The influence of the sample matrix on LC-MS/MS method development and analytical performance

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Chapter 2

Robust, high-throughput LC-MS/MS method for Therapeutic Drug Monitoring of cyclosporin A, tacrolimus, everolimus and sirolimus in whole blood

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ABSTRACT

The authors describe a fast, robust and straightforward LC-MS/MS method with the use of a single LC-MS/MS system for cyclosporin A, tacrolimus, sirolimus and everolimus in whole blood. The purpose of this method was to replace the immunoassay methods used in the laboratory of a hospital performing most organ transplantations (including heart, lung, liver, kidneys, bone marrow and intestinal track). Several LC-MS/MS methods have been described so far, however most of them require complicated on-line extraction procedures. The described LC-MS/MS method uses a chromatographic gradient in combination with protein precipitation as sample preparation. The chromatographic method is capable of separating otherwise interfering peaks, with an analysis time of 2.6 minutes. Analyses were performed on a triple quadrupole LC-MS/MS system, with a C_{18}-column held at 60°C. Sample preparation required only one precipitation/dilution step. Sirolimus and everolimus are prepared and measured separately from tacrolimus and cyclosporin A. During method development it was found that the use of zinc sulphate provides process efficiency results of about 100% for tacrolimus and cyclosporin A but only 81% and 87% for sirolimus and everolimus respectively. With the developed sample preparation without zinc sulphate for sirolimus and everolimus process efficiencies were 99% and 108% respectively. The methods have been fully validated and in a comparative study patient samples were analysed with immunoassay and our developed LC-MS/MS methods. In the comparative studies, correlations (R^2 values) of more than 0.85 were found between the immunoassay and the new LC-MS/MS patient blood levels. There was a systematic deviation in blood levels measured by LC-MS/MS compared to immunoassays for cyclosporin A (17% lower than with immunoassay) and everolimus (30% lower than with immunoassay). There appeared to be little or no systematic deviation for sirolimus and tacrolimus. The controls determined by the LC-MS/MS method over the last ten months showed CVs of no more than 8.0% for each of the four immunosuppressants. In conclusion the authors find the developed methods to be cost saving, more flexible, more sensitive and to have larger linear ranges than the previously used immunoassay methods. The methods are already used for over 20,000 patient samples in the daily routine, analysing approximately 70 patient samples per day.

INTRODUCTION

Allograft rejection by the recipient immune system still remains one of the most important obstacles in allogeneic organ transplantation. Various immunosuppressive drugs are currently used to suppress the immune system and prevent cytokine associated tissue damage. Cyclosporin A, tacrolimus, sirolimus and everolimus are successfully applied in kidney, heart, lung, pancreas, intestinal tract, skin and liver transplantations [1]. But their narrow therapeutic index requires caution regarding the dosages of these immunosuppressants. At low blood levels there is risk of rejection. At high blood levels serious side-effects can emerge (including nephro- and cardiotoxicity, neurological effects and elevated risk of infections [2, 3]). Because immunosuppressants exhibit a great degree of interindividual and intraindividual pharmacokinetic and pharmacodynamic variability (incomplete, unpredictable uptake, extensive metabolism and Cytochrome P450 related drug interactions), patient blood levels are complex and unpredictable [4]. In addition the therapeutic ranges of the different immunosuppressants are even dependent on the transplanted organ, the age of the patient, the co-medication and the period after transplantation [5]. Constant patient therapeutic drug monitoring (TDM) is therefore mandatory. At the moment, several pharmacokinetic markers are used, including (limited sampling) AUC monitoring, C2 (two hours after administration) and through blood levels [4]. Which one of the TDM approaches produces the optimal clinical outcome, is still under debate. As the number of organ transplantations grows and the post-transplant patients require lifelong medical treatment and lifelong TDM, the number of patient immunosuppressant blood samples will increase. At the same time these drugs are also used against a growing number of other diseases, such as steroid resistant nephrotic syndrome, psoriasis and other auto immune disorders [6]. This puts ever more pressure on TDM laboratories to develop methods that are fast, robust and accurate and can be used for larger series of patient samples.

Currently, several analytical methods have been developed for the determination of immunosuppressive drugs, among which the following immunoassays (IA): Fluorescence polarization immunoassay (FPIA), Microparticle Enzyme Immunoassay (MEIA), Enzyme Multiplied Immunoassay (EMIT), radioimmunoassay (RIA), Enzyme-Linked Immuno Sorbent Assay (ELISA) and HPLC-UV and HPLC-MS/MS methods [7].

Analytical methods based on IA for the measurement of immunosuppressive drugs are easily operated, but have a number of disadvantages. The analysis costs of IA techniques are relatively high (e.g. for tacrolimus and everolimus). The LLOQ of the IA techniques is often

not low enough, especially for tacrolimus in case of liver transplantation in young children. The IA techniques are often not selective enough. Cyclosporin A exhibits an extensive metabolism. These metabolites influence the analysis results because of the significant cross-reactivity in the IA [8]. The everolimus kit shows cross-reactivity with sirolimus [9, 10]. For patients who switch from sirolimus to everolimus medication the selectivity of the IA technique is insufficient. In addition, the everolimus IA performed in the authors' laboratory often produced rejected runs because of deviant quality control samples with 23 out 83 of the medium levels (11 µg/L) having a deviation over 15% (authors internal laboratory quality control programme). LC-MS/MS is at this moment generally accepted as the technique of choice because of its selectivity, sensitivity and flexibility [7]. Several LC-MS/MS methods have been described so far, yet most of them require on-line extraction procedures [11-18]. The on-line extraction procedures also require an additional pump, switch valve and trapping column, making the method more complicated and vulnerable to instrument problems. In other cases different LC-MS/MS configurations are required to analyse all four immunosuppressants [14, 18].

