GPCho) was assessed by monitoring common fragments to all GPChos in SIM mode over the entire chromatographic run. By applying a high declustering voltage (40 V) in the ionization source of the mass spectrometer, GPChos readily undergo in-source collision-induced dissociation (CID) and form the product ion trimethylammonium-ethylphosphate (*m/z* 184) that can further dissociate to trimethylammoniumethylate (*m/z* 104).[27] Stability of the samples had already been assessed in our previous work;[25] these experiments were therefore not repeated.

Quantification

Calibrators were prepared in human serum (Tennessee Blood Services). Each batch was tested before preparing calibrators, to ensure that it contained no traces of target analytes. An aqueous stock solution of all analytes was diluted in serum to obtain 8 calibrator levels. The calibration ranges were different for all analytes. APAP, APG, p-phenetidine and phenacetin were measured between 0.1

Table 2. MRM transitions and applied collision energies for all investigated substances and their corresponding internal standards. Values correspond to settings for QqQ instrument (parameters shown in italics were used for ion trap experiments)

Analyte	MRM transition	CE [V]	ISTD	MRM transition	CE [V]
APAP	*m/z* 152.1 ➔110.0, 65.0	25, 15	APAP-d3	*m/z* 155.1 ➔111.1, 65.0	25
	m/z 152 ➔ 110	*28*		*m/z 155 ➔ 111*	*28*
APG	*m/z* 328.1 ➔ 151.9	18	APG-d3	*m/z* 331.0 ➔ 154.8	18
	m/z 328 ➔ 152	*28*		*m/z 331 ➔ 155*	*27*
APS	*m/z* 231.9 ➔151.9, 109.9	18	APS-d3	*m/z* 234.9 ➔154.9, 110.9	18
APM	*m/z* 313.1 ➔207.9,165.8	20	APM-d5	*m/z* 318.1 ➔212.0, 167.8	20
APC	*m/z* 271.0 ➔182.0, 140.0	18	APC-d5	*m/z* 276.1 ➔186.7, 142.9	18
p-Phenetidine	*m/z* 138.1 ➔110.0, 93.0	20	p-Phenetidine-d5	*m/z* 143.1 ➔111.0, 93.0	18
Phenacetin	*m/z* 180.0 ➔138.1, 110.0	22	Phenacetin-d5	*m/z* 185.1 ➔143.1, 111.0	18
Imipramine	*m/z* 281.2 ➔ 86.0, 58.0	25	Imipramine-d3	*m/z* 284.2 ➔89.1, 61.1	25
Amitriptyline	*m/z* 278.2 ➔233.1, 90.9	20	Amitriptyline-d6	*m/z* 284.3 ➔233.1, 91.0	25
	m/z 278 ➔ 233	*28*		*m/z 284 ➔ 233*	*28*

and 100 μg/mL, APS between 0.03 and 50 μg/mL and APM, APC, imipramine and amitriptyline between 0.01 and 10 μg/mL. Internal standard concentrations are summarized in Table 3. All compounds were quantified by LC-MS/MS using multiple reaction monitoring (MRM). Calibration curves were constructed using analyte/internal standard peak area ratios. Unknown samples were quantified by comparing the ratio of the integrated area of the analyte and the corresponding internal standard to the calibration curve.

Accuracy

To evaluate performance and accuracy of the new extraction method, 68 patient samples were analyzed and values compared to results obtained for the same samples using a previously established LC-MS method,[25] which also lists information on ethics approval of the study. This experiment was conducted for the main target APAP as well as for the metabolites APG, APS, APC, and APM. Regression was performed using Passing-Bablok analysis.

Statistical analyses

Statistical analysis was carried out using the software JMP (SAS, Böblingen, Germany). For DoE, the user can chose screening or response surface designs, resembling qualitative or quantitative analyses, respectively. Screening designs are used to investigate a large number of experimental parameters using a minimized number of runs, providing information on the influence of each factor as well as some second-order factor interactions. Response surface designs aim at the optimization of the value of defined experimental parameters. These experimental parameters are described as factors in the DoE process that are divided into three categories. Firstly, continuous factors can take any value in a given interval; they are represented by continuous numbers (*e.g.* temperature, pH, volume…). Secondly, discrete factors only have particular values, but are not necessarily numeric (*e.g.* number of wash steps, type of magnetic particle…), and finally, Boolean factors are discrete factors that exhibit only 2 levels (yes or no). After the nature of the response was chosen (*e.g.* peak area of analyte in eluate fraction of the extraction procedure), a number of factors suspected of having an influence on this response were listed and their effect studied between two user-defined limits. The software generated a number of experiments that were conducted in a randomized order to avoid systematic errors. Analysis of results was then performed; a general regression model set up and optimized to fit the experimental data. Outliers were identified by the software and marked, to avoid misinterpretation of the data.

Table 3. Concentration of internal standard in samples

ISTD	Conc. [μg/mL]	Used for
APAP-d3	1.03	APAP
APG-d3	0.85	APG
APS-d3	1.57	APS
APM-d5	9.06	APM
APC-d5	6.55	APC
Phenacetin-d5	7.53	Phenacetin
p-Phenetidin-d5	7.67	p-Phenetidin
Imipramin-d3	7.53	Imipramin
Amitriptylin-d6	7.67	Amitryptilin

Results and discussion

Prior to the method optimization experiments, all parameters known or suspected to influence the extraction process were listed in the fishbone diagram shown in Figure 2. For the DoE software (see Experimental), two different screening designs were created. While this approach substantially reduced the number of required experiments, it did not jeopardize any analytical input information needed for determining the significance of the factors and their interactions. DoE approaches have been successfully applied in assay development.[28–30] Chromatographic separation of the analyte panel was performed as previously described.[25]

Development and optimization of the method

Screening design 1

For the first screening design, several types of magnetic beads were evaluated for extraction of three of the target analytes: APAP, APG, and amitriptyline. Since the main difficulty of the extraction procedure was simultaneous extraction of analytes with a wide range of polarities, these three analytes were chosen because they span the entire range of polarities within the target analyte panel: APG is the most polar compound; amitriptyline is non-polar and APAP is a 'medium' polarity analyte. Several types of bead chemistries were tested, including C_8/C_{18} saturated carbon chains for hydrophobic interactions, ion exchangers (carboxylic acid as cation and melamine as anion exchanger), polystyrene as well as 25-40 μm diameter hyper-crosslinked polystyrene for π-π interactions. Depending on the chemical nature of the extraction material, several factors that potentially influence adsorption and desorption of the target analytes such as pH, temperature, type of organic solvent *etc.* were tested in this design, which are marked in blue in the fishbone diagram in Figure 2. Particle (C_8/C_{18}, MF, hcPS or PS) and eluent type (acetonitrile, methanol or iso-propanol) were set as discrete factors. All other tested factors were continuous. Incubation pH was varied between 5 and 9 and incubation temperature was set to 4, 20, and 37°C, incubation time ranged from 5 to 55 min. The elution pH was also tested between 5 and 9 (depending on the pK_a of the surface groups on the beads), elution volume was varied from 30 to 90 μL, and the percentage of organic solvent in the elution solution was investigated between 20 and 80%. All other factors were kept constant. Ninety experiments were necessary for this screening design. The response was defined as the presence of a peak in the resulting chromatogram. Aqueous solutions of the target analytes at a concentration of 100 μg/mL were used. Five mg of particles suspended in 100 μL of 0.1 M phosphate buffer at pH 7 were added to 10 μL of sample. Different times and temperatures were tested for the incubation step of analyte and particles. Following incubation, the particles were washed twice with 200 μL buffer. The wash solutions were discarded. Eluates were collected after 10 min of incubation with the particles and measured *via* LC-MS/MS.

Screening design 2

The second screening design was aimed at the investigation of effects of polarity of the magnetic bead surface on extraction of polar substances. For this purpose, micro-particles carrying carboxylic acid groups were modified with different amounts of aliphatic C_8 chains. They were used to test the efficacy of extracting the three model target substances. Four different batches were compared: the original unmodified beads (D-0x), and batches modified with 25-fold (D-25x), 100-fold (D-100x), and 300-fold (D-300x) excess of

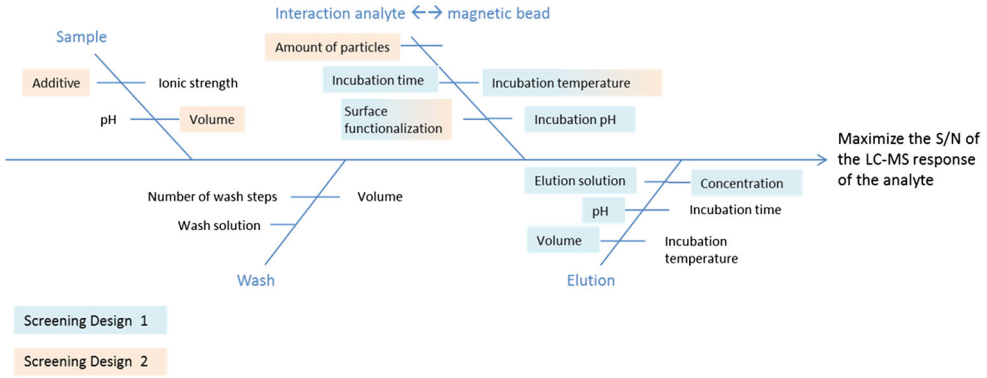

Figure 2. Fishbone diagram representing the investigated parameters related to the sample preparation protocol.

octyl amine. Additionally, other factors were included in the second screening design; they are marked in orange in Figure 2. They include particle type (discrete factor) and type of additive used prior to sample extraction and heating of the sample during incubation. The additive was defined as a continuous factor, where pure ethanol and *t*-butanol defined the limiting points and ethanol:*t*-butanol at 1:1 ratio served as center point. The response was defined as the recovery of the target substance in the elution fraction. The test system consisted of aqueous and serum samples containing approx. 12 μg/mL of the three target substances. Heating the samples to 60°C was also set as a continuous factor and was performed for 0, 10, and 20 min. The percentage of additive (water, ethanol or *t*-butanol) was tested at 0, 30, and 60% and the amount of particles added to the samples was 1, 1.5, and 2 *w/v*, corresponding to 0.5, 0.75, and 1.0 mg dry particles, respectively. Twenty microliters of aqueous solutions containing all three analytes at a concentration of approx. 10 μg/mL were used for each extraction.

Result screening design 1

The measurements for screening design 1 were carried out using an ion trap MS and statistically analyzed using DoE software (see Experimental). The following parameters exhibited a significant influence on the target response: particle type, type of organic eluent and percentage of organic solvent in the elution solution. Other factors did not influence the extraction efficiency of the target analytes significantly. Acetonitrile showed higher recoveries than methanol and iso-propanol, and a minimum of 65% acetonitrile was needed for elution of the target substances. Since the incubation pH was not a significant factor, the samples were diluted with water instead of buffer in subsequent experiments. Incubating for longer than 5 min did not show any further improvements of sensitivity. The observations regarding pH and equilibration time differed from previous results for extraction of highly hydrophobic compounds (antidepressants, phenolic compounds) using magnetic particles coated with ionic surfactants,[12,18,19] where pH and equilibration times exhibited significant influence on sample preparation. That influence was probably due to variations of adsorption of the highly hydrophobic drugs on the rather labile surface of hemimicelles formed by ionic surfactants as compared to the static and stable surface of the polystyrene particles used here. A further important observation from this screening design was that APG was very difficult to extract using functionalized magnetic beads as compared to the other target analytes. The recoveries in the eluate fractions for this substance were virtually zero in almost all preparations, except when using hyper-crosslinked polystyrene (hcPS) particles. Extraction of amitriptyline and acetaminophen could be achieved with hydrophobic particles, anion exchangers and hyper-crosslinked polystyrene particles.

Result screening design 2

The beads tested in the second screening design were derivatized with octyl chains to obtain mixed-mode extraction surfaces. The polarity of the four batches of magnetic beads D-0x, D-25x, D-100x, and D-300x decreased with increasing number of octyl chains bound to the surface. It was assumed that the combination of polar groups and non-polar alkyl chains would allow extraction of both hydrophilic and hydrophobic target analytes. Unfortunately, the representative substance for polar analytes, APG, was not extracted with any of the tested particles, under none of the tested conditions. In fact, the type of particle and the amount of particles per sample were marked as non-significant by the DoE software. The extraction efficiency of the original particles for APAP and amitriptyline was similar to the derivatized versions but the obtained peak areas for APAP were all very small, making these magnetic beads not suitable for the application. The solvents added to the samples prior to the extraction were assumed to favor the adsorption of polar analytes to the surface of the beads by decreasing the polarity of the liquid phase. Instead, a significant decrease in intensity of the target response was observed for the medium and non-polar analytes APAP and amitriptyline. The high percentage of organic solvent appeared to inhibit the adsorption of hydrophobic analytes to the magnetic beads without showing any improvement for the extraction of hydrophobic targets. Heating to 60°C during incubation with the particles also showed a negative effect on the recovery of the particles.

The two described screening designs allowed us to identify all relevant factors that exhibited strong influence on the outcome of the new sample preparation method. Unfortunately, the simultaneous extraction of the three target substances was difficult with most materials and only one of the eight tested particle types allowed simultaneous extraction of APAP, APG, and amitriptyline. The following experiments were therefore limited to hyper-crosslinked polystyrene particles. Factors that showed a significant effect were the type and percentage of organic eluent for elution, type and percentage of additive to samples prior to the extraction, as well as temperature for equilibration of the samples with the beads. Non-significant parameters for this sample preparation were sample pH, equilibration time with the particles, eluent volume and pH, elution time and degree of derivatization in case of modified Dynabeads particles. An amount of 0.5 mg of particles was

sufficient for extraction of the target substances. This amount depends on the size of the beads and has to be adjusted when larger particles are used.

Additional experiments were carried out to optimize the sensitivity of the method, without experimental design considerations. The relevance of the size of the particles was investigated by comparing hcPS particles (diameter 25-40 μm, non-magnetic) with larger beads (Optipore SD 2, diameter distribution 0.1-1 mm, magnetic). Considering the largest particles of each batch, the differences in diameters between both types corresponded to a factor of 500. Assuming that both types of beads have the same density, the specific surface area available per mg dry particles then decreased by a factor of almost 20. Since 0.5 mg small hcPS particles were sufficient for extraction of 20 μL of sample, this meant that at least 10 mg of the larger particles were needed for extraction of the same amount of compound. Considering that the larger particles (Optipore) have a higher density because of their magnetic core, the amount was further increased to 25 mg for extraction of each sample, to insure that enough binding sites are available for all target substances. In order to determine the absolute recovery of sample preparation with both bead sizes, a spiked solution containing 20 ng of analyte was processed with both particle types, using the optimized protocol obtained after both screening designs. Additionally, an external calibration curve was performed with aqueous solutions containing all three test analytes. No internal standard was used for these experiments. The recorded peak areas in the eluate fraction were compared to the calibration curve to determine the amount of target substance. The obtained value was divided by the initially spiked concentration to obtain the absolute recovery of the sample preparation step. The smaller beads yielded a recovery of 63% for APAP, >100% for APG and 25% for amitriptyline; the larger particles showed recoveries of 53% for APAP, 100% for APG and 49% for amitriptyline. Since no internal standard was used for these measurements, the imprecision of the results was high, especially for APG, which showed smaller response surface areas than the other analytes. This effect readily explains the over-recovery of APG seen with the first set of particles. No significant increase of sensitivity was provided by using the larger particles, but since they showed a more uniform distribution of recoveries for the three types of polarities, they were chosen for the sample preparation protocol.

