TECHNICAL NOTE 73680

Confident quantification of immunosuppressants in human whole blood by liquid chromatography-tandem mass spectrometry for clinical research

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Application benefits

- Quantification of four immunosuppressants in a single run
- Simple offline sample preparation using protein precipitation
- Robustness and sensitivity with entry-level triple quadrupole MS

Goal

Implementation of an analytical method for the quantification of cyclosporin A, tacrolimus, sirolimus, and everolimus in human blood on a Thermo Scientific™ TSQ Fortis™ triple-stage quadrupole mass spectrometer

Introduction

A robust LC-MS method for the quantification of cyclosporin A, everolimus, sirolimus, and tacrolimus in human whole blood for clinical research is reported. The method involves a simple protein precipitation step followed by injection of the supernatant on a Thermo Scientific™

Vanquish™ Flex Binary UHPLC system for chromatographic separation. A TSQ Fortis triple-stage quadrupole mass spectrometer with heated electrospray ionization (HESI) was used for detection. Data were acquired by selected reaction monitoring (SRM) using isotopically labeled internal standards for each analyte. Method performance was evaluated using the MS99200 ClinMass™ TDM Kit System – Immunosuppressants in Whole 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 all analytes.

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Experimental

Target analytes

- Cyclosporin A
- d_{12}-Cyclosporin A (internal standard)
- Everolimus
- $^{13}C_2,d_4$-Everolimus (internal standard)
- Sirolimus
- $^{13}C,d_3$-Sirolimus (internal standard)
- Tacrolimus
- $^{13}C,d_2$-Tacrolimus (internal standard)

The concentration ranges covered by the calibrators are reported in Table 1.

Sample preparation

Reagents included eight calibrators (including blank) and controls at five levels from RECIPE. Each compound was quantified using the corresponding isotopically labeled internal standard. Sample preparation consisted of a simple protein precipitation step. 220 µL of a precipitating reagent including internal standards were added to 100 µL of whole blood. Precipitated samples were vortex-mixed, then centrifuged. The supernatant was transferred to a clean vial for LC-MS/MS analysis.

Liquid chromatography

Chromatographic separation was performed using a Vanquish Flex Binary UHPLC system. The LC separation was achieved using mobile phases and an analytical column provided by RECIPE. Details of the analytical method are reported in Table 2. Total runtime was 1.9 minutes.

Mass spectrometry

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

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 all analytes. Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected immediately 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 (8833 LOT #1057 and 8903 LOT #1366), which were 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).

Table 1. Concentration ranges covered by calibrators

Analyte	Concentration range (ng/mL)
Cyclosporin A	26.4–1671
Tacrolimus	1.25–60.4
Sirolimus	1.47–69.8
Everolimus	1.49–69.5

Table 2. LC method description

	Time (min)	Flow rate (mL/min)	A (%)	B (%)
Gradient profile	0.00	0.9	90	10
	0.70	0.9	90	10
	0.80	0.9	5	95
	1.70	0.9	5	95
	1.80	0.9	90	10
	1.90	0.9	90	10
Injection volume (µL)				20
Column temperature (°C)				70

Table 3. MS settings

Source type	Heated electrospray ionization (H-ESI)
Vaporizer temperature	350 °C
Capillary temperature	320 °C
Spray voltage (positive mode)	3000 V
Sheath gas	56 AU
Sweep gas	1 AU
Auxiliary gas	9 AU
Data acquisition mode	Selected-reaction monitoring (SRM)
Collision gas pressure	2 mTorr
Cycle time	0.40 s
Q1 mass resolution (FWMH)	0.7
Q3 mass resolution (FWMH)	1.2

Data analysis

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

Results and discussion

The reported analytical method proved to be linear not only in the calibration range covered by the calibrators but also in a wider range obtained by diluting the lowest calibrator by a factor of 2. The lower limits of quantification (LLOQ) were 13.2 ng/mL for cyclosporin A, 0.75 ng/mL for everolimus, 0.74 ng/mL for sirolimus, and 0.63 ng/mL for tacrolimus, with correlation factors (R^2) always above 0.99. Representative chromatograms for the lowest calibrator are reported in Figure 1. Representative calibration curves for all compounds are reported in Figure 2.