In the authors' hospital laboratory, many urgent and outpatient clinical samples are analysed daily, divided over all four immunosuppressive drugs. Therefore the development of a fast and laboursaving method which uses one LC-MS/MS system was very desirable.

In this article the authors describe a newly developed fast, selective and robust LC-MS/MS method for the simultaneous determination of cyclosporin A, tacrolimus, sirolimus and everolimus in whole blood with little sample preparation, as a tool for therapeutic drug monitoring (TDM).

MATERIALS AND METHODS

Reagents

Everolimus and cyclosporin A were a kind gift of Novartis Pharmaceuticals (Basel, Switzerland), and sirolimus was kindly provided by Wyeth Pharmaceuticals in New York, USA. Ascomycin and tacrolimus were purchased from Sigma-Aldrich Inc (St. Louis, USA). Stock solutions were prepared in methanol and stored at -20°C. Analytical grade methanol, acetonitrile and zinc sulphate heptahydrate were purchased from Merck (Darmstadt, Germany). Purified water was purchased from Biosolve B.V. (Valkenswaard, the Netherlands).

Ammonium acetate was purchased from Acros (New Jersey, USA). Pooled blank whole blood with dipotassium edetate as an anticoagulant was a kind gift of Medisch Lab Noord (Groningen, the Netherlands).

Equipment and conditions

All experiments were performed on a Thermo Fisher Scientific (Waltham, MA, USA) triple quadrupole Quantum Access LC-MS/MS system with a Surveyor® MS pump and a Surveyor plus® autosampler with an integrated column oven. The Quantum Access mass selective detector was operated in electrospray positive ionisation mode and performed selected reaction monitoring (SRM). High purity nitrogen was used for the sheath gas and auxiliary gas and argon was used as collision gas. In the first quadrupole ammonium adducts $[M + NH_4]^+$ were selected for tacrolimus, sirolimus and everolimus, for cyclosporin A the single charged ions $[M + H]^+$ were selected. For all transitions, scan times, optimum tube lens and collision energy values are shown in table 1.

Table 1 The mass parameters at a scan width of 0.5 *m/z*

Immunosuppressant	Parent ion *m/z*	Product ion *m/z*	Scan time MS	CE V	TL V
Ascomycin	809.5	756.5	100	21	84
Tacrolimus	821.5	768.4	200	21	106
Cyclosporin A	1202.9	1184.8	400	30	176
Everolimus	975.6	908.5	100	18	89
Sirolimus	931.5	864.4	100	18	122

m/z: mass to charge ratio; CE: Collission energy in eVolts; TL tube lens value in eVolts.

Sirolimus and everolimus have their optimum capillary temperature set at 280°C and tacrolimus and cyclosporin A at 360°C. For all these immunosuppressants, the ion source spray voltage was set at 5,500 V, the sheath gas pressure at 55 Arbitrary Units (AU), the auxiliary gas pressure at 8 AU. The Surveyor plus® autosampler was set at 10°C and the integrated column oven was set at a temperature of 60°C. The mobile phase gradient consisted of methanol, purified water and an 0.2 mol/L ammonium formate buffer pH 3.5 which maintained constant at 5% during the gradient, see figure 1.

Analyses were performed on a 50 x 2.1 mm Hypurity® C_{18}, 3-µm analytical column from Interscience (Breda, the Netherlands) equipped with a separate 0.5 µm Varian frit filter

(Palo Alto, USA). Chromatographic separation was performed by means of a gradient with a flow of 0.5 mL/min and an analysis time of 2.6 minutes. The automatic switch valve in the mass selective detector was used to divert the first 0.8 minutes of flow to the waste, keeping material not retained on the column such as salts away form the spray source. In figure 1 a combined chromatogram and gradient table is shown for all four immunosuppressants, where the gradient line of methanol is corrected in time for its delay volume. Xcalibur® software version 2.0 SR2 was used for peak height integration for all components.

Time minutes	A %	B %	C %	Flow µL/minute
0.00	5.0	30.0	65.0	500
0.36	5.0	30.0	65.0	500
0.37	5.0	75.0	20.0	500
1.62	5.0	83.0	12.0	500
1.63	5.0	95.0	0.0	500
2.22	5.0	95.0	0.0	500
2.23	5.0	30.0	65.0	500
2.60	5.0	30.0	65.0	500

