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Quantification of immunosuppressants in human blood by liquid chromatography-tandem mass spectrometry for clinical research

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Keywords

Immunosuppressants, offline sample preparation, blood, mass spectrometry, TSQ Quantis

Application benefits

- Simple offline sample preparation by protein precipitation
- Four immunosuppressants in a single quantitative method

Goal

Implementation of an analytical method for the quantification of four immunosuppressants in human blood on a Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer.

Introduction

Therapeutic drug monitoring (TDM) of immunosuppressive drugs in organ-transplanted patients is an extremely important aspect to prevent toxicity or transplant rejection due to inadequate dosage. The commonly used immunoassay-based technology has been gradually undergoing replacement by liquid chromatography coupled to mass spectrometry, owing to its ability to offer higher sensitivity and specificity. In this report, an analytical method for the quantification of four immunosuppressants in human blood for clinical research is reported; the analysis includes tacrolimus, sirolimus, everolimus, and cyclosporine A. Blood samples are extracted by offline internal standard addition and protein precipitation. Extracted samples are injected onto a Thermo Scientific™ Transcend™ II TLX-2 system for online SPE and LC separation. Detection is performed on a TSQ Quantis triple quadrupole mass spectrometer with heated electrospray ionization by selected reaction

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monitoring (SRM) using one isotopically labeled internal standard for each analyte. Method performance was evaluated using the ClinMass® LC-MS/MS Complete Kit for Immunosuppressants in Blood from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response within the calibration ranges, carryover, accuracy, and intra- and inter-assay precision for each analyte.

Experimental

Target analytes

The analytes and corresponding internal standards and concentration ranges covered by the calibrators used are reported in Table 1.

Sample preparation

Reagents included seven calibrators (including blank) and five controls from RECIPE, as well as four isotopically labeled internal standards for the quantification. Samples of 100 µL of blood were protein precipitated using 220 µL of precipitating solution containing the internal standards. Precipitated samples were vortex-mixed and centrifuged, and the supernatant was transferred to a clean plate or vial.

Liquid chromatography

Online SPE and LC separation were achieved using mobile phases, SPE cartridge, and analytical column provided by RECIPE. A schematic representation of the LC configuration is reported in Figure 1. Details of the analytical method are reported in Table 2. Total runtime was 2.0 minutes.

Table 1. Concentration ranges covered by calibrators

Analyte	Internal Standard	Concentration Range (ng/mL)
Tacrolimus	$^{13}Cd_2$-Tacrolimus	1.30–44.1
Sirolimus	$^{13}Cd_3$-Sirolimus	1.51–49.8
Everolimus	$^{13}C_2d_4$-Everolimus	1.45–48.0
Cyclosporine A	d_{12}-Cyclosporine A	26.3–1287

