Short communication

Sensitive quantification of sirolimus and everolimus by LC–MS/MS with online sample cleanup

Daniel M. Mueller, Katharina M. Rentsch *

Institute for Clinical Chemistry, University Hospital Zurich, Raemistrasse 100, 8091 Zuerich, Switzerland

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ABSTRACT

Sirolimus and its derivative everolimus are widely used today as immunosuppressive agents for example in the transplantation medicine. The problematic pharmacokinetic behavior of those substances makes therapeutic drug monitoring mandatory. Therefore, a fast, simple and sensitive high-throughput procedure using online extraction with turbulent flow chromatography for the concurrent measurement of sirolimus and everolimus has been developed. 200 μl of whole blood was mixed with internal standard (23-desmethoxyrapamycin) and the precipitation solution and centrifuged. An aliquot of the supernatant was transferred into autosampler vials. 50 μl of the supernatant was injected into the LC system, where the analytes were extracted using turbulent flow chromatography and thereafter analyzed using reversed phase chromatography. Detection was done by atmospherical pressure chemical ionization (APCI) mass spectrometry in the negative ionization mode. The method has been fully validated and compared to a previously used method. The method was shown to be linear over the entire calibration range (2.2–43.7 μg/l for everolimus and 2.9–51.2 μg/l for sirolimus). The lower limit of quantification was 0.5 μg/l for both compounds. For within-day and between-day analysis, the CV's were <7.6% for everolimus and <8.7% for sirolimus, respectively. The accuracy was between 92.1% and 105% for everolimus and 96.1% and 106% for sirolimus. Recovery ranged between 46.3% and 50.6% for everolimus and 51.2% and 57.2% for sirolimus. The method was demonstrated to be free of matrix effects and comparable to the previously used method. The presented LC–MS/MS method, using turbulent flow chromatography online extraction, allows a fast, simple and reliable determination of everolimus and sirolimus.

1. Introduction

Sirolimus, originally isolated from *Streptomyces hygroscopicus* [1] and its derivative everolimus are widely used as immunosuppressants. Both drugs show the same mode of action: they form a complex with FKBP12 [2], which blocks the mammalian target of rapamycin, mTOR, thereby inhibiting its downstream signaling [3]. mTOR is thought to be a major regulator of cell growth and proliferation [3].

Newer applications include cancer therapy. Sirolimus-sensitive signaling pathways are known to be active in many cancer types [3], therefore opening a new therapy approach. Other new applications include also the treatment of autosomal-dominant polycystic kidney disease, where today no causal treatment is known [4].

Because of the problematic pharmacokinetic behavior of these substances with large inter- and intraindividual variations, no good correlation can be found between the dose administered orally and the achieved systemic concentrations [2,5]. Since an important elimination pathway is via the cytochrome P450 system (especially via 3A4 isoenzyme [2]) both drugs are prone to drug–drug interactions. For all these reasons, sirolimus and everolimus are ideal candidates for therapeutic drug monitoring (TDM). Since there is a good correlation between the area under the curve and the minimal concentration in steady state [5], TDM is done most conveniently by measuring only the trough concentration. Widely accepted reference values for therapeutic trough concentrations in whole blood have been established.

TDM on sirolimus and everolimus is widely done, which can also be seen by the large number of papers published using different quantification methods, as recently reviewed by Korecka et al. [6]. In principal, sirolimus and everolimus can be determined either with immunoassays or with chromatographic methods, mainly LC–MS/MS. Immunoassays have the drawback that they are not specific enough to only measure the unchanged substance. Therefore, results obtained with the immunoassays are generally higher as compared to those measured with chromatographic methods [7]. For methods using chromatography, there are few using UV detection [8]. Today, mostly tandem MS is used as detection method

* Corresponding author. Tel.: +41 44 255 20 90; fax: +41 44 255 45 90.
  E-mail address: rentsch@access.uzh.ch (K.M. Rentsch).