Transfer to complete panel of target substances

The next step was the transfer of the developed protocol to the entire panel of target substances. Serum samples containing all nine analytes were processed using the large hcPS particles. The optimized parameters from both screening designs were used in these experiments. The percentage of organic solvent in the elution step was determined by testing three water/ACN ratios. Results from the first screening design indicated that a minimum of 65% ACN was necessary for the complete elution of the target substances. The tested solutions were 60, 70, and 80% ACN in water. An increase in the signal intensities was observed between 60 and 70% ACN, but no clear benefit was seen for 80% ACN. Additionally, the peaks for the polar analytes eluting at the beginning of the chromatographic run showed significant tailing for 80% ACN during elution because retention on the column was strongly affected by the higher content of organic solvent. Larger dilution of the eluate prior to the injection would result in a loss of sensitivity; therefore, 70% ACN was chosen. The final protocol was transferred to the QqQ instrument for all further analyses. Two MRM transitions were implemented per analyte, because of the faster acquisition rates of QqQ *versus* ion trap. A volume of 25 μL serum sample as previously implemented[25] was first diluted with water and then reduced with 50 μL of a 350 mmol/L tris(2- carboxyethyl)phosphine (TCEP) solution under heating conditions at 37°C for 30 min, before adding 25 mg of dried magnetic particles. The mixture was allowed to equilibrate for 15 min. The washing step was performed with water before elution with 120 μL 70% ACN; 100 μL eluate was diluted 1:3 with water before injection into the LC-MS/MS system.

Reduction using TCEP was initially incorporated for detection of the antidote of acetaminophen, N-acetyl-L-cysteine (NAC). NAC strongly binds to serum proteins, by forming disulfide bonds that have to be reduced before the antidote can be detected. Good results for release of NAC from the serum proteins have previously been observed using TCEP;[25] therefore, this step was incorporated in the sample preparation protocol without further optimization.

NAC was very difficult to ionize using ESI, but showed slightly better efficiency in negative *versus* positive ionization mode. Initial experiments using the ion trap mass spectrometer did not provide sufficient sensitivity for quantification of NAC at the desired concentration levels, which did not allow us to verify whether the described sample preparation method can also be used for this substance. NAC was omitted from the following experiments.

Performance of the method

The performance of the new sample preparation method was assessed by determining the linearity, precision and recovery of the assay. Possible ion suppression effects from glycerophosphocholines (GPCho) were also investigated and compared to a clean-up procedure based on dilution and filtration of the samples. Sixty-eight native serum samples were used to carry out a method comparison with both sample preparation methods.

Linearity

To assess linearity, the regression coefficients were determined for all analytes in the concentration ranges described in the Experimental section. An eight-point calibration was carried out; the obtained regression equations as well as R^2 values are summarized in Table 4. APS exhibited the lowest value for R^2, which was due to the lower detection sensitivity for this analyte. APS is one of the most polar substances in the chosen analyte panel; therefore the affinity to the extraction phase was not as high as for non-polar substances. The peak areas were therefore much smaller and more prone to variations within the analytical system. Nevertheless, all values for R^2 were higher than our cut-off level set at 0.99. The method was therefore linear in the chose calibration ranges for all analyzed target substances.

Precision

Precision was determined using three spiked serum samples at three concentration levels. The concentrations of the analytes in each level as well as calculated CVs are indicated in Table 5. Each sample was processed and analyzed 6 times. Level 2 was analyzed in duplicate; both injections were averaged before calculation of the precision. Level 1 and 3 were only injected once into the LC-MS/MS system. The CVs were calculated for the mean of all 6 values.

The calculated CVs ranged from 2% to 9%. The highest imprecision was obtained for APG and APS, which was due to the low detection sensitivity. This was expected, since these are the two most polar substances of the analyte panel and the method exhibited decreased efficiency for increasing polarity. No useful data was obtained for APS for levels 2 and 3, because of a mishandling of

Table 4. Regression curves for the analytes and corresponding coefficients of regression (R^2)

Analyte	Regression curve	R^2
APAP	$y = 0.032x + 0.009$	0.9983
APG	$y = 0.0186x + 0.0016$	0.9965
APS	$y = 0.1808x - 0.0520$	0.9911
APC	$y = 0.6743x - 0.0045$	0.9970
APM	$y = 0.4963x + 0.0124$	0.9977
p-Phenetidin	$y = 0.09224x + 0.0010$	0.9968
Phenacetin	$y = 0.1453x + 0.0085$	0.9994
Imipramin	$y = 0.3511x - 0.0009$	0.9993
Amitriptylin	$y = 0.6417x + 0.0004$	0.9991

Table 5. Coefficients of variation calculated for 3 spiked serum samples at different concentrations with $n = 6$ preparations (except for imipramine and amitriptyline at Level 2)

Analyte	Level 1		Level 2		Level 3	
	[µg/mL]	CV	[µg/mL]	CV	[µg/mL]	CV
APAP	30	4%	17.4	2%	1.0	3%
APG	30	9%	17.2	2%	1.0	2%
APS	15	n.z.	8.1	7%	0.5	n.z.
APC	3.0	3%	1.7	3%	0.1	2%
APM	3.0	3%	1.7	3%	0.1	2%
p-Phenetidin	30	3%	17.4	5%	1.0	3%
Phenacetin	30	8%	16.8	2%	1.0	3%
Imipramin	3.0	6% (n = 5)	1.7	3%	0.1	4%
Amitriptylin	3.0	5% (n = 4)	1.7	2%	0.1	4%

the samples. Overall, the performance of the presented assay was comparable to other published methods for extraction of pharmaceutically relevant compounds from biofluids.[2,9,12,17–19]

Ion suppression

Compounds known to cause strong ion suppression in ESI-MS are glycerophosphocholines (GPCho).[31,32] Their influence was assessed by processing and injecting analyte-free serum into the LC-MS/MS system, as described in the Experimental section. Chromatograms for magnetic beads were compared to chromatograms obtained using the previously published LC-MS/MS method[25] (Figure 3). The profiles were very similar; the intensities for the two recorded *m/z* traces started to increase at ca. 12-13 min. The maximum intensity was reached at the end of the analysis, during the wash step of the gradient. Since the last analyte (amitriptyline) eluted from the column after 15 min, this experiment confirmed that the sample matrix did not interfere with the analysis. Comparison with the elution profile of the previous method also showed that extraction with magnetic particles did not improve on the elimination of phospholipids from the samples. Since no protein precipitation step was carried out prior to the extraction, GPChos were very abundant in the sample when the magnetic beads were added. They appear to have similar affinity to the extraction phase than the analytes, since they are highly recovered during the elution phase. Since GPChos were chromatographically separated from the target substances, their presence in the elution fraction did not pose a problem for this application.

Recovery

The recovery of the method was investigated by determining the distribution of the analytes over the entire workflow. For these experiments, three washing steps were carried out. The concentration of each target analyte was then determined in each of the four fractions: wash step 1, 2, and 3 and eluate. The supernatant of the first step could not be injected directly into the LC-MS/MS instrument, because of the high concentration of matrix components that would lead to a severe contamination of the ionization source. The results of this experiment are summarized in Table 6. The percentages shown correspond to the amount of substance found in each fraction compared to the sum of all fractions. This data shows clearly that only the first wash step had a significant influence on analyte recovery. APG, APS and APC were significantly represented in the first wash solution. Only 0-4% analyte were contained in the wash fractions 2 and 3. This confirms that one wash step was sufficient to remove components that were loosely (or not at all) bound to the magnetic beads. Another obvious effect was the clear relation between percentage of analyte found in the eluate fraction and its polarity. Non-polar analytes were more effectively retained than polar analytes. The relationship between log P values and amount found in the eluate fraction is shown in Table 6. The analytes are classified by decreasing polarity and their distribution in the four analyzed fractions is graphically displayed in Figure 4.

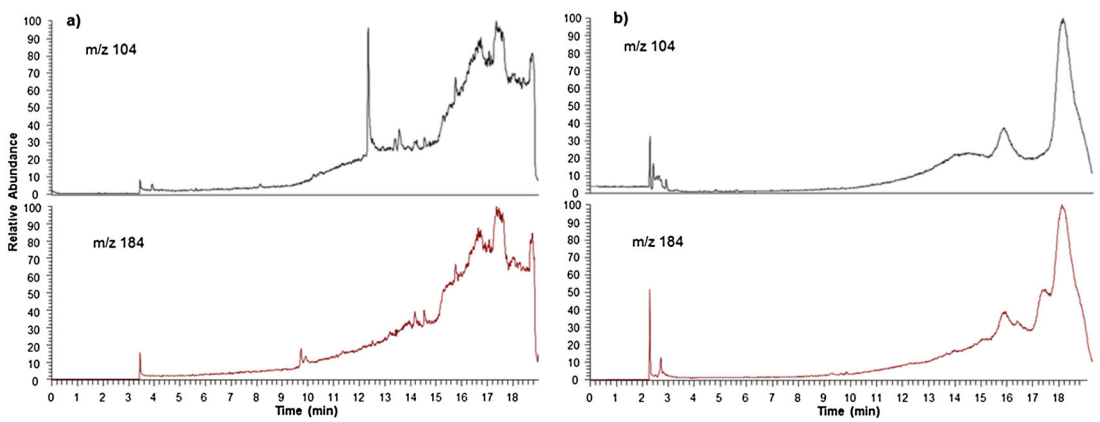

Figure 3. Extracted ion chromatograms using GPCho's in-source dissociation products (*m/z* 104 and 184) for two sample preparation methods: (a) dilution and filtration, and (b) extraction with magnetic beads.

Table 6. Recovery of the target compounds in each step of the workflow and log P values[a]

Analyte	Recovery				Log P
	Wash step 1	Wash step 2	Wash step 3	Eluate	
APAP	7%	3%	2%	87%	0.475 (±0.201)
APG	25%	4%	2%	68%	−1.811 (±0.434)
APS	28%	1%	0%	71%	−0.146 (±0.417)
APC	14%	7%	4%	75%	0.451 (±1.035)
APM	5%	0%	0%	95%	−0.575 (±1.043)
p-Phenetidine	6%	4%	4%	87%	1.445 (±0.219)
Phenacetin	2%	1%	0%	97%	1.655 (±0.222)
Imipramine	2%	0%	1%	97%	4.355 (±0.302)
Amitriptyline	1%	0%	1%	98%	4.41 (±0.266)

[a]Source: SciFinder, calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02.

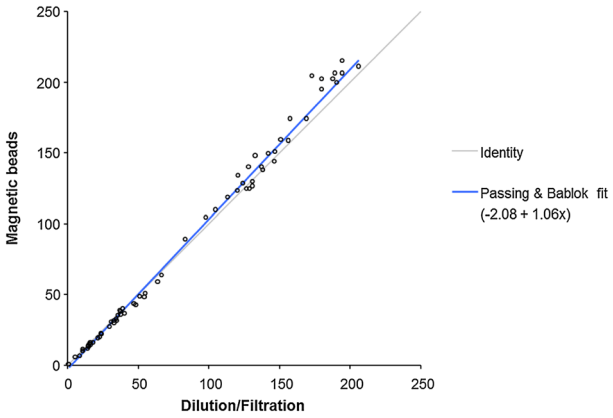

Figure 5. Comparison of the obtained values for APAP in 68 patient samples using the described sample preparation method in the present study and an established LC-MS/MS method.

Apart from APM, which behaved differently than expected from the log P value, the percentage found in the eluate fraction increased with increasing log P. Since a large error range is given for calculated log P value of APM, it is possible that this compound also fits the overall trend well.

Method accuracy, analysis of patient samples

The accuracy was evaluated by measuring 68 patient samples that had previously been analyzed using an established, fully validated LC-MS/MS method.[25] The obtained values for the main target APAP and for metabolites APG, APS, APM, and APC were compared. Unfortunately, no comparison was possible for the other analytes, since they are not metabolites of the main target but structurally similar components that could influence the result of the immunoassay. Their quantification in patient samples was therefore not seen as critical as the main target or its degradation products; no sample collection was performed for these compounds.

The result of the comparison for APAP is shown in Figure 5. The Passing-Bablok regression analysis gave very good correlation for both methods. Similar results were obtained for the metabolites; their correlation with the reference method for APAP is summarized in Table 7. Some samples exhibited metabolite concentrations below the LLOQ of the method; metabolite concentrations could therefore not be determined in all 68 patient samples. It was obvious from the data that the measured values correlated better for lower concentration values. This was due to the fact that samples outside the calibration range (>100 µg/mL) had to be diluted with analyte-free serum prior to sample clean-up. Since this step was not compensated by the internal standard, a higher imprecision was expected for samples with a higher concentration than the highest calibrator (>100 µg/mL for APAP). Nevertheless, the correlation between both methods was still excellent.

Table 7. Correlation of obtained results with magnetic particles sample preparation compared to the established reference method

Metabolite	Correlation to Ref.[25]	Sample number
APG	$y = 1.03x - 0.29$	60
APS	$y = 1.03x - 0.40$	59
APC	$y = 1.05x - 0.01$	61
APM	$y = 1.06x + 0.00$	61

To our knowledge, this work is the first to describe a generic method for simultaneous extraction of nine different target analytes from human serum using reversed-phase magnetic

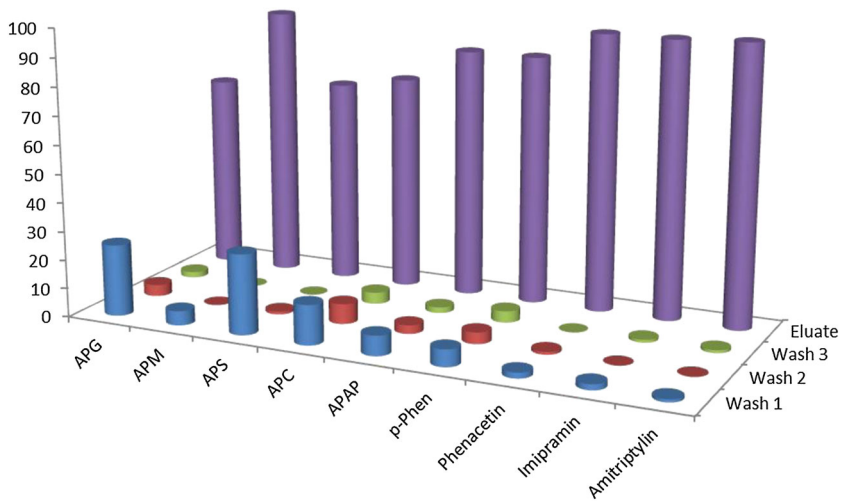

Figure 4. Graphic representation of the distribution of the target analytes in the analyzed fractions, in order of decreasing polarity.

particles. Because of the possibility for full automation, this method competes with online extraction methods such as RAM or Turboflow extraction.[33–39] The performance data shown here are generally comparable to Turboflow extractions; only few Turboflow assays have shown slightly better sensitivity[33] but required large volumes of organic solvent volumes and high eluent flow rates. Also, high carry-over effects were seen in those studies with little effect on phospholipid removal.[40,41]

Conclusion

The aim of the present work was the development of an improved sample preparation method for acetaminophen and its main metabolites, structurally related drugs, and the tricyclic antidepressants imipramine and amitriptyline in a complex biological matrix using magnetic particles. The experiments were planned using a design of experiment (DoE) statistical approach with three representatives analytes (APG, APAP, and amitriptyline), which covered the entire span of chemical polarities of the analyte panel. Generally, it was observed that extraction of the strongly polar analyte APG was more challenging than less polar analytes. Only one of the tested particle types allowed simultaneous extraction of the three model analytes and was used for analysis of the entire panel of targets substances. The new assay was compared to an established and validated LC-MS/MS reference method and showed very good correlation of the results for a collective of 68 native serum samples for APAP as well as its metabolites.

