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Simultaneous determination of four immunosuppressants by means of high speed and robust on-line solid phase extraction–high performance liquid chromatography–tandem mass spectrometry☆

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Abstract

In this study immunosuppressants, i.e. cyclosporin A (CyA), tacrolimus (TRL), sirolimus (SRL) and everolimus (RAD) were quantified in whole blood samples from immunosuppressant treated transplant recipients by an integrated on-line solid phase extraction–high performance liquid chromatography–tandem mass spectrometry (SPE–HPLC–MS/MS) system. This method has been developed to improve the following characteristics: speed, robust analysis, simultaneous determination and low cost. This can be achieved by the use of a perfusion column as an extraction cartridge in combination with a short HPLC column and highly selective and sensitive atmospheric pressure ionisation tandem mass spectrometry (API–MS/MS) in the multiple reaction monitoring (MRM) detection mode. This high throughput technique is perfectly appropriate for routine therapeutic drug monitoring (TDM) of organ transplanted patients.

Keywords: Cyclosporin A; Tacrolimus; Sirolimus; Everolimus

1. Introduction

Transplant recipients are treated lifelong with immunosuppressants to avoid organ rejection. However, all immunosuppressants have to be used within a narrow therapeutic window. Therefore, it is most important to regularly control drug level concentrations in blood by therapeutic drug monitoring (TDM). In the last years the number of transplant recipients permanently increases resulting in arising sample numbers which requires a fast, robust, selective and sensitive high throughput analysis of blood samples. Most commonly used immunosuppressants are cyclosporin A (CyA), tacrolimus (TRL) and sirolimus (SRL). Recently, everolimus (RAD) is approved to be an additional immunosuppressive drug in Europe.

Determination of immunosuppressants in blood samples has been shown in a multiplicity of recent publications. Nowadays the high performance liquid chromatography (HPLC) in combination with atmospheric pressure ionisation mass spectrometry (API–MS) or tandem mass spectrometry (API–MS/MS) [1–12] is preferably applied for the determination of immunosuppressants in whole blood samples. These methods will replace more and more the determination by means of enzyme-linked immunosorbent assay (ELISA) or microparticle enzyme immunoassay (MEIA) [13–17] in the future. For TDM of sirolimus and everolimus no commercial immunoassays are available. However, using ELISA or MEIA undesired cross reactivities between parent drug and its metabolites can be observed very often. In contrast, the LC–MS/MS technique is able to avoid such matrix effects by detecting compound specific masses (single ion monitoring, SIM) or mass transitions (multiple reaction monitoring, MRM).

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The highly selective and sensitive HPLC–MS coupling technique proved to be a suitable analysis method for the determination of widespread analytes in environmental, food and even pharmaceutical samples. Currently immunosuppressant drugs are frequently applied in combination allowing a dose reduction of each single component leading to significantly increased survival rates of transplant recipients [18]. The main advantages of HPLC–MS are simultaneous and sensitive determination of several analytes by using MRM detection mode of the MS/MS system, a short total analysis time, and by high throughput reducing the financial costs to a minimum for the single sample. Additionally, a simple off-line precipitation step followed by on-line SPE offers a fast sample preparation.

The used hardware equipment and its configuration is very important to achieve short total analysis time for coupling on-line SPE with HPLC. In the last years such technical devices, like the high throughput liquid chromatographic system (HTLC 2300, Cohesive Technologies, USA) or the Prospekt system (Spark Holland, The Netherlands), were commercially developed. The HTLC 2300 system is using the Turbulent Flow SPE and the Prospekt system is able to automatically change SPE cartridges. Both systems enables high throughput analysis systems. They were developed especially for pharmacokinetic applications [19–23] and were also introduced in environmental analysis of pesticides in water [24,25]. Unfortunately, these techniques are relatively cost-intensive.

Here we describe a high speed and very robust on-line SPE–HPLC–MS/MS system for high throughput multicomponent analysis of CyA, TRL, SRL and RAD in patient blood samples. A simple binary pump system is used in combination with a central switching valve at isocratic operation. The extremely short total analysis time of 2.5 min is realised by use of perfusion column as SPE cartridge and short HPLC column at high flow rates. Performance parameters of this method like limits of detection, lower limits of quantification (LLOQ), matrix effects, precision and accuracy of drug concentrations are evaluated in spiked normal blood samples and more than 2000 patient blood samples were analysed so far.

2. Experimental

2.1. Chemicals

Solvents used as eluents were methanol and water (J.T. Baker, HPLC-gradient grade, Deventer, The Netherland). Ammonium acetate (fractorpur grade) was purchased from Merck (Darmstadt, Germany) and acetic acid was applied from Riedel-de Haen (Hannover-Seelze, Germany). Both were added to high organic eluent A (see Section 2.5).