[9–11]. Within the last 10 years, several online extraction procedures have been published [12–23]. Most of them use either online solid-phase extraction (SPE) or an additional chromatographic column for extraction. With one exception [19] all authors describe the detection of sirolimus and/or everolimus as adduct ions after electrospray ionization.

This paper presents a simple and sensitive LC–MS/MS procedure using online extraction with turbulent flow chromatography and atmospheric pressure chemical ionization (APCI) as well as the direct detection of the negatively charged molecules for the concurrent measurement of sirolimus and everolimus.

2. Materials and methods

2.1. Chemicals and reagents

Sirolimus and 23-desmethoxyrapamycin were purchased from Sigma–Aldrich (Buchs, Switzerland), everolimus from Fluka (Buchs, Switzerland). Ammonium acetate was purchased from Scharlau (Taegerig, Switzerland) and formic acid from Merck (Darmstadt, Germany). Calibrators were obtained from Chromsystems (Munich, Germany) and quality control samples from Recipe (Munich, Germany).

LC–MS grade methanol and 2-propanol was purchased from Seelze GmbH (Seelze, Germany), acetonitrile from Romil (Cambridge, Great Britain), and acetone from Merck (Darmstadt, Germany). Purified water was obtained using a central water purification installation (Burkhalter AG, Worblaufen, Switzerland).

2.2. Standard solutions

Standard solutions for tuning the MS and optimizing the chromatography were dissolved in acetonitrile to reach a concentration of 10 μg/l. Lyophilized calibrators and quality control samples were dissolved according to the instructions of the manufacturers.

2.3. Patient samples

Patient samples sent to the laboratory for the quantification of sirolimus or everolimus were anonymously taken out of the archive and reanalyzed with the new method.

2.4. Sample preparation

200 μl of whole blood was mixed with 50 μl of the internal standard solution (0.05 μg/l 32-desmethoxyrapamycin in acetonitrile) and 300 μl of precipitation solution (methanol/acetonitrile 90/10, v/v). The samples were vortexed for approximately 30 s, put in an ultrasonic bath for 2 min and vortexed for 30 s. The solution was allowed to stand for 6 min, vortexed for 30 s and centrifuged at 10,000 rpm for 10 min at 10 °C.

200 μl of the supernatant was transferred into autosampler vials, which were stored at 10 °C until injection. The injection volume was 50 μl.

2.5. LC–MS/MS analysis

The HPLC system consisted of a TLX-1 HTLC system (Thermo Fisher Scientific, Basel, Switzerland), consisting of two Allegro pumps, an HTC PAL autosampler and built in switching valves. Extraction was done using a Cyclone column (0.5 × 50 mm), separation with a Hypersil Gold C18 column with 1.9 μm particle size (2.1 × 50 mm, both Thermo Fisher Scientific, Basel, Switzerland), placed in a column oven held at 70 °C. The mobile phases consisted of 10 mM ammonium acetate/methanol 95/5, v/v, containing 0.1% formic acid (eluent A), methanol/acetonitrile 50/50, v/v, containing 10 mM ammonium acetate and 0.1% formic acid (eluent B), and acetonitrile/2-propanol/acetone 1/1/1, v/v/v (eluent C). During the first 0.5 min the sample was loaded onto the extraction column using 75% of eluent A and 25% of eluent B. Afterwards, the elution onto the analytical column was carried out using 65% eluent A and 35% of eluent B. Thereafter, the extraction column was washed and the separation of the analyte was performed on the analytical column. After 3.25 min the analytical column was washed and thereafter re-equilibrated for 1.5 min. The gradient program is shown in Table 1.

As mass spectrometer, a TSQ Quantum Access Max (Thermo Fisher Scientific, Basel, Switzerland) was used. Analytes were ionized by atmospheric pressure chemical ionization (APCI) in negative mode. Vaporizer temperature was kept at 450 °C, capillary temperature at 250 °C, and the discharge current was set at 4 μA. Sheath gas pressure was held at 20 AU, auxiliary gas pressure at 5 AU.