Overall, the extraction with magnetic beads resulted in a considerable decrease of time needed for sample preparation as compared to the dilution and filtration clean-up approach described previously. Particle suspensions can be handled as liquids, allowing full automation of the sample preparation process. Moreover, smaller sample volumes can be used as compared to *e.g.* solid-phase or liquid-liquid extraction, because of the high specific surface of the beads. The described method is fast and simple, and can be applied to simultaneous extraction of analytes with a wide range of chemical polarities, making the extension of the described assay to other target substances readily possible. Magnetic beads also permit enrichment of matrix components such as proteins and lipids; to remove them from the sample or for analysis. Chemical modifications of the surface of the magnetic beads allow them to be adapted to many applications including DAT/TDM analyses, proteomics, waste water analysis *etc*.

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Publication 4

Simultaneous quantification of digoxin, digitoxin and their metabolites in serum using high-performance liquid chromatography tandem mass spectrometry

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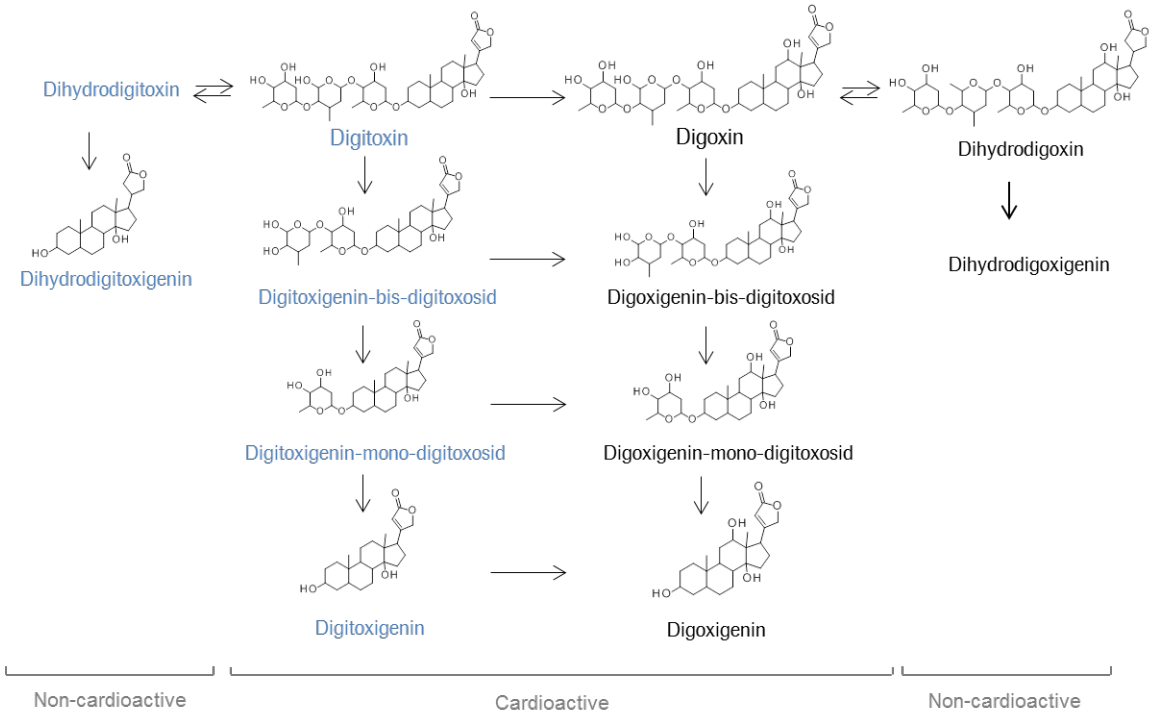

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Simultaneous quantification of digoxin, digitoxin, and their metabolites in serum using high performance liquid chromatography-tandem mass spectrometry

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The aim of this work was the development of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for simultaneous quantification of two cardiac glycosidic drugs in human serum, namely digoxin (DG) and digitoxin (DT), as well as several of their metabolites: digoxin-bis-digitoxose and digoxin-mono-digitoxose, digitoxin-bis-digitoxose and digitoxin-mono-digitoxose, digoxigenin, digitoxigenin, dihydrodigoxin and acetyl- and methyldigoxin. The assay also allowed for semi-quantitative analysis of two structurally similar compounds, deslanoside and lanatoside. As internal standards, deuterated analogues were synthesized for some of the target analytes. Highly abundant proteins were initially removed by protein precipitation using zinc sulfate before samples were extracted by supported liquid extraction. Chromatographic separation was achieved on a pentafluorphenyl stationary phase prior to electrospray ionization triple quadrupole mass spectrometry in multiple reaction monitoring mode. The assay allowed quantification of the analytes with lower limits of quantification between 0.04 and 2.0 ng/mL. Linearity was shown over the range 0.16–9.5 ng/mL for DG and its metabolites, and 1.6–95 ng/mL for DT and its metabolites with correlation coefficients $R>0.991$. The quantification range was determined as 1.1–8.9 ng/mL for DG and its metabolites and 12–90 ng/mL for DT and its metabolites. Within this range, DG and DT were determined with an accuracy of ±2% and precision <7% RSD. Trueness was confirmed by analyzing native samples and comparing the results to values obtained by a certified analysis laboratory. In conclusion, the assay represents a useful reference method for immunoassay-based digoxin and digitoxin tests, which are easily biased by the presence of metabolites of the target analytes or structurally similar substances.

Additional supporting info may be found in the online version of this article at the publisher's web site.

Keywords: digoxin; digitoxin; sample preparation; LC-MS/MS

Introduction

Digoxin (DG) and digitoxin (DT) are cardiac glycosides used for treatment of congestive heart failure and arrhythmias.[1,2] In addition, recent studies have shown promising effects of these drugs in the treatment of various cancers.[3–6] Since the therapeutic range of the substances is very narrow, intoxications from overdoses of these drugs are not uncommon.[7] Digoxin is the most frequently used cardiac glycoside;[8] digitoxin and other compounds such as methyldigoxin (MetDG), acetyldigoxin (AcDG), deslanoside or lanatoside C are also available for treatment. The absorption rate, pharmacokinetics and dosage of these drugs strongly differ from each other,[7] which makes it critically important to have reliable assays to monitor their levels.[9]

The metabolic degradations of digoxin and digitoxin are shown in Figure 1.[7] Even though their metabolisms appear to be similar, major differences with regards to toxicity have been observed between the two substances. These differences are related to the excretion route. After oral intake, digoxin undergoes extensive distribution in tissues and is mainly excreted as unchanged drug via the renal route;[10] small amounts of its cardioactive metabolites can also be found in urine. On the other hand, digitoxin is metabolized mainly in the liver rather than the kidneys as seen for digoxin. For this reason, digitoxin is preferably used in older patients or patients with renal failure.[11] Major differences are also observed for the binding affinity of digoxin and digitoxin to serum proteins,[12] ranging from 92–98% values for DT and its metabolites, but only 13–21% for DG and its metabolites. These numbers are important because tissue uptake is related to the free drug concentration, but not to the total drug concentration.[13] Digoxin is usually administered to generate serum concentration levels between 0.5 and 2 ng/mL,[14,15] digitoxin between 10 and 25 ng/mL.[16] Appropriate cut-off levels for digoxin and digitoxin monitoring assays are therefore at 2 and 25 ng/mL, respectively.

In addition to digoxin and digitoxin, several other cardiac glycosides are used for treatment. For example, methyldigoxin, which is eliminated via the renal route; 25% of the given dose is found unchanged in urine and 30% is metabolized to digoxin.[17] It is also found unchanged and demethylated in blood.[17] Lanatoside is also mainly metabolized via the renal route. It shows a poor absorption

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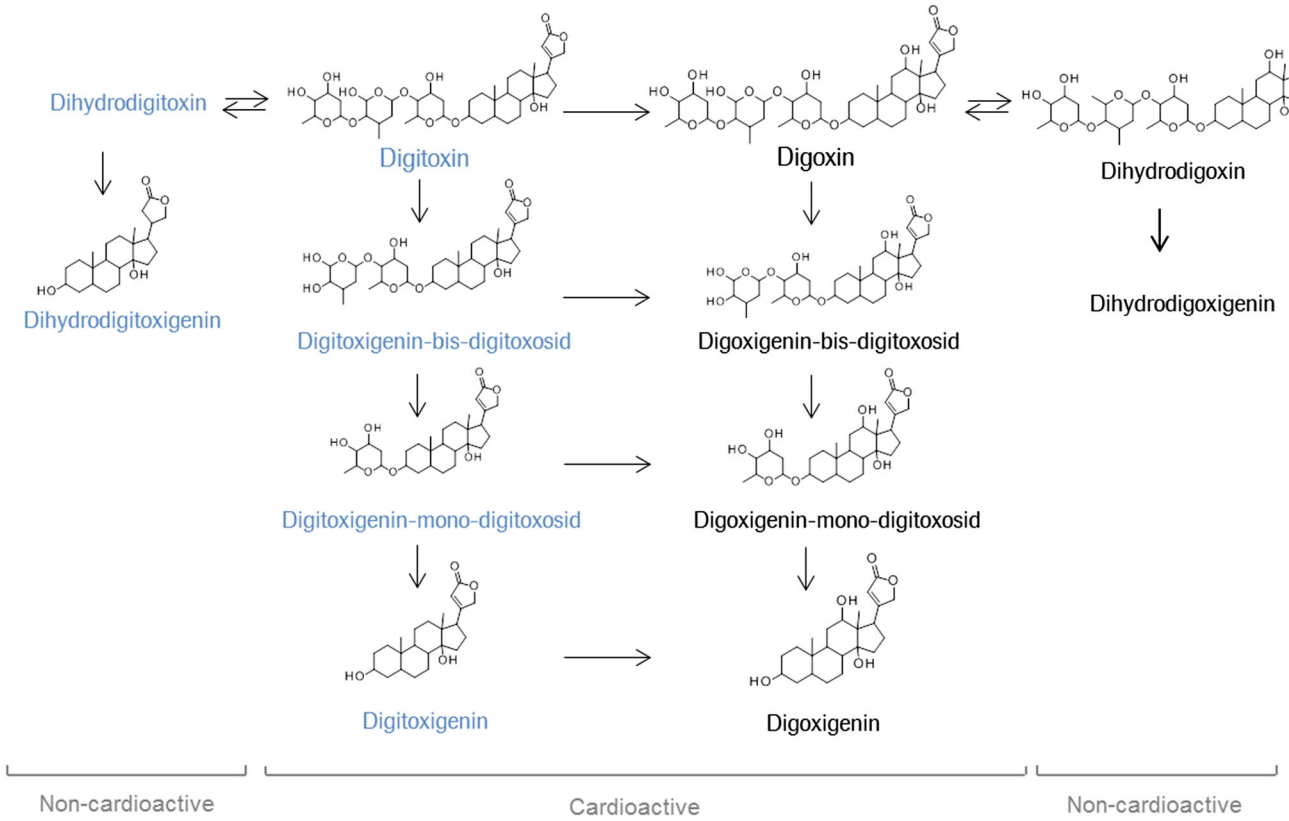

Figure 1. Metabolic pathway of digoxin and digitoxin.

rate when taken orally and is found as deslanoside (80%) and digoxin (17%) in urine.[7] Acetyldigoxin is used to enhance the resorption in the intestinal tract. After oral intake, it is quickly deacetylated and undergoes the same metabolism as digoxin.[18] In human blood, digoxin is found either after administration of the drug or as metabolite of deslanoside, digitoxin, acetyldigoxin, lanatoside C and methyldigoxin.[17,19]

The work presented here was aimed at the development of a sensitive assay for simultaneous quantification of digoxin, digitoxin, their metabolites digoxin-bis-digitoxose and digoxin-mono-digitoxose (DGbis and DGmono), digitoxin-bis-digitoxose and digitoxin-mono-digitoxose (DTbis and DTmono), digoxigenin (DGenin) and digitoxigenin (DTenin), dihydrodigoxin (H_2DG) and acetyldigoxin and methyldigoxin in human serum and plasma, to be used as a reference method for both a digoxin- and a digitoxin-specific immunoassay. Additionally, the method was also directed at semi-quantitative analysis of deslanoside and lanatoside. Several individual liquid chromatography-mass spectrometry (LC-MS) assays for digoxin and digitoxin[20–30] have been reported as well as in combination with their metabolites or related cardiac glycosides.[19,31–35] To our knowledge, no LC-MS/MS method has been described for simultaneous quantification of the above combination of drugs and their metabolites.

Experimental

Materials, reagents, and chemicals

Digoxin (DG), digitoxin (DT) and β-acetyldigoxin (AcDG) were purchased from European Pharmacopeia Reference Standards (Strasbourg, France). Metabolites digitoxin- (DTbis, DTmono) and digoxin-bis- and –mono-digitoxose (DGbis, DGmono), as well as digoxigenin (DGenin) and dihydrodigoxin (H_2DG) were old stocks from Boehringer Mannheim (Mannheim, Germany). Methyldigoxin (MetDG) was from Serva (Heidelberg, Germany); digitoxigenin (DTenin) and lanatoside from Sigma-Aldrich (Steinheim, Germany). Deslanoside was an USP Reference Standard. LC-MS grade solvents (methanol and acetonitrile) were purchased from Biosolve (Valkenvaard, the Netherlands). Dichloromethane (DCM), ethylacetate, lithium formate, methyl-tert-butylether (MTBE), 2-propanol (IPA), N,N-dimethylformamid (DMF), deuterium oxide and methanol-d_6 were from Sigma-Aldrich, chloroform (CHL) from Fisher Scientific (Schwerte, Germany), and triethylamine and formic acid from Merck (Darmstadt, Germany). Heptane and zinc sulfate were purchased from Fluka (Buchs, Switzerland). Human serum from Tennessee Blood Services (Memphis, TN, USA) was used as a negative calibrator matrix. Lyophilized serum samples from a round robin trial were obtained from the Referenzinstitut für Bioanalytik (RfB, Bonn, Germany) and used as quality control samples. Supported liquid extraction (SLE) cartridges Extrelut NT1 were from Merck (Darmstadt, Germany). Purified water was generated using a Waters Millipore Milli-Q-Plus purification system (Eschborn, Germany).

Synthesis of deuterated analogues

We adapted the deuteration protocol based on a procedure described in the literature.[21,36] Briefly, DG and its metabolites DGbis, DGmono and DGenin were deuterated simultaneously; DT, DTbis, DTmono and DTenin were labelled in a second batch. The substances were weighed in a reaction flask and dissolved in DMF. Four mL of DMF were used per 10 mg of substance.

Deuterium oxide (1.2 mL) and triethylamine (200 μL) were added and the mixture was allowed to react for 24 h at 70–80°C under N_2 atmosphere. The reaction mixture was then evaporated to dryness at 70°C under a stream of N_2. The dry residue was weighed, dissolved in deuterated methanol to give a concentration of ~1 mg/mL, and stored at 2–8°C. Characterization was performed by LC-MS and LC-MS/MS.

Sample preparation

A serum volume of 300 μL was used for each analysis. Eighty μL of a solution of the eight deuterated compounds were added to the samples and equilibrated at room temperature for 30 min while shaking on a thermomixer. Protein precipitation was then carried out using 700 μL of a $ZnSO_4$ solution (10% *w/v*). After mixing for 5 min, the samples were centrifuged for 15 min and supernatants extracted using SLE. After equilibrating the serum extracts on the SLE support for 10 min, 1 mL of dichloromethane (DCM)/isopropanol (IPA; 90:10 *v/v*; elution solvent 1) was added to the cartridges. After 10 min, elution was carried out using 3 mL of the elution solvent 1 and 3 mL of CHL/IPA 95:5 *v/v* (elution solvent 2). Both fractions were collected in the same vial. The combined eluates were then evaporated at 40°C under a gentle stream of N_2. The dry residues were reconstituted in 120 μL of methanol/water (30:70 *v/v*) and injected into the LC-MS system.

