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Short Communication

Deproteination of whole blood for LC–MS/MS using paramagnetic micro-particles

Dorien Van den Bossche [a,b], Nele Peersman [a], Koen Desmet [a,c], Pieter Vermeersch [a,c], Steven Pauwels [a,c,*]

[a] Clinical Department of Laboratory Medicine, University Hospitals Leuven, Leuven, Belgium
[b] Department of Pharmaceutical Sciences, KU Leuven, Leuven, Belgium
[c] Department of Cardiovascular Sciences, KU Leuven, Leuven, Belgium

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ABSTRACT

Objectives: Liquid chromatography tandem mass spectrometry has become increasingly popular in clinical laboratories over the last decade due to the inherent sensitivity and specificity of the technology. Nevertheless, full automation and hence application in routine laboratories is still hampered by laborious and difficult-to-automate sample pre-treatment protocols. Functionalized paramagnetic micro-particles could simplify sample pre-treatment and ease automation. We evaluated the applicability of a pre-commercial, straightforward paramagnetic micro-particle based kit for the lysis and deproteination of whole blood for the LC–MS/MS analysis of everolimus and compared the performance to our routine protein precipitation method.

Design and methods: Samples were prepared for LC–MS/MS everolimus analysis on an Acquity UPLC chromatographic system coupled to a TQD mass spectrometer (both Waters Ltd.) using a pre-commercial MagSi-TDMprep kit and a routine protein precipitation respectively. Both pre-treatment methods were validated for imprecision, accuracy, linearity, limit of quantification, matrix effect, recovery and process efficiency. A method comparison ($n = 63$) between both pre-treatment methods was performed.

Results: Imprecision and bias were within pre-defined criteria (15%) for both pre-treatment methods. Both methods were linear from 1.2 to 14.8 μg/L with a limit of quantification of 0.6 μg/L. Process efficiency was on average 65% for protein precipitation pre-treatment and was substantially higher for the MagSi-TDMprep method (85%). A Passing–Bablok regression showed no significant difference between the two pre-treatment methods.

Conclusion: For everolimus in whole blood, the MagSi-TDMprep sample pre-treatment was applicable and comparable to protein precipitation for LC–MS/MS with the possible advantage of easier automation.

Introduction

The use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) has increased substantially over the last decade. In clinical laboratories, popularity is still growing with new applications emerging regularly in different fields of laboratory medicine [1]. There is an ongoing discussion on the ability of mass spectrometry to replace immunoassays as user-friendly, random-access analyzers [2–4]. Recently, some significant steps towards the dream of automated LC–MS/MS have been published, particularly simplifications in the quantification method replacing traditional calibration curves in each run with simple direct quantification techniques [5,6]. Nevertheless, full automation is still impeded by other problems. Laborious sample pre-treatment protocols are one of the main obstacles still hampering the automation of LC–MS/MS methods. Most popular protocols like protein precipitation by organic solvents or salts, ultrafiltration or liquid–liquid extraction consist of steps like centrifugation, application of positive pressure, vacuum or nitrogen streams that are difficult to automate.

Functionalized paramagnetic micro-particles are already widely used in immunoanalyzers and DNA extraction protocols to streamline and automate the processes. Also for LC–MS/MS analyses the application of paramagnetic micro-particle-based technology has been proposed as a useful sample pre-treatment strategy, although reported methods are not straightforward and require multiple steps for washing or eluting [7–9]. Recently, an easy-to-use kit for the deproteination of serum samples for LC–MS/MS, based on protein denaturation by using a proprietary reagent containing paramagnetic micro-particles (MagnaMedics Diagnostics BV, Geleen, The Netherlands), was used for the LC–MS/MS analysis of amiodarone [10]. The process was very straightforward as only one particle-immobilization step was needed. The performance relative to other simple pre-treatment strategies was however not established.

We first evaluated the applicability of a pre-commercial, straightforward paramagnetic micro-particle based kit for the lysis and

* Corresponding author at: University Hospitals Leuven, Laboratory Medicine, Herestraat 49, 3000 Leuven, Belgium. Fax: +32 16 34 70 42.
E-mail address: steven.pauwels@uzleuven.be (S. Pauwels).