Selective reaction monitoring (SRM) was used for detection, using the deprotonated molecules of each molecule as precursor ions. Scan width was set to 1 m/z, scan time to 0.2 s and collision gas pressure was 1.5 mTorr. The following transitions were monitored: m/z 957.6 → 547.2 (collision energy 33 V) for everolimus, m/z 913.6 → 591.2 (collision energy 22 V) for sirolimus and m/z 883.3 → 517.4 (collision energy 30 V) for 23-desmethoxyrapamycin. One transition per analyte was regarded to be sufficient, since only few substances can be ionized in negative mode using APCI and since the masses of the analytes are quite high, therefore interferences with other compounds are unlikely. Also in most other methods published, only one transition per analyte is monitored [10,11,16].

2.6. Method validation

2.6.1. Linearity

The commercially available calibrators ranging from 2.2 μg/l to 43.7 μg/l for everolimus and 2.9 μg/l to 51.2 μg/l for sirolimus were prepared as described above. Standard curves were plotted as

Table 1
HPLC gradient program (5.75 min total run time).

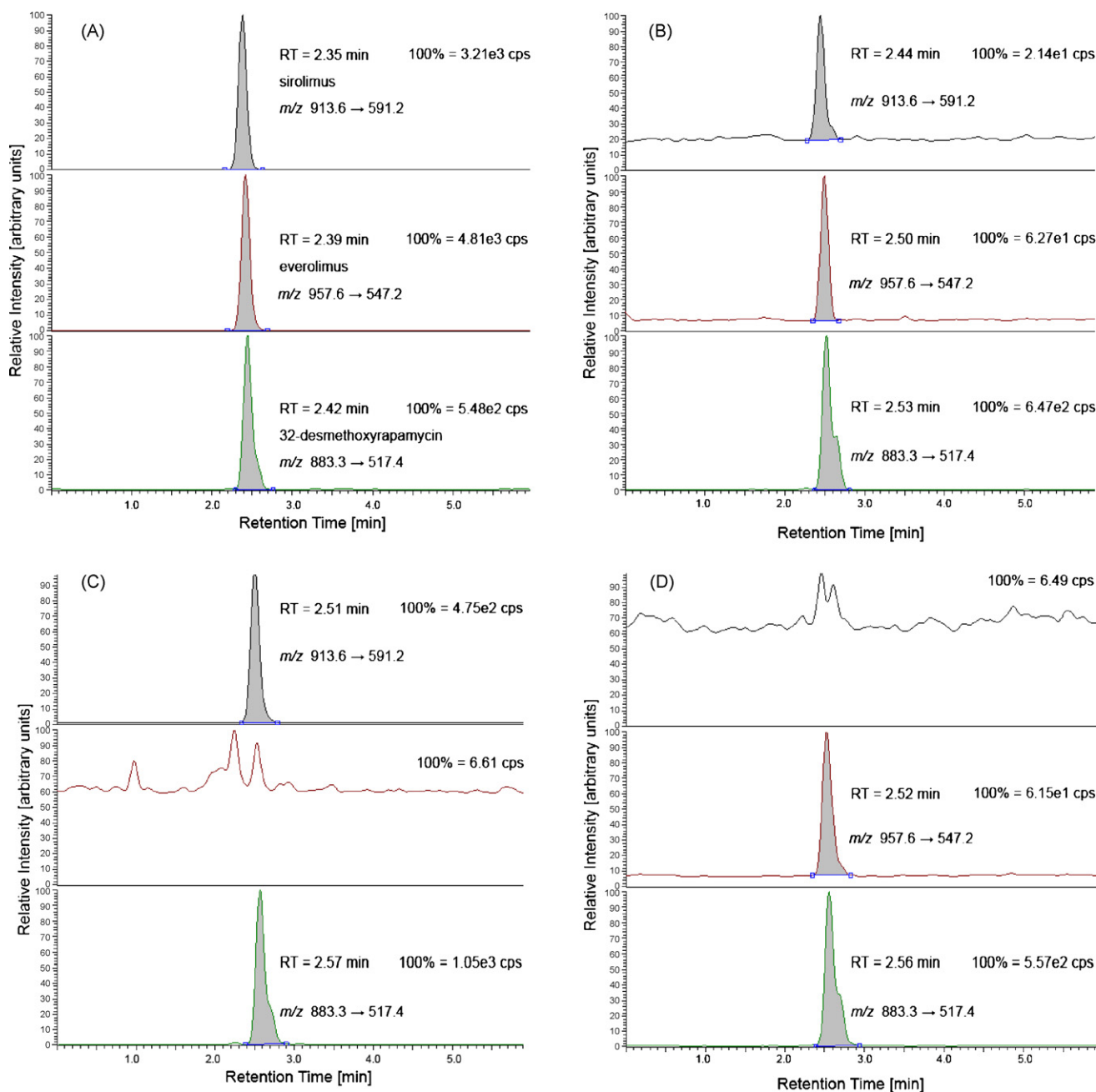| Time [min]	Loading pump				Eluting pump			
	Flow [ml/min]	%A	%B	%C	Flow [ml/min]	%A	%B	%C
0	2	75	25		0.75	75	25	
0.5	0.25	65	35		0.5	75	25	
1.58	2			100	0.75	25	75	
2.33	2		100		0.75	25	75	
2.58	2		100		0.75	25	75	
3.25	2		100		0.75			100
4.25	2		100		0.75	5	95	
5	2	75	25		0.75	75	25	
5.75	2	75	25		0.75	75	25	