Quantification

Calibrators were prepared in human serum provided by Tennessee Blood Services (Memphis, TN, USA). Each lot was tested before preparing the calibrators, to ensure that no traces of the target analytes were detected. An aqueous stock solution containing all target substances was diluted in serum to obtain 8 calibrator levels. The calibration ranges were different for digoxin and digitoxin; DG, DGbis, DGmono, DGenin, H_2DG, AcDG and MetDG were measured between 0.2 and 8 ng/mL and DT, DTbis, DTmono, DTenin, deslanoside and lanatoside were measured between 2.0 and 80 ng/mL. The following deuterated substances were used as internal standards: DG-d_3 (1.0 ng/mL; used for DG, H_2DG, AcDG and MetDG), DGbis-d_3 (0.85 ng/mL), DGmono-d3 (0.85 ng/mL), DGenin-d_3 (1.6 ng/mL), DT-d_3 (9.1 ng/mL; used for DT, deslanoside and lanatoside), DTbis-d_3 (6.6 ng/mL), DTmono-d_3 (7.5 ng/mL) and DTenin-d_3 (7.7 ng/mL). All compounds were quantified by multiple reaction monitoring (MRM) except for DTenin, which was monitored in selected ion monitoring (SIM mode). Calibration curves were constructed using analyte/internal standard peak area ratios.

Instrumentation

The chromatographic system consisted of Dionex (Germering, Germany) U3000 binary pump, column oven and autosampler. The analytes were separated on a Phenomenex (Aschaffenburg, Germany) Kinetex PFP column (2.6 μm, 150×3.0 mm, 100 Å). The mobile phase was water/70 μmol/L lithium formate/0.1% formic acid (eluent A) and methanol/water (95:5 v/v)/70 μmol/L lithium formate/0.1% formic acid (eluent B). Gradient elution was performed at 300 μL/min and 40 °C. The gradient started with 50% B, increased to 80% B in 9 min and then to 100% B in 3 min. The final solvent composition was maintained for 2 min, before reconditioning the column at the initial solvent composition for 3 min; the total run time was 17 min. The mass spectrometer was a Thermo Scientific (Bremen, Germany) TSQ Vantage triple quadrupole equipped with a heated electrospray ionization source (HESI). The sprayer voltage was 3500 V; capillary temperature, 320 °C; vaporizer temperature, 350°C. Nitrogen was used as sheath gas (30 psi), and auxiliary gas (10 psi). Collision cell pressure (Argon) was 1.5 mTorr. Multiple reaction monitoring (MRM) transitions, optimized collision energies (CE) and S-Lens HF amplitudes for each transition are summarized in Table S1 (*Supporting Information*). To increase mass spectral acquisition speed and number of measured data points across each peak, the chromatographic run was divided into five segments. The first segment (0–5.5 min) contained DGenin, DGenin-d_3, DG-mono and DG-mono-d_3, using a declustering potential (DCV) of 16 V. During the second segment (5.5 - 7 min), transitions for DG-bis, DG-bis-d_3 and deslanoside were monitored. The third segment (7–8.6 min) was divided into 2 scan events, one dedicated to DG, DG-d_3, H_2DG and lanatoside and the other to DTenin, DTenin-d_3 with DCV of 20 V. DT-mono, DT-mono-d_3, MetDG and AcDG were measured in the fourth segment (8.6–9.8 min), and DT-bis, DT-bis-d_3, DT and DT-d_3 in the final event (9.8–17 min). During the final two min, the polarity was switched to negative mode, to avoid charging effects in the mass spectrometer. The first 3 and last 4 min of each run were directed into the waste to avoid contamination of the source by salts and residues of matrix components contained in the sample extracts.

Method validation

System suitability test

Both sensitivity of the system and retention time of analytes were checked in the system suitability test prior to any measurement. For this test, a methanol solution of all analytes at the concentration of the lowest calibrator (0.2 ng/mL for digoxin and metabolites and 2 ng/mL for digitoxin and metabolites) was injected into the LC-MS system. The segments of the chromatographic run were adapted to the retention times of the analytes to make sure that peaks were not truncated. Signal-to-noise ratios of analytes were expected to be above 10:1 to ensure sufficient sensitivity of the system.

Accuracy, precision

For determination of accuracy and precision, four concentration levels were used. Accuracy was determined by comparing calculated and theoretical concentrations using three replicates. For intra-assay precision, six replicates were processed and analyzed in duplicate. The coefficient of variation (CV) was calculated for the mean of all six preparations using the averaged values of both injections. For inter-assay precision, the same experiment was repeated on a different day. Samples were analyzed using a HPLC column with different batch number and CV was determined using all twelve preparations. The concentrations of fortified serum samples for the determination of precision and accuracy are shown in Table 1.

Ion suppression, matrix effects

To ensure that matrix components were not interfering with quantification of the target substances, an ion suppression experiment using post-column infusion was carried out according to Bonfiglio *et al.*[37] and the extent of matrix effects was investigated according to Matuszewski *et al.*[38] For this, three samples were prepared: a spiked serum sample, a blank serum sample that was spiked post-extraction and a reference solution in methanol containing all

Table 1. Concentration levels of spiked serum samples used for determination of accuracy and precision

Analyte	Concentration [ng/mL]			
	Level XLow	Level Low	Level Medium	Level High
DG	0.23	1.13	4.35	8.85
DG-bis	0.22	1.10	4.23	8.62
DG-mono	0.22	1.09	4.22	8.59
DGenin	0.22	1.12	4.32	8.81
H_2DG	0.23	1.13	4.35	8.85
AcDG	0.22	1.10	4.26	8.68
MetDG	0.18	0.89	3.44	7.00
DT	2.30	11.5	44.4	90.4
DT-bis	2.24	11.2	43.2	88.0
DT-mono	2.20	11.0	42.5	86.5
DTenin	2.24	11.2	43.2	88.0
Deslanoside	2.24	11.2	43.2	88.0
Lanatoside	2.24	11.2	43.2	88.0

analytes. The concentration was equivalent to the medium concentration level (Table 1). Additionally, possible interferences from glycerophosphocholines (GPChos) were assessed by in-source multiple reaction monitoring (IS-MRM).[39] Two common product ions for GPCho's (triethylammonium-ethyl phosphate (*m/z* 184) and trimethylammoniumethylate (*m/z* 104) were monitored over the entire chromatographic run time at high declustering voltage (40 V) to induce in-source fragmentation. A possible transfer of this method to different matrices such as plasma collected in lithium-heparin or EDTA tubes was also investigated; spiked serum samples were sequentially diluted using two plasma pools and theoretical values were compared to measured concentrations.

Specificity, transfer to other matrices

Specificity of the method was verified by analyzing analyte-free serum. Possible transfer of the method to plasma samples was assessed using the low concentration level. Two aliquots of serum were diluted 1:3, 1:1 and 3:1 with a plasma-pool collected in EDTA and another collected in lithium heparin tubes. The original sample and the three dilutions in each plasma-pool were analyzed and plotted against their theoretical concentration. Linear regression was performed for both matrices; attention was paid to calculated slopes and coefficients of regression.

Linearity, sensitivity

For assessment of linearity, the calibration range was extended down to 80% of the lowest and up to 120% of the highest calibrator. The coefficient of regression *R* was desired to be higher than 0.99 for sufficient linearity. The lower limits of quantification (LOQ) and detection (LOD) were determined by sequential dilution and measurement of the lowest calibrator level. The LOQ was determined as the concentration with signal-to-noise ratio larger than 10; LOD was defined as the concentration with signal-to-noise ratio >3.

Stability

Stability of spiked samples at -20°C was investigated as well as stability of processed extracts in the thermostatted autosampler at 2-8°C. For these experiments, fortified serum samples were measured immediately after preparation, after 2 d, 2 and 12 wk. Processed serum samples were left in the autosampler for several days and measured repeatedly to determine whether any degradation of analytes occurred. Also, stability of the deuterated internal standards was assessed in terms of H/D back exchange.

Application to analysis of patient and spiked samples

To verify the trueness of the method, two different studies were conducted at two different laboratories. Twenty spiked samples and 9 anonymized pooled patient samples containing digoxin in the range from 0.2 to 5 ng/mL, and 8 anonymized pooled patient samples containing digitoxin in the range from 9 to 75 ng/mL were analyzed. Aliquots were sent to an external laboratory (Referenzinstitut für Bioanalytik, RfB) for quantitation using a certified reference method.

Results and discussion

The objective of this work was the development and validation of an LC-MS/MS reference assay for accurate quantification of digoxin (DG) and digitoxin (DT) from human serum, as well as 10 metabolites of these drugs. In addition, two structurally related compounds that are known to significantly cross-react with DG and DT-specific immunoassays were included. Because of the low therapeutic concentration levels of DG and DT, the serum samples were preconcentrated using a sample workup protocol comprising protein precipitation and liquid-liquid extraction (LLE). LC-MS/MS analysis was then performed in ESI positive mode using lithium adduct ions of the investigated substances.

Optimization of LC-MS/MS conditions

Optimizing the MS system was initially performed using methanol stock solutions of the analytes, by infusing solutions into the HPLC flow. In these experiments, it was observed that the cardiac glycosides generated both $[M+H]^+$ and $[M+Na]^+$ adduct ions, the ratio of which strongly depended on experimental conditions. To favor formation of a single ion species for sensitive multiple reaction monitoring (MRM) analysis, several monovalent cations were compared as mobile phase additives and their effect on the response behavior of the target analytes evaluated. Ammonium, lithium, sodium and cesium were tested as ionic adducts. The most intense signals were recorded for the cesium adducts of the investigated drug substances. Unfortunately, $[M+Cs]^+$ adducts tend to exclusively eliminate the metal cation under collision-induced dissociation (CID) conditions,[40,41] thus greatly limiting specificity. The $[M+Cs]^+ \rightarrow [Cs]^+$ transition was therefore not utilized in our further work. Of the other cations, ammonium exhibited only low affinity for the analytes. Lithium and sodium ions yielded comparable results; lithium was finally chosen as pairing reagent because of slightly higher signal intensities of $[M+Li]^+$ over $[M+Na]^+$.

Instrument-specific parameters such as electrospray voltage, capillary and vaporizer temperatures, as well as auxiliary and sheath gas pressures were then optimized for each analyte individually for the highest signal-to-noise ratios (see Experimental), in addition to MS/MS specific operating settings (Table S1, *Supporting Information*).

The most labile bonds of cardiac glycosides are the glycosidic bonds, which break at low collision energies under CID conditions. Consequently, for DG, DT and their metabolites, the most intense MRM transition was $[M+Li]^+ \rightarrow 130^+$, corresponding to formation

of the isolated digitoxose ring. Unfortunately, this transition lacked specificity and was therefore not used here as molecules comprising a steroidal skeletal structure with one or several sugar molecules attached are quite common for a wide range of compounds. The above MRM transition could therefore potentially lead to false positive and/or inaccurate results. We chose the more specific transition $[M+Li]^+ \rightarrow [M+Li\text{-digitoxose}]^+$, despite its lower yield. This precursor/product ion combination would still lead to problems if the investigated compounds coeluted in HPLC or flow injection experiments because in-source fragmentation could occur and cause the labile glycosidic bond to break, which would lead to the formation of metabolites in the ionization source and thus bias the analysis. Baseline HPLC separation was therefore a necessity for the assay; in our optimized separation, all compounds eluted with ~1.5 min spacing between each peak (Figure 2).

Careful optimization of chromatographic conditions was also important because the *m/z* of several analytes differed by only 2 u, which led to isotope pattern overlaps. The main isotope of lithium is $^{[7]}$Li (92.5% natural abundance), while the second naturally occurring $^{[6]}$Li isotope occurs at 7.5% abundance. For H_2DG (*M*=782 g/mol) and DG-d_3 (*M*=783 g/mol), this effect caused interferences. In the MRM chromatographic trace of H_2DG, a second peak was recorded corresponding to the $^{[6]}$Li adduct of DG-d_3. In addition, the MRM trace of DG-d_3 exhibited a signal for the $^{[13]}$C isotope peak of H_2DG. Adequate chromatography resolution was therefore crucial to distinguish between signals from target analyte and isotopic cross-talk (Figure 3).

Unfortunately, DTenin exhibited a very resistant structure under CID conditions, and remained intact up to high CID energies, when it eventually and suddenly dissociated into many unspecific product ions, which limited MRM detection sensitivity. We therefore chose the $[M+Li]^+ \rightarrow [M+Li]^+$ transition for DTenin, which was, of course, very unspecific but it eliminated or reduced potentially coeluting isobaric interferences.

Optimization of sample preparation

A two-stage sample preparation protocol was implemented to achieve the highest possible detection sensitivity of the assay. First, protein-precipitation was carried out to release protein-bound analytes and to remove high-abundant proteins, followed by separation of the target analytes from residual matrix components by liquid-liquid extraction (LLE).

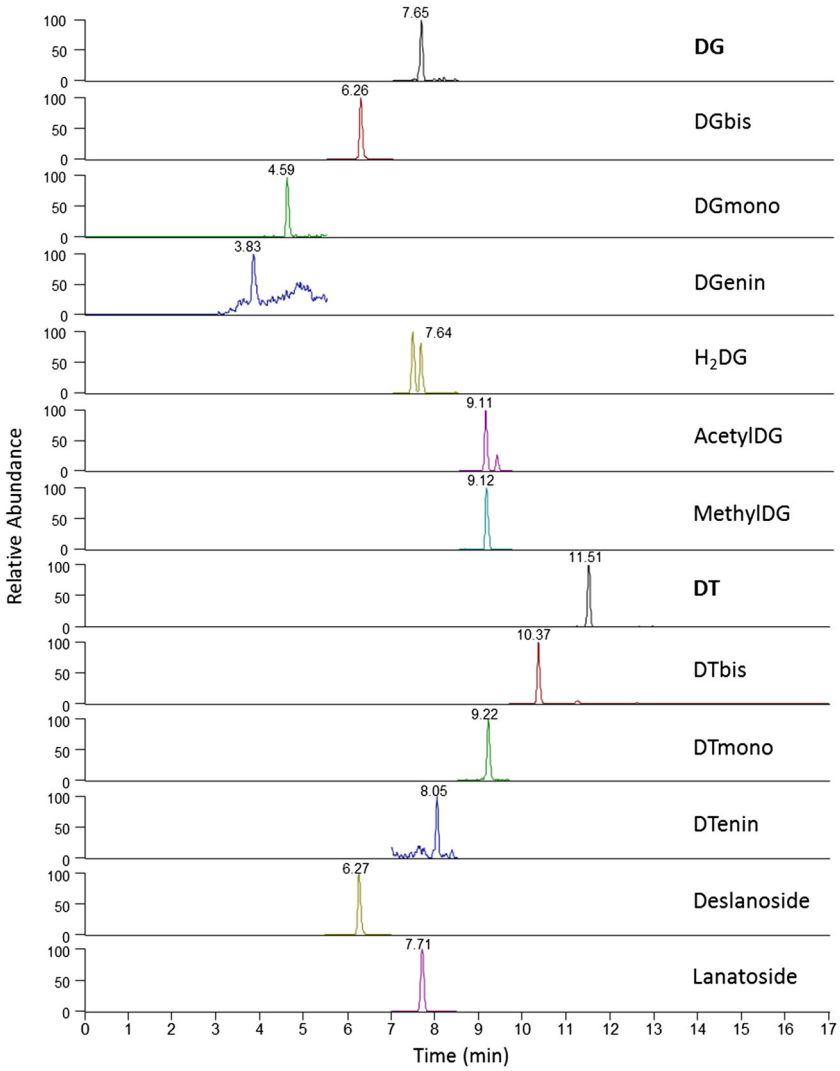

Figure 2. MRM traces of the investigated analytes after HPLC separation of the lowest calibrator level (DG and metabolites, 0.2 ng/mL; DT and metabolites, 2 ng/mL).

