Quantitation of Immunosuppressant Drugs in Blood Utilizing Second Generation Exactive High Resolution Accurate Mass Spectrometer and TraceFinder Software

Kristine Van Natta[1], Marta Kozak[1], Subodh Nimkar[1], William Clarke[2]

[1]Thermo Fisher Scientific, San Jose, CA, [2]Johns Hopkins Medical Institute, Baltimore, MD

Overview

Purpose: Evaluate second generation Thermo Scientific Exactive™ high performance bench-top mass spectrometer for analysis of immunosuppressant drugs in whole blood for clinical research use only utilizing the Thermo Scientific ClinSpec Immunosuppressant Test kit and samples previously analyzed by a clinically validated method on a triple stage quadrupole mass spectrometer.

Methods: Whole blood samples were processed by precipitation with $ZnSO_4$/ methanol. Samples were injected onto an HPLC under gradient conditions and detected on an Exactive Plus mass spectrometer.

Results: Standards and controls from test kit passed all method parameters for linearity recovery. This method showed excellent correlation with a clinically validated triple stage quadrupole method.

Introduction

Immunosuppressant drugs are used to prevent rejection of transplanted organs.

The ClinSpec™ Immunosuppressant Test kit was developed for use by research laboratories to analyze Tacrolimus, Sirolimus, Everolimus, and Cyclosporine A in whole blood specimens by LC-MS/MS. It consists of six different calibrator levels, and up to 5 quality control levels, internal standard and extraction reagent. Here we are using the same kit to analyze for these compounds using the Exactive Plus high resolution accurate mass (HR/AM) mass spectrometer.

Methods

Sample Preparation

Samples are prepared per instructions in the ClinSpec kit. Mix 50 µl of blood with 150 µL of 40mM $ZnSO_4$ in 66% methanol containing internal standards Ascomycin and Cyclosporine D. Shake for 30 minutes at room temperature. Centrifuge at 13,000 rpm for 10 minutes. Transfer supernatant to and autosampler vial, cap, and inject 50 µL onto HPLC system.

Liquid Chromatography

The HPLC used is a Thermo Scientific Accela 600 pump with Accela™ open autosampler. Mobile phases are 10 mM ammonium formate with 0.1% formic acid in water (A) and methanol (B) and acetonitrile:1-propanol:acetone (45:45:10) (C). The HPLC column used is a Javelin C18 guard column, 5 µm, 10 x 2.1 mm maintained at 80 °C run under the gradient shown in Figure 1. Divert valve is set to waste from 0 to 0.7 minutes, to mass spectrometer from 0.7 to 1.2 minutes and back to waste from 1.2 to 2 minutes.

FIGURE 1. HPLC gradient for Immunosuppressant analysis

Start (min)	Sec	Flow (µL/min)	%A	%B	%C
0.00	15	800	70	30	
0.25	15	800		100	
0.50	36	800		100	
1.10	24	1000			100
1.50	20	1000	70	30	
1.83	10	800	70	30	
2.00	1	800	70	30	

Mass Spectrometry

Compounds are detected on a Exactive Plus high performance bench-top mass spectrometer equipped with an Orbitrap mass analyzer. A schematic diagram of the Exactive Plus instrument is illustrated in Figure 2. An atmospheric pressure chemical ionization (APCI) probe was used as an ion source.

The instrument was operating in positive full-scan mode at a resolution setting of 70,000 (FWHM) at *m/z* 200. The Exactive Plus has an S-lens for improved ion transmission. The performance and robustness of the Automatic Gain Control (AGC) is improved using a C-Trap charge detector (CTCD). Relevant scan and source parameters are shown in Figure 3 and 4.

FIGURE 2. Schematic diagram of the Exactive Plus high resolution accurate mass benchtop mass spectrometer.

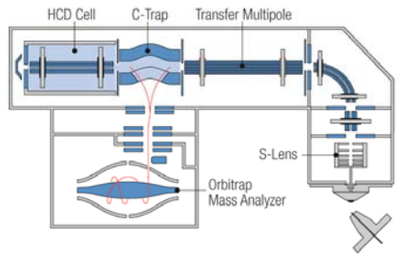


FIGURE 3. Scan Parameters for Exactive Plus Mass Spectrometer

Parameter	Value
Scan range	800-4000
Fragmentation	none
Resolution	70,000
Polarity	Positive
Microscans	1
Lock Masses	Off
AGC Target	1e6
Max Inject Time	200 ms

FIGURE 4. Source Parameters for APCI Probe.

Parameter	Value
Sheath Gas	15
Aux gas	17
Sweep gas	1
Discharge current	4.6 µA
Capillary temp	275 °C
S-Lens RF Level	75
Vaporizer Temp	300 °C

Method Validation

Validation consisted of analyzing replicates of quality controls along with a calibration curve on multiple days. We also analyzed donor samples previously analyzed with a clinically validated method utilizing a triple stage quadrupole instrument and compared the results.

Data Analysis

Data is acquired and processed using Thermo Scientific TraceFinder™ software. Ascomycin is used as internal standard for Tacrolimus, Sirolimus, and Everolimus. Cyclosporine D is used as internal standard for Cyclosporine A. All of the compounds form ammoniated adducts with exact masses listed in Figure 5. Chromatograms for individual compounds are extracted from the full-scan data with a mass tolerance of 5 ppm.

FIGURE 5. Exact masses for ammoniated adducts of immunosuppressant drugs.

