Quantitation of Immunosuppressant Drugs in Whole Blood Using the Prelude-SPLC System and TSQ Endura Mass Spectrometer for Research

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Key Words
Immunosuppressant drugs, Prelude SPLC, TSQ Endura

Goal
To develop a rapid, sensitive, selective, and robust LC-MS/MS method to determine the concentrations of cyclosporine A, tacrolimus, sirolimus, and everolimus in whole blood.

Introduction
LC-MS/MS-based methods have advantages due to their selectivity and low cost compared to traditional immunoassay-based methods. In addition, unlike immunoassays, LC-MS/MS-based methods are ideally suited to the analysis of multiple compounds in a single analytical run. In this report, a single analytical run was used to precisely and accurately measure the levels of four immunosuppressant drugs in blood for research. This was accomplished by using a sample preparation and liquid chromatography (SPLC)-MS/MS system, which combines online sample extraction powered by Thermo Scientific™ TurboFlow™ technology with chromatographic separation. The Thermo Scientific™ Prelude SPLC™ system features two independent channels of sample preparation and liquid chromatography. Thus, the chromatographic methods on the Prelude SPLC system can be executed in parallel, either with a different method on each channel or the same method on both channels. Two channel multichannel operation on the Prelude SPLC system is automatically optimized into one mass spectrometer for serial detection, which improves mass spectrometer utilization time, increases throughput, and reduces analysis cost. The Prelude SPLC syringe pumps and high pressure, low-volume gradient mixing provide enhanced HPLC performance with improved peak shape and resolution as well as stable retention times, compared to the dual piston reciprocating pumps.

Methods
Sample Preparation
A 200 µL aliquot of whole blood sample was mixed with 300 µL of zinc sulfate solution (0.1 M) in a 1.5 mL centrifuge tube and vortexed for 30 seconds. The mixture was further processed by adding 500 µL of methanol (Fisher Chemical brand) containing internal standards (40 ng/mL D_{12}-cyclosporine A and 4 ng/mL $^{13}CD_2$tacrolimus). The sample was immediately vortexed for another 30 seconds. The entire mixture was centrifuged at 4000 RCF for 10 minutes. A 40 µL sample was analyzed.

Liquid Chromatography
SPLC-MS/MS analysis was conducted using a Prelude SPLC powered by TurboFlow technology coupled to a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer. The processed sample was directly injected onto a Thermo Scientific™ Cyclone-P™ column (0.5 x 50 mm, Part Number: CH-953289) for online sample cleanup. This step was followed by chromatographic separation on a Thermo Scientific™ Accucore™ C8 column (3 x 30 mm, 2.6 µm particle size, Part Number: 17226-033030). The Cyclone-P TurboFlow column was maintained at room temperature while the Accucore C8 column was maintained at 70 °C. The total run time was 5 minutes and the total solvent consumption was 8.1 mL per sample, including online sample extraction and chromatographic separation. Figure 1 shows the SPLC method profile.

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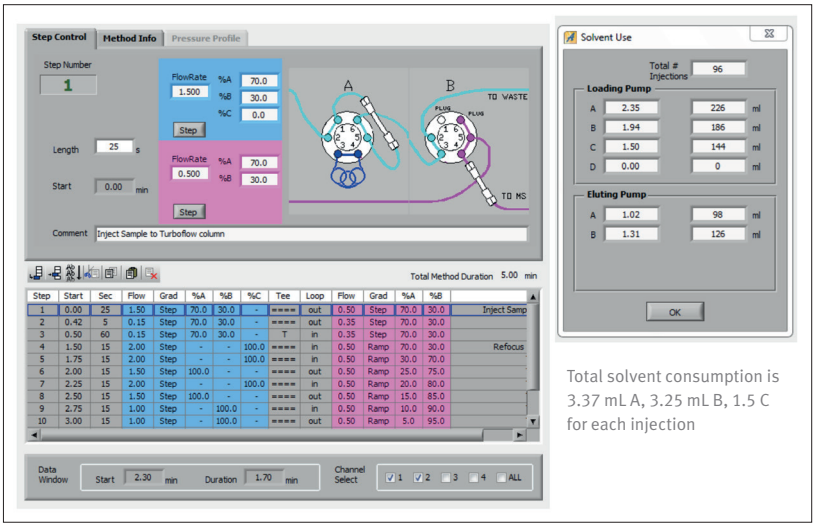

Total solvent consumption is 3.37 mL A, 3.25 mL B, 1.5 C for each injection

Figure 1. SPLC method profile

MS Method

Analyte detection was performed on a TSQ Endura MS equipped with a heated electrospray ionization (HESI) source. Table 1 shows MS conditions and Table 2 shows the selected-reaction monitoring (SRM) transitions for all four drugs and the two internal standards.