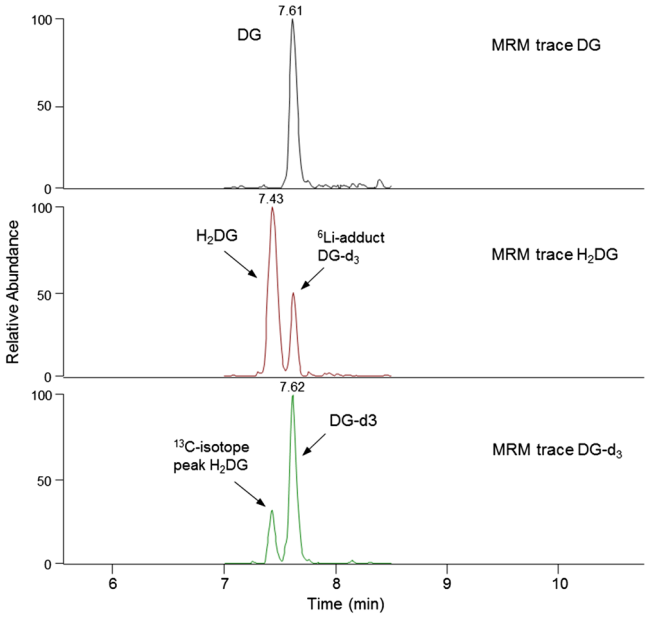

Figure 3. MRM traces of DG, H_2DG and DG-d_3 at 0.6, 0.6, and 1 ng/mL.

First, several reagents were evaluated for protein precipitation. Methanol, a mixture of methanol and ethanol, acetonitrile and a concentrated zinc sulfate solution (10% *w/v*) were compared. Since similar signal-to-noise ratios were obtained for methanol and $ZnSO_4$ in subsequent LC-MS/MS experiments for the majority of analytes, the phospholipid content of blank serum extracts precipitated with methanol and $ZnSO_4$ were compared according to Little *et al.*[39] The results demonstrated that the $ZnSO_4$ extract contained significantly lower amounts of phospholipids as compared to methanol extracts (Figure 4); $ZnSO_4$ was therefore chosen for all further experiments. Protein removal efficiency of $ZnSO_4$ in water or in organic solvents has been investigated in other studies and provided also good results for serum and plasma samples.[42,43]

For the second step of the sample preparation, several organic solvents were tested for LLE of the target analytes: for example, ethylacetate, heptane, DCM, CHL, methyl-*t*-butylether (MTBE) and isopropanol (IPA). Different mixtures of solvents were also compared to achieve the highest possible recovery of all target analytes simultaneously. The highest recoveries for all analytes were obtained for the combination of DCM/IPA 90/10 (*v/v*) and CHL/IPA 95/5 (*v/v*) as extraction solvent.

Manual LLE procedures are generally tedious and time consuming, in particular when several extraction steps are required per sample, as was the case in our application. We therefore transferred the protocol to SLE cartridges after the optimal extraction solvent composition had been established. Concentrations between 27% and 35% of DG and its metabolites and 8–15% of DT and its metabolites were recovered in the eluates after the two-step sample preparation workflow as compared to an aqueous reference solution. These differences are in accordance with the higher affinity of DT to serum proteins as compared to DG.

Validation

Accuracy/precision

Calculated recoveries were 83%, 100%, 98%, and 102% for DG and 65%, 100%, 102%, and 97% for DT (for the xlow, low, medium, and high levels, respectively; see Table 2). Calculated CVs for intra-assay precision were 9.1%, 7.0%, 4.8%, and 3.4% for DG and 17%, 7.0%, 6.9%, and 6.3% for DT (for xlow, low, medium and high levels, respectively, Table 6). CVs for inter-assay precision were 19%, 8.8%, 7.0%, and 8.1% for DG and 32%, 7.7%, 7.0%, and 9.7% for DT (for xlow, low, medium and high concentration levels, respectively; Table 3). Intra- and inter-assay precision were not determined

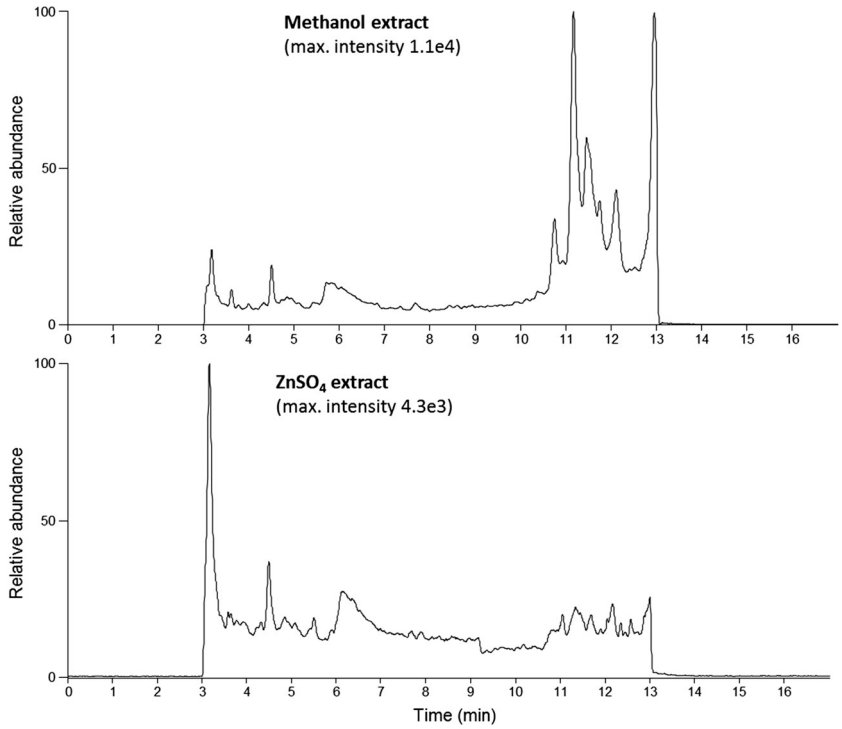

Figure 4. Chromatographic traces of *m/z* 184 and *m/z* 104 for two serum extracts, which were precipitated with MeOH (*upper trace*) or $ZnSO_4$ (*lower trace*).

Table 2. Recoveries for DG, DT and their metabolites based on spiked serum samples

Level Analyte	XLow (*n*=3)		Low (*n*=3)		Medium (*n*=3)		High (*n*=3)	
	Spiked conc. [ng/mL]	Measured conc. [ng/mL]	Spiked conc. [ng/mL]	Measured conc. [ng/mL]	Spiked conc. [ng/mL]	Measured conc. [ng/mL]	Spiked conc. [ng/mL]	Measured conc. [ng/mL]
DG	0.23	0.19 (83%)	1.1	1.1 (100%)	4.4	4.3 (98%)	8.9	9.1 (102%)
DGbis	0.22	0.16 (73%)	1.1	1.1 (100%)	4.2	4.2 (100%)	8.6	8.3 (97%)
DGmono	0.22	0.19 (86%)	1.1	1.1 (100%)	4.2	4.0 (95%)	8.6	7.8 (91%)
DGenin	0.22	0.17 (77%)	1.1	1.2 (109%)	4.3	4.2 (98%)	8.8	8.3 (94%)
H_2DG	0.23	0.15 (65%)	1.1	1.0 (91%)	4.4	4.9 (111%)	8.9	10 (115%)
AcDG	0.22	0.19 (86%)	1.1	1.1 (100%)	4.3	4.1 (96%)	8.7	9.0 (104%)
MetDG	0.18	0.15 (83%)	0.89	0.87 (97%)	3.4	3.2 (94%)	7.0	7.2 (103%)
DT	2.3	1.5 (65%)	12	12 (100%)	44	45 (102%)	90	87 (97%)
DTbis	2.2	1.8 (82%)	11	12 (109%)	43	45 (105%)	88	87 (99%)
DTmono	2.2	1.8 (82%)	11	11 (100%)	43	41 (95%)	867	85 (98%)
DTenin	2.2	2.1 (95%)	11	11 (100%)	43	40 (93%)	88	84 (95%)
Deslanoside	2.2	2.4 (109%)	11	14 (127%)	43	64 (149%)	88	106 (120%)
Lanatoside	2.2	2.4 (109%)	11	11 (100%)	43	50 (116%)	88	102 (116%)

Table 3. Precision

Level Analyte	XLow		Low		Medium		High	
	Intra-assay (n=6)	Inter-assay (n=12)	Intra-assay (n=6)	Inter-assay (n=12)	Intra-assay (n=6)	Inter-assay (n=12)	Intra-assay (n=6)	Inter-assay (n=12)
DG	9.1%	19%	7.0%	8.8%	4.8%	7.0%	3.4%	8.1%
DGbis	10%	24%	4.0%	4.7%	3.1%	5.9%	3.5%	8.2%
DGmono	20%	27%	8.1%	5.9%	3.2%	6.4%	5.8%	7.3%
DGenin	4.4%	23%	2.9%	4.8%	2.5%	1.8%	6.6%	4.8%
H_2DG	21%	45%	22%	18%	8.1%	8.2%	12%	15%
AcDG	10%	19%	8.6%	25%	12%	13%	3.9%	22%
MetDG	10%	23%	11%	9.4%	13%	11%	3.8%	12%
DT	17%	32%	7.0%	7.0%	6.9%	7.7%	6.3%	9.7%
DTbis	12%	23%	8.4%	9.2%	9.4%	10%	9.4%	13%
DTmono	6.3%	21%	7.7%	12%	7.5%	6.1%	14%	11%
DTenin	3.7%	13%	5.1%	4.3%	3.9%	3.7%	3.1%	3.0%

for deslanoside and lanatoside, since only semi-quantitative analysis was required for these substances.

The lowest level investigated showed a significantly higher bias than the other samples, both with regards to accuracy and precision. Since linearity was shown over this concentration range, the lowest level was maintained as calibrator level, but the quantification range was limited to 1.1–8.9 ng/mL for DG and its metabolites and to 12–90 ng/mL for DT and metabolites.

Linearity/sensitivity

The calibration ranges were different for DG and DT and their respective metabolites; they were chosen according to expected concentration levels in human subjects. Calculated coefficients of regression *R* were >0.991 for all investigated compounds, demonstrating good linearity in the investigated calibration ranges. The determined LOD and LOQ values for all target analytes are summarized in Table 4.

Table 4. LOD and LOQ

Analyte	LOD	LOQ
DG	0.08 ng/mL	0.12 ng/mL
DGbis	0.04 ng/mL	0.04 ng/mL
DGmono	0.16 ng/mL	0.20 ng/mL
DGenin	0.20 ng/mL	0.20 ng/mL
H_2DG	0.04 ng/mL	0.08 ng/mL
AcDG	0.04 ng/mL	0.04 ng/mL
MetDG	0.03 ng/mL	0.06 ng/mL
DT	0.40 ng/mL	0.40 ng/mL
DTbis	0.40 ng/mL	0.40 ng/mL
DTmono	0.39 ng/mL	0.79 ng/mL
DTenin	1.2 ng/mL	2.0 ng/mL
Deslanosid	0.40 ng/mL	0.40 ng/mL
Lanatosid	0.40 ng/mL	0.40 ng/mL

Stability

It was shown that spiked serum calibrators could be stored for 2 weeks at -20°C; after 13 wk the measured values differed significantly (4–20%) from the original concentrations. The stability of DG in processed samples was shown for 12 d, whereas significant deviations from the initial value were observed for DT after 2 d. These values should be considered when analyzing patient samples. Stability of the deuterated internal standards was investigated

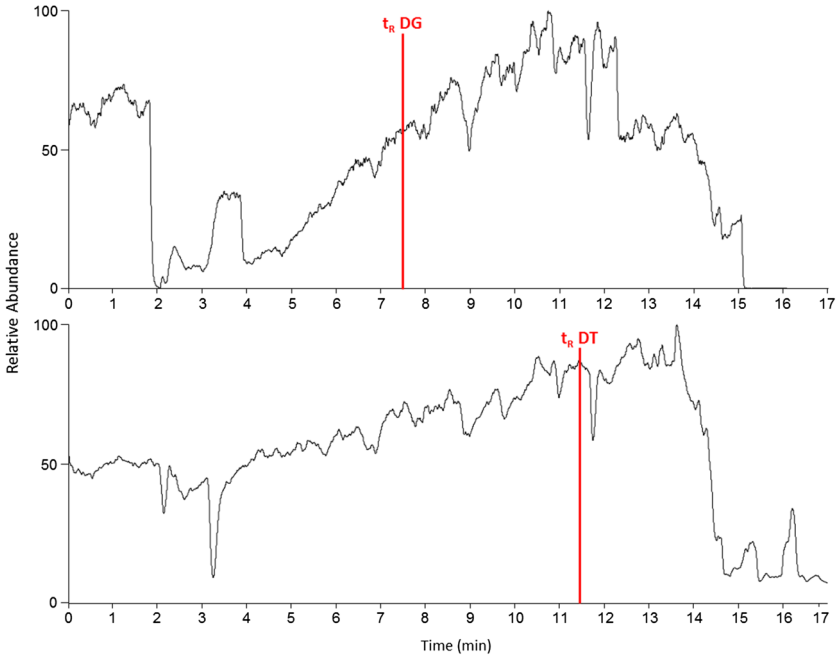

Figure 5. Post-column infusion of DG and DT during analysis of processed matrix blank.

by analyzing three blank serum samples with addition of internal standard solution. If significant H/D back exchange of the deuterated analogues was to occur, we expected signals for the native substances in the blank serum extracts. No such effect was observed for any of the deuterated analytes. Calibrators and samples were analyzed in duplicate and run consecutively, to allow investigation of the stability of the internal standard by monitoring possible decreases of peak areas during the entire duration of the analyses. It was shown that the peak areas of the internal standards remained constant over the entire sequence.

Specificity, transfer to plasma

Specificity of the method was assessed by measuring a pool of analyte-free serum in triplicate and verifying that no signal coming either from the matrix or the internal standard was recorded at the expected retention time of the analytes. No interference was obvious for any of the analyzed compounds.

Linear regression of sample dilutions with lithium heparin-plasma showed slopes of 0.91 and 0.97 for DG and DT, respectively, whereas the values for their metabolites were between 0.74 and 0.98. Correlation coefficients *R* varied between 0.963 and 0.999. In EDTA plasma, slopes were 0.96 and 0.95 for DG and DT, respectively; slopes for their metabolites ranged from 0.74 to 1.03 with coefficients of variation *R* between 0.987 and 1.00. Transferability of the method to plasma for DG and DT was shown, with slightly better results in EDTA compared to lithium heparin plasma but the quantification of some metabolites can be biased when analyzed in a different matrix.

Ion suppression, matrix effects

The influence of the matrix was further investigated by setting up post-column infusion experiments (see Experimental). The obtained chromatograms for the target analytes DG and DT are shown in Figure 5. No detrimental ionization effects were observed for any of the target substances.