No significant carryover was observed for any analyte, with no signal detected in the blank injected immediately after the highest calibrator.

The data showed good accuracy with the percentage bias between nominal and average back-calculated concentration for these control samples always being between -15.6% and 5.9%, as reported in Table 4. The %CV for intra-assay precision was always below 15.2% for all analytes at all levels (Table 5). The maximum %CV for inter-assay precision including all analytes was 16.7%.

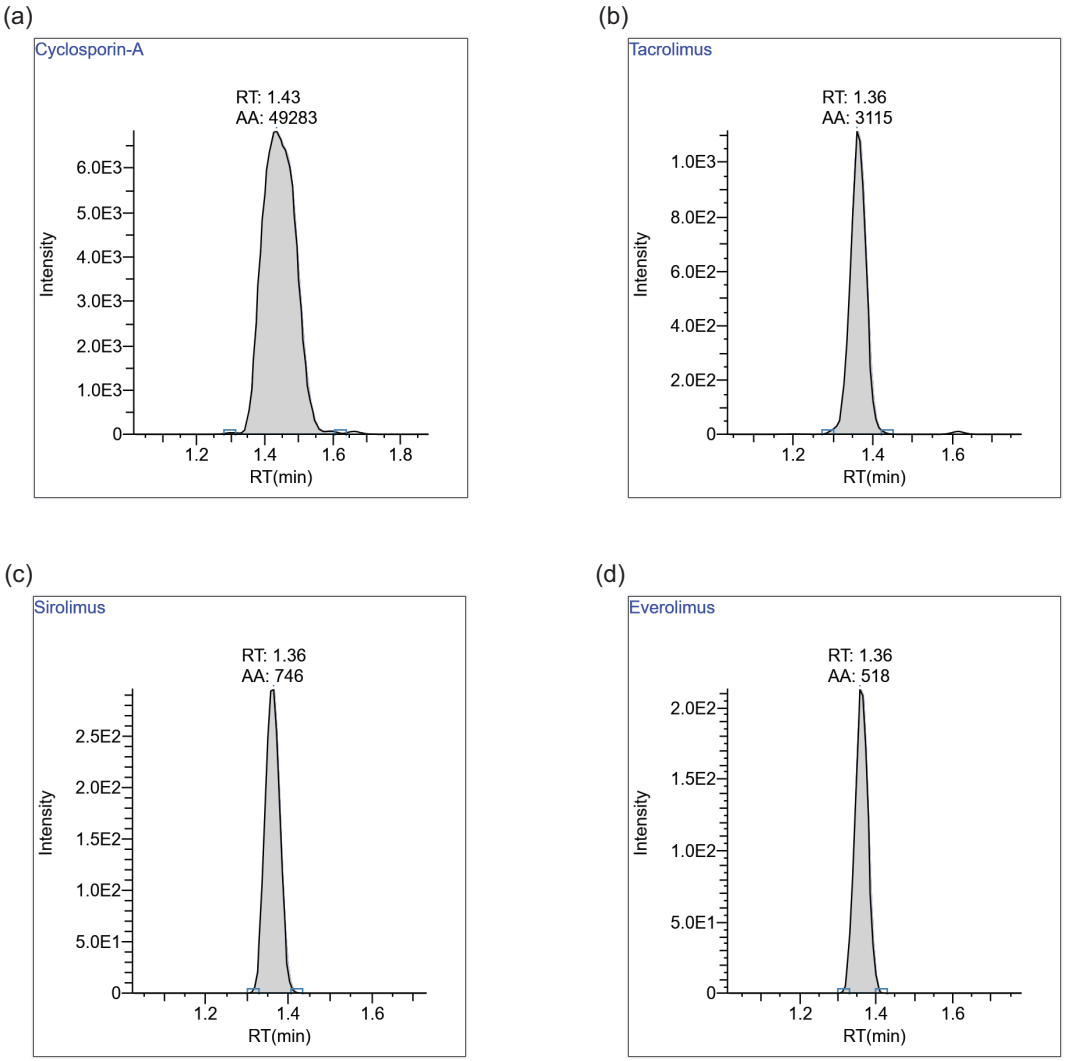

Figure 1. Representative chromatograms of the lowest calibrator at LLOQ level for (a) cyclosporin A, (b) tacrolimus, (c) sirolimus, and (d) everolimus

