Analysis of Immunosupressant Drugs in Whole Blood Using a High-Efficiency Method on an Affordable High-Resolution Mass Spectrometer

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Application Note 632

Key Words
Q Exactive Focus, tacrolimus, cyclosporin A, sirolimus, everolimus, clinical research

Goal
To evaluate the performance of the Thermo Scientific™ Q Exactive™ Focus hybrid quadrupole-Orbitrap mass spectrometer as a quantitative platform for high-efficiency HPLC-MS analysis of immunosuppressant drugs (tacrolimus, everolimus, sirolimus and cyclosporin A) in whole blood for clinical research.

Introduction
Clinical research labs commonly use selective and cost-efficient LC-MS techniques for the analysis of immunosuppressant drugs. Usually, the quantitative method is developed on a triple quadrupole mass spectrometer. Here, we evaluated a high-efficiency method implemented on a Q Exactive Focus hybrid quadrupole-Orbitrap mass spectrometer for improved selectivity when compared to the conventional solution.

Methods
Sample Preparation
Samples were processed by protein precipitation. Briefly, 150 µL of 0.1 M $ZnSO_4$ and 250 µL of methanol containing internal standards (10 ng/mL ascomycin, 250 ng/mL cyclosporin D) were added to 100 µL of blood. Samples were vortexed and centrifuged, and the supernatant was injected into an analytical column.

Calibration Standards and QC samples
Calibration standards and QC samples were purchased from ChromSystems Diagnostics (Table1).

Table 1. Concentrations of calibration standards and QC samples.

Analyte	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6
	Concentration (ng/mL)					
Tacrolimus	2.35	5.96	11.9	17.6	24.6	42.2
Sirolimus	2.27	6.12	11.9	18.4	27.9	46.1
Everolimus	2.27	5.94	11.7	18.1	25.1	45.4
Cyclosporin A	26.1	123	294	485	764	942

Analyte	QC1	QC2	QC3	QC4
	Concentration (ng/mL)			
Tacrolimus	2.6	7.3	16.7	34.2
Sirolimus	2.9	10.1	20.4	38.5
Everolimus	2.3	4.4	8.5	28.8
Cyclosporin A	53.0	276	514	1111

Liquid Chromatography
A two-minute gradient elution was performed using a Thermo Scientific™ Dionex™ UltiMate™ 3000RS liquid chromatography pump with an OAS autosampler. Mobile phases consisted of 10 mM ammonium formate with 0.1% formic acid in water and methanol (Fisher Chemical™ Optima™ grade) for phases A and B, respectively.

Mass Spectrometry
Compounds were detected on a Q Exactive Focus benchtop Orbitrap mass spectrometer equipped with a Thermo Scientific™ Ion Max™ source and a HESI-II probe. Data were acquired in parallel-reaction monitoring (PRM) mode. In this mode, a single precursor ion was selected in the quadrupole with an isolation width of 2.0 *m/z* and fragmented in the HCD cell using an optimized compound-specific collision energy. The resulting MS/MS product ion spectrum was detected in the Orbitrap detector at a resolution of 17,500 (FWHM at *m/z* 200).

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Method Evaluation

The limits of quantitation (LOQ) and linearity ranges were evaluated by collecting calibration curve data in quintuplicate in three different batches. Method precision and accuracy were evaluated by running a calibration curve and quintuplicate replicates of quality controls on three different days. Matrix effects were evaluated by spiking previously analyzed blood in triplicate from six different donors and calculating the average %Recovery. This method developed on the Q Exactive Focus mass spectrometer was cross-correlated with the clinical research collaborator method developed on triple quadrupole mass spectrometer using donor samples containing tacrolimus and cyclosporin A.

Data Analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ software. For each analyte, the most abundant fragment from the MS/MS spectrum was selected as the quantifying ion. The resulting chromatograms were extracted and reconstructed with a mass accuracy of 5 ppm for quantification.
Figures 1a–1d show representative MS/MS spectra for selected analytes with quantifying ion specified.

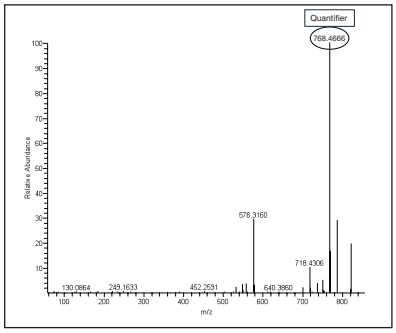

Figure 1a. MS/MS spectrum for tacrolimus with quantifying ion identified.

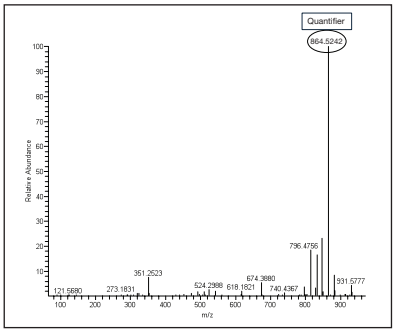

Figure 1b. MS/MS spectrum for sirolimus with quantifying ion identified.