The investigation of matrix effects according to Matuszweski *et al.*[38] showed a significant influence of the matrix on quantitation of the target substances; for DG and its metabolites 43% to 66% was observed, and 85% to 95% for DT and its metabolites.

Phospholipid analysis of the serum extracts according to Little *et al.* showed that residual GPCho's present in serum extracts eluted mostly at the end of the chromatogram, after the elution of the target compound with the highest affinity for the separation column (DT) and therefore did not bias the analysis of the target substances. (Chromatograms not shown.)

Application to analysis of patient and spiked samples

The results of two laboratories were compared (see Experimental); linear regression analysis showed the following coefficients: slope 1.1 for DG and 0.89 for DT, intercept -0.08 for DG and 0.28 for DT with coefficients of regression *R*=0.987 in both cases. Considering the small number of samples, the comparison of both methods demonstrated good correlation and confirmed the trueness of our method.

Conclusions

Digoxin and digitoxin are frequently used for treatment of cardiac dysfunctions and can lead to severe side-effects and possibly death in case of an overdose. The presented LC-MS/MS assay allowed simultaneous quantitation of digoxin, digitoxin and several of their metabolites in human serum. Extensive sample preparation, which combined protein precipitation and supported liquid extraction, was needed to achieve the required sensitivity. The method was fully validated; it showed good results in terms of accuracy, precision, linearity and sensitivity. The transferability of the method to EDTA and lithium heparin was shown for the two main analytes and several of their metabolites. Good correlation with an established reference method for digoxin and digitoxin was also demonstrated, for spiked and native samples.

Immunoassay-based tests, which allow very short analyses times for quantification of therapeutic drugs, are easily biased by presence of metabolites or structurally similar compounds. The purpose of the presented LC-MS/MS assay was the use as single reference method for an immunologic test for DG and a separate test for DT. The described LC-MS/MS assay did not achieve the performance of a certified reference measurement procedure, mainly related to higher imprecision. This shortcoming could be improved, for example by using $^{[13]}$C-labelled stable isotope standards instead of the deuterated analogs prepared by H/D exchange here. Sensitivity could be further increased by transferring the assay to a latest generation MS platform or by increasing sample volumes. In addition, the assay could be specifically optimized for only the two main target substances, DG and DT, and omitting the full metabolite panel as shown in the present work. The latter would increase the sensitivity, but would obviously entirely change the target application of the assay.

Despite the limitations, the assay presented in this study allowed for fast and accurate quantification of the target compounds and provides useful information on metabolite profiles in patient samples.

Acknowledgements

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site.

Supporting Information

Simultaneous quantification of digoxin, digitoxin and their metabolites in serum using high performance liquid chromatography-tandem mass spectrometry

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Table S1. MRM transitions, collision energies (in parentheses, CE [V]) and S-Lens potentials for the investigated compounds and their corresponding internal standards (IS).

Compound	MRM transition	S-Lens	IS	MRM transition
DG	m/z 787.4 à 657.4, 527.3 (45)	293	DG-d_3	m/z 790.4 à 530.4, 660.4 (45)
DG-bis	m/z 657.3 à 527.3, 379.1 (40)	205	DG-bis-d_3	m/z 660.7 à 382.0, 530.4 (40)
DG-mono	m/z 527.2 à 379.2, 317.1 (25)	167	DG-mono-d_3	m/z 530.4 à 320.1, 381.9 (25)
DGenin	m/z 397.2 à 379.2, 351.2, 317.2 (15)	113	DGenin-d_3	m/z 400.1 à 319.9, 382.1 (15)
H_2DG	m/z 789.4 à 659.4, 529.3, 371.0 (47)	297	DG-d_3	m/z 790.4 à 530.4, 660.4 (45)
DT	m/z 771.4 à 641.4, 511.3 (45)	273	DT-d_3	m/z 774.4 à 514.3, 644.4 (45)
DT-bis	m/z 641.3 à 511.3, 363.1 (38)	205	DT-bis-d_3	m/z 644.4 à 514.4, 366.4 (45)
DT-mono	m/z 511.3 à 381.2, 363.2 (34)	165	DT-mono-d_3	m/z 514.4 à 366.1, 384.1 (34)
DTenin	m/z 381.1 à 381.1 (25)	120	DTenin-d_3	m/z 384.1 à 384.1 (25)
AcDG	m/z 829.4 à 657.4, 527.3 (45)	305	DG-d_3	m/z 790.4 à 530.4, 660.4 (45)
MetDG	m/z 801.4 à 657.4, 527.3 (43)	280	DG-d_3	m/z 790.4 à 530.4, 660.4 (45)
Deslanoside	m/z 949.4 à 298.9, 429.0 (60)	292	DT-d_3	m/z 774.4 à 514.3, 644.4 (45)
Lanatoside	m/z 991.5 à 280.9, 471.1 (60)	295	DT-d_3	m/z 774.4 à 514.3, 644.4 (45)

Publication 5

Rapid quantification of digitoxin and its metabolites using differential ion mobility spectrometry-tandem mass spectrometry

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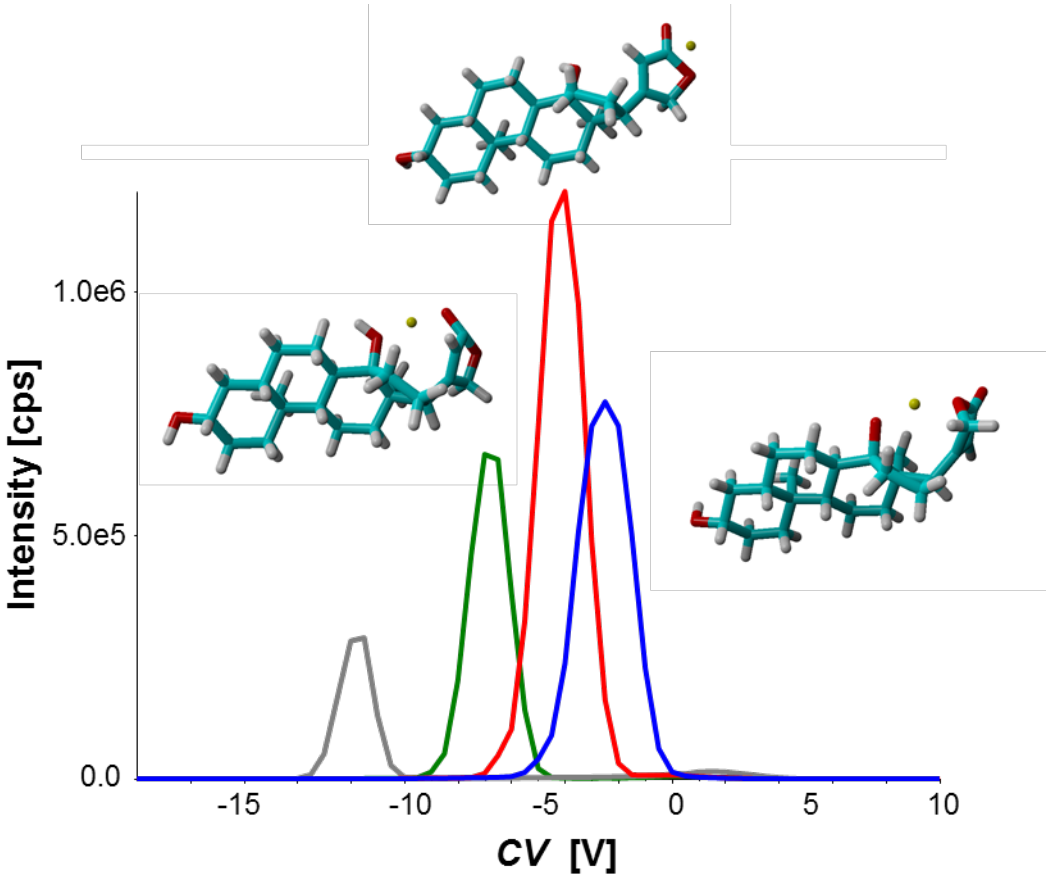

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Rapid Quantification of Digitoxin and Its Metabolites Using Differential Ion Mobility Spectrometry-Tandem Mass Spectrometry

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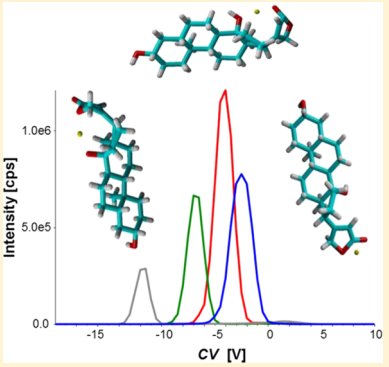
Supporting Information

ABSTRACT: This study focuses on the quantitative analysis of the cardiac glycoside drug digitoxin and its three main metabolites digitoxigenin-bisdigitoxose, digitoxigenin-monodigitoxose, and digitoxigenin using electrospray ionization-differential ion mobility spectrometry-tandem mass spectrometry (ESI-DMS-MS/MS). Despite large molecular weight differences, gas-phase separation of the four compounds in the DMS drift cell was not possible, even by utilizing additional volatile chemical modifiers. Baseline separation was achieved after adduct formation with alkali metal ions, however, and efficiency was shown to improve with increasing size of the alkali ion, reaching optimum conditions for the largest cesium ion. Subsequently, an assay was developed for quantification of digitoxin and its metabolites from human serum samples and its analytical performance assessed in a series of proof-of-concept experiments. The method was applied to spiked human serum pools with concentration levels between 2 and 80 ng/mL. After a short reversed-phase chromatographic step for desalting the sample, rapid DMS separation of the analytes was carried out, resulting in a total run time of less than 1.5 min. The instrumental method showed good repeatability; the calculated coefficients of variation ranged from 2% to 13%.



Ion mobility spectrometry (IMS) separates ionized analytes based on their mobilities in an electric field in the gas phase.[1] The mobility depends on its mass, charge, and shape.[2] In classical IMS, ions are separated in a drift cell filled with inert buffer gas at atmospheric pressure in a constant low electric field. Ions passing through the buffer gas are subjected to a number of collisions and these processes will be influenced by the collision cross sections (CCS) of the analyte ions. Ions of different size and shape are thus separated in the drift tube. Differential ion mobility spectrometry (DMS) and field asymmetric ion mobility spectrometry (FAIMS) separate ions based on changes of ion mobility in alternating electric fields.[3] An asymmetric electric waveform (separation voltage, SV), comprising a short high voltage (10−30 kV/cm) and a longer low voltage (<1 kV/cm) component, is applied to the drift tube. Ions are separated based on their mobility differences between high and low fields. Ions oscillate perpendicularly to the gas flow and only pass through the drift tube if their net transversal motion equals zero. Similar to a quadrupole mass analyzer, ions with a particular mobility can be guided through the drift tube and reach the detector by applying a compound-specific compensation voltage (CV).[4] If the compensation voltage is scanned through a wider voltage range, the differential ion mobility spectrum of a sample is obtained, which finds its analogy in the full scan mass spectrum of the quadrupole MS. Keeping SV and CV constant at a voltage specific for a certain analyte makes the DMS device act as a filter, to increase signal-to-noise (S/N) and specificity. This is similar to the selected ion monitoring (SIM) mode of the quadrupole MS.

Significant improvements of DMS separation efficiency are obtained by adding polar chemical modifiers to the buffer gas.[5−9] These modifiers alter the drift behavior of ions as a result of dynamic clustering/declustering processes that increase the difference between high and low field drift velocities. During the low field, ions form clusters with modifier molecules, resulting in larger CCS than "naked" ions and resulting in slower movement. During the high field, clusters are dissociated and mobilities increase.[10,11] Schneider et al. systematically demonstrated enhancement effects for a wide range of chemical modifiers (e.g., isopropanol, ethyl acetate, acetonitrile), thus reducing considerably the number of modifiers that have to be tested for a specific application.[5,11] Beneficial effects of different modifiers have been shown for small molecules,[12] pharmacologically active substances,[13] and chiral molecules, such as amphetamine-type compounds.[14] Further improvements have been achieved by implementing multicomponent modifiers, which increased peak capacity and sensitivity for detection of low molecular weight drugs and allowed fine-tuning separations in specific applications.[15] Drift times were also strongly influenced in ESI-IMS-MS after adding nitrobenzene as a modifier, which has the ability to form large clusters with small target ions.[16] We have recently shown that

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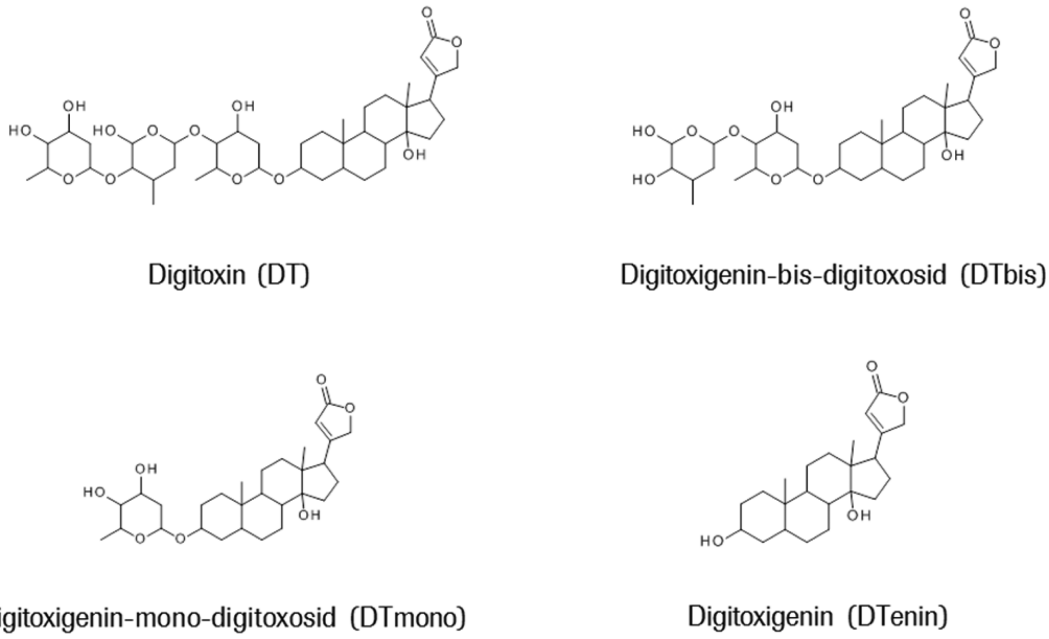

Figure 1. Chemical structures of the target analytes.

CV values in DMS for a wide range of chemically diverse compounds can be very accurately predicted with use of alcohol modifiers based on readily available descriptors such as proton affinity and gas phase acidity of the modifier molecules.[17]

Several studies have successfully demonstrated the application of ion mobility spectrometry for quantification or structure elucidation of small molecules in complex biological samples.[3,18−22] Generally, addition of a DMS filter allows eliminating chemical interferences and therefore considerably reduces the chemical noise.[23] Sample pretreatment can then be reduced to a minimum or be completely eliminated, as was shown by Porta et al., who performed direct analysis of cocaine and tramadol from post-mortem tissues.[24] Hall et al. achieved quantitation of five drug metabolites in urine using direct infusion of extracted samples into a DMS-MS system.[25] DMS-MS has also been shown to be very useful for fast separation of cocaine and its metabolites from common adulterants.[26] Metabolite separation (e.g., propranolol[7] or morphine[27] and their glucuronide metabolites) was achieved within milliseconds. This is significantly faster than common LC-MS assays, which usually take several minutes for chromatographic separation and detection.