Table 1. MS conditions

Ionization	Heated electrospray ionization
Vaporizer temp	450 °C
Capillary temp	200 °C
Spray voltage	3500 V
Sheath gas	52 AU
Auxiliary gas	20 AU
Data acquisition mode	Selected-reaction monitoring (SRM)
Chrom filter peak width	3 s
Collision gas pressure	2 mTorr
Cycle time	0.2 s
Q1 (FWMH)	0.7
Q3 (FWMH)	0.7
SRM parameters	Refer to Table 2

Table 2. SRM Parameters

Compound Name	Q1 (*m/z*)	Q3 (*m/z*)	RF Lens	Collision Energy
Tacrolimus	821.6	768.5	224	24
$^{13}CD_2$-Tacrolimus	824.6	771.6	224	24
Cyclosporine A	1202.8	425.4	250	58
D_{12}-Cyclosporine A	1214.8	437.4	250	58
Sirolimus	931.7	864.5	250	23
Everolimus	975.5	908.5	224	23

Calibrators and Controls

Whole blood calibrators for the immunosuppressant drugs and quality control (QC) samples were purchased from ChromSystems Instruments & Chemicals GmbH. Vendor instructions were followed to reconstitute the lyophilized calibrators and controls.

Results and Discussion

Data were acquired and processed with Thermo Scientific™ TraceFinder™ software version 3.1. Figure 2 shows a representative chromatogram of the calibration standard at the lowest level. All calibration curves were linear with R^2 values greater than 0.9943. All of the QC samples were within 20% of the manufacturer-specified concentrations (Table 3). Table 4 shows the linearity range and R^2 values, and Figure 3 shows representative calibration curves for all four drugs. Figure 4 shows the extracted ion chromatogram along with the calculated concentrations for tacrolimus and sirolimus from different donor samples.

Table 3. Accuracy of QC samples

Sirolimus	Theoretical Amount	Calculated Amount	Difference (%)
QC1	2.90	2.66	-8.41
QC2	10.1	10.8	6.78
QC3	20.4	22.4	9.63
QC4	38.5	35.8	-7.09
Cyclosporin A	**Theoretical Amount**	**Calculated Amount**	**Difference (%)**
QC1	53.0	56.9	7.37
QC2	276	320	15.8
QC3	514	500	-2.75
QC4	1110	1190	6.77
Everolimus	**Theoretical Amount**	**Calculated Amount**	**Difference (%)**
QC1	2.30	2.21	-3.83
QC2	4.40	3.80	-13.6
QC3	8.50	9.42	10.8
QC4	28.8	27.1	-5.89
Tacrolimus	**Theoretical Amount**	**Calculated Amount**	**Difference (%)**
QC1	2.60	3.04	16.8
QC2	7.30	7.66	4.86
QC3	16.7	17.4	4.08
QC4	34.2	32.3	-5.60

Table 4. Linearity ranges

Compound Name	Linear Range (ng/mL)	R^2
Tacrolimus	2.1–38.5	0.9971
Cyclosporine A	23.3–919	0.9951
Sirolimus	2.3–46.1	0.9943
Everolimus	2.2–41.1	0.9973