Fig. 1. Representative chromatograms of (a) a standard supplemented with 43.7 μg/l for everolimus and 51.2 μg/l for sirolimus; (b) a spiked sample at the lower limit of quantification with 0.5 μg/l for everolimus and sirolimus; (c) a patient sample with 4.1 μg/l of sirolimus and (d) a patient sample with 0.8 μg/l of everolimus.

the peak area ratio (compound/internal standard) versus the concentration and corrected by subtracting blank peak area ratios. To assess linearity, the line of best fit was determined by least square regression. Calculation of the concentration of samples with concentrations below the first calibration standard was performed by proportional conversion using the respective peak area ratios of the first calibration standard.

2.6.2. Imprecision, accuracy, limit of quantification and recovery

For the determination of the between-day and within-day imprecision and accuracy, commercially available quality control samples were prepared as described above. Quality control (QC) samples of 3 levels were analyzed five times on the same day (within-day precision) and once on 5 different days (between-day precision).

The lower limit of quantification (LLOQ) of the different analytes was determined by calculating the coefficient of variation (CV) of different samples with decreasing concentrations. The last concentration where the CV was <20% and the accuracy ±20% was taken as limit of quantification.

Recovery was determined with the 3 quality control levels by comparing the peak areas of spiked whole blood samples with the peak areas of spiked aqueous samples with the same concentration, which were directly injected onto the analytical column, omitting the online extraction step.