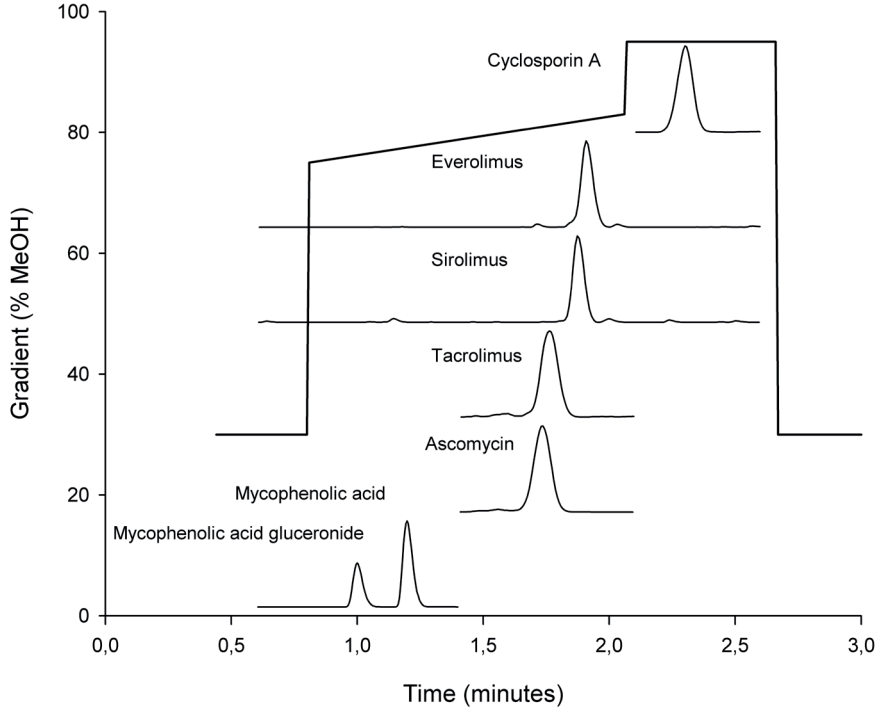

Figure 1 Typical ion chromatograms for all immunosuppressants and the methanol gradient line corrected for its delay time.
Chromatographic gradient. A: 0.2 M ammonium formate buffer pH 3.5; B: methanol; C: purified water.

Sample preparation

An aliquot of 200 µL whole blood is transferred into a glass 2.0 mL HPLC screw top vial (Varian, Palo Alto, USA). Protein precipitation reagent with internal standard ascomycin (an ethyl analogue of tacrolimus, structurally related to tacrolimus, everolimus and sirolimus) is added to monitor sample injection and preparation. Sirolimus and everolimus are prepared and analysed separately from cyclosporin A and tacrolimus. For sirolimus and everolimus 500 µL methanol: acetonitrile 50:50% v/v containing 100 µg/L ascomycin is used as the protein precipitation reagent. For cyclosporin A and tacrolimus precipitation is performed by adding 500 µL methanol containing 100 µg/L ascomycin, followed by 25 µL 2.4 mol/L zinc sulphate in water. The further procedure is identical for both sample preparations. After precipitation, the vials are vortexed for 1 minute (Multitube vortexer, Labtek corporation Ltd., New Zealand) and stored at -20°C for at least 10 minutes. The samples are precipitated and vortexed in batches of approximately 50 vials. Afterwards the vials are again vortexed for 1 minute, centrifuged at 11,000 *g* for 5 minutes and 5 µL of the clear upper layer of supernatant is automatically injected.

Method validation

For validation, blank human whole EDTA blood was spiked with immunosuppressant stock or standard solutions to yield the required concentrations. Validation was performed with a maximum tolerated bias and CV for the lower limit of quantification (LLOQ) of 20% and 15% for the other validation concentrations [19]. For each validation concentration bias and CV were calculated per run. Within-run, between-run and overall CVs were calculated with the use of one-way ANOVA. Eight calibration points were used to determine linearity on three separate days. To maximise sample throughput, calculation of concentrations by the use of response factors are preferred in the authors' laboratory. Response factors are calculated using the highest calibrator. To validate this practice linearity for the eight calibration points is to be validated without the use of a weighting factor, and response factors are used to calculate accuracy and precision. For determination of accuracy, precision and over-curve dilution (OC), all concentrations were prepared and measured in five-fold in three separate runs on separate days. The over-curve pool for dilution consisted of a single concentration of at least two times the concentration of the higher limit of quantification (HLOQ), which was ten times diluted during sample preparation. Stabilities of the immunosuppressants were assessed at room temperature in whole blood, in the autosampler at 10˚C and during three freeze-thaw-cycles.

To assess process efficiencies organic solutions with the composition of a processed sample containing water instead of whole blood were prepared. Sufficient stock solution was spiked to obtain concentrations identical to a whole blood sample of 10 µg/L for sirolimus, everolimus and tacrolimus, and 250 µg/L for cyclosporin A after sample preparation. These solutions were analysed in triplicate. Ion suppression was tested by infusing a stock solution to the gradient flow with a T-piece and injecting multiple processed blank whole blood samples [20]. The whole blood samples used for ion suppression tests were from 2 individuals and one pooled batch which was also used for the preparation of the validation pools. Also processed samples using blank water instead of whole blood were injected to monitor changes in signal sensitivity during the gradient. Carry-over was monitored during method development and validation.

Correlation studies

In the correlation studies, all immunosuppressive patient samples were analysed by our conventional (IA) and new (LC-MS/MS) methods. These studies were executed in order to achieve a more realistic view on the expected differences in immunosuppressant blood-levels due to drug metabolism in our patient population, measured by IA and LC-MS/MS. The conventional analyses were performed on the following Abbott auto-analysers (Abbott diagnostics, USA): cyclosporin A on an AxSym® by means of FPIA, tacrolimus and sirolimus on an IMx by means of MEIA, everolimus on a TDxFLx by means of FPIA. All IA analyses were performed with Abbott assay kits except for everolimus where Seradyn® assay kits (Seradyn Inc. USA) were used. The methods were compared by simple linear regression and Passing Bablok using the statistical software Analyse-it®.