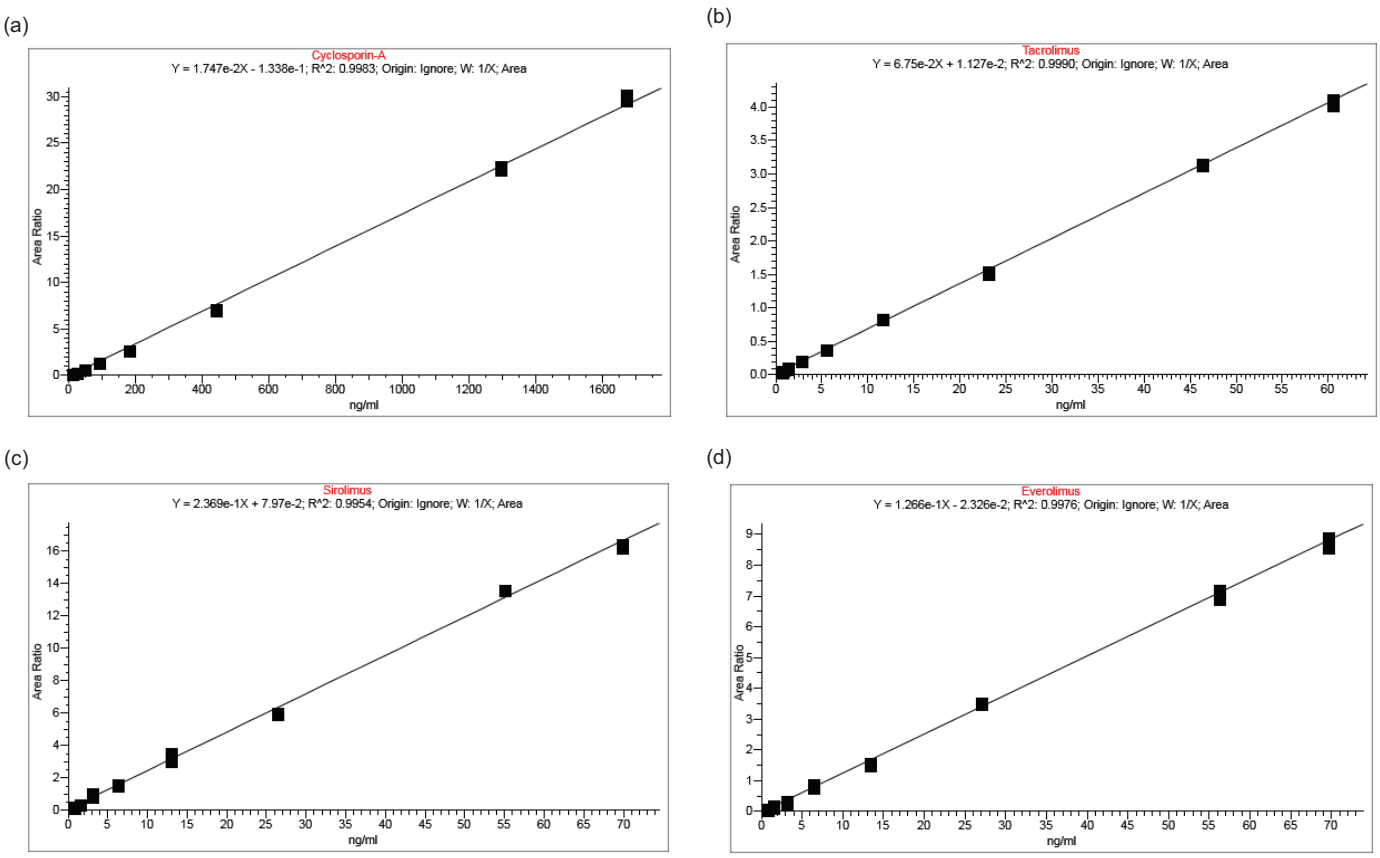

Figure 2. Representative calibration curves for (a) cyclosporin-A, (b) tacrolimus, (c) sirolimus, and (d) everolimus

Table 4. Analytical accuracy results (n=5) for controls 8833 LOT #1057 and 8903 LOT #1366