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deproteination of whole blood for the LC–MS/MS analysis of everolimus (MagSi-TDMprep). We also positioned the performance of the paramagnetic micro-particle extraction to our routine simple protein precipitation method.

Materials and methods

Chemicals and solutions

The primary stock solutions of everolimus (Sigma-Aldrich, Steinheim, Germany) and *d4*-everolimus (Toronto Research Chemicals, Toronto, Canada) (internal standard (IS)) with a concentration of 1 mg/mL were prepared in methanol and acetonitrile respectively and stored at −80 °C. Five calibrators (1.2, 2.1, 3.9, 7.6 and 14.8 μg/L) were prepared by spiking drug-free human EDTA whole blood (routine left-over). Three quality control (QC) levels were purchased from Chromsystems (Gräfelfing, Germany). Acetonitrile and methanol were purchased from BioSolve (Valkenswaard, The Netherlands). Analytical grade formic acid was acquired from Sigma-Aldrich, analytical grade ammonium acetate from Merck (Darmstadt, Germany). HPLC-grade water was generated using a Milli-Q-water-purification system (Millipore, Molsheim, France).

Sample preparation and LC–MS/MS conditions

Samples were prepared by a pre-commercial MagSi-TDMprep kit on the one hand and by simple protein precipitation on the other hand. The MagSi-TDMprep kit for whole blood contained a suspension of proprietary surface modified paramagnetic micro-particles, a proprietary denaturation reagent and a proprietary lysis reagent. 100 μL lysis reagent was added to 50 μL EDTA whole blood, the samples were immediately vortexed and kept for 1 min at room temperature. Subsequently, 25 μL of the paramagnetic micro-particle suspension was added. The tubes were vortexed and kept at room temperature for 3 min. In the last step, the proteins were denatured by 500 μL protein denaturation reagent containing IS (concentration 1.7 μg/L). The tubes were then placed in a magnetic particle separator (MagnaMedics MM-separator M12 + 12) in order to separate the micro-particles and proteins from the supernatant. 200 μL of the supernatant was transferred into HPLC-vials for LC–MS/MS analysis and 20 μL was injected in the chromatographic system.

Our routine protein precipitation procedure consisted of the addition of 300 μL of a 55:45 (*v*/*v*) methanol:acetonitrile mixture containing internal standard (concentration 2.5 μg/L) to 50 μL whole blood. After the addition, samples were vortexed and incubated for 20 min at room temperature. Then, samples were vortexed again, centrifuged (10 min at 16,100 *g*) and 20 μL of the supernatant was injected in the chromatographic system.

Chromatographic separation was carried out on an Acquity UPLC system (Waters Ltd.). Only a pre-column (Phenomenex Security Guard Cartridge (C18, 4 × 3.0 mm)), maintained at 50 °C, was used as stationary phase. The mobile phase was a mixture of methanol (buffer A) and water (buffer B) containing 0.1% formic acid and 2 mM ammonium acetate. Linear gradient elution starting from 50% buffer A augmenting to 100% buffer A at 0.60 min and back to 50% buffer A at 2.00 min was used. The total run time was 3.00 min. The flow was set at 0.60 mL/min. Mass spectrometric analysis was performed using a tandem mass spectrometer (TQD, Waters Ltd.) equipped with an electrospray ionization source operating in the electrospray-positive mode. The source and desolvation temperature were set at 150 °C and 450 °C. Capillary voltage was set at 3.50 kV, cone voltage at 24 V, collision energy at 16 eV. Everolimus and IS were measured by multiple reaction monitoring (MRM) monitoring of the respective ammonium adducts. The following MRM transitions were monitored: *m*/*z* 975.5 → 908.3 for everolimus and *m*/*z* 979.5 → 912.3 for IS.

Everolimus was quantified by means of calibration to each run, using a weighted ($1/\chi^2$) least square linear regression in QuanLynx software (Waters Ltd, Watford, UK) of the 5 calibrators.

Analytical validation

The LC–MS/MS method using the MagSi-TDMprep pre-treatment protocol was validated for imprecision, accuracy, linearity, limit of quantification (LOQ), matrix effect, recovery and process efficiency.

Within-run imprecision was determined by running left-over patient samples ten-fold at three concentration levels (2.1 μg/L, 4.2 μg/L and 6.8 μg/L). Between run imprecision was determined by analyzing the same samples in ten separate runs spread over 5 days. A maximum CV of 15% was tolerated [11].

