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Quantification of Immunosuppressants in Human Whole Blood by Online SPE Liquid Chromatography Tandem Mass Spectrometry for Clinical Research

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ABSTRACT

Purpose: Implementation of an analytical method for clinical research for the quantification of cyclosporin A, everolimus, sirolimus and tacrolimus in human whole blood.

Methods: The method involves a simple protein precipitation step followed by online solid-phase extraction (SPE) using a Thermo Scientific™ Transcend™ II system; a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer with heated electrospray ionization is used for detection by selected reaction monitoring (SRM) using isotopically labeled internal standards for each analyte. Method performance was evaluated using the MS1100 ClinMass® LC-MS/MS Complete Kit for Immunosuppressants in Whole Blood, advanced – On-Line Analysis from RECIPE®.

Results: The method involves a minimal sample preparation and meets research laboratory requirements for sensitivity, linearity of response, accuracy and precision.

INTRODUCTION

An analytical method for clinical research for the quantification of cyclosporin A, everolimus, sirolimus and tacrolimus in human whole blood is reported. The method involves a simple protein precipitation step followed by online SPE using a Thermo Scientific Transcend II system; a Thermo Scientific TSQ Endura triple quadrupole mass spectrometer with heated electrospray ionization operated in positive mode is used for detection by selected reaction monitoring (SRM) using isotopically labeled internal standards for each analyte. Method performance was evaluated using the MS1100 ClinMass LC-MS/MS Complete Kit for Immunosuppressants in Whole Blood, advanced – On-Line Analysis from RECIPE, to obtain limits of quantification, linearity ranges, accuracy and intra- and inter-assay precision for each analyte.

MATERIALS AND METHODS

Sample Preparation

Reagents included calibrators and controls from RECIPE at seven (including blank) and three different levels, respectively, covering the concentrations reported in Table 1. Each analyte was quantified using a corresponding isotopically labeled internal standard. Sample clean-up was performed by a simple preliminary protein precipitation with internal standard addition followed by on-line SPE on a Transcend II system.

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Table 1. Nominal concentrations (ng/mL) for (a) calibrators and (b) quality controls used for the evaluation of the analytical method performance

(a)

	Calibrator 1	Calibrator 2	Calibrator 3	Calibrator 4	Calibrator 5	Calibrator 6	Calibrator 7
Cyclosporin A	0.00	25.8	49	95.7	181	439	1243
Everolimus	0.00	1.45	2.9	6.01	12.6	24.9	49.4
Sirolimus	0.00	1.62	3.21	6.43	13.4	26.3	52.9
Tacrolimus	0.00	1.37	2.86	5.66	11.7	23.2	45.1

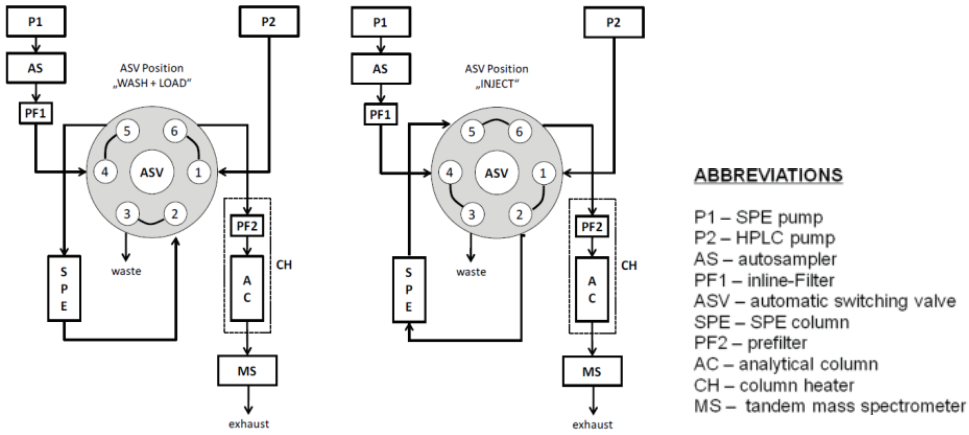
(b)

	Control 1	Control 2	Control 3
Cyclosporin A	62.5	132	258
Everolimus	3.28	6.67	13.3
Sirolimus	3.64	11.2	18.9
Tacrolimus	3.34	10.6	18.2

Liquid Chromatography

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

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



Mass Spectrometry

Analytes and internal standards were detected by SRM on a TSQ Endura triple quadrupole mass spectrometer with heated electrospray ionization operated in positive mode; MS conditions are reported in Table 2. Two SRM transitions for each analyte were included in the acquisition method for quantification and confirmation, respectively (Table 3).

