TECHNICAL NOTE 66003

Quantification of immunosuppressants in human blood by LC-HRAM mass spectrometry for clinical research

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Application benefits

- Increased accuracy of method by implementation of a comprehensive ClinMass® kit for sample preparation
- High-resolution mass spectrometry for improved selectivity
- Robust, sensitive hardware enables increased confidence in data
- Simple offline sample preparation by protein precipitation

Goal

Implementation of an analytical method for the quantification of four immunosuppressants in human blood on a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer.

Introduction

Therapeutic drug monitoring (TDM) research of immunosuppressive drugs in organ-transplant recipients is an extremely important aspect to prevent intoxication 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, due to its ability to offer higher sensitivity and specificity, thus providing more accurate results. The use of high resolution allows for selectivity and sensitivity even in full scan (Full MS) mode. The additional use of fragmentation in Parallel Reaction Monitoring (PRM) mode provides enhanced specificity to the analytical method.

In this study, an analytical method for clinical research for the quantification of four immunosuppressants in human blood is reported; the analysis includes 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™ Vanquish™ Flex Duo UHPLC system for online solid-phase extraction (SPE) and LC separation. Detection was performed using a high-resolution accurate mass (HRAM) Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with heated electrospray ionization (HESI) either by Full MS or by PRM using an isotopically labelled internal standard for each analyte of interest. Method performance was evaluated using the ClinMass® LC-MS/MS Complete Kit for Immunosuppressants in Blood (MS1100) from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response within the calibration ranges, lower limit of quantification (LLOQ), carryover, accuracy, trueness of measurement, and intra- and inter-assay precision for each analyte.

Experimental

Target analytes

Target analytes included tacrolimus, sirolimus, everolimus, and cyclosporine A and the corresponding stable-isotope labelled internal standards. Different batches of calibrators with slightly different calibration ranges were used for the two acquisition modes (Table 1).

Sample preparation

Reagents included seven calibrators (including blank) and two controls from RECIPE (MS8833 batch #1509), as well as a mix of four isotopically labelled 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

A Vanquish Flex Duo UHPLC system configured as a dual channel instrument for both LC-only and online SPE applications (Figure 1) was used for online sample extraction and chromatographic separation. The online SPE channel was used in this case, utilizing the mobile phases, SPE cartridge, and analytical column provided by RECIPE. Details of the analytical method are reported in Table 2. Total runtime was 2.0 minutes.

Mass spectrometry

Analytes and internal standards were detected using either Full MS or PRM acquisition mode on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with heated electrospray ionization operated in positive ion mode. A summary of the MS conditions is reported in Table 3.

Table 1. Target analytes, corresponding internal standards, and calibration ranges

Analyte	Internal Standard	Full Scan mode (MS9933 #2430)	PRM mode (MS9933 #1428)
Tacrolimus	$^{13}Cd_2$-Tacrolimus	1.25–42.9	1.30–43.4
Sirolimus	$^{13}Cd_3$-Sirolimus	1.38–44.6	1.44–47.3
Everolimus	$^{13}C_2d_4$-Everolimus	1.22–45.6	1.31–46.1
Cyclosporine A	d_{12}-Cyclosporine A	21.7–1265	22.7–1258