Investigated immunosuppressants are listed in Table 1. SRL was a kind gift from Wyeth-Ayerst (Princeton, USA). CyA and RAD were kindly donated by Novartis (Basle, Switzerland). TRL was a kind gift of Fujisawa Pharmaceuticals (Osaka, Japan). Ascomycin was purchased from Sigma–Aldrich (Steinheim, Germany) and cyclosporin D (CyD) was a kind gift of Novartis (Basle, Switzerland). Ascomycin was used as internal standard (IS) for SRL, RAD and TRL. CyD was applied as IS for CyA.

$ZnSO_4$ (Titrisol) was obtained from Merck (Darmstadt, Germany). CyA, TRL, SRL, RAD, Ascomycin and CyD were used in methanolic stock solutions at a concentration level of 1 mg ml^{-1} and stored at −20 °C.

2.2. Calibrators and quality control samples

Healthy donors' EDTA-treated whole blood, obtained from the local blood bank, were used for calibrators and quality control samples. For calibration these samples were spiked with immunosuppressants within the concentration range between 1 and 50 ng ml^{-1} for SRL, RAD, TRL (1, 2, 5, 10, 15, 50 ng ml^{-1}) and between 10 and 1000 ng ml^{-1} for CyA (10, 50, 100, 150, 500, 1000 ng ml^{-1}). In detail 100 ml EDTA-treated whole blood of the healthy donor was spiked with 100 μl of a methanolic combined solution containing 50 μg ml^{-1} SRL, TRL, RAD and 1000 μg ml^{-1} CyA as the highest combined calibrator concentration. Spiked blood was further diluted with EDTA-treated whole blood to get the lower calibrators and quality control samples. All calibrators and quality control samples were aliquoted in 100 μl batches and immediately stored at −20 °C until

Table 1
Investigated immunosuppressants and MRM transitions employed for ESI–MS/MS detection, declustering potential (DP) and collision energy (CE) for API 3000, retention time for phenyl-hexyl HPLC column and peak number key (see Fig. 3)

	Cyclosporin A (CyA)	Tacrolimus (TRL)	Sirolimus (SRL)	Everolimus (RAD)	Ascomycin internal standard	Cyclosporin D (CyD) internal standard
MRM-Transition I (quantifier)	1219.95/1203.15	821.63/768.65	931.64/864.75	975.71/908.75	809.61/756.65	1233.94/1217.25
MRM-Transition II (qualifier)	1219.95/1185.05	975.71/908.75	931.64/882.85	975.71/858.75	809.61/564.55	1233.94/1199.05
DP (V)	56	56	51	51	56	51
CE (V)	33	31	23	25	31	29
Retention time (min)	2.13	1.99	2.01	2.03	1.99	2.17
Peak number	5	1	3	4	2	6

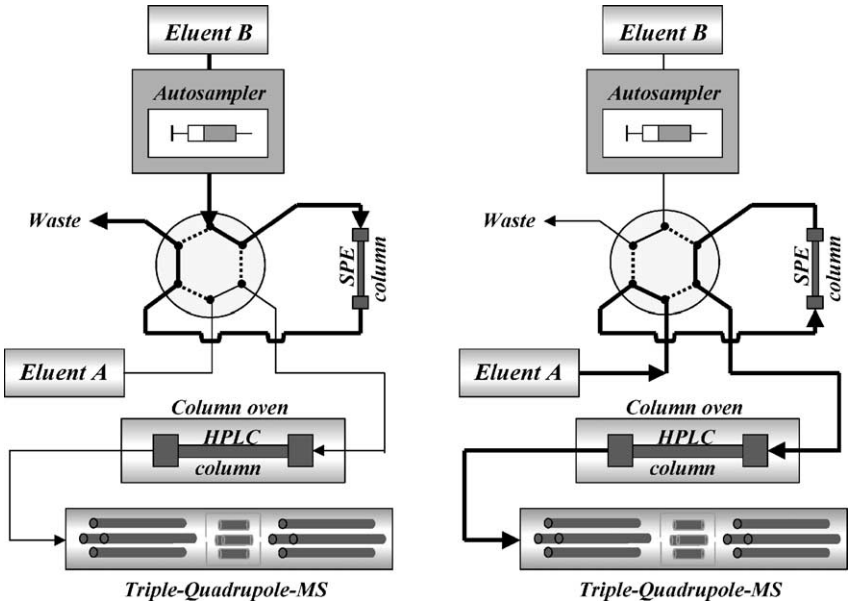

Fig. 1. Schematic view of the developed on-line SPE–HPLC–MS/MS system (left side: sample enrichment and rinsing step from 0.0 to 1.0 min, right side: analyte elution and transfer to HPLC column and analysis by means of API–MS/MS from 1.1 to 2.3 min).

analysis. Batches were stable for at least 6 months. One hour before sample preparation one batch of calibrators and quality control samples was thawed.

