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TECHNICAL NOTE 65439

Confident quantitation for every user: 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, multi-channeling

Application benefits

- Simple offline sample preparation by protein precipitation
- Easy to implement quantitative method for four immunosuppressants in the same run
- Best-in-class sensitivity with mid-range triple quadrupole MS

Goal

Implementation of an analytical method for the quantification of four immunosuppressants (tacrolimus, sirolimus, everolimus, and cyclosporine A) in human blood on a Thermo Scientific™ TSQ Quantis™ triple quadrupole mass spectrometer.

Introduction

Drug monitoring of immunosuppressive drugs in organ-transplanted patients is critical for prevention of toxicity or transplanted organ rejection due to inadequate dosage. While immunoassay has been the preferred technology, it has been gradually replaced by liquid chromatography coupled to mass spectrometry, which offers higher selectivity and specificity. In this report, an analytical method covering four commonly monitored immunosuppressants in human blood for clinical research is reported. The analytes include tacrolimus, sirolimus, everolimus, and cyclosporine A. Blood samples were extracted by offline internal standard addition and protein precipitation. Extracted samples were injected onto a Thermo Scientific™ Transcend™ LX-2 system. Detection

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by mass spectrometry was performed using a TSQ Quantis triple quadrupole mass spectrometer with heated electrospray ionization by selected-reaction monitoring (SRM) using one isotopically labeled internal standard for each analyte. Method performance was evaluated using calibrators and controls obtained from Chromsystems Instruments & Chemicals GmbH (Gräfelfing, Germany) and checked for 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.

Table 1. Concentration ranges covered by calibrators

Analyte	Internal Standard	Concentration Range (ng/mL)
Tacrolimus	$^{13}Cd_2$-Tacrolimus	2.38–43.9
Sirolimus	d_3-Sirolimus	2.40–48.1
Everolimus	d_4-Everolimus	2.19–41.8
Cyclosporine A	d_4-Cyclosporine A	24.7–929

Sample preparation

Reagents included seven calibrators (including blank) and four controls from Chromsystems, as well as four isotopically labelled internal standards for the quantification of immunosuppressants in blood. Samples of 100 µL of blood were protein precipitated using 150 µL of 0.1 M $ZnSO_4$ and 200 µL of methanol containing the internal standards. Precipitated samples were vortex-mixed and centrifuged,and the supernatant was transferred to a clean plate or vial.

Liquid chromatography

A Transcend LX-2 system was used. The system consists of two separate, parallel UHPLC channels connected to a single mass spectrometer (Figure 1). This configuration enhances productivity by efficiently utilizing the mass spectrometer idle time. In our case, each batch was run by staggering the injections on both channels, effectively doubling the sample throughput compared to a single-channel system. LC separation was achieved on a Thermo Scientific™ Hypersil GOLD™ analytical column 50 × 2.1 mm (1.9 µm) kept at 60 °C. Mobile phases A and B consisted of water and methanol, respectively, both containing 10 mM ammonium formate and 0.1 % formic acid. Mobile phase C consisted of a mix of acetonitrile, acetone, and 2-propanol 60/20/20 (v/v/v). Details of the analytical method are reported in Table 2. Total runtime was 3.5 minutes.

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 ion 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.

Figure 1. Thermo Scientific Transcend LX-2 system

Table 2. LC method description

Gradient Profile				
Time (min)	**Flow Rate (mL/min)**	**A (%)**	**B (%)**	**C (%)**
0.00	0.6	95	5	0
0.08	0.6	95	5	0
0.50	0.6	90	10	0
0.51	0.6	0	100	0
1.50	0.6	0	100	0
1.51	0.6	0	0	100
2.50	0.6	0	0	100
2.51	0.6	95	5	0
3.50	0.6	95	5	0
Other Parameters				
Injection volume (µL)		25		
Column temperature (°C)		60		