2.6.3. Matrix effects

Ion suppression was evaluated according to the method described by Bonfiglio et al. [24]. A solution containing everolimus, sirolimus and 32-desmethoxyrapamycin (each 10 μg/l) was
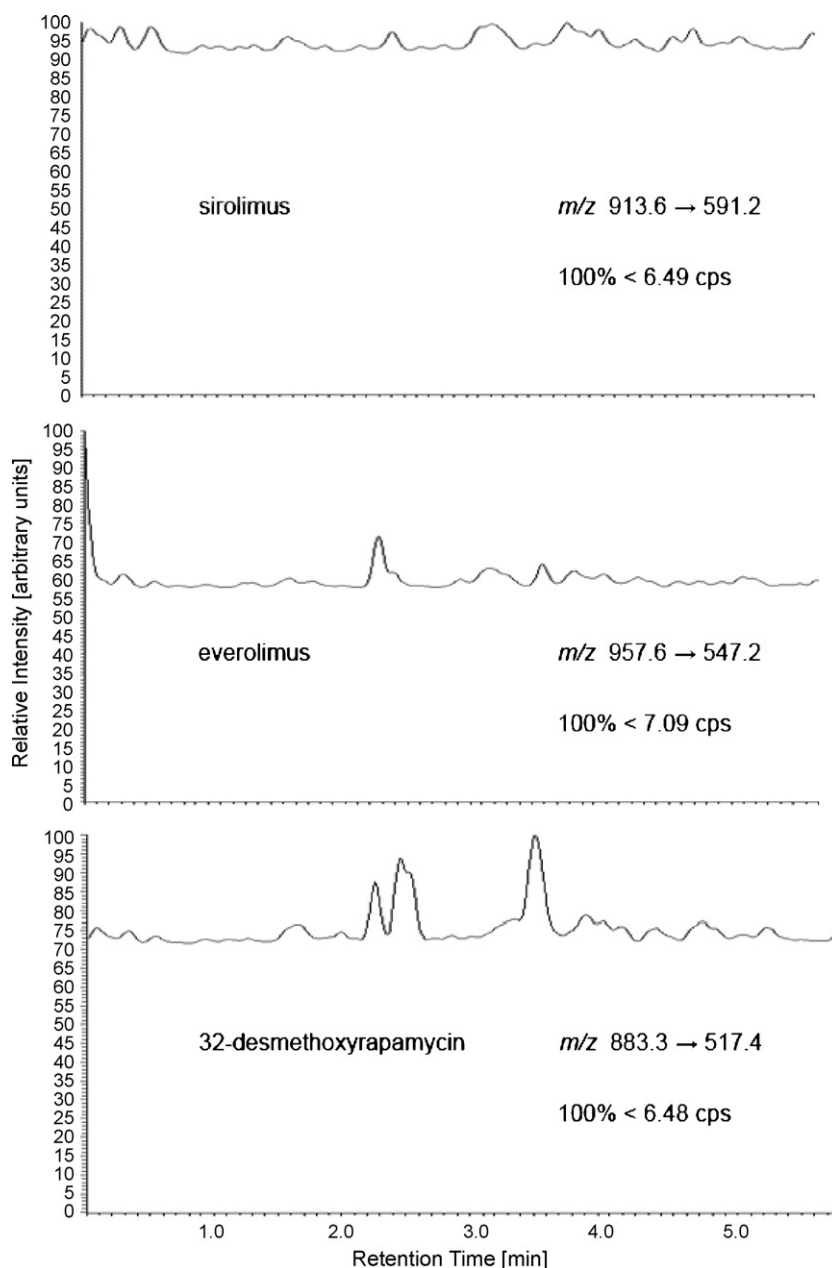
Fig. 2. Example of the effect of the whole blood matrix on the signal intensities of everolimus, sirolimus and 32-desmethoxyrapamycin determined by direct infusion of an analyte solution via a T-piece during injection of a blank whole blood matrix from a person not taking sirolimus or everolimus. The observed fluctuations of the baseline are on the intensity level of noise.

infused into the column effluent via a T-valve at a flow-rate of 5 μl/min. 5 precipitated whole blood samples of different persons not taking sirolimus or everolimus were injected by the autosampler. The resulting chromatograms were evaluated for regions showing ion suppression or enhancement.

2.6.4. Stability of the precipitated samples

The stability of the precipitated samples over the whole concentration range was assessed with all 3 QC levels for 15 h in the cooled autosampler stack (10 °C).

2.6.5. Comparison to the existing method

Patient samples which were already analyzed with an existing, validated offline method [25] were reanalyzed with the new online method.