2.3. Sample preparation

One hundred microlitres of EDTA-treated whole blood samples were treated with 200 μl precipitation reagent (methanol/0.2 M $ZnSO_4$ (80/20, v/v)) including 20 ng ml^{-1} Ascomycin and 100 ng ml^{-1} CyD in polypropylene tubes. Samples were immediately vortexed (20 s and centrifuged for 15 min at 20,800 × *g* at 4 °C. Supernatant of 150 μl were transferred into a 96 flat bottom well plate (Nunc GmbH & Co., Wiesbaden, Germany) and closed with a 96 lid plate (Costar Storage Mat III^{TM}, Omnilab GmbH, Gehrden, Germany). During analysis samples were kept accurately at 20 °C using a temperature controlled autosampler to allow constant experimental conditions.

2.4. Instruments

The instrument set up is shown in Fig. 1. The configuration used here is consisting of a binary pump system with separately operated pump heads A and B (Perkin-Elmer Series 200 Micro Pump, Juegesheim, Germany). In addition, a temperature controlled autosampler (Perkin-Elmer Series 200 Autosampler) fitted with a six-port valve (Rheodyne series 7125) and a 100 μl sample loop were used. Column switching is performed via a software controlled Valco 10-port valve, however, only six ports are required as shown in Fig. 1.

On-line enrichment is performed by a Perfusion-column (POROS R1/20, 2.1 mm × 30 mm, 20 μm particle size, Applied Biosystems, Darmstadt, Germany). The HPLC column was a short phenyl-hexyl column (Phenomenex Luna 5 μm Phenyl Hexyl, 2 mm × 50 mm, Aschaffenburg, Germany). Furthermore, a convenient HPLC-column oven (Perkin-Elmer Series 200 Column oven) is used for heating. The API 3000 mass spectrometer (triple quadrupole, Applied Biosystems/MDS Sciex Concord, Canada) equipped with TurboIonspray source (ESI) allows high selective and sensitive detection in MRM detection mode.

2.5. On-line SPE–HPLC coupling

The analyte enrichment on SPE column and rinsing step is shown in Fig. 1 (left side). The pump B supplied enrichment eluent B (H_2O/MeOH (50/50, v/v)) and is required for sample introduction and rinsing step at a flow rate of 2700 μl min^{-1} for 1 min (0.1–1.1 min). At zero time an aliquot of 25 μl supernatant of each prepared sample was injected. Simultaneously eluent A (MeOH/H_2O 97/3, 10 mM NH_4Oac, 0.1% acetic acid) was introduced to HPLC–MS/MS at a flow rate of 300 μl min^{-1}. Table 2 shows the timetable for the pump configuration.

Table 2
Timetable for pump configuration

Step	Total time (min)	Flow rate eluent A (μl min^{-1})	Flow rate eluent B (μl min^{-1})
0	0.0	300	900
1	0.1	300	2700
2	1.1	300	2700
3	1.2	300	900
4	2.5	300	900

Pump A: MeOH/water 97/3 (v/v), 10 mM NH_4Oac, 0.1 acetic acid and pump B: water/MeOH 50/50 (v/v).

The SPE elution and analyte transfer to HPLC column is performed by switching the valve (right side in Fig. 1) after 1.1 min. The developed configuration offers a complete and fast SPE elution in backflush mode and analyte transfer to HPLC column and tandem mass spectrometer by means of eluent A at 300 μl min^{-1}, which is well compatible to the TurboIonspray source. High organic content of the eluent A (97% MeOH) is desirable to achieve a narrow elution profile and to minimise the band broadening. However, eluent A is not able to completely separate the analytes using the phenyl-hexyl HPLC column. The high selective MS/MS detection in MRM (precursor/product ion) detection mode is well convenient for simultaneous detection of more than one analyte without any retention time differences. The retention times of the immunosuppressants are shown in Table 1. During this analysing step the flow rate of eluent B was reduced to 900 μl min^{-1} for solvent saving. After 2.3 min the switching valve was switched back to the start position (left side in Fig. 1) and the start eluent composition was pumped for re-equilibration. A total analysis time of 2.5 min including on-line SPE, HPLC and API–MS/MS detection was obtained for all four immunosuppressants and two used internal standards.