Accuracy was evaluated by comparing the measured concentration to the concentration indicated on the insert of the commercial controls (three levels, 1.8 μg/L, 3.2 μg/L and 6.0 μg/L). A maximum bias of 15% was tolerated [11].

Linearity was evaluated by the correlation coefficient (R^2) of the calibration curve (criterion $R^2 > 0.99$).

LOQ was defined as the lowest concentration with a CV < 20% and a S/N > 10 [12]. LOQ was determined by serially diluting (1 in 2) the lowest calibrator (1.2 μg/L) with blank whole blood and determining between-run precision (at 0.6 μg/L and 0.3 μg/L). Between-run precision was determined by performing 10 runs on 6 different days for each dilution.

Recovery, matrix effect and process efficiency were determined by spiking methanol:water 1:1 (*v*/*v*) at 5.0 μg/L everolimus (A), by spiking the same concentration after extraction of blank whole blood (6 different matrices) (B) and in whole blood before extraction (6 different matrices) (C). Matrix effect was calculated as a percentage by the formula: Peak area B/Peak area A × 100. Recovery is calculated as a percentage by the formula: Peak area C/Peak area B × 100; process efficiency as Peak area C/Peak area A. All analyses were performed in triplicate.

We compared the results of the analytical validation of the method using paramagnetic particles as pre-treatment with the results obtained for our routine protein precipitation method, validated with similar methodology and criteria.

The use of left-over patient blood was in accordance with the ethical standards of the responsible institutional committee on human experimentation.

Method comparison

63 patient samples were analyzed using both protein precipitation and paramagnetic particles as sample pretreatment. A Passing–Bablok regression and a Bland–Altman plot were performed in Microsoft Excel Analyse it (version 2.26) to evaluate the difference.

Results

Fig. 1 shows representative chromatograms at 0.6 μg/L everolimus in whole blood of the routine protein precipitation method and the MagSi-TDMprep method. The retention time was 0.9 min for both methods. Results of the analytical validation and comparison to our routine protein precipitation method are presented in Table 1. Fig. 2 shows the Passing–Bablok regression comparing the protein precipitation and the MagSi-TDMprep pre-treatment method everolimus results. The regression equation shows an intercept of −0.20 μg/L with a 95% confidence interval of −0.55 to 0.01 and a slope of 1.00 with a 95% confidence interval of 0.93–1.10. The intercept was not significantly different from 0 and the slope not significantly different from 1. The Bland–Altman plot (Fig. 2) shows a non-significant bias with relatively normal scatter in context with the concentrations measured by both methods.