Table 3. MS settings

Source type:	Heated electrospray ionization (HESI)
Vaporizer temperature:	400 °C
Capillary temperature:	350 °C
Spray voltage (positive mode):	3500 V
Sheath gas:	50 AU
Sweep gas:	2 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
Source fragmentation:	10.0 V

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 four different levels provided by Chromsystems (MassCheck® 0081 lot #2317) 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 four 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 four 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 linear 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. The correlation factor (R^2) was always above 0.999 for all the analytes. Representative chromatograms for the lowest calibrator for all the analytes and their internal standards are reported in Figure 2. Representative calibration curves (run in duplicate) are reported in Figure 3.

Negligible carryover was registered for all the analytes with no peak detected in the blank injected after the highest calibrator.

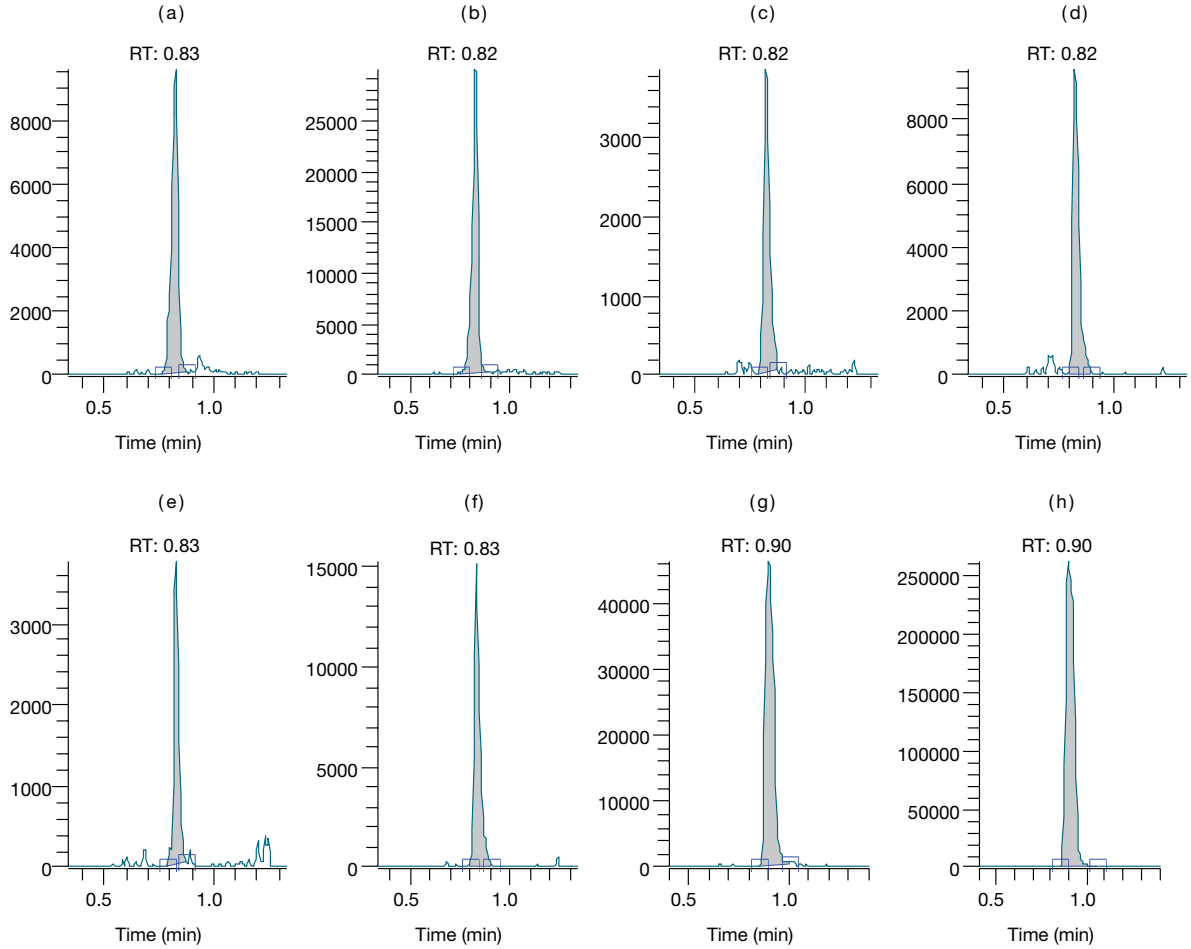

Figure 2. Representative chromatograms of the lowest calibrator for (a) tacrolimus, (b) $^{13}Cd_2$-tacrolimus, (c) sirolimus, (d) d_3-sirolimus, (e) everolimus, (f) d_4-everolimus, (g) cyclosporine A, and (h) d_4-cyclosporine A

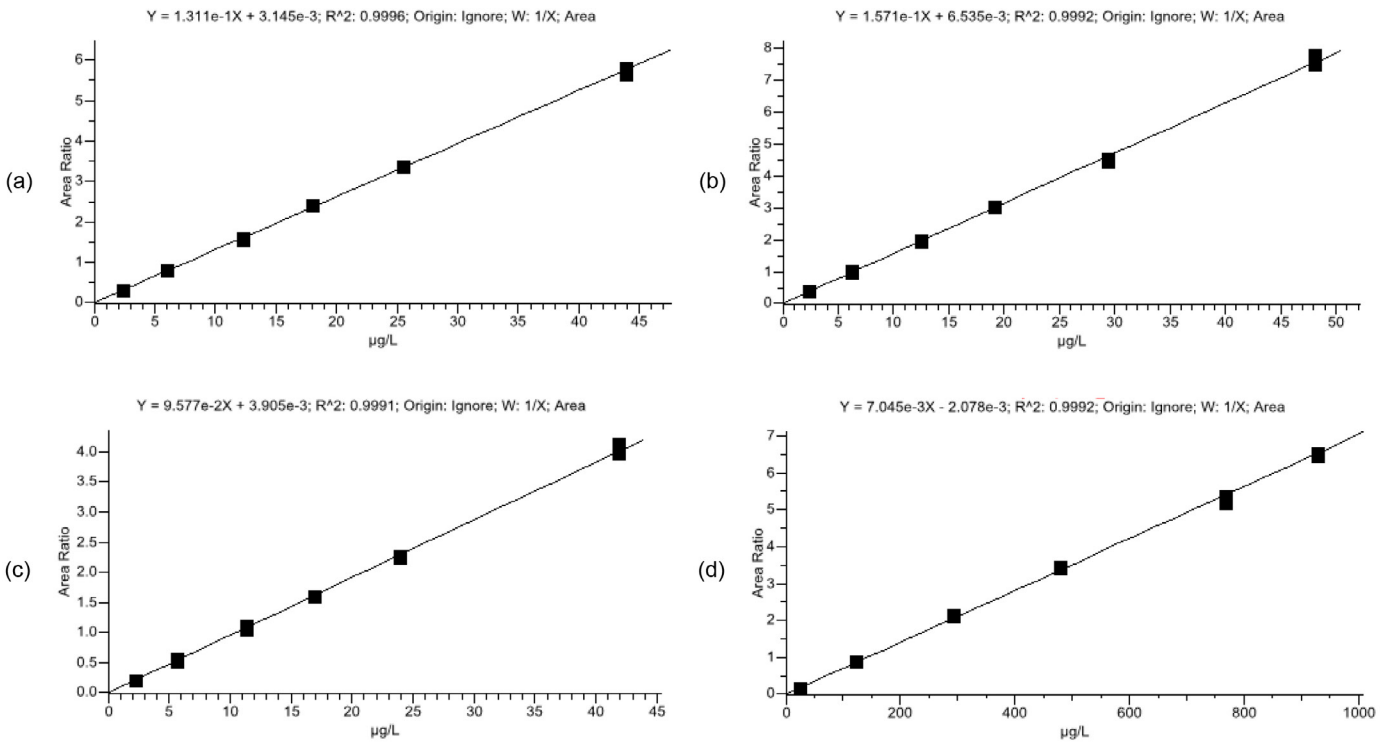

Figure 3. Representative calibration curves for (a) tacrolimus, (b) sirolimus, (c) everolimus, and (d) cyclosporine A – day 1