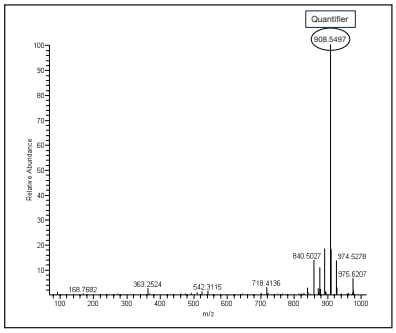

Figure 1c. MS/MS spectrum for everolimus with quantifying ion identified.

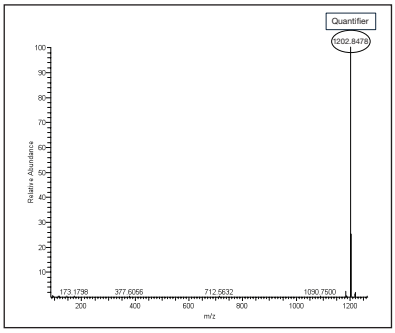

Figure 1d. MS/MS spectrum for cyclosporin A with quantifying ion identified.

Results

LOQs were defined as the lowest concentrations that had back-calculated values within 20% and %RSD for five replicates within 20%. Using these criteria, limits of quantitation for all analytes were equal to concentrations of the lowest calibration standards in the ChromSystems kit and ranged from 2.27 ng/mL to 26.1 ng/mL.

Calibration ranges determined by the concentrations of kit calibrators were linear for all analytes.

Figures 2a–2d show representative calibration curves for all four analytes along with chromatograms for the lowest calibration standard. Calibration standard precision (n=5) was better than 10% and accuracy was within ±7%.

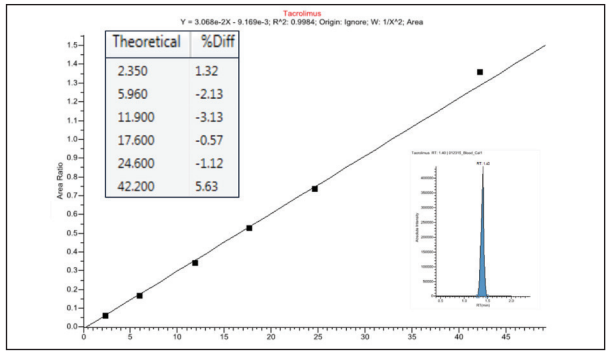

Figure 2a. Representative calibration curve and chromatogram of the lowest calibration standard for tacrolimus.

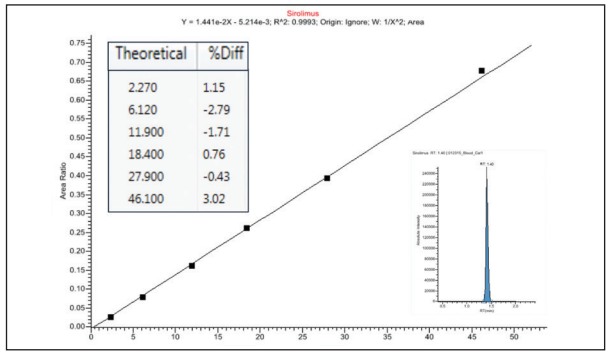

Figure 2b. Representative calibration curve and chromatogram of the lowest calibration standard for sirolimus.

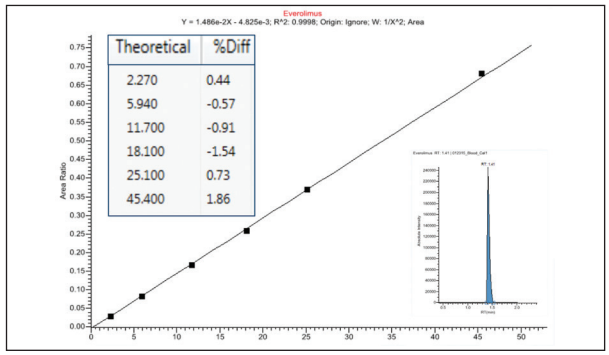

Figure 2c. Representative calibration curve and chromatogram of the lowest calibration standard for everolimus.

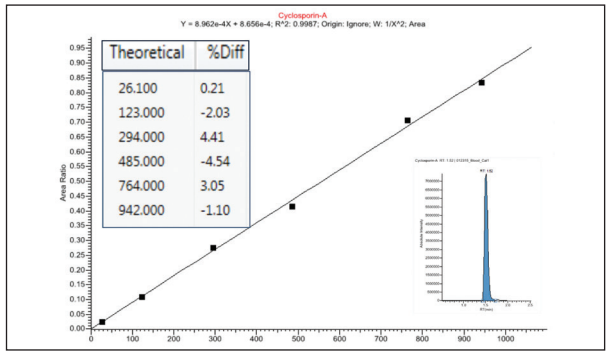

Figure 2d. Representative calibration curve and chromatogram of the lowest calibration standard for cyclosporin A.