2.6. API–MS/MS conditions

Two positive ion mode MRM transitions for each immunosuppressants and internal standard (one qualifier and one quantifier) were detected and are listed in Table 1 including declustering potential (DP) and collision energy (CE), respectively. All analytes gave $[M + NH_4]^+$ as the most intensive precursor ion. The dwell time was set to 40 ms for each MRM transition. TurboIonspray interface settings and collision gas pressure were manually optimized (IS voltage: 5500 V, temperature: 300 °C, nebulizer gas: 12 psi, curtain gas: 10 psi). Tandem-MS was performed using nitrogen as collision gas.

3. Results and discussion

3.1. Method performance

Most publications using LC–MS(/MS) technique usually referred to total analysis times of approximately 5 or more minutes. The total analysis time of 2.5 min by the on-line SPE–HPLC–MS/MS system offers a desirable short analy-

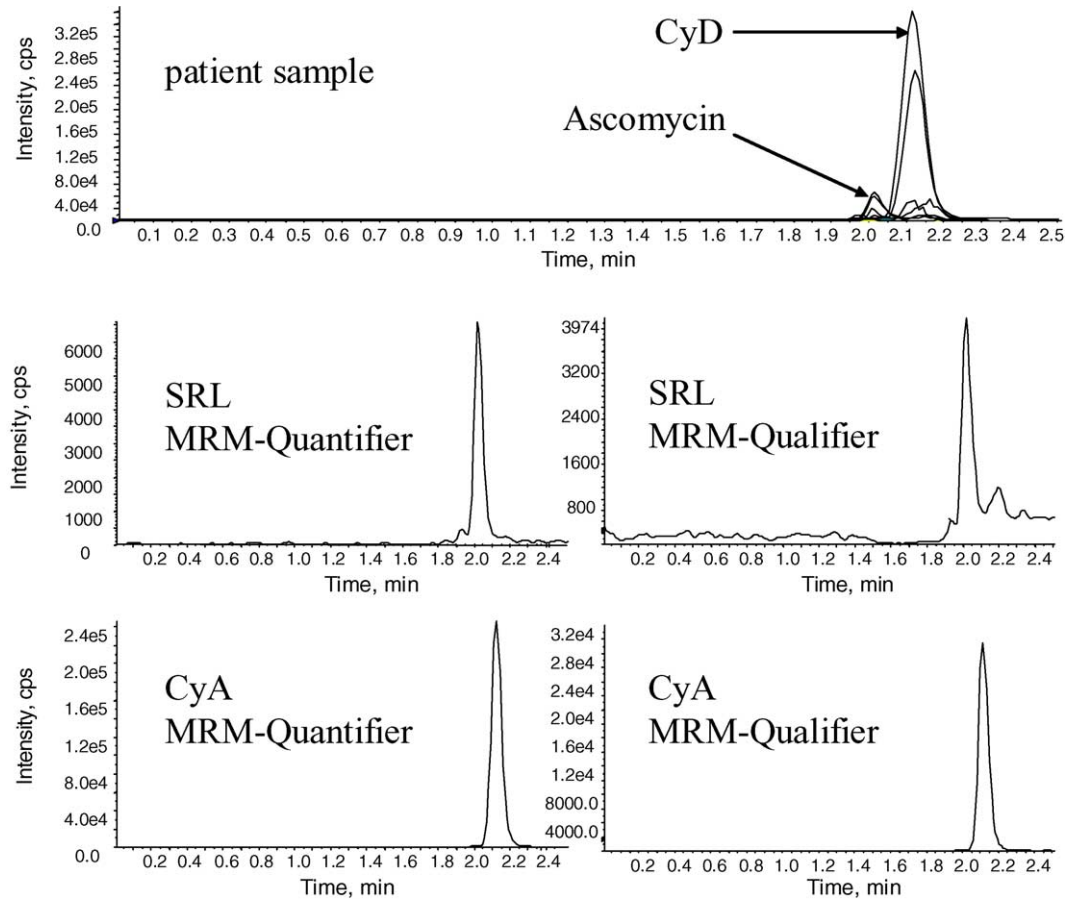

Fig. 2. Chromatogram of a real patient sample using combination therapy of CyA and SRL above: extracted ion chromatogram (XIC) including Ascomycin ($c = 20$ ng ml^{-1}) and CyD ($c = 100$ ng ml^{-1}) as internal standards below: MRM-transitions of SRL and CyA (quantifier and qualifier).