Table 2. MS conditions

Source type	Heated electrospray ionization (HESI) in positive mode
Vaporizer temp	400° C
Ion Transfer Tube temp	250° C
Discharge current	4500 V
Sheath gas	50 AU
Sweep gas	0 AU
Auxiliary gas	15 AU
Data acquisition mode	Selected reaction monitoring (SRM)
Chrom filter peak width	3.0 s
Collision gas pressure	1.5 mTorr
Cycle time	0.400 s
Q1 (FWMH)	0.7
Q3 (FWMH)	0.7

Test Method

The method performance was evaluated by obtaining limits of quantification, linearity ranges, accuracy and intra- and inter-assay precision for each analyte. Analytical accuracy was evaluated in terms of trueness of measurement using the Proficiency Test Samples #601-22 and #601-62 from INSTAND e.V. prepared and analyzed on five different days in single runs each day. Intra-assay precision was evaluated in terms of percentage coefficient of variation (%CV) using the controls at three different levels in replicates of eight (n=8) prepared and analyzed in one batch. Inter-assay precision was evaluated on the same controls in replicates of three (n=3) prepared and analyzed on five different days.

Figure 2. Liquid chromatography method description including online SPE

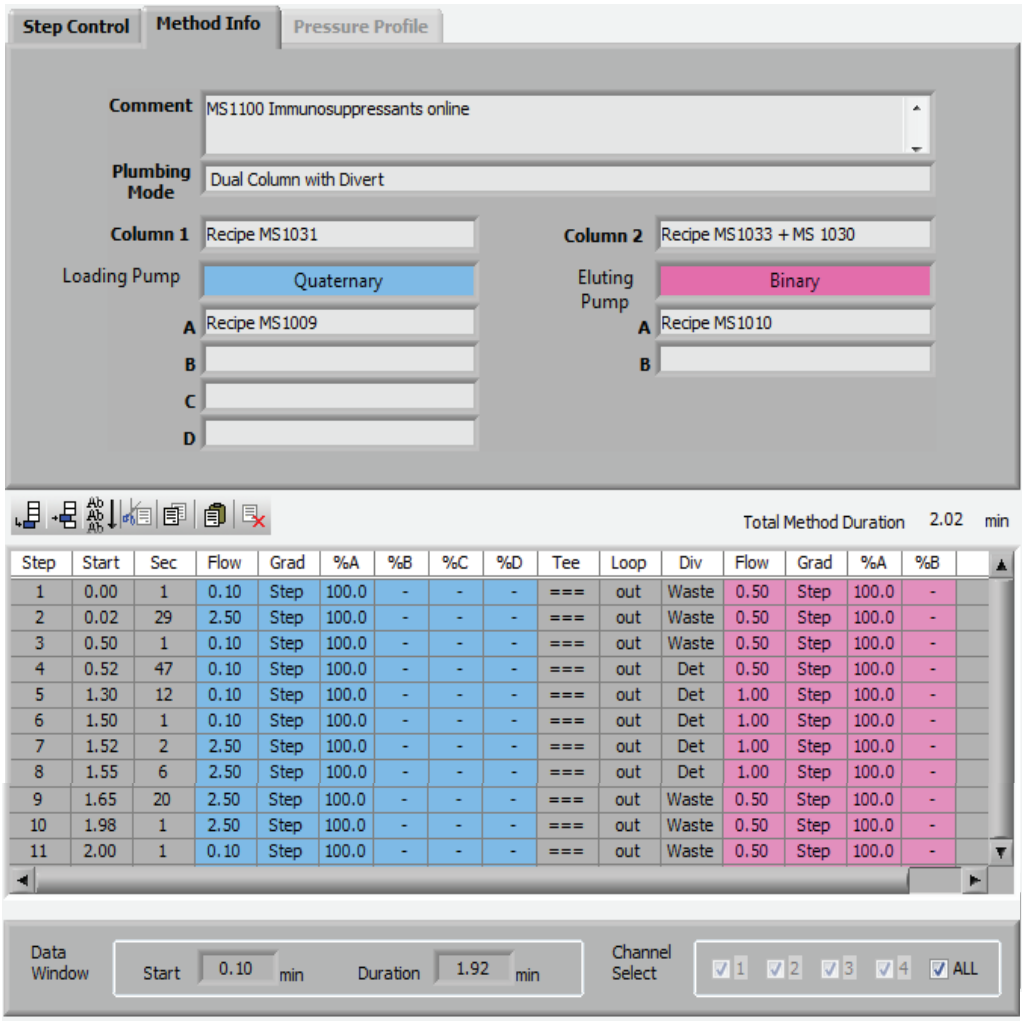


Table 3. SRM transitions and collision energies

Analyte	Precursor (m/z)	Product (m/z)	Collision Energy (V)
Cyclosporin A	1219.9	1185.0	28.1
		1203.0	12.4
d_{12}-Cyclosporin A	1231.9	1197.0	28.1
		1215.0	12.4
Everolimus	975.7	908.6	16.0
		926.6	10.3
$^{13}C_2d_4$-Everolimus	981.7	914.6	16.0
		932.6	10.3