RESULTS

Method

In the developed methods sirolimus and everolimus are measured separately from tacrolimus and cyclosporin A, but under the same chromatographic conditions. Sirolimus and everolimus require an optimum capillary temperature of 280°C and a sample preparation without zinc sulphate. Whereas tacrolimus and cyclosporin A require an optimum capillary temperature of 360°C, and a sample preparation with zinc sulphate. One efficient and fast

chromatographic gradient is developed for all four immunosuppressants where a column temperature of 60°C and a high column flow of 0.5 mL/min on a 2.1 mm diameter column results in short runtimes of 2.6 minutes. In spite of these short runtimes otherwise interfering peaks of sirolimus and everolimus are chromatographically separated and excellent peak shapes are obtained for all immunosuppressants, as can be seen in the chromatograms in figure 1 and 2.

Method validation

During method development it was found that the use of zinc sulphate provides process efficiency results of 103% for tacrolimus and 110% for cyclosporin A but of only 81% and 87% for sirolimus and everolimus respectively. With the developed sample preparation without zinc sulphate for sirolimus and everolimus process efficiencies were 99% and 108% respectively. In table 2 the average peak heights and process efficiency results are displayed for everolimus.

The ion-suppression tests show that none of the immunosuppressants have ion suppression at their elution times. In figure 3 representative ion suppression plots are given for both sample preparations. Ion suppression plots are practically the same for all four immunosuppressants. The ion-suppression figure shows 2 significant ion suppression areas. One at the injection

Table 2 Process efficiency results for everolimus and sirolimus at 10 µg/L

Protein precipitation reagents	Sample	Average peak heights cps	CV %	Process efficiencies %
Everolimus				
Methanol : Acetonitrile 50:50% v/v	Blood	17,217	5	108
	Process efficiency	16,002	4	
Methanol and zinc sulphate 2.4 M	Blood	12,986	5	87
	Process efficiency	14,949	5	
Sirolimus				
Methanol : Acetonitrile 50:50% v/v	Blood	14,906	6	99
	Process efficiency	15,035	7	
Methanol and zinc sulphate 2.4 M	Blood	11,112	8	81
	Process efficiency	13,710	7	

The average peak heights of four processed blood samples are compared to the average peak heights of four process efficiency samples. Process efficiency samples are of the same composition as processed whole blood samples, where whole blood has been replaced by water.