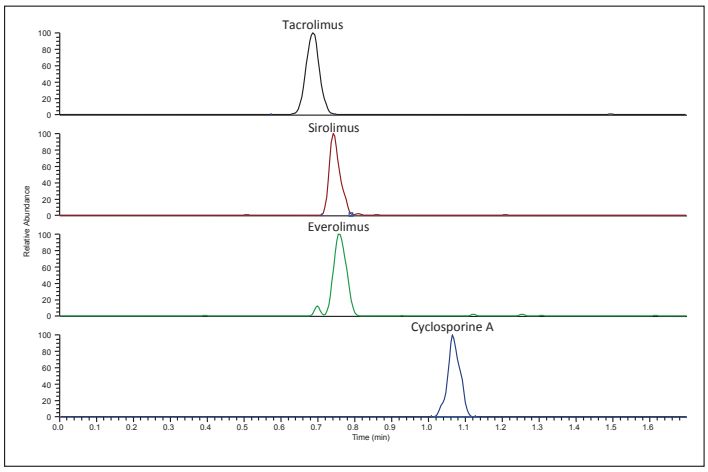

Figure 2. Chromatograms of the calibration standard at the lowest level

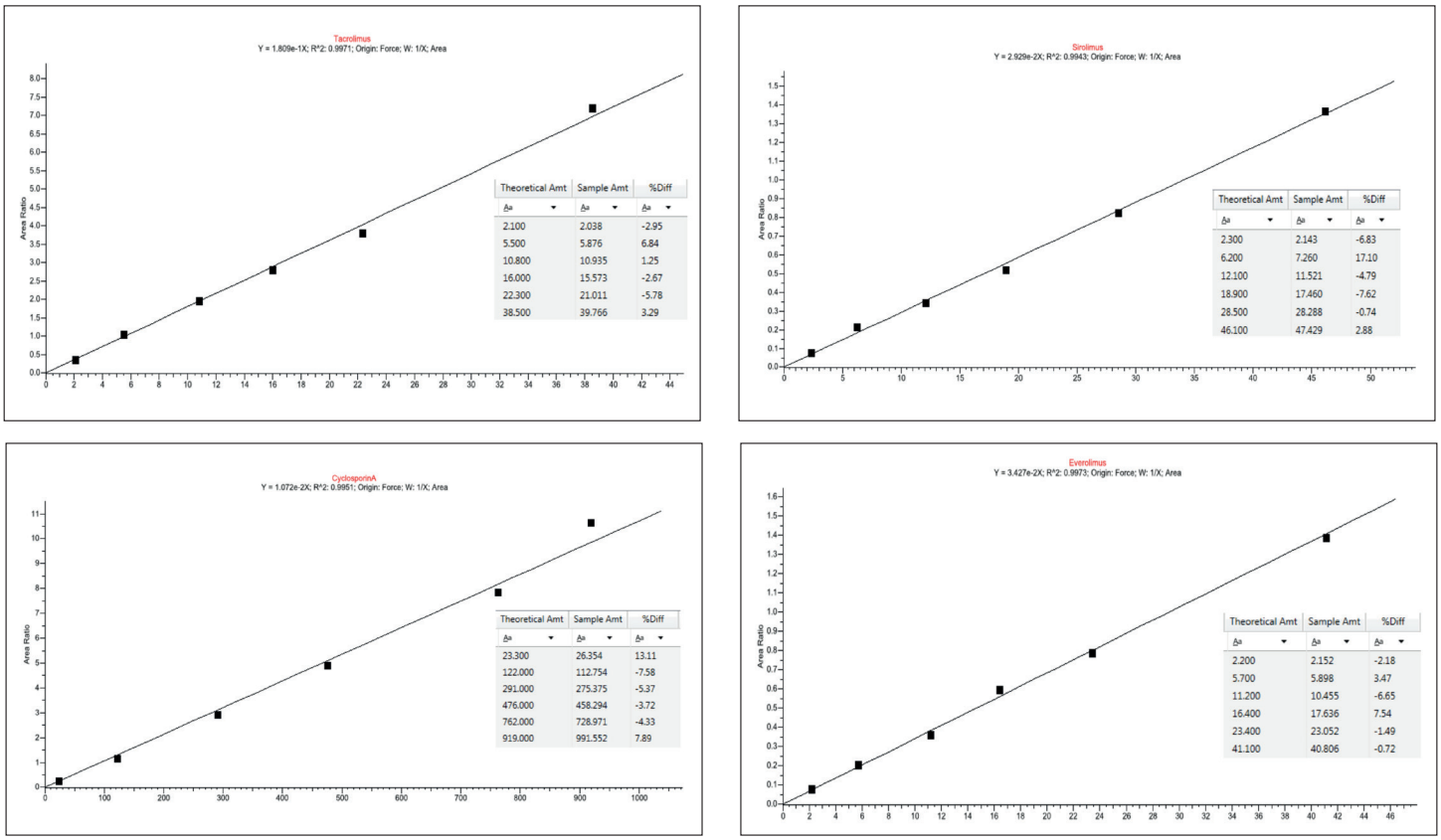

Figure 3. Calibration curves of all four ISD drugs

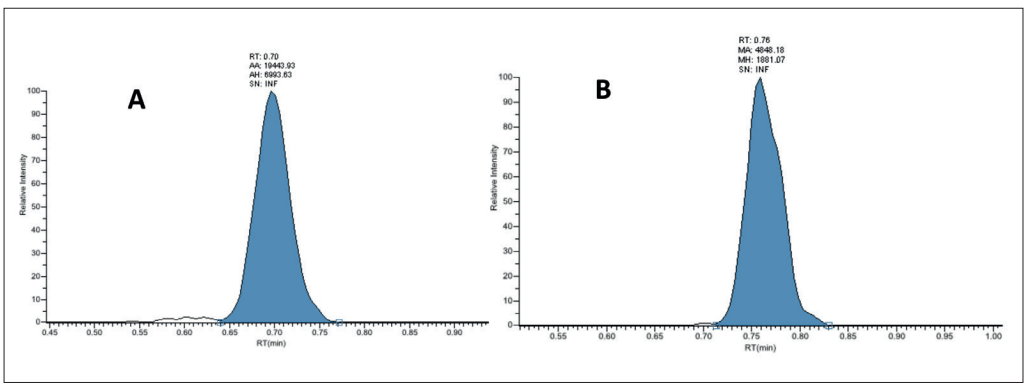

Figure 4. A: Chromatogram of tacrolimus quantifying peak from a donor at a calculated concentration of 13.6 ng/mL in whole blood (15.1 ng/mL reported from immunoassay); B: Chromatogram of sirolimus quantifying peak from a donor at a calculated concentration of 22.3 ng/mL in whole blood (22.4 ng/mL reported from immunoassay).

Conclusion

Using the Prelude SPLC system, a high-throughput and robust method was developed for the precise and accurate measurement of immunosuppressant drugs in blood for research. This method met analytical laboratory precision and accuracy criteria. Prelude SPLC system provides automated online sample cleanup and two-channel operation, thus minimizing the sample preparation steps and increasing the sample throughput. The total length of the SPLC run is 5 minutes with a data acquisition window of 1.75 minutes. With the two multiplexing channels on the Prelude SPLC, analytical throughput is 576 samples in 24 hours.

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