3. Results

3.1. LC–MS/MS analysis

Representative chromatograms of everolimus, sirolimus and 32-desmethoxyrapamycin are depicted in Fig. 1. Since the fragmentation of the substances did not show any common fragments, a chromatographic separation of the 3 compounds was not necessary. Especially at the peak of the internal standard, a shoulder – due to different isomers – could be detected. As the reproducibility of the peak area of the internal standard was satisfying, this shoulder was not considered to be an analytical problem.

3.2. Method validation

3.2.1. Linearity

The standard curve was found to be linear over the whole calibration range (2.2–43.7 μg/l for everolimus and 2.9–51.2 μg/l for

Table 2
Imprecision and accuracy data.

Compound	Theoretical concentration, μg/l	Measured concentration, μg/l ($n=5$)	Imprecision CV, %	Accuracy (%)
				Everolimus
Within-day	0.5	0.4	20.0	88.6
	3.34	3.51	7.35	105
	10.6	9.82	5.97	92.6
	18.2	16.8	5.42	92.1
Between-day	0.5	0.5	17.3	100
	3.34	3.12	7.59	93.5
	10.6	10.8	2.55	102
	18.2	17.8	5.60	97.6
Sirolimus				
Within-day	0.5	0.6	17.7	120
	3.64	3.52	8.70	96.8
	11.2	10.8	7.75	96.1
	18.9	20.1	5.06	106
Between-day	0.5	0.5	18.7	92.9
	3.64	3.87	3.79	106
	11.2	11.6	2.44	104
	18.9	19.6	1.38	104

sirolimus), showing very good reproducibilities and correlation coefficients >0.995 ($n=5$) for both analytes.

3.2.2. Imprecision, accuracy, limit of quantification and recovery

The results of imprecision experiments are summarized in Table 2. For everolimus, within-day and between-day imprecision was <7.6%, for sirolimus <8.7%. All those results are within the ranges requested by the FDA for bioanalytical method validation [26].

The accuracy was between 92.1% and 105% for everolimus and 96.1% and 106% for sirolimus. Also these values are within the requested ranges by the FDA.

The lower limit of quantification, defined as the lowest concentration having a CV ≤ 20% and accuracy between 80% and 120%, was 0.5 μg/l for both compounds (Table 2).

The recovery, measured at 3 different concentrations over the whole calibration range (each level $n=5$), ranged between 46.3% and 50.6% for everolimus and 51.2% and 57.2% for sirolimus.

3.2.3. Matrix effects

In Fig. 2, one example of the effect of the whole blood matrix on the signal intensities of everolimus, sirolimus and 32-desmethoxyrapamycin is shown. The experiment was done with a total of 5 different whole blood samples. In the relevant timeframes, no significant matrix effects were observed in all 5 analyzed whole blood samples.

3.2.4. Stability of the precipitated samples

The peak ratio (analyte/internal standard) varied between 86.9% and 105% for everolimus (each level $n=5$) and 98.2% and 108% for sirolimus (each level $n=5$) of the initial value after storing the precipitated samples for 15 h in the cooled autosampler tray at 10 °C.

3.2.5. Comparison to the existing method

Patient samples which were analyzed for sirolimus ($n=104$) or everolimus ($n=102$) as routine TDM in our laboratory were reanalyzed with the method described here. The results are depicted as Bland–Altman plot in Fig. 3. Generally, a good correlation between the offline and the online method was observed ($r=0.90$ for everolimus and $r=0.92$ for sirolimus). Some outliers are most likely due to problems with the internal standard which were sometimes observed with the old offline method, and are no longer present with the new online method.