Table 3
Method performance parameters determined for spiked blood samples according to Guidance for Industry [26]

	Cyclosporin A (CyA)	Tacrolimus (TRL)	Sirolimus (SRL)	Everolimus (RAD)
LOD (ng ml^{-1})	1.3	0.1	0.1	0.1
LLOQ (ng ml^{-1})	10.0	1.0	1.0	1.0
Linearity[a] (R^2)	0.9999	0.9997	0.9998	0.9998
Recovery[b] (%)				
Concentration 1	103	91	92	94
Concentration 2	98	88	91	92
R.S.D. (%), intra-day[c]	4.6	10.2	9.3	10.3
R.S.D. at LLOQ (%), intra-day[d]	4.4	12.5	11.0	11.5
R.S.D. (%), inter-day[e]	12.3	13.5	12.3	10.9
Accuracy (%)[f]	99.2	96.9	98.9	103.1

[a] c = 10–1000 ng ml^{-1} (CyA), c = 1–50 ng ml^{-1} (SRL, RAD, TRL).
[b] c_1 = 100 ng ml^{-1} (CyA), c_1 = 10 ng ml^{-1} (SRL, RAD, TRL); c_2 = 500 ng ml^{-1} (CyA), c_2 = 100 ng ml^{-1} (SRL, RAD, TRL).
[c] Pooled spiked blood sample: c = 50 ng ml^{-1} (CyA), c = 5 ng ml^{-1} (SRL, RAD, TRL) (n = 100).
[d] Spiked blood samples (lowest calibrator): c = 10 ng ml^{-1} (CyA), c = 1 ng ml^{-1} (SRL, RAD, TRL) (n = 40).
[e] Spiked blood sample: c = 50 ng ml^{-1} (CyA), c = 5 ng ml^{-1} (SRL, RAD, TRL) (n = 40).
[f] For CyA, SRL and TRL: participation in proficiency test, for RAD: laboratory in-house control standards (n = 40).

sis time, which is important for high throughput in clinical applications. Fig. 2 shows a typical chromatogram of a real patient sample by on-line SPE–LC–MS/MS analysis. This patient was treated with a combination therapy of SRL and CyA. The quantifier and qualifier for SRL and CyA were extracted for better illustration. Signals of qualifiers are less intensive compared to quantifiers. Furthermore, in the upper chromatogram (Fig. 2) the internal standards Ascomycin and CyD can be identified.

Table 3 contains system performance parameters like limits of detection (LOD, signal-to-noise ratio 3:1), lower limits of quantification (LOQ), squared correlation coefficients (R^2), recovery rates, relative standard deviation values (R.S.D.) and accuracy values determined for spiked blood samples. The validation of the method was carried out considering Guidance for Industry [26]. The samples for determination of precision and accuracy were handled exactly as patient samples, calibrators and quality control samples (see Section 2.3).

Limits of detection are in the subnanogram per millilitre range, 0.1 ng ml^{-1} for SRL, RAD and TRL and 1.3 ng ml^{-1} for CyA, which is well suitable to TDM of patient samples. The analyte responses at LLOQ are 10 times higher compared to LOD, which is in accordance to Guidance for Industry. For all immunosuppressants the method is linear within the range of concentrations tested (1–50 ng ml^{-1} for SRL, Rad, TRL and 10–1000 ng ml^{-1} for CyA).

Recovery experiments were performed at two concentration levels of all four immunosuppressants in combination (c_1 = 100 ng ml^{-1} CyA, c_1 = 10 ng ml^{-1} SRL, TRL, RAD and c_2 = 500 ng ml^{-1} CyA, c_2 = 100 ng ml^{-1} SRL, TRL, RAD) by comparing the peak areas for extracted samples after online SPE–HPLC–MS/MS with unextracted standards after direct MS/MS representing 100% recovery. The two chosen concentration were adjusted in respect to wanted and highest concentrations found in patients. The data in Table 3 confirmed that no breakthrough of the analytes were detected by using an enrichment eluent of methanol/water (50/50, v/v) in combination with the perfusion column.

Furthermore, precision and reproducibility of a method can be assessed by calculation of R.S.D. In Table 3 R.S.D. values are shown for intra-day precision of measurements in repetition of a pooled spiked blood sample at patient level concentration (c = 50 ng ml^{-1} for CyA, c = 5 ng ml^{-1} for SRL, RAD, TRL, n = 100). Additionally, measurements in repetition of spiked blood samples at LLOQ (c = 10 ng ml^{-1} for CyA, c = 1 ng ml^{-1} for SRL, RAD, TRL, n = 40) were performed. All R.S.D. values are within 15% at patient level concentration and at LLOQ demonstrating the robustness

Table 4
Immunosuppressant concentrations of a spiked blood sample in comparison to additional spiked blood samples with 10-fold higher concentration of CyA, TRL, SRL and RAD

	Concentration (ng ml^{-1}) (%)[a]	Spiked with CyA 1000 ng ml^{-1} (%)[a]	Spiked with TRL 100 ng ml^{-1} (%)[a]	Spiked with SRL 100 ng ml^{-1} (%)[a]	Spiked with RAD 100 ng ml^{-1} (%)[a]
CyA	106.6 ± 1.9	n.d.	107.4 ± 2.3	108.2 ± 2.3	108.1 ± 2.2
TRL	10.5 ± 4.5	10.6 ± 5.4	n.d.	11.1 ± 3.8	10.6 ± 4.3
SRL	12.6 ± 6.0	11.4 ± 4.6	13.1 ± 6.2	n.d.	13.6 ± 5.9
RAD	10.2 ± 5.7	10.1 ± 3.7	10.4 ± 6.1	10.6 ± 5.9	n.d.