The data demonstrated outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the used control samples ranging between -6.3% and 1.2% (Table 4). The %CV for intra-assay precision was always below 7.3% for all the analytes. The maximum %CV for inter-assay precision including all the analytes was 6.8%. Results for intra- and inter-assay precision are reported in Table 5.

Table 4. Analytical accuracy results for controls MassCheck® 0081 lot #2317

Analyte	Control	Nominal Concentration (ng/mL)	Average Calculated Concentration (ng/mL)	Bias (%)
Tacrolimus	CTRL1	2.86	2.68	-6.3
	CTRL2	7.15	7.41	-4.1
	CTRL3	15.4	15.7	-4.9
	CTRL4	32.9	33.2	-4.7
Sirolimus	CTRL1	2.76	2.67	-3.3
	CTRL2	9.89	9.52	-3.8
	CTRL3	19.5	18.5	-5.2
	CTRL4	39.3	37.4	-4.9
Everolimus	CTRL1	2.60	2.52	-3.0
	CTRL2	4.79	4.73	-1.3
	CTRL3	9.63	9.06	-5.9
	CTRL4	32.7	30.7	-6.1
Cyclosporine A	CTRL1	49.7	47.2	-5.1
	CTRL2	248	251	1.2
	CTRL3	487	470	-3.4
	CTRL4	1100	1067	-3.0

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	CTRL1	2.65	1.4	2.56	1.1	2.83	4.2	2.68	5.1
	CTRL2	7.24	1.1	6.98	1.9	8.02	2.4	7.41	6.4
	CTRL3	15.4	0.9	15.1	4.5	16.6	4.8	15.7	5.6
	CTRL4	33.0	1.4	32.0	2.7	34.5	1.9	33.2	3.8
Sirolimus	CTRL1	2.54	1.5	2.64	5.2	2.82	4.2	2.67	5.7
	CTRL2	9.17	2.3	9.23	3.9	10.15	1.0	9.52	5.4
	CTRL3	18.4	3.1	17.3	1.8	19.7	4.2	18.5	6.2
	CTRL4	37.9	5.4	34.5	1.1	39.6	2.7	37.4	6.8
Everolimus	CTRL1	2.43	2.0	2.54	6.1	2.59	4.5	2.52	5.1
	CTRL2	4.57	7.3	4.71	7.2	4.90	3.7	4.73	6.5
	CTRL3	9.16	5.0	8.69	1.1	9.32	6.2	9.06	5.4
	CTRL4	32.5	4.5	28.7	1.1	30.9	3.4	30.7	6.3
Cyclosporine A	CTRL1	46.8	3.0	47.5	2.8	47.2	2.8	47.2	2.7
	CTRL2	249	3.1	244	5.1	260	2.0	251	4.2
	CTRL3	487	4.2	442	2.0	482	5.5	470	5.9
	CTRL4	1140	0.3	982	0.8	1078	2.0	1067	6.4

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Conclusions

A robust, reproducible, and sensitive LC-MS method for clinical research for quantification of immunosuppressants in human blood was developed and implemented. The method was analytically validated on a Transcend LX-2 system connected to a TSQ Quantis triple quadrupole mass spectrometer. Calibrators and controls from Chromsystems were used. The data obtained with the described method successfully met sensitivity, reliability, accuracy, and precision expectations typically found in clinical research laboratories.

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