Quantitation of Immunosuppressant Drugs in Blood Using a Second-Generation High-Resolution, Accurate-Mass Mass Spectrometer

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Key Words
Immunosuppressant, tacrolimus, sirolimus, everolimus, cyclosporine A, Exactive Plus, TraceFinder, ClinSpec, clinical research, FK-506

Goal
Evaluate the combined use of a second-generation Thermo Scientific™ Orbitrap™-based high-resolution mass spectrometer and an immunosuppressants test kit for clinical research analysis of immunosuppressant drugs in whole blood. Apply the method to analysis of samples previously analyzed using a validated method on a triple-stage quadrupole mass spectrometer and compare the results.

Introduction
The Thermo Scientific™ ClinSpec™ Immunosuppressant Test kit was developed for clinical research liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis of tacrolimus, sirolimus, everolimus, and cyclosporine A in whole blood specimens. The kit consists of six different calibrator levels, up to five quality control levels, internal standard and extraction reagent. Here, the kit is used with a second-generation high-resolution, accurate-mass (HR/AM) mass spectrometer to analyze for these compounds. The results are compared to results previously obtained using a validated method and a triple-stage quadrupole mass spectrometer.

Experimental

Sample Preparation
Samples were prepared per the package insert in the ClinSpec kit.[1] Briefly, whole blood samples were processed by precipitation with $ZnSO_4$/methanol solution containing internal standards ascomycin and cyclosporine D. The samples were shaken for 30 minutes at room temperature before being centrifuged at 13,000 rpm for 10 minutes. The supernatant was transferred to an autosampler vial, capped, and 50 µL was injected onto the HPLC system.

Liquid Chromatography
Chromatographic separation was performed using a Thermo Scientific™ Accela™ 600 HPLC pump and Thermo Scientific™ Hypersil GOLD™ Javelin™ guard column, (10 x 2.1 mm, 5 µm particle size), maintained at 80 °C. Mobile phases A and B consisted of 10 mM ammonium formate with 0.1% formic acid in water and methanol, respectively. Mobile phase C was acetonitrile/1-propanol/acetone (45:45:10). The total run time was 2 minutes.

Mass Spectrometry
Samples were analyzed with a Thermo Scientific™ Exactive™ Plus high-performance benchtop mass spectrometer equipped with an Orbitrap mass analyzer. 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 of 70,000 (FWHM) at *m/z* 200. Relevant scan and source parameters are shown in Tables 1 and 2.

Table 1. Scan parameters for Exactive Plus mass spectrometer

Scan Parameter	Value
Mass range:	*m/z* 800–4000
Resolution:	70,000
Polarity:	Positive
Microscans:	1
Lock mass:	Off
AGC target:	1×10^6
Max inject time:	200 msec

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Table 2. Source parameters for APCI probe

Source Parameter	Value
Sheath gas:	15
Aux gas:	17
Sweep gas:	1
Discharge Current:	4.6 kV
Capillary temperature:	275 °C
S-Lens voltage:	75 V
Vaporizer temperature:	300 °C

Validation

Validation consisted of analyzing replicates of quality controls along with a calibration curve on multiple days. We also analyzed donor samples previously analyzed using a validated method on a Thermo Scientific™ TSQ Access MAX™ triple-stage quadrupole mass spectrometer and compared the results.

Data Analysis

Data was acquired and processed using Thermo Scientific™ TraceFinder™ software. Ascomycin was used as internal standard for tacrolimus, sirolimus, and everolimus. Cyclosporine D was used as internal standard for cyclosporine A. All of the compounds form ammoniated adducts (Table 3). Extracted ion chromatograms (XIC) for individual compounds were reconstructed from the full-scan data with a mass tolerance of 5 ppm. Figure 1 shows representative chromatograms for analytes at their respective LOQs and internal standards.

Table 3. Exact masses of the ammoniated adducts of the immunosuppressant drugs and internal standards

Compound	*m/z*	Compound	*m/z*
Ascomycin	809.5158	Everolimus	975.6152
Tacrolimus	821.5158	Cyclosporine A	1219.8752
Sirolimus	931.5890	Cyclosporine D	1233.8908