[a] n = 10

and reproducibility of the developed method. Furthermore, acceptable R.S.D. values are achieved for inter-day precision analysing spiked blood samples ($c = 50\,\mathrm{ng\,ml^{-1}}$ for CyA, $c = 5\,\mathrm{ng\,ml^{-1}}$ for SRL, RAD, TRL).

The samples for accuracy determination of CyA, SRL and TRL were obtained from Dr. David Holt proficiency test, Analytical Unit, Cardiac and Vascular Sciences, St. George's Hospital Medical School, London, UK. Proficiency test for RAD is not available today and we determined the accuracy on the basis on laboratory in-house standard controls. The accuracy in Table 3 show the mean value of 40 different proficiency tests obtained by comparing on-line SPE–HPLC–MS/MS results to the true concentration of the analyte. This demonstrates the accuracy of the immunosuppressants drug analysis by means of on-line SPE–HPLC–MS/MS.

During the past half year we have analysed about 2000 real patient blood samples (80% SRL and CyA after single or mostly after combination therapy, 18% CyA and 2% RAD). About 1000 blood samples can be analysed using one perfusion column for on-line SPE and one phenyl-hexyl HPLC column at 60 °C without any efficiency losses neither in system performance nor hardware like increased column back pressure due to clogging, peak broadening or peak tailing emerged from performance loss of the HPLC column.

3.2. Matrix and carryover effects

The ability to solve matrix problems in an efficient way is an essential feature of the method described here. Using on-line SPE in front of HPLC–MS/MS analysis results in a better matrix component elimination compared to direct HPLC–MS/MS analysis.

Fig. 3 compares analysis of spiked blood sample by means of on-line SPE–HPLC–MS/MS with analysis of the same blood sample by means of HPLC–MS/MS. For sim-