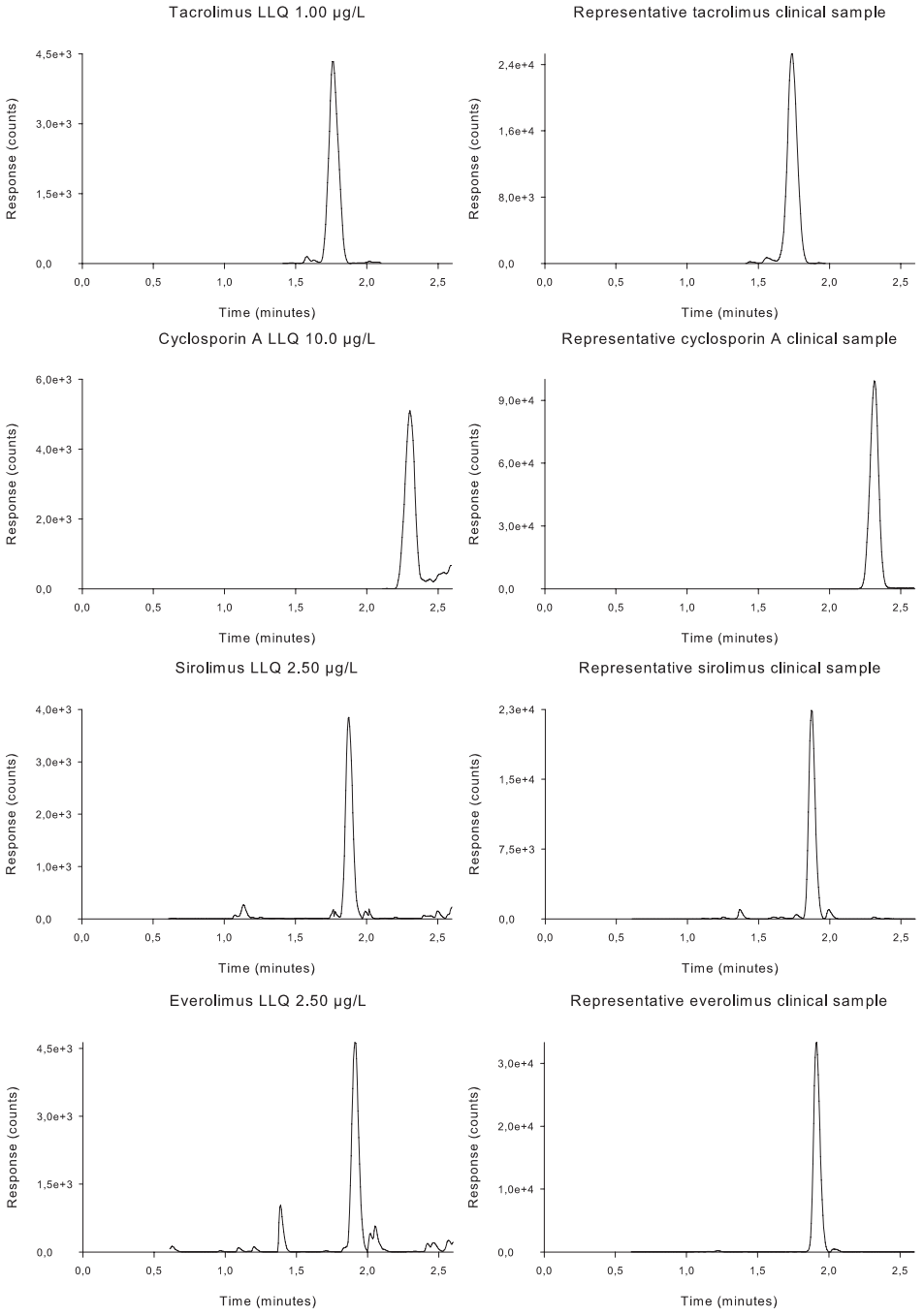

Figure 2 Ion chromatograms of LLOQs and representative clinical samples of tacrolimus, cyclosporin A, sirolimus and everolimus.

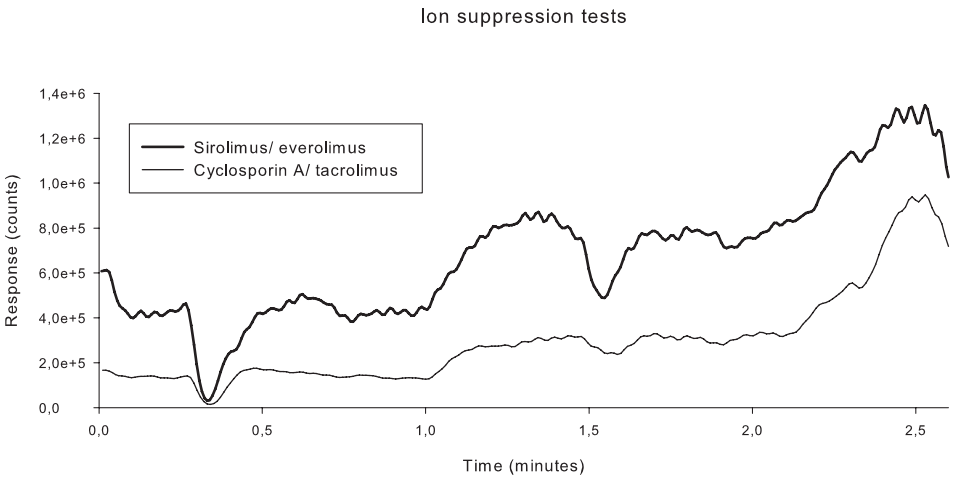

Figure 3 Representative ion suppression chromatograms for all four immunosuppressants.
The ion suppression plots from top to bottom correspond with the order of immunosuppressants in the legend.

front and one at 1.5 minutes. Both suppression areas are well separated from the eluting peaks. During the runtime the sensitivity rises due to the increase of the methanol in the gradient, as can be seen in the gradient shown in figure 1. Ascomycin, added to monitor sample injection and preparation, showed little variation in peak height (about 4% within a run of approximately 60 injections) and proved to be suitable as an internal standard. Linearity has been assessed without the use of a weighting factor. Regression coefficients are: R^2=0.9978 for tacrolimus, R^2=0.9877 µg/L for cyclosporin A, R^2=0.9938 for everolimus and R^2=0.9967 for sirolimus. The validation results for accuracy and precision are all well within the maximum tolerated bias and CV (20% for the LLOQ, 15% for the other concentrations), as summarized in table 3. During validation, overall CV and bias proved to be less than 14% for all LLOQs and less than 12% for the other concentrations. The LLOQ is 1.00 µg/L for tacrolimus, 10.0 µg/L for cyclosporin A and 2.50 µg/L for both everolimus and sirolimus. The over-curve dilution of ten times the over-curve pool showed to have a bias and CV well within 10% for all components. During whole blood stability evaluation, cyclosporin A, everolimus, sirolimus and tacrolimus proved to be stable at room temperature for at least 72 hours. Processed samples proved to be stable in the autosampler at 10°C for at least 69 hours for all four immunosuppressants. Freeze-thaw stability was determined during three cycles and for none of the immunosuppressants a trend showing instability was observed. No carry-over peaks were present in blanks following the HLOQ and no interfering peaks were present in blank whole blood samples.