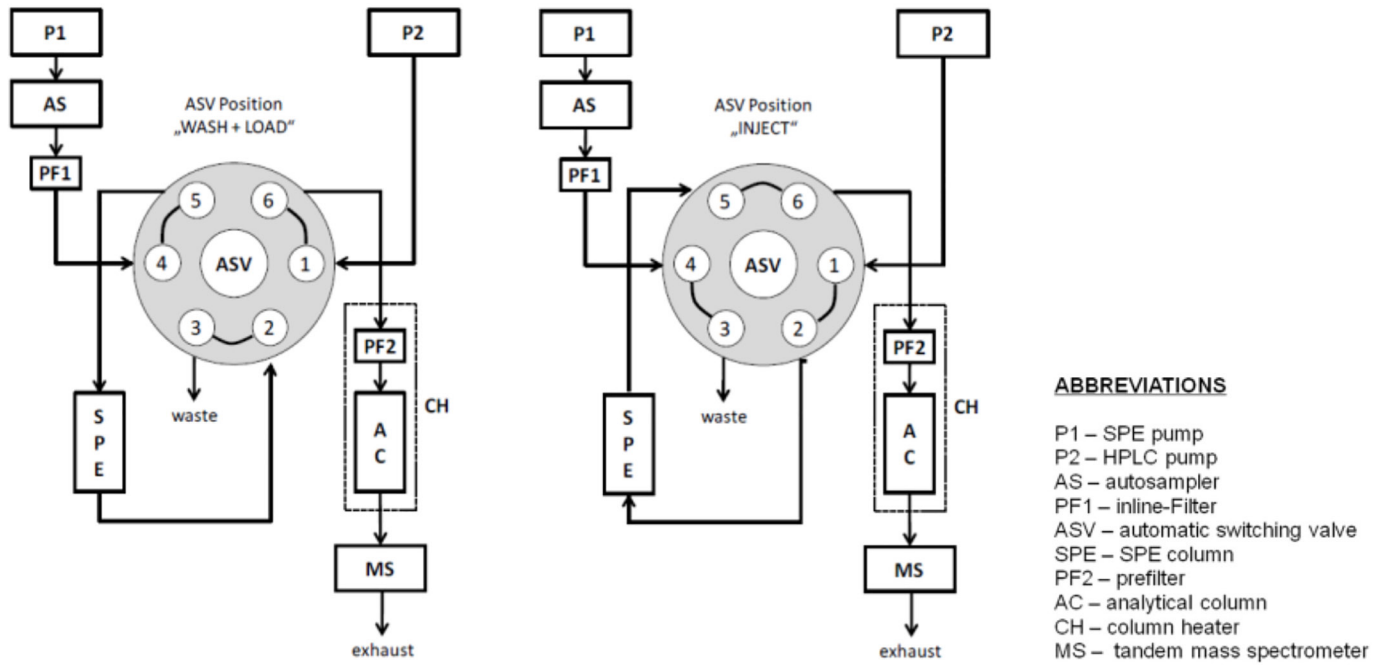

Figure 1. Schematic representation of the Transcend II system configuration used for on-line SPE

Table 2. LC method description

Parameter	Setting					
	Time (min)	ASV Position	Pump P1 Flow Rate (mL/min)	Event SPE Column	Pump P2 Flow Rate (mL/min)	Event Analytical Column
Gradient profile	0.00	Load	0.1		0.5	
	0.01		2.5	Loading		Equilibration
	0.50	Inject	2.5	Elution		Loading
	0.51		0.1			Separation
	1.30				0.5	
	1.35				1.0	
	1.50		0.1			
	1.51		2.5			
	1.55				1.0	
	1.65	Load		Equilibration	0.5	Equilibration
	1.99		2.5			
	2.00		0.1		0.5	
Injection volume	50 µL					
Column temperature	60 °C					

Mass spectrometry

Analytes and internal standards were detected by SRM on a TSQ Quantis triple quadrupole mass spectrometer with heated electrospray ionization operated in positive mode. A summary of the MS conditions is reported in Table 3. Two SRM transitions for each analyte were included in the acquisition method for quantification and confirmation, respectively.

Table 3. MS settings

Parameter	Setting
Source type	Heated electrospray ionization (HESI)
Vaporizer temperature	400 °C
Capillary temperature	350 °C
Spray voltage (positive mode)	3500 V
Sheath gas	45 AU
Sweep gas	1 AU
Auxiliary gas	10 AU
Data acquisition mode	Selected-reaction monitoring (SRM)
Collision gas pressure	1.5 mTorr
Cycle time	0.300 s
Q1 mass resolution (FWMH)	0.7
Q3 mass resolution (FWMH)	0.7

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration ranges, carryover, accuracy, and intra- and inter-assay precision for each analyte. Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected just after it. Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations using quality control samples at five different levels provided by RECIPE (MS8833 batch #1057 and MS8903 batch #1366) prepared and analyzed in replicates of five on three different days. Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at five different levels in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at five levels in replicates of five prepared and analyzed on three different days).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software.

Results and discussion

A quadratic interpolation with 1/x weighting was used for all the analytes. The percentage bias between nominal and back-calculated concentration was always within ±10% for all the calibrators in all the runs. Representative chromatograms for the lowest calibrator for all the analytes and their internal standards are reported in Figure 2. Representative calibration curves are reported in Figure 3.