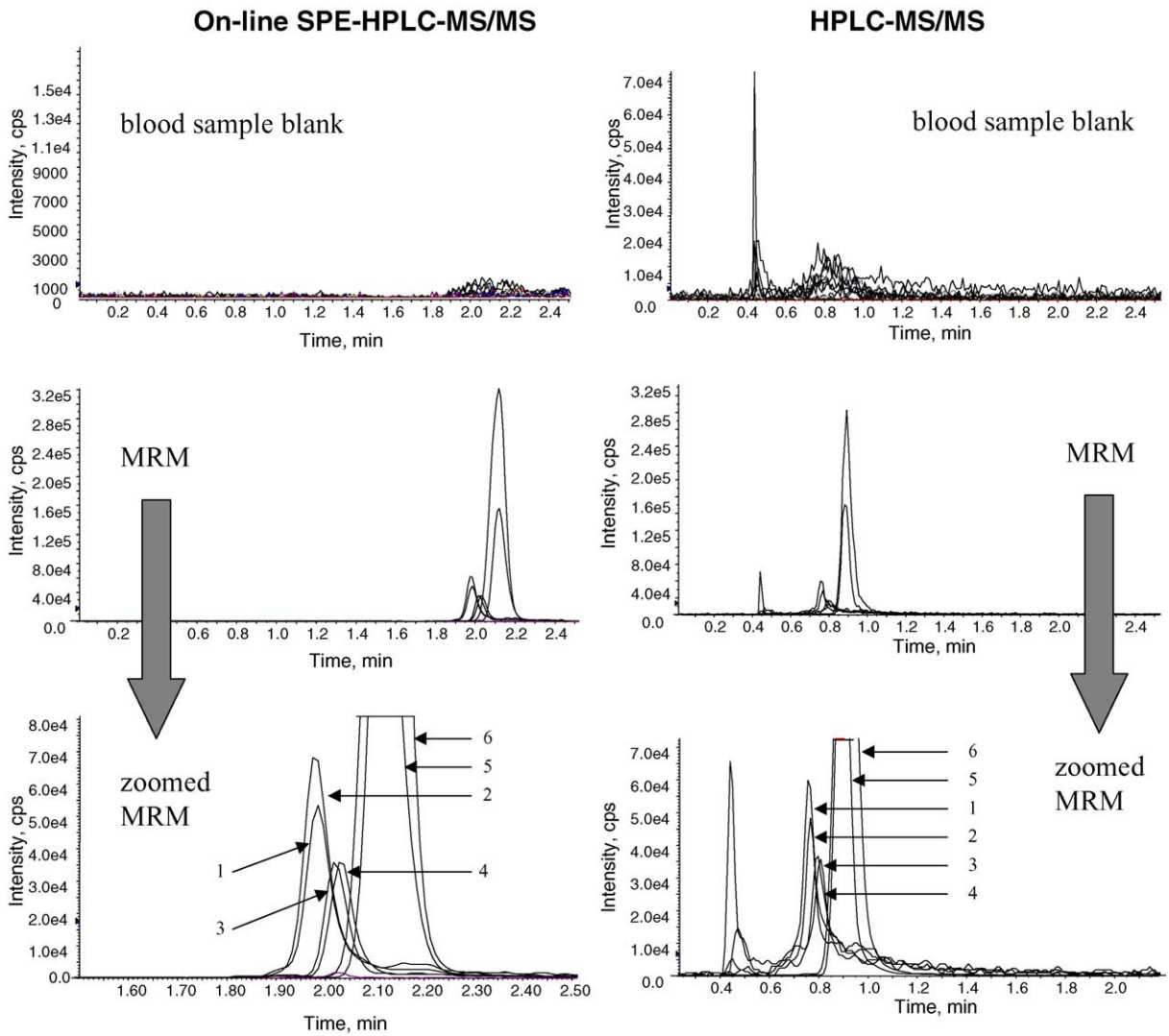

Fig. 3. Chromatograms show no matrix effects with on-line SPE–HPLC–MS/MS (left side) compared to HPLC–MS/MS (right side) obtained from blood sample blank (above) and sample of spiked whole blood (below) (1) TRL, (2) Ascomycin, (3) SRL, (4) RAD, (5) CyA, (6) CyD (SRL, RAD, TRL: $c = 10\,\mathrm{ng\,ml^{-1}}$, CyA: $c = 100\,\mathrm{ng\,ml^{-1}}$, Ascomycin: $c = 20\,\mathrm{ng\,ml^{-1}}$, CyD: $c = 100\,\mathrm{ng\,ml^{-1}}$).

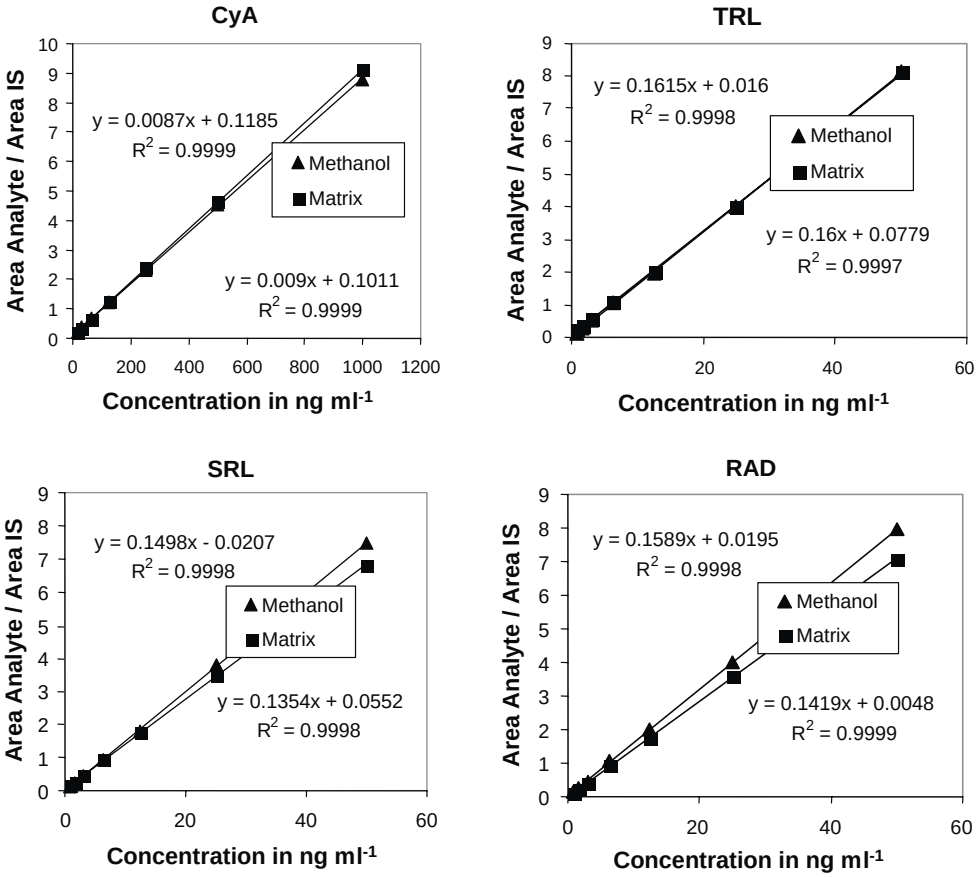

Fig. 4. Comparison of immunosuppressant calibration curves resulted from spiked methanolic (precipitating reagent) and blood samples (SRL, RAD, TRL: $c = 5$–$50\,\mathrm{ng\,ml^{-1}}$, CyA: $c = 10$–$1000\,\mathrm{ng\,ml^{-1}}$, Ascomycin: $c = 20\,\mathrm{ng\,ml^{-1}}$, CyD: $c = 100\,\mathrm{ng\,ml^{-1}}$).