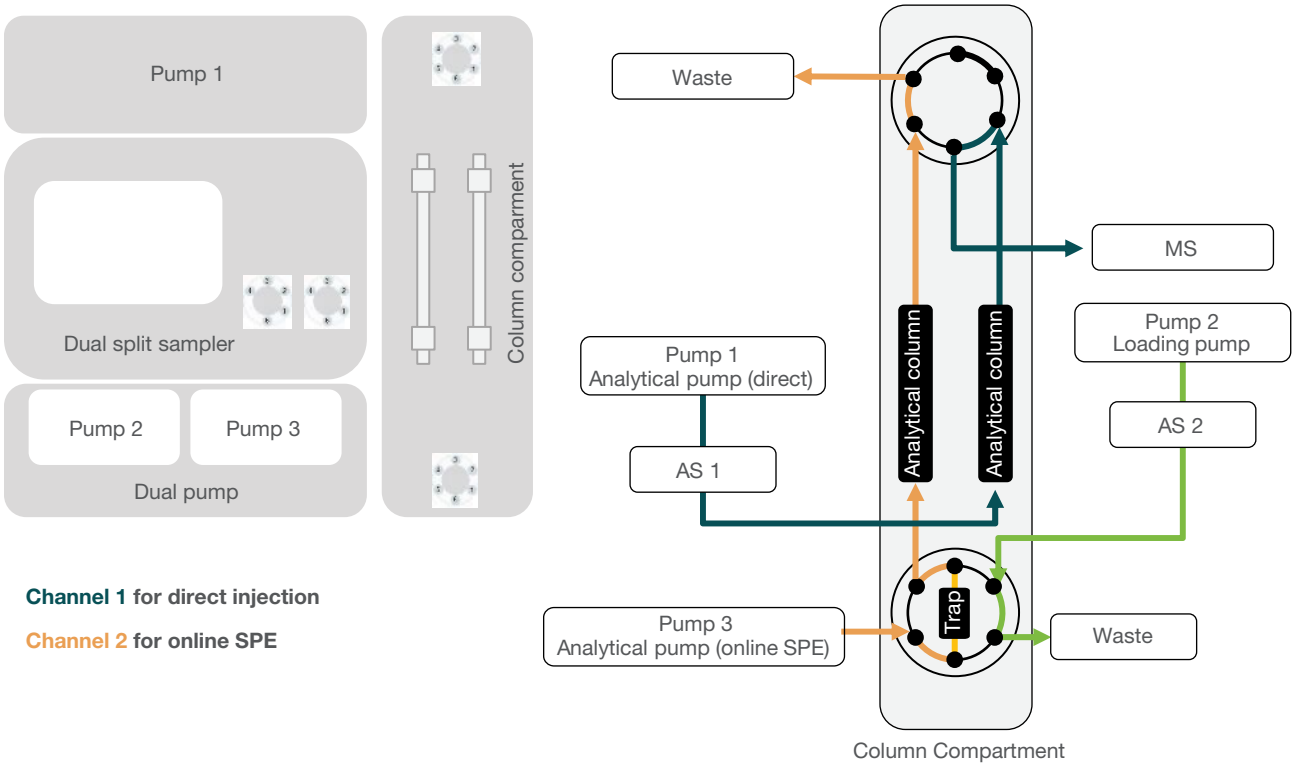


Figure 1. Schematic representation of the Vanquish Duo UHPLC system setup

Table 2. LC method description

Gradient profile					
Time (min)	**SPE valve**	**Pump 2 flow rate (mL/min)**	**Event SPE column**	**Pump 3 flow rate (mL/min)**	**Event analytical column**
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
1.20				0.5	
1.25				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		0.0		0.1	
Other parameters					
Injection volume				50 µL	
Column temperature				60 °C	

Table 3. MS settings

Ion source parameters	
Source type	HESI-II
Spray voltage - Positive (V)	2,000
Sheath gas (Arb)	40
Aux gas (Arb)	15
Sweep gas (Arb)	1
Ion transfer tube temp. (°C)	320
Vaporizer temp. (°C)	250
S-Lens RF level	60
Full Scan settings	
Resolution (at *m/z* 200)	70,000
Scan range (*m/z*)	800–1,250
AGC target	1e6
Maximum IT (ms)	100
PRM Settings	
Resolution (at *m/z* 200)	17,500
Isolation window (*m/z*)	2.0
AGC target	2e4
Maximum IT (ms)	50
Fixed first mass (*m/z*)	500
Stepped normalized collision energy	10, 15, 20

Method evaluation

The method performance was evaluated in terms of linearity of response, LLOQ, carryover, accuracy, trueness of measurement, and intra- and inter-assay precision for each analyte.