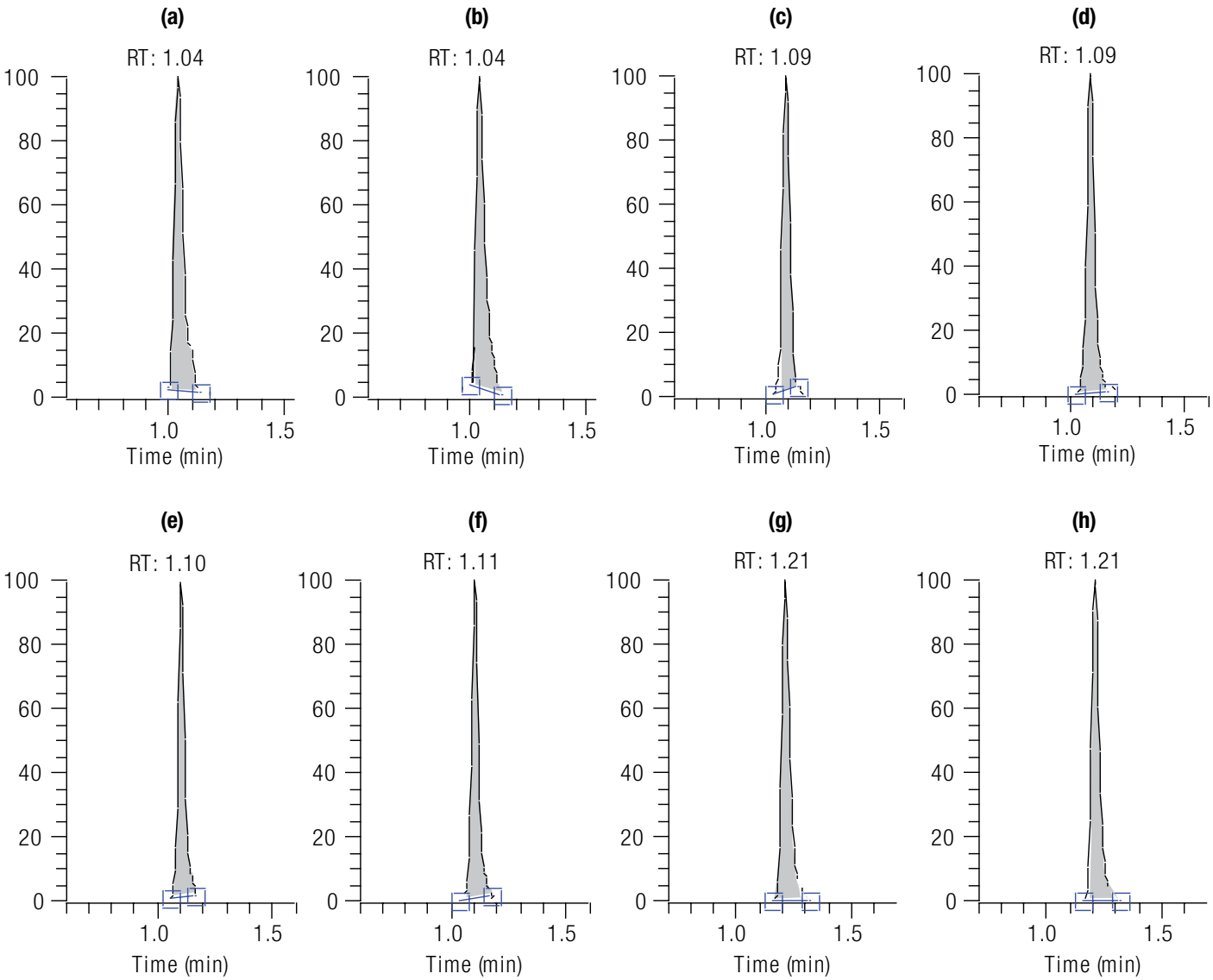

Figure 2. Representative chromatograms of the lowest calibrator for (a) tacrolimus, (b) $^{13}C_2d_4$-tacrolimus, (c) sirolimus, (d) $^{13}Cd_3$-sirolimus, (e) everolimus, (f) $^{13}C_2d_4$-everolimus, (g) cyclosporine A, and (h) d_{12}-cyclosporine A