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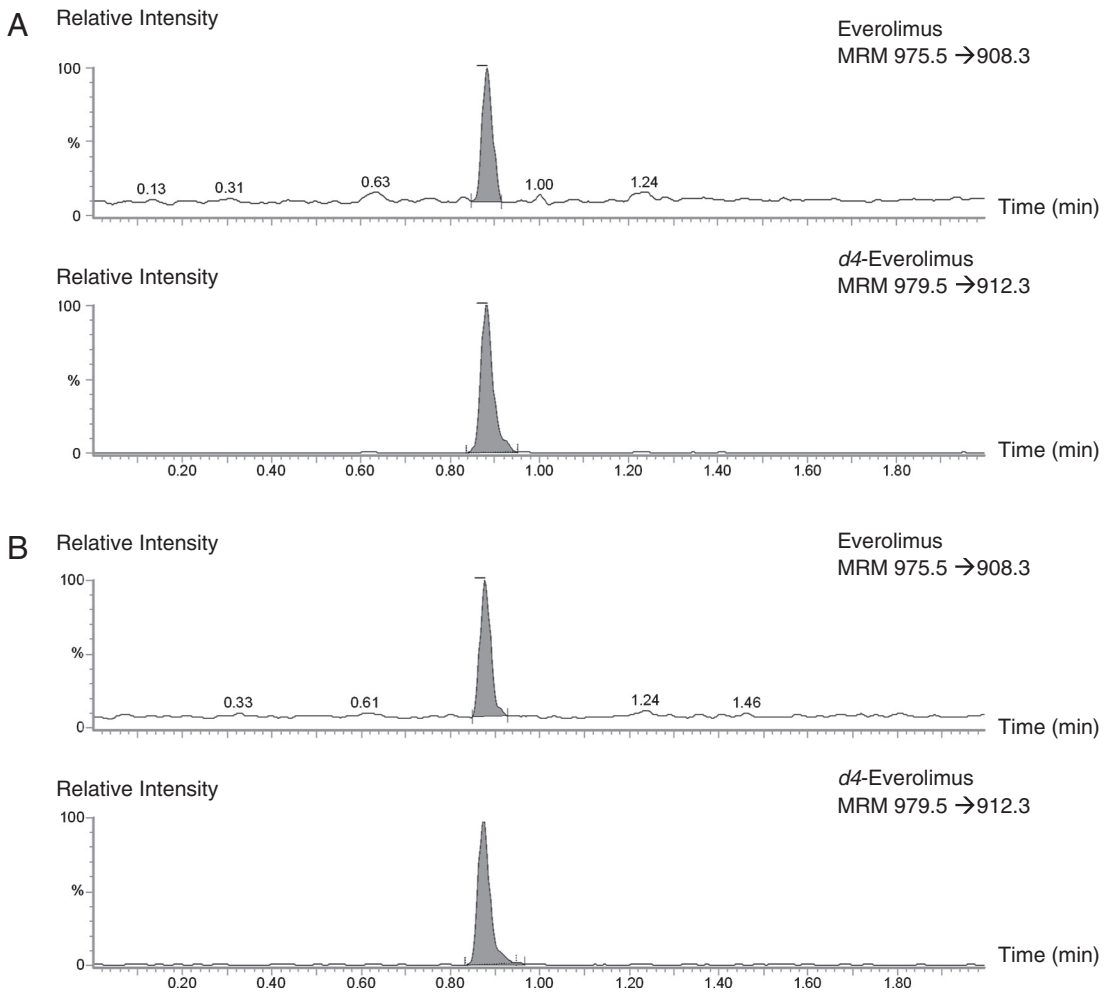

Fig. 1. Representative chromatograms at 0.6 μg/L everolimus in whole blood of the MagSi-TDMprep method (Panel A) and the routine protein precipitation method (Panel B).

Discussion

We are the first to evaluate the applicability of a pre-commercial kit for lysis and deproteination of whole blood as sample pre-treatment for LC–MS/MS analysis. The analytical validation results were within pre-defined criteria and comparable to the results obtained for our routine protein precipitation method for everolimus, except for matrix effect. Our results show a substantial lower matrix effect using the MagSi-TDMprep sample pre-treatment as compared to simple protein precipitation. This effect might, however, be caused by the larger dilution using the MagSi-TDMprep method. For the paramagnetic particle based protocol a dilution of 1 in 13.5 is used compared to a 1 in 7 dilution for the routine protein precipitation method. The internal standard compensated adequately for the matrix effect and recovery.

Table 1
Analytical validation results for our routine protein precipitation and MagSi-TDMprep pre-treatment method for everolimus quantification in whole blood using LC–MS/MS.

	Protein precipitation	MagSi-TDMprep
Within-run precision (CV range)	4–12%	6–14%
Between-run precision (CV range)	7–11%	8–13%
Accuracy (% range)	91–102%	86–91%
LOQ	0.6 μg/L	0.6 μg/L
Linear range	1.2–14.8 μg/L	1.2–14.8 μg/L
Recovery (mean % (range))	95% (94–97%)	98% (89–103%)
Matrix effect (mean % (range))	67% (66–69%)	85% (68–102%)
Process efficiency (mean % (range))	65% (64–66%)	85% (68–96%)

We further investigated on the origin of the difference in matrix effect by modifying our routine method to the same dilution factor as the MagSi-TDMprep method (1 in 13.5) and determining the matrix effect on two different matrices (blank EDTA blood). Briefly, we added 625 μL instead of 300 μL of a 55:45 (*v*/*v*) methanol:acetonitrile mixture containing internal standard to 50 μL of whole blood. The matrix effect using our modified routine method was 91% for the first and 60% for the second matrix. For the MagSi-TDMprep method, similar matrix effects (90% and 68% respectively) were observed indicating the diminished matrix effect is largely caused by dilution effects. As peak areas using our routine protein precipitation (dilution 1 in 7) were still higher than peak areas for the MagSi-TDMprep pre-treatment (dilution 1 in 13.5), the decreased matrix effect does not completely offset the increased dilution effect (lower analyte concentration injected). Our routine protein precipitation method was still slightly more sensitive, although LOQ criteria were also only met up till 0.6 μg/L everolimus.

