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

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ABSTRACT

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

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

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

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

1. Introduction

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

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

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

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

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

2. Methods and results

2.1. Chemicals and reagents

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

2.2. HPLC–MS/MS conditions

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

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

2.3. Sample preparation

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

3. Results

3.1. Validation

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Discussion

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

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

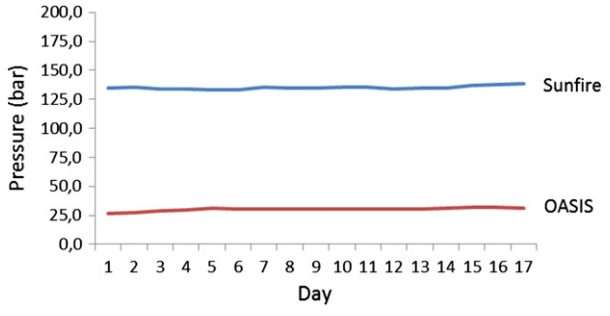

Fig. 1. Back-pressure of analytical column (Sunfire) and on-line extraction column (OASIS) during method development and validation involving nearly 600 sample injections.

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

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

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

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

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

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