Analyte	Control	Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)
Cyclosporin A	Level I (LOT #1057)	57.0	52.8	-7.4
	Level II (LOT #1057)	119	105	-11.6
	Level III (LOT #1057)	237	200	-15.6
	Level IV (LOT #1366)	680	633	-6.9
	Level V (LOT #1366)	1544	1500	-2.8
Tacrolimus	Level I (LOT #1057)	3.46	3.38	-2.4
	Level II (LOT #1057)	7.08	7.28	2.9
	Level III (LOT #1057)	14.6	15.0	3.1
	Level IV (LOT #1366)	27.1	27.5	1.6
	Level V (LOT #1366)	52.7	53.1	0.7
Sirolimus	Level I (LOT #1057)	3.69	3.89	5.5
	Level II (LOT #1057)	11.9	12.5	5.0
	Level III (LOT #1057)	20.3	20.7	2.0
	Level IV (LOT #1366)	29.8	31.5	5.6
	Level V (LOT #1366)	58.5	62.0	5.9
Everolimus	Level I (LOT #1057)	3.68	3.41	-7.3
	Level II (LOT #1057)	11.5	11.4	-0.7
	Level III (LOT #1057)	19.3	20.0	3.9
	Level IV (LOT #1366)	29.7	28.9	-2.8
	Level V (LOT #1366)	59.4	59.0	-0.7

Table 5. Analytical intra- and inter-assay precision results (n=15) for controls 8833 LOT #1057 and 8903 LOT #1366

Analyte	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3			
		Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)
Cyclosporin A	Level I (LOT #1057)	49	1.6	56	1.0	54	3.4	52.8	6.1
	Level II (LOT #1057)	105	0.9	108	2.2	103	3.2	105	3.1
	Level III (LOT #1057)	203	1.7	197	1.6	197	6.4	200	3.4
	Level IV (LOT #1366)	634	0.8	655	1.8	601	7.0	633	4.5
	Level V (LOT #1366)	1508	1.3	1582	4.1	1410	4.1	1500	5.8
Tacrolimus	Level I (LOT #1057)	3.26	8.7	3.35	9.5	3.52	7.5	3.38	8.6
	Level II (LOT #1057)	7.32	6.0	7.16	5.3	7.37	6.7	7.28	5.8
	Level III (LOT #1057)	15.2	1.4	14.9	1.7	15.0	7.0	15.0	4.0
	Level IV (LOT #1366)	28.7	2.7	27.9	3.3	26.1	6.9	27.5	5.8
	Level V (LOT #1366)	52.2	2.8	56.2	4.4	50.7	5.5	53.1	6.0
Sirolimus	Level I (LOT #1057)	3.70	10.7	4.14	14.0	4.31	14.8	3.89	16.7
	Level II (LOT #1057)	13.5	8.8	11.0	15.2	13.0	11.7	12.5	14.1
	Level III (LOT #1057)	21.7	14.9	20.1	9.7	20.2	9.3	20.7	11.5
	Level IV (LOT #1366)	31.4	5.7	30.7	10.1	32.2	11.5	31.5	9.0
	Level V (LOT #1366)	63.4	6.5	63.6	5.3	58.9	10.4	62.0	7.8
Everolimus	Level I (LOT #1057)	3.41	5.7	3.31	11.9	3.51	13.9	3.41	10.6
	Level II (LOT #1057)	12.1	9.7	11.0	8.3	11.1	10.1	11.4	9.8
	Level III (LOT #1057)	20.9	8.9	19.4	6.1	19.9	4.2	20.0	7.0
	Level IV (LOT #1366)	30.6	2.8	29.0	7.0	28.3	5.4	28.9	8.8
	Level V (LOT #1366)	61.5	2.9	60.3	5.9	52.7	2.5	59.0	7.8

Conclusion

A robust, reproducible, and reliable method for the quantification of cyclosporin A, everolimus, sirolimus, and tacrolimus in human whole blood was developed using liquid chromatography coupled to tandem mass spectrometry for clinical research. The method was analytically validated using a Vanquish Flex Binary UHPLC system connected to a TSQ Fortis triple-stage quadrupole mass spectrometer. Simple, robust protein precipitation was used for purification of the samples. The data obtained with the described method successfully met sensitivity, reliability, accuracy, and precision expectations typically demanded by clinical research laboratories.

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