Compound	m/z	Compound	m/z
Ascomycin	809.5158	Everolimus	975.6152
Tacrolimus	821.5158	Cyclosporine A	1,219.8752
Sirolimus	931.5890	Cyclosporine D	1,233.8908

Results

Linearity

All compounds were linear within the test kit calibrator ranges of 1-30 ng/mL for Tacrolimus, Sirolimus and Everolimus and 10–1500 ng/mL for Cyclosporine A. R-values were all greater than 0.99 (R^2 > 0.98). Figure 6 shows representative chromatograms reconstructed with 5 ppm mass window at 1 ng/mL for Tacrolimus, Sirolimus and Everolimus and 10 ng/mL for Cyclosporine A. Figure 7 shows representative calibration curves for all compounds. Standards backcalculated to within 6.3% for Tacrolimus, 10.9% for Sirolimus, 14.7% for Everolimus and 7.5% for Cyclosporine A.

Quality Controls

Quality control samples analyzed in this study show good recovery and reproducibility. Imprecisions as given by %CV are better than those given in the product insert for all compounds and levels tested except for Everolimus, which still compare favorably to the test kit. Figure 8 shows validation statistics for this study with comparison to %CV given in ClinSpec product insert.

FIGURE 6. Reconstructed chromatogram with 5 ppm mass window of lowest standard for Tacrolimus (a), Sirolimus (b), Everolimus (c), Cyclosporine A (d), and internal standards Ascomycin (e) and Cyclosporine D (f).

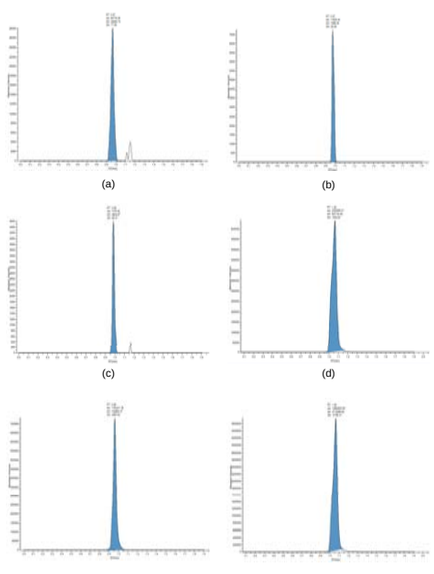


FIGURE 7. Representative calibration curves for immunosuppressant drugs. Standards backcalculated to within 6.3% for Tacrolimus, 10.9% for Sirolimus, 14.7% for Everolimus and 7.5% for Cyclosporine A.

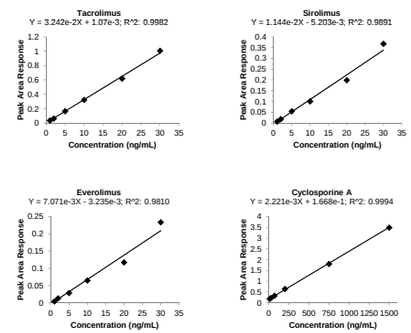


FIGURE 8. Results of Quality Control samples. %CV compare data collected in this study to statistics from product insert.

Tacrolimus	QC1	QC2	QC3
ng/mL	3	12	25
Mean	2.90	11.4	24.5
Std Dev	0.19	0.3	1.0
%Diff	–3.20	–4.60	–1.85
% CV*	6.6/10.3	2.2/6.1	4.2/5.9
n	13	13	11

Sirolimus	QC1	QC2	QC3
ng/mL	3	12	25
Mean	3.02	10.8	21.3
Std Dev	0.39	0.8	1.0
%Diff	0.691	–10.1	–14.7
% CV*	12.9/13.7	7.6/9.0	4.7/8.3
n	13	13	11

Everolimus	QC1	QC2	QC3
ng/mL	3	12	25
Mean	3.28	10.7	23.7
Std Dev	0.61	1.0	1.9
%Diff	–9.17	–10.9	–5.18
% CV*	18.6/14.1	9.5/9.5	8.1/7.7
n	13	13	11

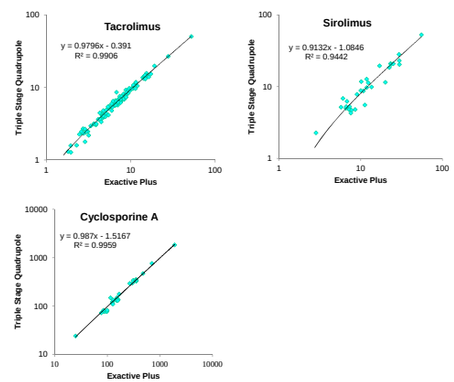
Cyclosporine A	QC1	QC2	QC3	QC4
ng/mL	30	125	375	700
Mean	31.5	121	381	757
Std Dev	2.2	6	15	29
%Diff	5.12	–3.20	1.71	8.10
% CV*	7.1/7.7	4.9/5.9	4.0/6.6	3.8/6.2
n	13	13	11	13

* %CV from this study/%CV from product insert

Cross Validation Samples

Donor samples previously analyzed with a clinically validated method utilizing a triple stage quadrupole mass spectrometer were reanalyzed on the Exactive Plus. A total of 114 samples containing Tacrolimus, 34 containing Sirolimus, and 32 containing Cyclosporine A were analyzed. No donor sample values were available for Everolimus. Figure 9 shows the correlation between the two methods. All slopes were greater than 0.9, indicating good agreement between the two methods. R-squared values were also greater than 0.99 for Tacrolimus and Cyclosporine A and greater than 0.94 for Sirolimus.

FIGURE 9. Comparison of results from Exactive Plus with clinically validated method on a triple stage quadrupole mass spectrometer. Data and linear fit shown on log-log scale.



Conclusion

- Assay shows good linearity across required calibration ranges.
- Controls indicate good method precision and robustness.
- The Exactive Plus give comparable results to a clinically validated triple quadrupole method showing the suitability of Orbitrap technology for routine quantitation of whole blood samples for use by clinical research laboratories.

References

1. Thermo Scientific ClinSpec Immunosuppressants Test product insert

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