One major advantage of DMS is its ability to distinguish isobaric and isomeric compounds.[7,28−33] The mobility of ions can also be influenced by complex formation prior to DMS. Several types of cations (alkali ions,[34,35] ammonium,[36] silver,[37] copper,[38] transition metals[39]) have been added to enhance DMS separation. Depending on the geometry of the formed complexes, the CCS values of the resulting ion clusters, and hence their mobilities, are strongly influenced.

In the present work, we investigated complex formation reactions for the cardiac glycoside digitoxin (DT) and its three main metabolites (digitoxin-bisdigitoxosid (DTbis), digitoxin-monodigitoxosid (DTmono), and digitoxigenin (DTenin)) (Figure 1) using the alkali ions Li^+, Na^+, K^+, Rb^+, and Cs^+, and their beneficial effect on specificity of DMS separation. Subsequently, we developed a rapid assay for quantitative analysis of the four compounds from human serum and assessed its analytical performance in a series of proof-of-concept experiments.

EXPERIMENTAL SECTION

Reagents and Chemicals. Acetonitrile was LC-MS grade from Biosolve (Valenswaard, Netherlands); acetone, butanol, cyclohexane, diethyl ether, ethanol, ethyl acetate, and formic acid were from Merck (Darmstadt, Germany). Isopropanol, cesium formate, lithium formate, rubidium carbonate, and sodium formate were from Sigma-Aldrich (Steinheim, Germany), and digitoxin reference standard was from European Pharmacopeia Reference Standards (Strasbourg, France). Metabolites digitoxin-bis- and monodigitoxose were old stock from Boehringer (Mannheim, Germany); digitoxigenin was from Sigma-Aldrich. Potassium formate was obtained from Fluka (Neu-Ulm, Germany). Organic-free water was generated with a Waters Millipore Milli-Q-Plus purification system (Eschborn, Germany).

Instrumentation. An AB Sciex (Concord, Ontario, Canada) SelexIon DMS device was used on AB Sciex 5500 QTRAP and 6500 QTRAP quadrupole linear ion trap (QqLIT) mass spectrometers. Electrospray ionization (ESI) in positive ion mode was performed with the Turbo-V ESI source of these instruments at +5.5 kV prior to DMS, with the heat injectors turned off. Zero air was used as gas 1 (30 psi) and 2 (10 psi), nitrogen as curtain gas (18 psi), and helium as collision gas (medium); DP was set to 80 V, EP to 10 V. The liquid chromatography system was an Agilent (Waldbronn, Germany) 1290 ultrahigh performance LC equipped with two binary pumps, Infinity autosampler, and thermostat column compartment. LC desalting was carried out with a Waters (Eschborn, Germany) XTerra C_{18} column (2.1 × 50 mm, 3.5 μm). For selected reaction monitoring (SRM), the following transitions were chosen: m/z 898 → 133, m/z 767 → 133, m/z 637 → 133, and m/z 507 → 133 for DT, DTbis, DTmono, and DTenin, respectively, which corresponded to the $[M+Cs]^+ \rightarrow [Cs]^+$ dissociations. The collision gas pressure was set to

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"medium" and optimized collision energies were 29, 27, 27, and 21 V for DT, DTbis, DTmono, and DTenin, respectively.

DMS Optimization. For qualitative analyses, standard solutions of the analytes at 3 μg/mL in methanol:water (50:50 v/v + 0.1% formic acid) were used. Modifiers for DMS separation were water, cyclohexane, diethyl ether, ethyl acetate, acetonitrile, methanol, ethanol, 2-propanol, and 1-butanol at 1.5%. For DMS of analyte/cation adducts, the following monovalent cation salts were added to the standard solutions: Li-formate, Na-formate, K-carbonate, Rb-formate, and Cs-formate at 1 mmol/L. The DMS temperature was set to "medium" (225 °C). Separation improved at high separation voltages (SV = 3.5−4 kV); as the highest setting of 4 kV gave electrical discharges, SV was reduced to 3.8 kV. Separation was further improved by turning DMS resolution enhancement on, which introduced a countercurrent gas flow to increase collision rate and accentuate differences of ion mobilities. As this parameter also caused signal loss at higher values, it was set to "medium" (25 L/h). The DMS offset was adjusted to −3 V.

Quantitative DMS-MS/MS Analysis. For quantitative analysis, spiked human serum samples containing the four DT substances were analyzed. The concentrations of the serum calibrators were 2, 4, 6, 8, 20, 60, and 80 ng/mL for all analyzed substances. Initially, serum samples were precipitated with methanol. In further experiments, a combination of protein precipitation with methanol and liquid/liquid extraction with dichloromethane and chloroform was performed. Serum extracts were injected into the LC system for desalting using an isocratic mobile phase (methanol:water 80/20 + 0.1 mmol/L cesium formate). The flow rate was 400 μL/min. A 10-μL sample was injected and analyzed by DMS-MS/MS; each analysis was performed in duplicate. After the desalting step, DMS analysis was performed in two ways. First, characteristic CV values were chosen for each analyte (−3.0, −4.5, −6.8, and −11.6 V for DT, DTbis, DTmono, and DTenin, respectively), as additional filter prior to SRM transitions. Alternatively, DMS spectra were recorded between −15 and +2 V (using a 0.25 V step interval). For this setup, the delay between injection of the sample and start of the CV scan was carefully optimized and set to 40 s. Total runtime of this assay including the LC desalting step was 1.3 min.

For determining accuracy, an additional chromatography step was performed prior to DMS: eluent A (water + 0.1 mmol/L cesium formate), eluent B (methanol:water + 0.1 mmol/L cesium formate) using the same LC column. The gradient started with 40% B, increased to 100% B within 2.5 min, and was held for 1 min, before reconditioning the column at the initial solvent composition for 2 min. The total run time was 4.5 min.

RESULTS AND DISCUSSION

The goal of this study was the development of an analytical assay for digitoxin and its three main metabolites from human serum samples based on rapid gas-phase separation of the analytes by differential ion mobility spectrometry-tandem mass spectrometry (DMS-MS/MS) without preceding liquid chromatography.

DMS Separation of Digitoxin and Its Metabolites. Initially, the electrospray ionization (ESI) conditions of digitoxin compounds were optimized by infusion experiments. All compounds exhibited two main species in their ESI mass spectra; viz., $[M + H]^+$ and $[M + Na]^+$ ions, reflecting the affinity of these compounds for alkali metal ions. To focus the ion current solely into one species, lithium was initially added in large excess to the analyte solutions, resulting in exclusive $[M+Li]^+$ lithiated species; namely, m/z 771 for DT, m/z 641 for DTbis, m/z 511 for DTmono, and m/z 381 for DTenin.

DMS separation of these lithiated species was performed next, with the goal of achieving sufficient resolving power for the analytes in the gas phase without compromising detection sensitivity. In the first set of experiments, DMS measurements were carried out without a gas phase chemical modifier; this procedure did not provide baseline separation, however (DMS spectra not shown). Different polar chemical modifiers were then evaluated to increase resolution in the DMS spectra.[5,40−43] We explored ethyl acetate, acetonitrile, isopropanol (IPA), and several linear alcohols ranging from methanol to butanol. The measured compensation voltages (CV) at the peak maxima for the digitoxin variants using these modifiers are summarized in Table 1. As is evident from the data, acetonitrile and ethyl acetate did not improve separation efficiency as compared to experiments without modifier. For the alcohols, on the other hand, resolution increased with increasing length of the alkyl chain; however, the signal intensities started to drop for higher alcohols. This effect was likely caused by ion suppression effects in the DMS cell from proton transfer reactions to the alcohol modifiers, as proton affinities of alcohols increase with chain length.[44] The optimum modifier in our experiments for lithium adducts was isopropanol, as a compromise between efficiency and detection sensitivity. The data in Table 1 clearly show though that DMS baseline separation was not achieved for DT and its metabolites with use of the combination of lithium adduct ions and isopropanol as chemical modifier.

Table 1. Measured CV Values for DT, DTbis, DTmono, and DTenin Using Different Chemical Modifiers and Li^+ As Counter Ion

	CV [V]			
modifier	DT	DTbis	DTmono	DTenin
no modifier	5.7	4.3	5.2	6.9
acetonitrile	6.3	6.4	5.0	6.1
ethyl acetate	6.0	6.6	4.8	6.2
methanol	1.1	0.6	−0.9	−3.1
ethanol	0.5	−0.7	−1.8	−3.9
isopropanol	−0.2	−0.8	−2.1	−4.7
butanol	−0.9	−3.8	−2.8	no signal

Effects of Alkali Ions on DMS Migration Behavior. To further increase the limited resolving power and separation efficiency in the experiments above, alkali metal ions of increasing size were used as counterions (DT variants exhibited strong affinity for alkali metal ions as explained above). Alkali ion adducts have previously been used to modify separations of other compounds such as diastereomers (using classical IMS[34]), disaccharide isomers (using FAIMS[36]), and flavonoid diglycoside isomers,[37] but no systematic size specific effects were reported in these applications.

In our experiments, $[M+X]^+$ adducts with alkali ions were readily formed by the DT variants with all investigated alkali metal ions ($X = Li^+, Na^+, K^+, Rb^+, Cs^+$). The smaller DTenin and DTmono species also formed stable $[M+X+2\cdot IPA]^+$ adduct ions that survived the transport region after the DMS drift cell and were detected for Li^+ and Na^+ (with lower intensities for K^+, Rb^+, and Cs^+), while DT and DTbis were only seen as $[M+X]^+$ species for all alkali metal ions. The increased stability of

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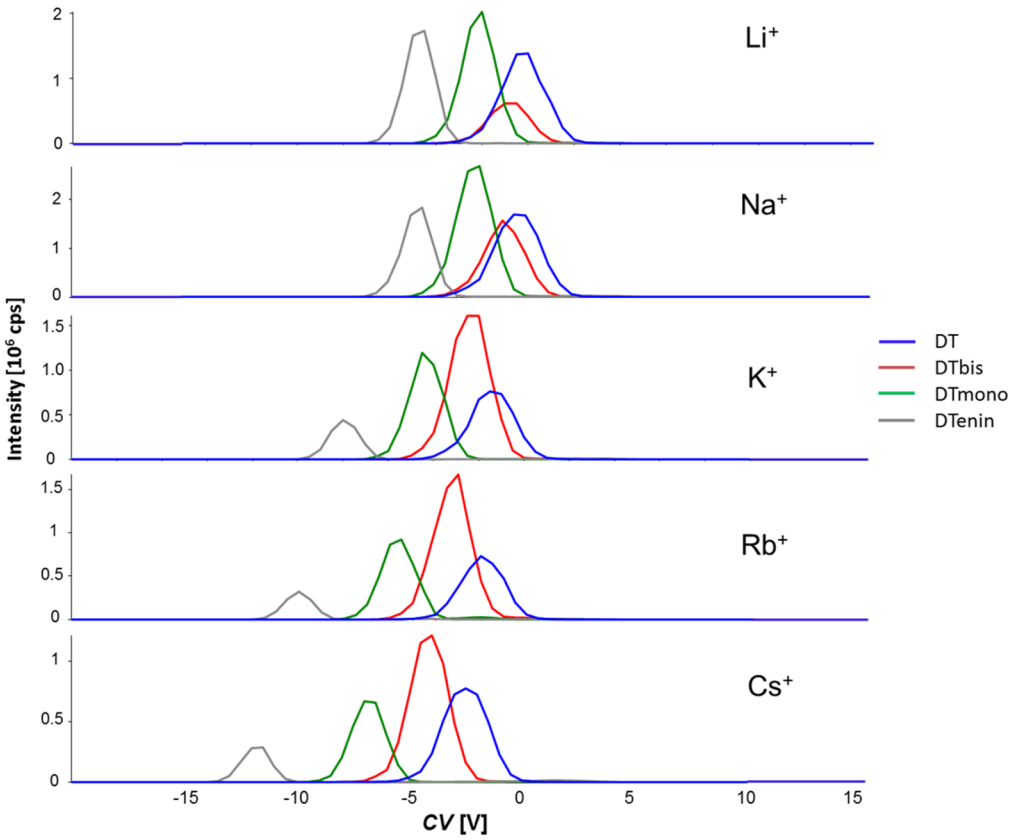

Figure 2. Differential mobility spectra of DT and its metabolites using different alkali metal ions and isopropanol as a DMS modifier. Note: Changes of relative intensities of the analytes were the result of the used ion species for detection; for DTenin and DTmono $[M+X+2{\cdot}IPA]^+$ ions were monitored for Li^+ and Na^+; $[M+X]^+$ was used for the other cations.

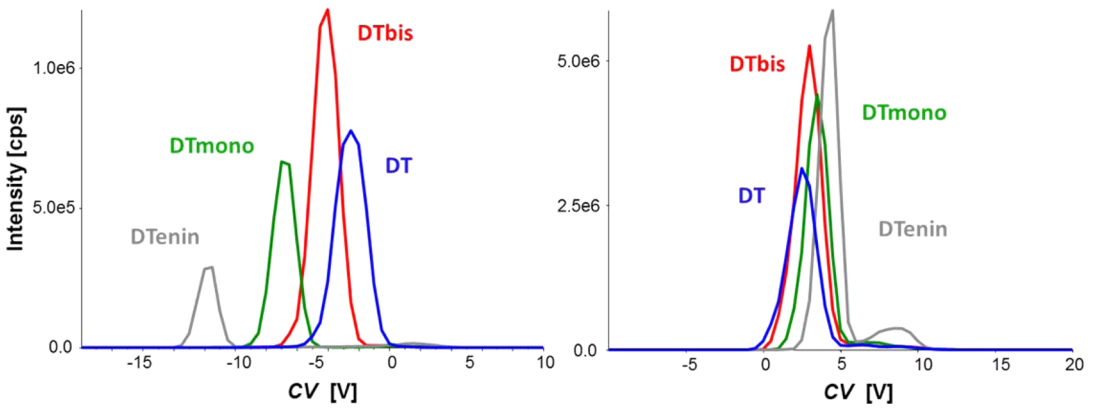

Figure 3. Differential mobility spectra of the analytes DT, DTbis, DTmono, and DTenin using Cs^+ adducts with isopropanol as modifier (left) and without modifier (right).