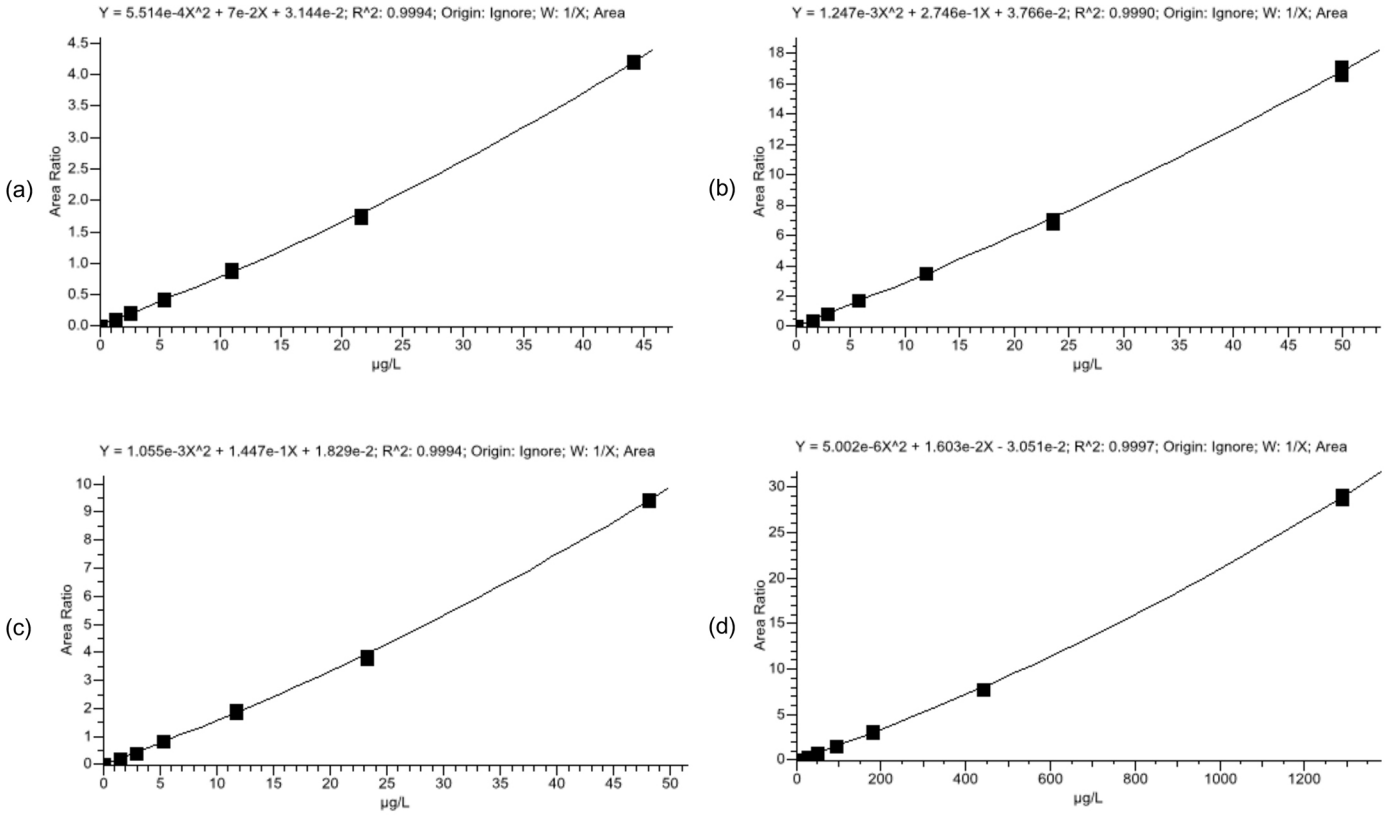

Figure 3. Representative calibration curves for (a) tacrolimus, (b) sirolimus, (c) everolimus, and (d) cyclosporine A

The maximum carryover was 0.2% for cyclosporine A; negligible carryover was registered for the other analytes with no peak detected in the blank injected after the highest calibrator.

The data demonstrated outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the control samples ranging between -7.3% and 5.9% (Table 4). The %CV for intra-assay precision was always below 7.1% for all the analytes. The maximum %CV for inter-assay precision including all the analytes was 6.1%. Results for intra- and inter-assay precision are reported in Table 5.