In our study the protocol for MagSi-TDMprep pre-treatment was performed manually. This was more complicated than protein precipitation pre-treatment. The paramagnetic micro-particle protocol is, however, developed for full automation which will be easier to accomplish as no difficult pre-treatment steps like centrifugation, vacuum, pressure or nitrogen stream are needed. Also, the precision will probably benefit from full automation. However, using this technology, other issues can arise. For example, the magnetic sample separator

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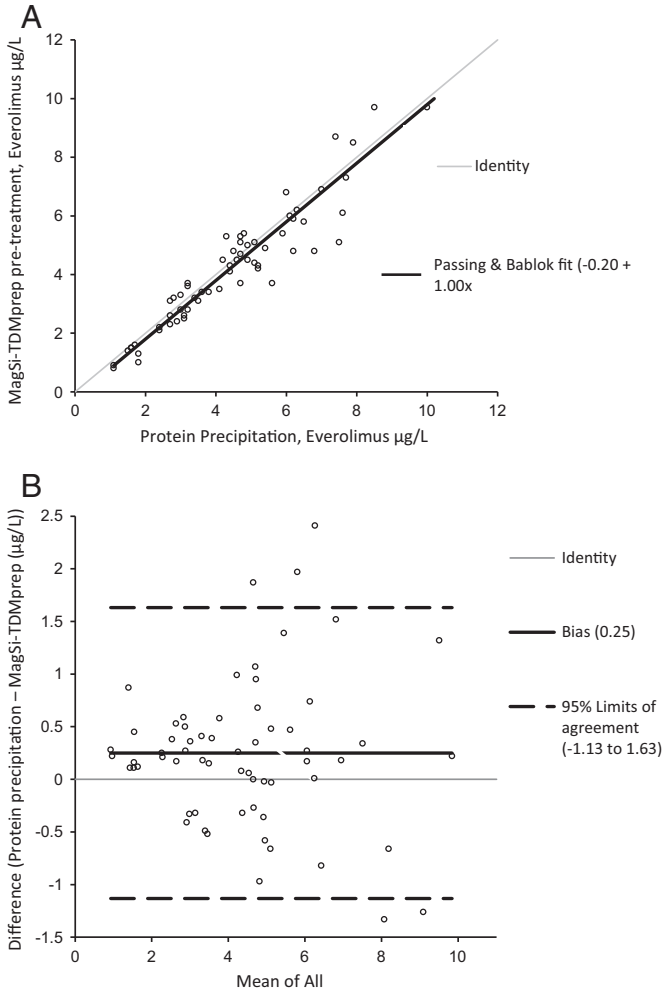

Fig. 2. The Passing–Bablok regression (Panel A) and Bland–Altman plot (Panel B) for 63 patient results for everolimus in whole blood using protein precipitation and MagSi-TDMprep as sample pre-treatments.

needs to be powerful enough to rapidly and adequately separate the paramagnetic particles from the supernatant.

Recently, a kit for the deproteination of serum samples for LC–MS/MS, based on protein denaturation by using a proprietary reagent containing paramagnetic micro-particles from the same manufacturer (MagnaMedics Diagnostics BV, Geleen, The Netherlands), was used for the LC–MS/MS analysis of amiodarone [10]. In this paper, the deproteination of serum by paramagnetic micro-particles was followed by online solid phase extraction. As solid phase is a powerful sample clean-up method, the stand-alone potential of the paramagnetic particles potential was not established. Also, no comparison with respect to other popular and widely used extraction techniques like protein precipitation was performed. Lastly, serum contains less possible interfering substances as whole blood.

In conclusion, for everolimus in whole blood, the MagSi-TDMprep sample pretreatment was applicable and comparable to protein precipitation as LC–MS/MS sample pre-treatment with the possible advantage of easier automation.

Conflicts of interest

All authors declare no conflicts of interest.

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