$[M+X+2{\cdot}IPA]^+$ ions of DTenin and DTmono was probably due to stronger electrostatic interactions, stabilized by coordination with solvent molecules.[45,46]

Because of the significant structural and molecular weight differences of the four studied analytes (M = 765, 635, 505, and 375 g/mol for DT, DTbis, DTmono, DTenin, respectively, Figure 1), we originally expected these compounds to exhibit very different transport behaviors during DMS, even in their native form as protonated molecules (after ESI), without the need for any cation modifications. As seen above, this was not the case, however, and adduct formation with alkali metal ions was essential (Figure 2). Coordination of molecules with alkali metal ions can have a significant impact on the conformation of the compound.[47] The interactions of alkali ions with the digitoxin compounds would likely involve coordination to both the steroid core as well as the flexible carbohydrate chain. Carbohydrates composed of several sugar rings have been studied by other groups and have been shown to be very complex, due to the flexible structure of these substances and the multiple electronegative sites that coordinate with the cations.[35,48,49] Importantly, computational experiments have to consider the chemical modifier in DMS, as both absolute compensation voltage values and separation efficiencies were strongly enhanced when IPA was used in the DMS cell (Figure 3), as often seen when polar modifiers are added.[5−9] The mechanisms of interaction of analytes with modifier molecules

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are very complex and not always fully understood.[13,50] A computational description of all interactions of the analytes with the alkali cations and modifier was outside the scope of this study

Most importantly, adduct formation influenced DMS migration behavior of the target analytes significantly, as seen in Table 2 (for comparison purposes, CV values for the target

Table 2. CV, Resolution (R_s)[a] and Separation Efficiency (Peak Width, fwhm)[b] for DT, DTbis, DTmono, and DTenin As a Function of Different Alkali Counter Ions (Modifier: Isopropanol)

	CV [V]			
counter ion	DT	DTbis	DTmono	DTenin
Li^+	−0.2	−0.8	−2.1	−4.7
Na^+	−0.3	−1.0	−2.3	−4.8
K^+	−1.4	−2.4	−4.3	−8.0
Rb^+	−2.0	−3.3	−5.7	−10.0
Cs^+	−2.5	−4.1	−6.8	−11.8
	resolution R_S for adjacent peak pairs			
counter ion	DT/DTbis	DTbis/DTmono	DTmono/DTenin	
Li^+	0.3	1.0	2.2	
Na^+	0.4	0.9	1.9	
K^+	0.6	1.2	2.6	
Rb^+	1.0	1.9	5.1	
Cs^+	1.1	2.2	5.9	
	peak width (fwhm) [V]			
counter ion	DT	DTbis	DTmono	DTenin
Li^+	2.3	2.2	1.8	1.6
Na^+	2.3	2.0	1.9	1.6
K^+	2.3	2.2	1.9	1.6
Rb^+	2.3	2.0	1.9	1.5
Cs^+	2.4	2.0	1.9	1.5

[a]Resolution for gas-phase species in the CV space was calculated analogous to chromatography peaks: $R_s = 2(CV_2 - CV_1)/(w_{b2} + w_{b1})$, where CV_1 and CV_2 and w_{b1} and w_{b2} are the compensation voltages of the peak maxima and peak widths at baseline, respectively, of the two adjacent peaks. [b]Efficiencies expressed as fwhm (full-width-at-half-maximum) values.

substances without modifier are summarized in the Supporting Information, Table S1). There is a clear trend of increased resolving power with the size of the counterion (Figure 2), with the highest resolving power obtained for the heaviest cesium adduct ions. The calculated resolution (R_s) increased from 0.3, 1.0, and 2.2 (for Li^+), to 1.1, 2.2, and 5.9 (for Cs^+), in the separation of DT/DTbis, DTbis/DTmono, and DTmono/DTenin peak pairs, respectively, while efficiency remained similar for all counterions (Table 2). The relative intensities of the four analytes depended on the monitored ion species. DTenin and DTmono were detected as $[M+X+2\cdot IPA]^+$ ions for Li^+ and Na^+, and as $[M+X]^+$ for the other cations, which explains differences of intensity ratios seen for the four compounds with Li^+ and Na^+ versus K^+, Rb^+, and Cs^+.

Quantitative DMS-MS/MS Assay for Human Serum Samples. Using the optimized DMS and ionization parameters, a DMS-LC-MS/MS method was developed to allow separation of serum samples directly in the DMS cell after a short chromatographic step. This very rapid LC run was initially performed *only* to remove salts and other ionic or highly polar interferences, as well as to focus the analytes prior to DMS-MS/MS, giving coelution of the four target analytes after only 1 min run time.

The actual separations of the four compounds were conducted in the gas phase of the DMS cell with isopropanol as gas phase modifier and cesium ions were added to the mobile phase to enhance resolution and efficiency as described in the previous sections (Figure 3). To improve specificity of the assay, compound-specific transitions for single reaction monitoring (SRM) were chosen (see the Experimental Section), corresponding to $[M+Cs]^+ \rightarrow [Cs]^+$ dissociations. The same SRM transition has been previously used for an LC-MS/MS reference method for quantitation of digitoxin in serum[51] and showed robust and reproducible results, even though overall specificity is limited because of the somewhat generic product ion. Cs^+ adducts generally tend to only lose the metal cation under collision-induced dissociation (CID) conditions.[46,52]

To implement the method for analysis of patient samples, fortified human serum samples with concentration levels between 2 and 80 ng/mL were used for the experiment. Samples were precipitated with methanol to remove high-abundant proteins and extracts directly injected into the DMS-MS/MS system after a short reversed-phase C_{18} isocratic run. The specific CV values for the four analytes (Table 2) were used as a DMS filter, to increase specificity in concert with the SRM transitions. For method calibration, peak areas were integrated and plotted against spiked concentration levels. Lack of sensitivity in the lower concentration range and an isobaric interference overlapping with DTenin resulted in a slightly lower regression slope and higher intercept for this analyte (the corresponding regression curves are shown in the Supporting Information, Figure S1). Obviously, protein precipitation was not sufficient to remove all interfering compounds from the serum samples. Therefore, liquid/liquid extraction was additionally evaluated for cleanup, to see whether DTenin analysis can be improved, if required. Duplicate analyses were performed for each sample. In this second method, we chose to scan the CV voltage during data acquisition instead of using fixed CV values for each SRM transition. Because of the fast CV scanning procedure, this alternative data acquisition routine had little effect on the overall separation time, but slightly affected repeatability (see below). An example of a differential ion mobility spectrum of a serum sample after this sample preparation procedure is shown in the Supporting Information (Figure S2). Correlation of peak areas and spiked concentration levels exhibited linearities similar to the data above but the intercept in the calibration curve for DTenin was indeed smaller, indicating that the additional liquid/liquid extraction can remove the interference if required (see the Supporting Information, Figure S3). In our subsequent experiments, we decided to accept the slightly less linear method for DTenin to avoid the time-consuming additional liquid/liquid extraction step.

Trueness of the DMS-MS/MS method was investigated by analyzing three fortified serum samples containing all analytes (6, 20, and 40 ng/mL; low, medium, and high, respectively) and comparing the measured values to results obtained after adding a liquid chromatography step (see the Experimental Section). Recovery values for direct DMS-MS/MS versus LC-DMS-MS/MS are shown in Table 3. Values ranged from 95% to 117%. The high bias for DTenin at the lowest concentration was, again, the result of the interference remaining in the extract

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Table 3. Trueness of the Method Expressed As Recovery of the Direct DMS-MS/MS Method As Compared to the LC-DMS-MS/MS Method

	recovery [%]		
analyte	low	medium	high
DT	111	95	99
DTbis	100	107	103
DTmono	104	117	99
DTenin	56[a]	102	110

[a]Higher bias caused by interference that remained in the extract from insufficient cleanup.

from insufficient cleanup, which was effectively removed by chromatography prior to DMS.

To determine the influence of DMS on the intensity of background chemical noise signals, a blank serum extract was measured with and without DMS filter. As a result, background noise was reduced 25-fold when DMS was included in the MS/MS assay and 10-fold when it was included in the LC-MS/MS assay. These results clearly demonstrate the large gain of specificity when using differential ion mobility as part of the assay.

The lower limit of quantification (LLOQ) of the assay was defined as the lowest concentration of the calibration range (=2 ng/mL), with a required repeatability of 20% RSD or less at this level. To verify that the assay was capable of quantifying the analytes at this calibrator level, coefficients of variation were calculated for all four analytes at the LLOQ for the mean of six injections. Coefficients of variation calculated for DT, DTbis, DTmono, and DTenin at 2 ng/mL were 19%, 18%, 12%, and 14%, respectively; however, a chromatographic separation should be included in the assay if quantification of DTenin is intended at the low level (see explanation above). Lower concentrations could be readily quantified for some of the target substances. The therapeutic range of digitoxin lies between 10 and 25 ng/mL,[53] however; consequently, there was no need to extend the calibration range to levels below 2 ng/mL.

Instrumental method repeatability was investigated by determining coefficients of variation for all analytes at three concentration levels: 60, 20, and 4 ng/mL. RSD values were calculated from measuring the mean of six injections of prepared serum samples for DT, DTbis, DTmono, and DTenin, respectively. In comparison, RSD values were also determined for peaks integrated from differential ion mobility spectra. The obtained coefficients of variation are summarized in Table 4, ranging from 6% to 13% in CV scan mode for the four analytes, and from 2% to 9% using fixed CV values. Since no internal standard was used for this proof-of-concept analysis, the imprecision was slightly higher than usually seen in LC-MS/MS assays of drugs. Nevertheless, these values were excellent considering the short analysis time of <1.5 min of complex serum samples. Both fixed CV and CV scan methods were implemented for analysis of patient samples, showing slightly better performance with the fixed CV method in terms of method repeatability. A CV scan offers the advantage of direct visualization of the DMS separation and can give information on possible interferences by observing peak distortions. It is also not affected by possible drifts of optimal CV values for the target substances. On the other hand, a careful optimization of the delay time between injection and start of the CV scan is required. The use of a specific CV for each SRM transition allows slightly faster and more sensitive analyses, but requires regular tuning of the CV values for the target components, as CV shifts occur during DMS operation from buildup of contamination in the drift cell.

Table 4. Coefficients of Variation for DT, DTbis, DTmono, and DTenin at Three Concentration Levels: High, Medium, and Low

	coefficient of variation (n = 6) [%] (CV scan)		
analyte	low	medium	high
DT	8	12	8
DTbis	7	10	13
DTmono	9	11	13
DTenin	6	6	11
	coefficient of variation (n = 6) [%] (fixed CV values)		
analyte	low	medium	high
DT	6	8	4
DTbis	7	6	6
DTmono	4	8	3
DTenin	2	9	3

A number of LC-MS assays have been reported in the literature, specifically for digitoxin,[51,54] as part of a broader analyte panel,[55−58] or for combinations with its metabolites.[59,60] Several of these assays have achieved better precision and sensitivity than those obtained here, as a result of more extensive sample preparation and carefully optimized chromatographic separation. As well, a speed-optimized UHPLC method, in particular using parallel columns, might achieve similar throughput rates. The presented DMS-MS/MS technique does not intend to replace these LC-MS/MS assays, but rather offers an easy, fast, and reliable alternative in cases where the performance of a reference method is not needed.

CONCLUSIONS

This work has presented differential ion mobility-mass spectrometry separation of the cardiac glycoside drug digitoxin and its main metabolites digitoxigenin-bisdigitoxoside, digitoxigenin-monodigitoxoside, and digitoxigenin. Gas-phase DMS separation of the digitoxin compounds was not possible using the protonated molecules after ionization—even when using a chemical modifier to enhance separation—despite the compounds' large differences of molecular weight. We have shown that adduct formation with alkali metal ions had significant impact on the transport behavior and resolution in the DMS cell, however, readily allowing separation of the digitoxin compounds. Optimum conditions were achieved with use of cesium ions and isopropanol as clustering modifier. In a series of proof-of-concept experiments, the analytical performance was assessed in terms of linearity, sensitivity, accuracy, and instrument method repeatability of the method for serum samples. It was shown that direct DMS/MS-MS analysis of complex biological samples was readily possible if a sample preparation step was included in the workflow, similar to comparable chromatography assays. The use of isotope internal standards for the drugs will undoubtedly improve precision of this assay further and will be implemented in the future.

Most recently published studies on differential ion mobility spectrometry have focused on separation of isobars and isomers, which are often difficult to analyze by mass spectrometry and often coelute in liquid or gas chromatographic methods. As shown in this study, DMS can also be used

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on its own for separation of mixtures. A similar approach would probably work well to replace ballistic chromatography (e.g., TurboFlow or RapidFire), or for direct ionization techniques that do not allow separation, such as matrix-assisted laser desorption/ionization (MALDI) or desorption electrospray ionization (DESI), or in many other areas such as drugs of abuse testing (DAT) or therapeutic drug monitoring (TDM).

■ ASSOCIATED CONTENT

Supporting Information

Table S1 giving the measured *CV* values for $[M + X]^+$ adducts; Figure S1 showing the regression curves from protein precipitated serum; Figure S2 showing the differential mobility spectrum of a spiked serum extract; and Figure S3 showing the calibration curves from serum after protein precipitation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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VIII. Curriculum Vitae

Education

2011 – 2015 Roche Diagnostics GmbH, Penzberg, Germany

PhD research, subject "Development of mass spectrometric methods for the quantification of small molecules from the DAT/TDM field in biological samples with special focus on new sample preparation methods" under the supervision of Prof. Volmer from Saarland University, Saarbrücken.

2004 – 2011 University of Saarland, Saarbrücken, Germany and ECPM (European School of Chemistry, Polymers and Materials) Strasburg, France

French-German Chemistry studies and specialization in analytical chemistry and materials sciences.

Master Thesis ("Diplomarbeit") at Roche Diagnostics GmbH, Penzberg, Germany under the supervision of Prof. Volmer from Saarland University, Saarbrücken, Germany with the subject "Development of an LC-MS/MS reference method for the sensitive quantitation of Δ^9-tetrahydrocannabinol and its main metabolite in oral fluid"

Degrees: German "Diplomchemiker" and French: "Diplôme d'ingénieur", Grade: 1.8 (gut)

2004 *Abitur* and *Baccalauréat* (equivalent to High School Diploma) of the Deutsch Französisches Gymnasium (DFG) in Saarbrücken, Germany

Practical experience

2009 Dec – 2010 Jan *Vertiefungsarbeit* in the research group of Prof. Gregor Jung, Universität des Saarlandes, Saarbrücken, Germany

Development of a mass spectrometric method to analyze Bodipy-dyes

2009 Jul – Oct Internship in the research group of Prof. Richard G. Blair, University of Central Florida, Orlando, USA

Low temperature synthesis of SiI_4 using high-energy ball mills. Characterization of the solid state hydrolysis of cellulose by analyzing the change in degree of polymerization.

March 2009 *Travaux pratiques d'option* in the research group of Dr. Alain Van Dorsselaer, Laboratoire de Spectrométrie de Masse Bioorganique, Strasbourg, France

Proteomic analysis of interspecies transplants of human colorectal cancer cells

2008 Jul – Aug Internship at the Institute for New Materials Sciences, Saarbrücken, Germany

Development and optimization of a method to isolate aragonite particles from nacre. Characterization using regular and transmission electron microscopy.

IX. Scientific contributions

Publications

S. M. Hick, C. Griebel, D. T. Restrepo, J. H. Truitt, E. J. Buker, C. Bylda, R. G. Blair, Mechanocatalysis for biomass-derived chemicals and fuels. *Green Chemistry*, **2010**, 12, 468-474.

C. Bylda, A. Leinenbach, R. Thiele, U. Kobold, D.A. Volmer,, Development of an electrospray LC-MS/MS method for quantification of Δ^9-tetrahydrocannabinol and its main metabolite in oral fluid. *Drug Test. Anal*., **2012**, 4, 668-674.

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C. Bylda, V. Velichkova, J. Bolle, R. Thiele, U. Kobold, D.A. Volmer, Magnetic beads as an extraction medium for simultaneous quantification of acetaminophen and structurally related compounds in human serum, *Drug Test. Anal*., **2014**, DOI: 10.1002/dta.1708.

C. Bylda, R. Thiele, U. Kobold, D.A. Volmer, Simultaneous quantification of digoxin, digitoxin and their metabolites in serum using high-performance liquid chromatography tandem mass spectrometry, *Drug Test. Anal*., **2015**, DOI: 10.1002/dta.1781.

C. Bylda, R. Thiele, U. Kobold, A. Bujotzek, D.A. Volmer, Rapid quantification of digitoxin and its metabolites using differential ion mobility spectrometry-tandem mass spectrometry, *Anal. Chem*., **2015**, DOI: 10.1021/ac503187z.

Oral presentations

„Entwicklung einer LC-MS/MS-Referenzmethode zur sensitiven Bestimmung von THC und dessen Hauptmetaboliten aus Speichel“

DGKL LC-MS Anwendertreffen, 30. September 2011, Kloster Banz, Germany.

“Multiplex quantification of Acetaminophen and structurally related substances in human serum”

DGKL LC-MS Anwendertreffen, 6. November 2012, Kloster Banz, Germany.