Table 4. Analytical accuracy results for control MS8833 batch #1057 and MS8903 batch #1366

Analyte	Control	Nominal Concentration (ng/mL)	Average Calculated Concentration (ng/mL)	Bias (%)
Tacrolimus	Level_I (LOT #1057)	3.46	3.57	3.2
	Level_II (LOT #1057)	7.08	7.22	1.9
	Level_III (LOT #1057)	14.6	14.6	-0.7
	Level_IV (LOT #1366)	27.1	26.8	-4.1
	Level_V (LOT #1366)	52.7	50.4	-0.8
Sirolimus	Level_I (LOT #1057)	3.69	3.83	3.8
	Level_II (LOT #1057)	11.9	11.9	0.2
	Level_III (LOT #1057)	20.3	20.0	0.5
	Level_IV (LOT #1366)	29.8	29.0	-6.0
	Level_V (LOT #1366)	58.5	56.4	5.9
Everolimus	Level_I (LOT #1057)	3.68	3.76	2.2
	Level_II (LOT #1057)	11.5	11.4	-0.8
	Level_III (LOT #1057)	19.3	19.0	-0.8
	Level_IV (LOT #1366)	29.7	28.7	-4.6
	Level_V (LOT #1366)	59.4	55.6	-0.1
Cyclosporine A	Level_I (LOT #1057)	57.0	59.2	3.9
	Level_II (LOT #1057)	119	119	0.0
	Level_III (LOT #1057)	237	235	-0.7
	Level_IV (LOT #1366)	680	663	-7.3
	Level_V (LOT #1366)	1544	1453	3.3

Table 5. Analytical intra- and inter-assay precision results for control MS8833 batch #1057 and MS8903 batch #1366

Analyte	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3			
		Average Calculated Conc. (ng/mL)	CV (%)	Average Calculated Conc. (ng/mL)	CV (%)	Average Calculated Conc. (ng/mL)	CV (%)	Average Calculated Conc. (ng/mL)	CV (%)
Tacrolimus	Level_I (LOT #1057)	3.53	1.7	3.55	7.1	3.62	4.0	3.57	4.6
	Level_II (LOT #1057)	7.21	5.5	7.17	3.6	7.27	5.8	7.22	4.7
	Level_III (LOT #1057)	14.1	4.5	14.4	3.1	15.3	1.1	14.6	4.7
	Level_IV (LOT #1366)	27.3	3.9	26.3	2.9	26.7	6.0	26.8	4.5
	Level_V (LOT #1366)	51.1	3.2	50.8	1.9	49.2	2.4	50.4	2.9
Sirolimus	Level_I (LOT #1057)	3.95	2.5	3.73	5.5	3.81	6.3	3.83	5.2
	Level_II (LOT #1057)	12.2	5.1	11.4	4.4	12.2	6.6	11.9	6.0
	Level_III (LOT #1057)	19.3	4.7	19.7	3.3	21.0	4.1	20.0	5.3
	Level_IV (LOT #1366)	29.4	6.2	28.2	2.3	29.4	5.4	29.0	5.1
	Level_V (LOT #1366)	56.1	5.9	55.9	2.4	57.2	3.1	56.4	3.9
Everolimus	Level_I (LOT #1057)	3.86	2.7	3.63	5.7	3.79	5.4	3.76	5.2
	Level_II (LOT #1057)	11.7	4.2	10.9	3.7	11.6	3.8	11.4	4.8
	Level_III (LOT #1057)	18.7	3.6	18.1	3.4	20.1	2.1	19.0	5.4
	Level_IV (LOT #1366)	29.5	5.9	28.0	3.2	28.5	4.2	28.7	4.8
	Level_V (LOT #1366)	56.8	3.7	55.0	1.1	54.9	2.0	55.6	2.8
Cyclosporine A	Level_I (LOT #1057)	59.7	3.5	57.5	7.1	60.4	3.9	59.2	5.2
	Level_II (LOT #1057)	122	4.7	112	4.0	123	5.7	119	6.1
	Level_III (LOT #1057)	230	3.6	226	3.7	249	1.2	235	5.2
	Level_IV (LOT #1366)	692	6.7	641	2.0	656	5.1	663	5.8
	Level_V (LOT #1366)	1478	5.8	1430	2.7	1449	2.1	1453	3.9

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Conclusions

A robust, reproducible, and sensitive liquid chromatography-tandem mass spectrometry method for clinical research for quantification of immunosuppressants in human blood was developed and implemented. The ClinMass LC-MS/MS Complete Kit from RECIPE was used. The method was analytically validated on a Transcend II system connected to a TSQ Quantis triple quadrupole mass spectrometer. The method described here offers quick and simple offline protein precipitation with concomitant internal standard addition. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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