Table 3 Validation results of all five immunosuppressants

Immunosuppressant	regression coefficient linear range (µg/L)	Concentration (µg/L)	Within-run CV (%)	Between-run CV (%)	Overall CV (%)	Overall Bias (%)
Cyclosporin A	0.9877 (10–2,000)	LLOQ (10.0)	13.3	0.0	13.3	-2.5
		LOW (50.0)	6.3	0.0	6.3	2.7
		MED (800)	2.9	2.4	3.8	0.4
		HIGH (1,600)	2.1	3.9	4.4	1.1
		DIL (4,000)	4.5	1.3	4.6	1.0
		F/T stab	2.0	6.0	6.3	1.0
		RT stab 74 h	1.5	n/a	n/a	-2.4
		AS stab 69 h	3.7	n/a	n/a	11.9
Everolimus	0.9938 (2.50–50.0)	LLOQ (2.50)	8.3	2.8	8.8	-11.9
		LOW (5.00)	8.0	2.0	8.2	-2.2
		MED (15.0)	5.4	4.9	7.3	1.7
		HIGH (40.0)	5.3	0.0	5.3	0.8
		DIL (100)	7.9	5.7	9.7	-2.0
		F/T stab	5.3	0.0	5.3	1.0
		RT stab 72 h	2.4	n/a	n/a	-4.9
		AS stab 72 h	4.0	n/a	n/a	-2.0
Sirolimus	0.9967 (2.50–50.0)	LLOQ (2.50)	9.4	0.0	9.4	-5.6
		LOW (5.00)	6.3	2.8	6.9	1.0
		MED (15.0)	4.5	4.7	6.5	-3.0
		HIGH (40.0)	3.0	2.0	3.6	-6.6
		DIL (100)	7.5	2.4	7.9	-7.0
		F/T stab	4.0	3.0	5.0	-7.0
		RT stab 72 h	2.0	n/a	n/a	0.9
		AS stab 72 h	5.9	n/a	n/a	-9.7
Tacrolimus	0.9978 (1.00–50.0)	LLOQ (1.00)	9.2	4.6	10.3	1.3
		LOW (5.00)	7.4	0.0	7.4	11.4
		MED (15.0)	4.6	4.5	6.4	4.7
		HIGH (40.0)	3.3	3.8	5.0	2.0
		DIL (100)	5.0	3.9	6.3	0.0
		F/T stab	3.8	3.9	5.5	4.0
		RT stab 72 h	3.6	n/a	n/a	7.4
		AS stab 72 h	3.6	n/a	n/a	5.8

LLOQ, LOW, MED and HIGH are the validation concentrations used for accuracy and precision; DIL is the dilution pool, which is ten times diluted prior to sample preparation; F/T stab. = freeze and thaw stability after three cycles; RT stab.xx h = stability in whole blood at room temperature for xx hours; AS stab.xx h = stability in the autosampler at 10°C for xx hours after sample preparation.

Correlation study

In the comparative study (see figure 4) the linear regression equations showed good correlations between the old MEIA and FPIA and the new LC-MS/MS patient blood levels: cyclosporin A R^2=0.9642 (n=374); tacrolimus R^2=0.8594 (n=219) sirolimus R^2=0.9273 (n=27); everolimus R^2=0.9663 (n=57). Correlations were assessed by calculating a linear regression line with 95% confidence intervals. The linear regression equations for LC-MS/MS and IA