4. Discussion

The method described allows a fast, precise and sensitive quantification of everolimus and sirolimus in whole blood samples. Since online sample preparation is used, the method is suitable for high-throughput measurements, significantly reducing the turnaround time of patient samples. Only few manual steps are needed, therefore handling errors and variability due to manual pretreatment can be reduced. Another advantage of the method is the small sample
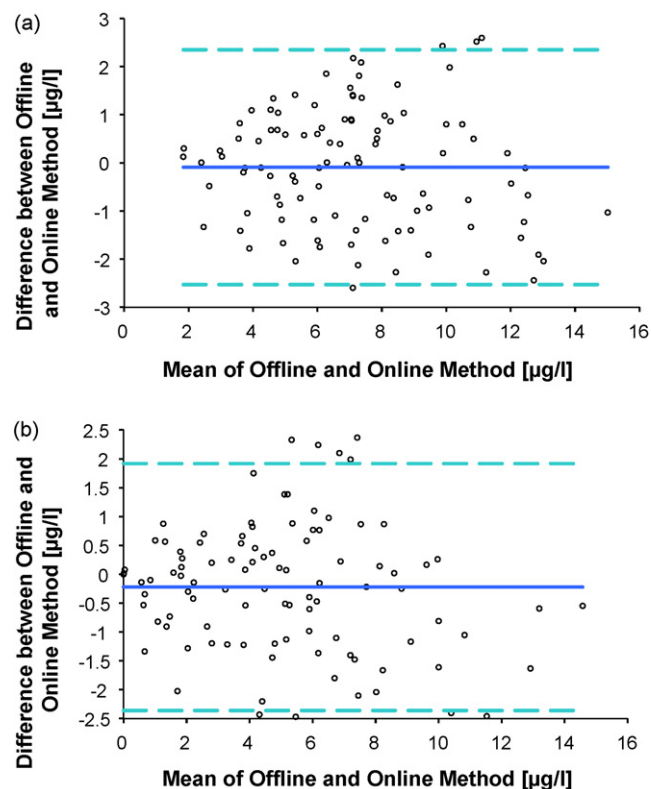

Fig. 3. Bland–Altman plots for the comparison between the offline and the online method (a) sirolimus ($n=104$); (b) everolimus ($n=102$). The dashed line indicates the 95% confidence interval, the solid line the bias of the online method versus the offline method.

volume needed, which allows determining both drugs in less than 0.5 ml EDTA blood.

The selected run time of the method is longer than in most online sample cleanup procedures [6,11,12,16–23]. The reason for this is the finding that longer cleaning and equilibration times lead to a more robust method, not showing any carryover and with significantly extended column lifetime (>600 injections).

Most LC–MS/MS methods used for the quantification of immunosuppressants are using ESI [9–18,20–22], most often in positive mode detecting ammonium or sodium adducts. Only a few authors describe a method using APCI [19,27]. As APCI in general is less prone to matrix effects than ESI, we already used APCI in our established offline method [25]. We still could apply negative APCI detecting the deprotonated molecules as precursor ions in the online method. We did not observe any matrix effects or specificity issues during the validation study and after the introduction of the method into the routine laboratory.

There are different approaches using online extraction for immunosuppressant drugs. Often online coupled SPE was performed [14,18,20,22]. These methods used sample volumes of 10–250 μl whole blood with resulting LLOQs in the range of 0.1–2.5 μg/l. In 2 former publications turbulent flow chromatography has been used as extraction method [12,21]. Only one paper presenting a turbulent flow extraction procedure [12] describes the analytical performance of the method using 50 μl of whole blood with an LLOQ of 0.4 μg/l. The method described here uses 200 μl whole blood and has a LLOQ of 0.5 μg/l for both analytes, which is comparable with the above mentioned methods. The precision and accuracy of all published methods for the quantification of everolimus and/or sirolimus are comparable.

Since the introduction of the method in the routine lab, over 800 samples were analyzed for everolimus, and more than 550 samples for sirolimus. The method has proved to be robust and the analysis time for batches with about 20 samples was reduced approximately 50%. In the meantime we have participated 6 times at external proficiency-testing schemes (D. Holt, UKNEQAS) and passed all 18 samples successfully. The mean bias was 3.87% for sirolimus and −5.51% for everolimus.

In summary, the presented method using turbulent flow chromatography for online extraction allows a fast and simple determination of everolimus and sirolimus on a daily base, characterized by a good reproducibility and accuracy.

Acknowledgements

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