plification only the quantifier of each analyte are detected. Spiked samples containing all four immunosuppressants within physiological concentrations ($c = 5\,\mathrm{ng\,ml^{-1}}$ for SRL, RAD, TRL; $c = 50\,\mathrm{ng\,ml^{-1}}$for CyA; $c = 20\,\mathrm{ng\,ml^{-1}}$ for Ascomycin; $c = 100\,\mathrm{ng\,ml^{-1}}$ for CyD). In the zoomed chromatogram (Fig. 3, below) the peaks for all analytes can be identified. The correlation of peak number and analyte including corresponding retention time is summarised in Table 1. The comparison of the blood sample blank between HPLC–MS/MS and on-line SPE–HPLC–MS/MS in Fig. 3 shows a 10-fold noise reduction from around 1×10^4 to 0.1×10^4 cps and an elimination of unspecific peaks, e.g. 0.45 min by SPE–HPLC–MS/MS coupling.

Therefore, both SPE and HPLC are important for efficient matrix minimisation and cannot be omitted. Using analysis time longer than 2.5 min for on-line SPE–HPLC–MS/MS indicated no additional matrix effects through increases in base line, which could lead to carryover effects in the following analysis. For this reason a total analysis time of 2.5 min is sufficient for this method.

Furthermore, immunosuppressants are extensive metabolized in the body. All metabolites are more polar and have shorter retention times in comparison to the parent drugs and display no potential source to produce interferences.

In addition, both spiked methanolic precipitating reagent samples and spiked blood calibrators samples were comparatively analysed (Fig. 4) to investigate matrix effects by on-line SPE–HPLC–MS/MS. The calibration curves of the investigated immunosuppressants show good agreement in slopes for each analyte giving evidence for the high efficiency of matrix treatment. Nevertheless, quantification should be performed generally by means of spiked blood samples calibrators and controls to ensure a comparable matrix influence.

As shown in Table 1 and illustrated in Figs. 2 and 3 the analytes were not chromatographically separated from each other in accordance to short total analysis time. Therefore, it is important to show that a high concentrated analyte does not influence the response to other components. Combined spiked whole blood samples at wanted patient levels ($106\,\mathrm{ng\,ml^{-1}}$ CyA, $10\,\mathrm{ng\,ml^{-1}}$ TRL, $12\,\mathrm{ng\,ml^{-1}}$ SRL, $10\,\mathrm{ng\,ml^{-1}}$ RAD) were analysed as concentration reference value. Furthermore, aliquotes of spiked whole blood samples were additionally spiked with a 10-fold higher concentration of a different immunosuppressant ($1000\,\mathrm{ng\,ml^{-1}}$ CyA, $100\,\mathrm{ng\,ml^{-1}}$ SRL, TRL, RAD). In Table 4 the concentration results for all spiked samples are summarised and the comparison show an excellent agreement of the determined

concentrations for every single analyte. Thus, there are no ion suppression effects of one analyte by another.

Carryover effect studies were performed in the validation of the method performance by direct analysis of methanolic sample blanks after measuring spiked blood samples. On-line enrichment with aqueous solvent part higher than 60% H_2O (eluent B) has been provided analyte carryover effect in the followed blank analysis. Therefore, we used an eluent of 50/50 H_2O/MeOH (v/v) for on-line SPE without breakthrough of the analytes to avoid carryover effects.

4. Conclusion

The on-line SPE–HPLC–MS/MS method described here allows the fast, sensitive, reliable and simultaneous determination of presently interested immunosuppressants CyA, TRL, SRL and RAD in whole blood samples. Fast on-line SPE on perfusion column in combination with a short phenyl-hexyl HPLC column and highly selective MS/MS detection in MRM mode turned out to be highly capable to reduce analysis time and to manage matrix problems in a very efficient manner. The system is easy to handle and robust for routine analysis at concentration ranges in subnanogram per millilitre, which is underlined by the investigated performance parameters. More than 2000 real patient blood samples were analysed by the descibed method. It is very thinkable that the described method can be adopted for TDM of other drugs in the clinical routine analysis.

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