Sirolimus	931.7	846.6	18.0
		864.6	15.3
$^{13}Cd_3$-Sirolimus	935.7	846.6	18.0
		864.6	15.3
Tacrolimus	821.6	576.4	22.1
		768.6	19.8
$^{13}Cd_2$-Tacrolimus	825.6	580.4	22.1
		772.6	19.8

Data Analysis

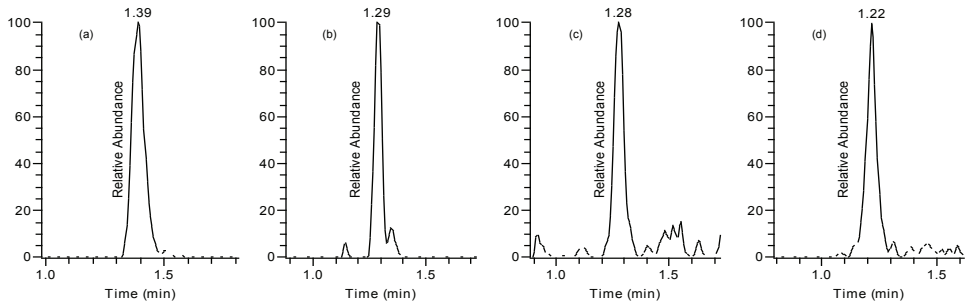
Data were acquired and processed using Thermo Scientific™ TraceFinder™ 3.3 software.

RESULTS

Linearity Ranges and Limits of Quantification

The 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 up to 10-fold. The lower limits of quantification (LLOQ) were 14.0 ng/mL for cyclosporin A, 0.85 ng/mL for everolimus, 1.87 ng/mL for sirolimus and 0.31 ng/mL for tacrolimus, with correlation factors (R^2) always above 0.99. Representative chromatograms for the lowest calibrator are reported in Figure 3. Representative calibration curves for each analyte are reported in Figure 4.

Figure 3. Representative chromatograms for the lowest calibrator for (a) cyclosporin A, (b) everolimus, (c) sirolimus and (d) tacrolimus



Accuracy

As far as analytical accuracy is concerned, the percentage bias between nominal and average back-calculated concentration for these control samples was always between -21.5% and 15.1%. Results are reported in Table 4.

Intra- and Inter-assay Precision

The %CV for intra-assay precision was always below 9.9% for all the analytes at all levels (Table 5). The maximum %CV for inter-assay precision including all the analytes was 12.9% (Table 6).

Figure 4. Representative calibration curves for (a) cyclosporin A, (b) everolimus, (c) sirolimus and (d) tacrolimus