Five additional calibration levels were used to determine linearity of response and LLOQ—one (MS9028 #1457) above the highest calibrator and four by 20-fold dilution of the lowest calibrator with blank matrix; five-level controls (MS8833 #1509 and MS8903 #1428) were also used. A full set of calibrators (eleven levels) and controls (five levels) were extracted in replicates of five (n=5), injected in a single batch, and all used for the linear interpolation. The LLOQ was set as the lowest level that could be determined with a CV < 20% across the entire batch of samples.

Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator (MS9028) 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 the quality control samples at two different levels provided by RECIPE, prepared and analyzed in replicates of five on three different days.

Trueness of measurement was also evaluated as percentage bias using certified external quality controls (IP444 for Full MS and IP434 for PRM) from LGC (United Kingdom), prepared and analyzed in replicates of five on a single day.

Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at two 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 two levels in replicates of five prepared and analyzed on three different days).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 5.1 software.

Results and discussion

A linear response with 1/× weighting with a correlation factor (R^2) always above 0.99 was obtained for all the analytes in both acquisition modes, not only in the calibration range covered by the calibrators, but also down to a LLOQ reported in Table 4. The percentage bias between nominal and back-calculated concentration was always within ±10% for all the calibrators in all the runs. Representative chromatograms for everolimus, cyclosporine A, and their internal standards at the LLOQ in both acquisition modes are depicted in Figure 2. Representative calibration curves for the same analytes in the concentration range covered by the kit are shown in Figure 3.

No carryover was registered; no peak was detected in the blank sample following the highest calibrator for any analyte.

The data presented in this report demonstrate the outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the used control samples ranging between -1.9% and 4.9% (Table 5).

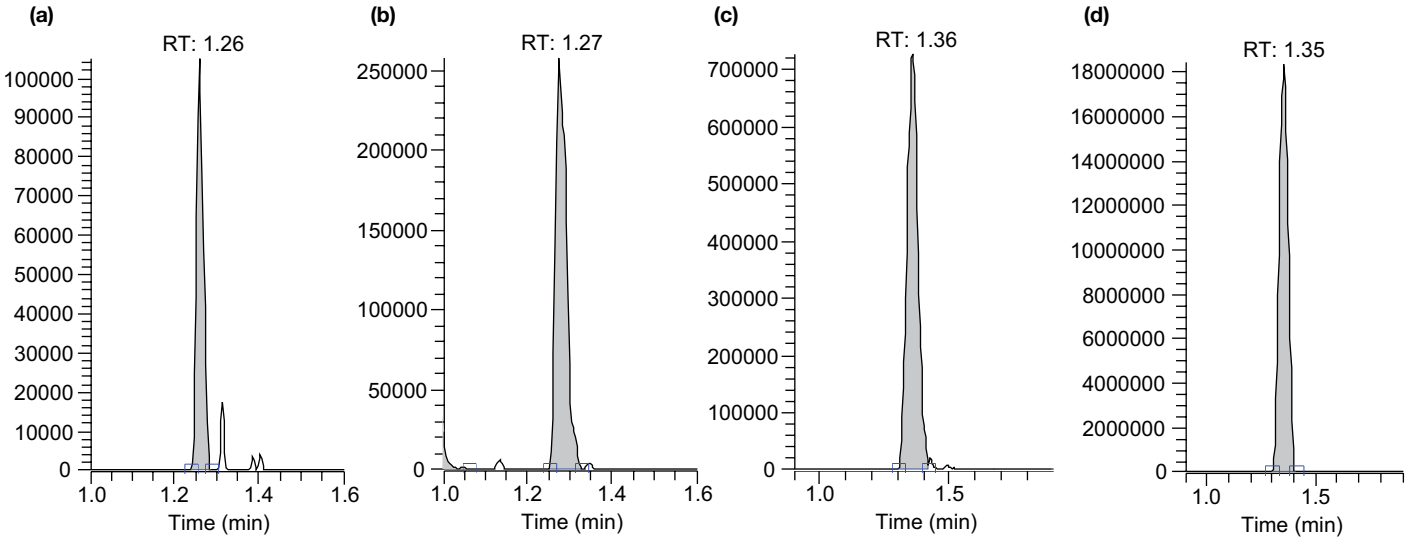
Good accuracy was also obtained from the evaluation of trueness of measurement of the external quality control samples, with experimental values always within the required acceptance ranges (Table 6).