Intra-assay and inter-assay precision were better than 7% for all analytes (Table 2a and Table 2b). Accuracy was within ±17% for the lowest QC sample and within ±10% for remaining QC samples.

Table 2a. Intra-assay precision.

Analyte	QC1	QC2	QC3	QC4
	%RSD			
Tacrolimus	4.1 – 5.0	3.7 – 5.6	2.6 – 4.2	3.9 – 5.1
Sirolimus	2.3 – 5.5	3.4 – 4.5	3.2 – 6.1	4.3 – 5.0
Everolimus	3.5 – 4.6	2.0 – 4.3	3.2 – 5.7	2.1 – 5.2
Cyclosporin A	2.2 – 4.2	2.8 – 4.9	3.9 – 6.8	4.6 – 5.1

Table 2b. Inter-assay precision.

Analyte	QC1	QC2	QC3	QC4
	%RSD			
Tacrolimus	4.6	4.5	3.8	4.2
Sirolimus	4.2	3.7	4.2	4.2
Everolimus	5.9	4.5	4.5	3.9
Cyclosporin A	3.5	3.5	5.1	4.6

Limited matrix effects were observed. %Recovery in 5 donor samples, calculated as the ratio between analyte peak area in blood matrix and analyte peak area in solvent, ranged from 82% to 112% (Table 3).

Table 3. %Recovery in spiked donor blood samples.

Analyte	Blood-1	Blood-2	Blood-3	Blood-4	Blood-5
	%Recovery				
Tacrolimus	96.9	107	109	107	108
Sirolimus	94.3	93.2	103	103	104
Everolimus	104	98.6	112	109	108
Cyclosporin A	90.8	91.5	101	101	82.0

Cross-correlation for the triple quadrupole MS and Q Exactive Focus MS methods for tacrolimus and cyclosporin A were linear with slopes of 1 and coefficient of correlations better than 0.97 (Figure 4a and Figure 4b).

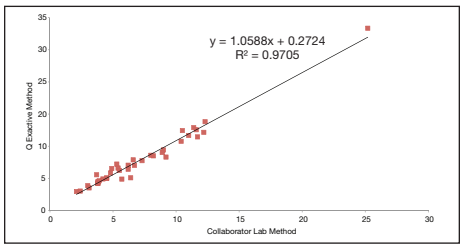

Figure 4a. Tacrolimus: correlation between collaborator lab method and method developed on Q Exactive Focus MS.

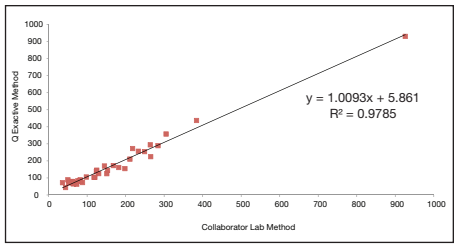

Figure 4b. Cyclosporin A: correlation between collaborator lab method and method developed on Q Exactive Focus MS.

Method efficiency can be improved four times with a four-channel Thermo Scientific™ Transcend™ II LC system, resulting in the analysis of 59-60 samples per hour (Figure 5).

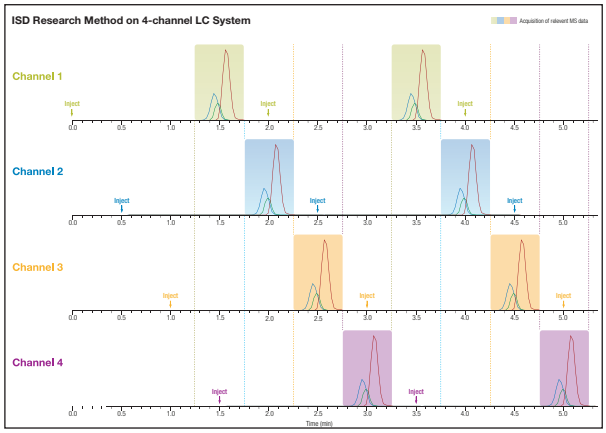

Figure 5. Execution of the method on a four-channel LC system.

Conclusion

We demonstrated a simple, high-efficiency method for analysis of four immunosuppressant drugs in whole blood implemented on a Q Exactive Focus high-resolution mass spectrometer for clinical research applications. The method evaluation results met clinical research lab requirements and correlated well with data collected using a triple quadrupole mass spectrometer. We proved that the Q Exactive Focus MS is a suitable mass spectrometer for quantitative analysis of immunosuppressant drugs in blood samples for clinical research applications.

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