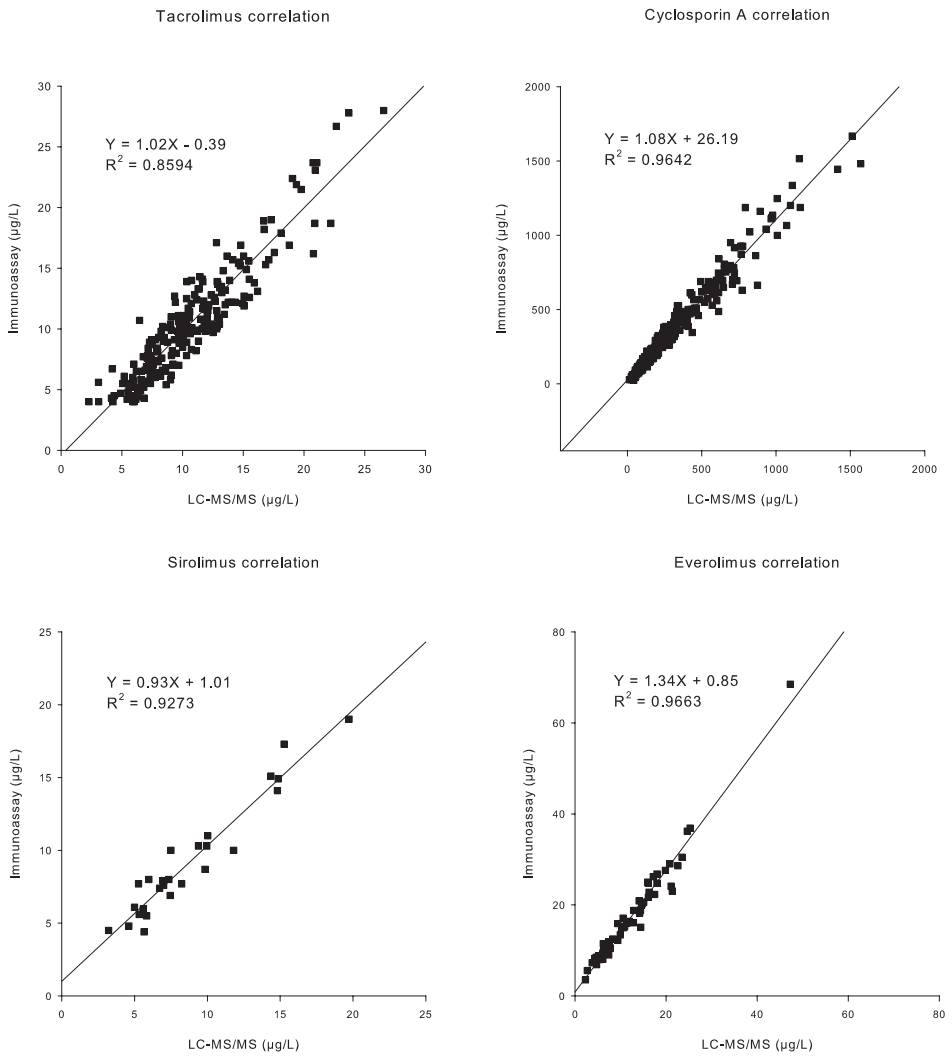

Figure 4 The correlation plots of cyclosporin A (IA by FPIA), tacrolimus (IA by MEIA), sirolimus (IA by MEIA) and everolimus (IA by FPIA) results obtained from patient samples analysed with the developed LC-MS/MS method (x) and the IA method (y).

obtained in the correlation study are the following (x = LC-MS/MS, y = IA): for cyclosporin A y = 1.08x + 26.19; tacrolimus y = 1.02x - 0.39; sirolimus y = 0.93x + 1.01; and for everolimus y = 1.34x + 0.85. The Passing Bablok regression showed similar results: cyclosporin A y = 1.15x + 11.46; tacrolimus y = 1.09x - 1.20; sirolimus y = 0.97x + 0.60; and everolimus y = 1.32x + 1.03. There was a systematic deviation in blood levels measured by LC-MS/MS compared to IA for cyclosporin A and everolimus. For cyclosporin A the concentrations measured with LC-MS/MS were found to be approximately 17% lower than with IA. And for everolimus the concentrations measured with LC-MS/MS were approximately 30% lower. There appeared to be little or no differences for sirolimus and tacrolimus.

Sample throughput

With the developed chromatographic gradient a high sample throughput of 20 samples per hour is achieved. Preparation of 50 samples in one batch takes approximately 32 minutes (15 minutes of pipetting, a total of 2 minutes vortexing, 10 minutes at -20°C and 5 minutes centrifuging).

Since the use of our LC-MS/MS method more than 20,000 patient samples have been analysed. Cyclosporin A and tacrolimus are the most common analyses of the four immunosuppressants, they form about 95 percent of the analyses. The remaining 5 percent are sirolimus and everolimus analyses. The controls used in our runs are monitored in our internal quality control program. Here the robustness of our LC-MS/MS method can be seen by the CVs of the controls used for the analysis during 10 months. The control levels are 10 µg/L for tacrolimus, sirolimus and everolimus, and 250 µg/L for cyclosporin A, all common therapeutic levels. The CV for tacrolimus is 5.3% (n=1,145), were 29 control values exceeded the acceptance criteria of ±15% bias. The CV for cyclosporin A is 7.4% (n=1,121), were 65 control values exceeded the acceptance criteria of ±15% bias. The CV for sirolimus is 7.9% (n=155), were 6 control values exceeded the acceptance criteria of ±15% bias. For everolimus the CV is 8.0% (n=153), were 8 control values exceeded the acceptance criteria of ±15% bias. The authors' laboratory also participates in the International Proficiency Testing Scheme. The method has passed every test since implementation.

DISCUSSION

This method was developed for high throughput TDM of tacrolimus, cyclosporin A, sirolimus and everolimus, while still being chromatographically selective and robust. With our system sirolimus and everolimus are measured separately from tacrolimus and cyclosporin A for three reasons. First, sirolimus and everolimus have a significant different optimum capillary temperature (280°C) than tacrolimus and cyclosporin A (360°C). Secondly, sirolimus and everolimus require a different sample preparation (without zinc sulphate). Thirdly the separate analysis of sirolimus and everolimus keeps the number of scan masses minimal and this has a positive effect on the signal to noise ratio.

The appropriate sample preparation in combination with well designed chromatography is acknowledged as a key factor for LC-MS/MS sample analyses, especially with a whole blood sample matrix [18]. Our research has shown that the use of zinc sulphate provides lower process efficiencies for sirolimus and everolimus. With the authors' sample preparations, process efficiency tests show about 100% for all four immunosuppressants. The process efficiency tests and the ion suppression tests prove that there is no negative influence of the matrix on the analytical results.

The authors' gradient is designed to give chromatographic separation of interfering peaks of especially sirolimus and everolimus. An adjacent peak can be seen in each sirolimus and everolimus chromatogram, as shown in figure 2.

During method development especially the LLOQ peak shapes and peak heights showed significantly less variation between injections when these peaks were chromatographically separated (data not shown). The gradient elution separates cyclosporin A from the other immunosuppressants allowing the use of scan segments in the instrument method, which has a positive effect on the signal to noise ratio and ultimately on the LLOQ.