The %CV for intra-assay precision was always below 5.4%. The maximum %CV for inter-assay precision was 6.5%. Results for intra- and inter-assay precision are reported in Table 7 and Table 8, respectively.

Table 4. Analytes and corresponding LLOQ

Analyte	LLOQ (ng/mL)	
	Full Scan mode	PRM mode
Tacrolimus	0.625	0.650
Sirolimus	0.690	0.288
Everolimus	0.244	0.262
Cyclosporine A	2.17	2.27

Full Scan mode



PRM mode

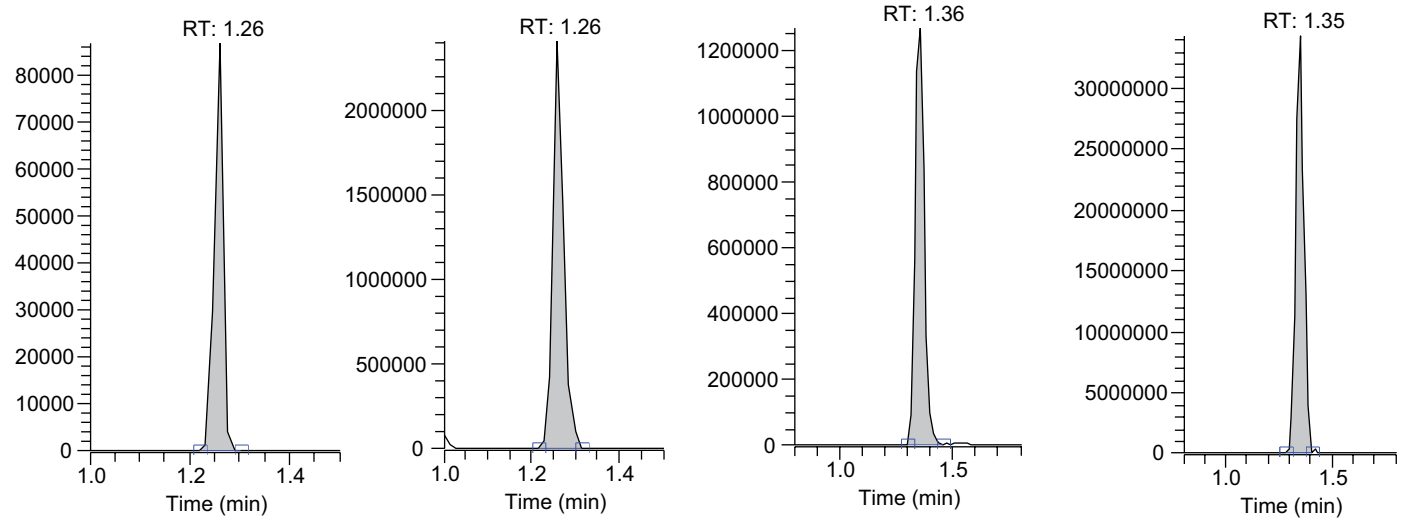


Figure 2. Representative chromatograms at the LLOQ for (a) everolimus, (b) $^{13}C_2d_4$-everolimus, (c) cyclosporine A and (d) d_{12}-cyclosporine A in Full Scan and PRM acquisition modes