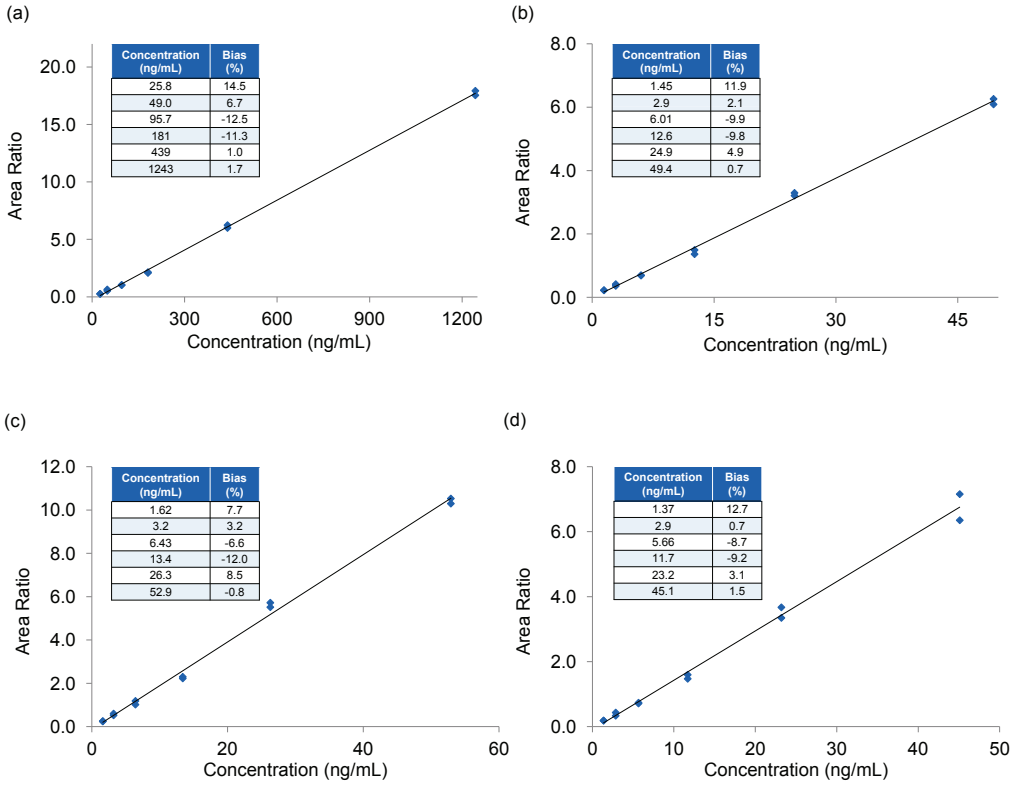


Table 4. Analytical accuracy results

Analyte	Control	Nominal Concentration (ng/mL)	Experimental Concentration (ng/mL)	CV (%)	Bias (%)
Cyclosporin A	601 (03/2016) 22	72.9	63.0	13.3	-13.5
	601 (10/2015) 62	256	201	15.8	-21.5
Everolimus	601 (03/2016) 22	0.19	N/A	N/A	N/A
	601 (10/2015) 62	8.55	7.36	13.5	-13.9
Sirolimus	601 (03/2016) 22	3.64	4.19	19.2	15.1
	601 (10/2015) 62	0.16	N/A	N/A	N/A
Tacrolimus	601 (03/2016) 22	3.83	3.69	9.6	-3.5
	601 (10/2015) 62	7.85	7.03	12.6	-10.5

Table 5. Intra-assay precision results

Analyte	MS8830 #519		MS8831 #519		MS8832 #519	
	Average Concentration (ng/mL)	CV (%)	Average Concentration (ng/mL)	CV (%)	Average Concentration (ng/mL)	CV (%)
Cyclosporin A	54.8	4.1	111	4.6	210	1.5
Everolimus	3.75	9.9	12.7	9.9	20.1	7.0
Sirolimus	3.54	7.9	14.1	3.8	23.4	5.6
Tacrolimus	3.62	6.2	7.63	6.4	14.9	6.3

Table 6. Inter-assay precision results

Analyte	MS8830 #519		MS8831 #519		MS8832 #519	
	Average Concentration (ng/mL)	CV (%)	Average Concentration (ng/mL)	CV (%)	Average Concentration (ng/mL)	CV (%)
Cyclosporin A	52.9	6.7	105	9.8	200	8.1
Everolimus	3.79	11.2	11.8	11.9	19	8.2
Sirolimus	4.32	12.9	12.9	12.6	21.5	11.1
Tacrolimus	3.57	9.7	7.47	11.9	14.6	7.5

CONCLUSIONS

A liquid chromatography tandem mass spectrometry method for clinical research for the quantification of cyclosporin A, everolimus, sirolimus and tacrolimus in human whole blood using the MS1100 ClinMass LC-MS/MS Complete Kit for Immunosuppressants in Whole Blood, advanced – on-line Analysis from RECIPE was implemented and analytically validated on a Transcend II system connected to a TSQ Endura triple quadrupole mass spectrometer.

The method involves a minimal sample preparation and meets research laboratory requirements for sensitivity, linearity of response, accuracy and precision.

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