Compared with our previous IA methods, the LLOQ of all immunosuppressive drugs was improved (except for everolimus; new LLOQ: 2.50 µg/L versus 2.00 µg/L). In the introduction the bad performance of the everolimus IA in the authors' laboratory was mentioned. This everolimus IA could only be validated when the average was calculated of duplicate analyses, although the LLOQ is slightly higher with the developed LC-MS/MS method. The robustness and method CV, based upon single analysis results, are improved in comparison with the everolimus IA. With the use of LC-MS/MS, linearity was increased which minimises the dilutions of high concentration samples and makes the method more suitable for daily clinical

practice. The IA and new (LC-MS/MS) validated linearity ranges are respectively: 4.00–30.0 µg/L versus 1.00–50.0 µg/L for tacrolimus, 25–800 µg/L versus 10–2,000 µg/L for cyclosporin A, 2.0–40.0 µg/L versus 2.50–50.0 µg/L for everolimus, 3.30–30.0 µg/L versus 2.50–50.0 µg/L for sirolimus. The large linear ranges are very important because more and more (high) C2 levels are analysed beside through levels. The comparative MEIA and FPIA / LC-MS/MS study revealed correlations of R2 values more than 0.85 between patient blood levels determined by IA and LC-MS/MS for all immunosuppressants. As expected, cyclosporin A and everolimus blood levels measured by LC-MS/MS were systematically lower due to significant IA cross reactivity with metabolites and/or structural analogues. The cross-reactivity of sirolimus with the everolimus IA method for example is 74% to 68% [9, 10]. The effect of metabolites, structural analogues, type of organ transplantation and patient metabolism make a good correlation study of IA versus LC-MS/MS difficult. For cyclosporin A (which shows extensive metabolism and metabolite cross reactivity with the IA) the concentrations measured with LC-MS/MS were found to be approximately 17% lower than with IA. Here the intercept is the main reason for this deviation, having more influence at low concentrations. Similar results for cyclosporin A were found in another comparison study performed by Napoli et al. showing average results which were 12% lower [21]. For everolimus the concentrations measured with LC-MS/MS were approximately 30% lower. In this correlation study the steeper slope caused overall everolimus deviations, while the large intercept has more influence near the LLOQ concentrations. The comparison results of Salm et al. for everolimus show similar results which were 24% lower when measured with LC-MS/MS [22]. These differences between the IA and the LC-MS/MS methods are predominantly due to the low selectivity and low robustness of the IA method. The correlation study of sirolimus and tacrolimus showed little or no difference between LC-MS/MS and IA. Other comparison studies performed to investigate the difference between LC-MS/MS and IA results for tacrolimus and sirolimus show varying results between studies [23]. For tacrolimus, Cogill et al. found results to be 15.6% lower when measured with LC-MS/MS compared to MEIA [24]. A correlation study of Wang et.al found no significant difference between LC-MS/MS and MEIA for tacrolimus and sirolimus [23]. Other comparison studies showed LC-MS/MS results to be 11.5% and 15% lower for sirolimus when measured with LC-MS/MS compared to MEIA [25, 26]. These varying correlation results between comparison studies may be caused by differences in patient populations.

In the authors' laboratory the total costs of sample throughput performed with LC-MS/MS showed to be less then the costs of IA (see table 4). For IA the purchase of the reagent kits

Table 4 Approximate yearly costs of the analyses with the three IA systems (AxSym, TDxFLx and IMx) versus the developed LC-MS/MS method

Yearly costs	Total for the IAs	LC-MS/MS
Appararatus	20,000	50,000
Maintenance	20,000	20,000
Reagents/ consumables	150,000	10,000
Technician(s)	50,000	50,000
Total	240,000	130,000

Calculations experienced in the authors' laboratory. Based on 15,000 samples analyzed per year. Prices in euros including VAT. Apparatus costs for the LC-MS/MS is based on 250,000 euro for purchase and 50,000 euro depreciation per year during 5 years.

contributed most to the costs of the IA. With IA the amount of samples analyzed have no great influence on the costs per sample. The costs of the LC-MS/MS mainly consisted of the purchase and maintenance of the system. Here an increase in samples does mean a decrease in the costs per sample. The LC-MS/MS method proved to be more labour efficient, but required higher educated technicians. This resulted in approximately the same technician costs, as mentioned in table 4. Based on table 4 it would be profitable to replace the IAs with the developed LC-MS/MS method starting from approximately 8,000 samples a year. In the authors' laboratory the developed LC-MS/MS method showed to save approximately 110,000 euro per year.

CONCLUSION

The method described here shows a straightforward LC-MS/MS method without the use of additional components and complicated method setups, but with a fast sample preparation and chromatography which maximises analysis speed without compromising in the separation of otherwise interfering peaks. Our research has shown that the use of zinc sulphate and methanol in the sample preparation gives process efficiency results of about 100% for tacrolimus and cyclosporin A, while for sirolimus and everolimus about 100% process efficiency is only achieved without the use of zinc sulphate. This has resulted in the use of two preparation methods allowing the simultaneous analysis of cyclosporin A and tacrolimus or sirolimus and everolimus with a single LC-MS/MS system. The method is fully validated and showed to be selective, fast and robust. This is proven by our analysis of approximately 70 patient samples daily, with a total number exceeding 22,000 patient samples over the

last year. In addition control values of all immunosuppressants obtained during bio-analysis have a CV of no more then 8.0 percent per immunosuppressant. With 20 injections per hour, during a working day of 8 hours 160 injections can be performed and when necessary patient samples can be analysed during the night, making this method fast enough for the expected increase of patient samples in the future. With this new method, preparation time, analysis time, consumables and chemicals are diminished in comparison to IA.

In the authors' laboratory the developed LC-MS/MS system proved to save approximately 110,000 euro per year.

This all provides better and faster patient care at lower costs. The fast sample preparation, short run times and simultaneous detection of immunosuppressive drugs allow high sample throughput at maximal flexibility, making the method ideal for therapeutic drug monitoring.

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