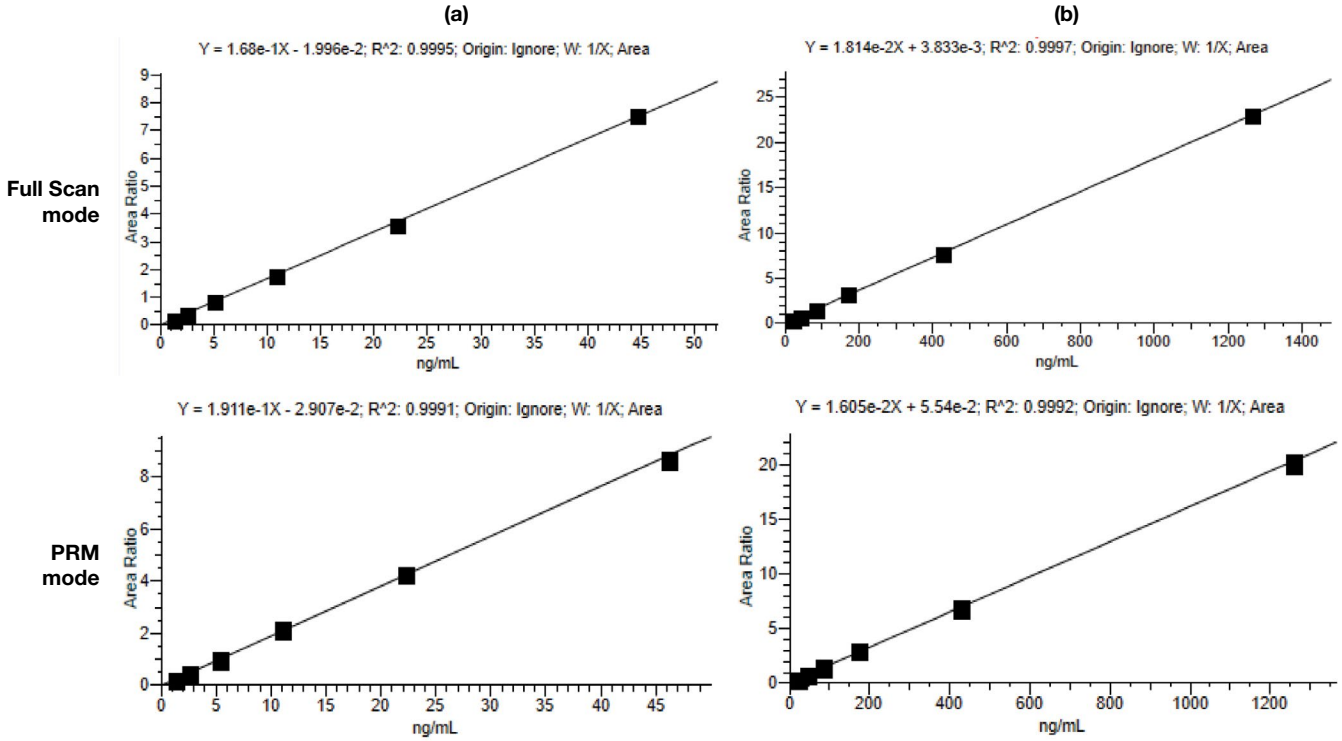


Figure 3. Representative calibration curves for (a) everolimus and (b) cyclosporine A

Table 5. Analytical accuracy results for controls MS8833 batch #1509

Analyte	Level I					Level III				
	Nominal concentration (ng/mL)	Full Scan mode		PRM mode		Nominal concentration (ng/mL)	Full Scan mode		PRM mode	
		Average calculated concentration (ng/mL)	Bias (%)	Average calculated concentration (ng/mL)	Bias (%)		Average calculated concentration (ng/mL)	Bias (%)	Average calculated concentration (ng/mL)	Bias (%)
Tacrolimus	3.49	3.51	0.6	3.43	-1.9	14.4	14.6	1.3	14.5	0.5
Sirolimus	3.20	3.23	0.8	3.28	2.4	17.3	17.7	2.4	17.8	2.8
Everolimus	3.34	3.38	1.1	3.43	2.8	17.9	18.1	0.9	17.6	-1.9
Cyclosporine A	51.0	52.0	1.9	51.3	0.6	204	210	3.2	214	4.9

Table 6. Analytical accuracy results for external quality controls (IP444 for Full Scan mode and IP434 for PRM mode)

Analyte	Control	Full Scan mode			PRM mode		
		Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)	Nominal concentration (ng/mL)	Average calculated concentration (ng/mL)	Bias (%)
Tacrolimus	CIC/TAC A	4.30	4.45	3.4	7.80	7.97	2.1
	CIC/TAC B	8.76	9.11	3.9	1.70	1.64	-3.8
	CIC/TAC C	16.0	16.1	0.5	26.8	25.3	-6.1
Everolimus	EVE A	21.9	18.9	-13.5	3.70	3.30	-12.2
	EVE B	5.00	4.26	-14.7	7.80	6.95	-12.3
	EVE C	4.40	3.45	-21.5	17.0	16.1	-5.4
Sirolimus	SIR A	7.57	7.31	-3.4	4.89	4.68	-4.6
	SIR B	7.57	7.20	-4.9	5.86	5.83	-0.6
	SIR C	7.60	7.26	-4.5	79.2	81.7	3.1
Cyclosporine A	CIC/TAC A	1592	1420	-10.8	925	798	-16.0
	CIC/TAC B	372	335	-9.9	123	122	-0.9
	CIC/TAC C	76.0	69.5	-8.5	175	171	-1.8

Table 7 Analytical intra-assay precision results for control MS8833 batch #1509

Analyte	Acquisition mode	Level I						Level III					
		Day 1		Day 2		Day 3		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 (%)	Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)
Tacrolimus	Full Scan	3.34	2.8	3.58	1.5	3.62	4.5	14.0	2.4	15.1	4.1	14.6	3.7
	PRM	3.38	3.3	3.45	3.9	3.45	3.5	14.5	2.3	14.7	2.6	14.3	1.6
Sirolimus	Full Scan	3.03	3.3	3.30	1.1	3.35	3.9	17.2	5.3	18.5	4.4	17.5	3.5
	PRM	3.29	3.4	3.26	2.5	3.28	1.4	17.9	2.1	18.0	1.8	17.5	2.8
Everolimus	Full Scan	3.15	3.0	3.60	2.2	3.37	5.4	17.8	4.3	18.7	3.8	17.7	4.7
	PRM	3.45	3.9	3.52	1.7	3.32	1.9	17.3	3.1	17.9	2.6	17.5	1.5
Cyclosporine A	Full Scan	48.5	2.7	53.2	3.0	54.2	4.8	202	5.1	218	2.8	211	5.4
	PRM	52.9	2.9	51.2	4.0	49.8	2.5	218	1.5	211	1.0	213	1.8

Table 8. Analytical inter-assay precision results for control MS8833 batch #1509

Analyte	Acquisition mode	Level I		Level III	
		Average calculated concentration (ng/mL)	CV (%)	Average calculated concentration (ng/mL)	CV (%)
Tacrolimus	Full Scan	3.51	4.7	14.6	4.6
	PRM	3.43	3.5	14.5	2.3
Sirolimus	Full Scan	3.23	5.3	17.7	5.2
	PRM	3.28	2.4	17.8	2.4
Everolimus	Full Scan	3.38	6.5	18.1	4.8
	PRM	3.43	3.5	17.6	2.7
Cyclosporine A	Full Scan	52.0	6.0	210	5.4
	PRM	51.3	3.9	214	2.0

Conclusions

An HRAM mass spectrometry-based method (utilizing a Vanquish Duo UHPLC system connected to a Q Exactive Plus hybrid quadrupole-Orbitrap MS) is reported here, demonstrating the power of Orbitrap technology in performing accurate qualitative analyses and routine quantitation with high efficiency. A liquid chromatography-HRAM mass spectrometry method for clinical research was developed and implemented for the quantification of four immunosuppressants in human blood. The ClinMass LC-MS/MS Complete Kit for Immunosuppressants in Blood from RECIPE was used. The method incorporates a quick and simple offline protein precipitation step with concomitant internal standard addition followed by online SPE. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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