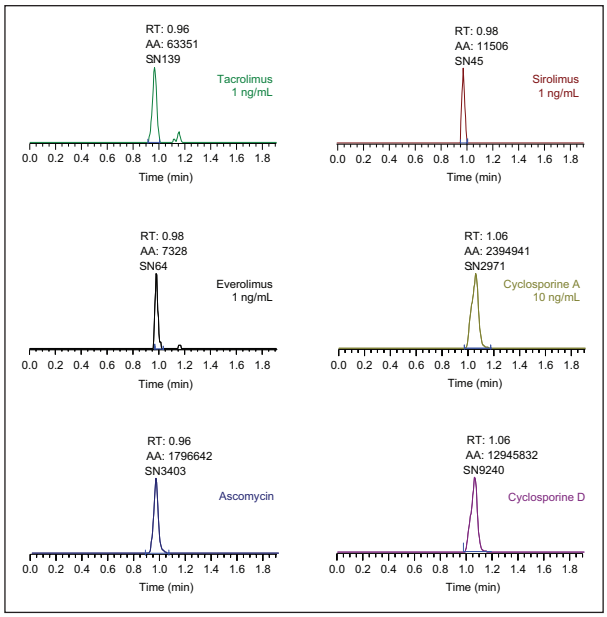

Figure 1. Extracted ion chromatograms with 5 ppm mass windows for analytes tacrolimus, sirolimus, everolimus (each at 1 ng/mL), and cyclosporine A (10 ng/mL), and internal standards ascomycin and cyclosporine D

Results and Discussion

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. Figure 2 shows representative calibration curves for all compounds. Standards back-calculated to within 6.3% for tacrolimus, 10.9% for sirolimus, 14.7% for everolimus, and 7.5% for cyclosporine A.

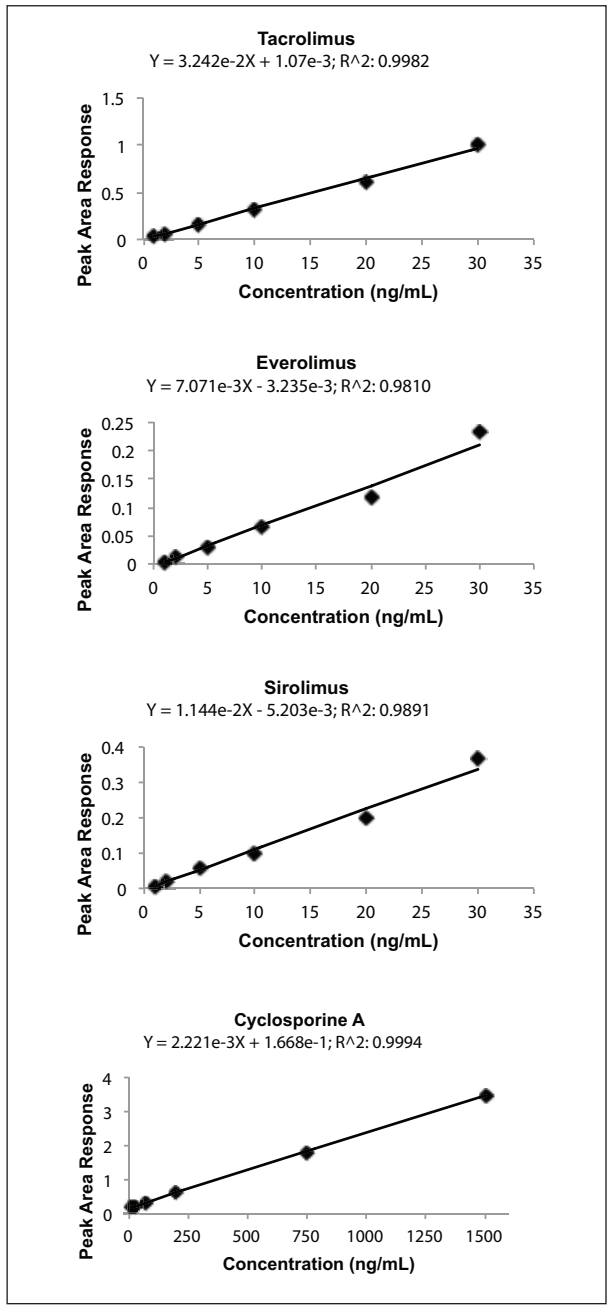

Figure 2. Representative calibration curves for immunosuppressant drugs

Quality Controls

Quality control samples analyzed in this study showed good recovery and reproducibility. Table 4 shows validation statistics for quality controls analyzed in this study. Imprecisions, as given by %CV, were also better than those given in the package insert for all compounds and levels tested except for those for everolimus, which still compared favorably to the test kit (data not shown).

Table 4. Mean %bias and %CV of quality controls

Analyte	Control 1 (3/30)*	Control 2 (12/125)*	Control 3 (25/375)*	Control 4 (0/700)*
Tacrolimus	-3.20/6.60	-4.60/2.22	-1.85/4.17	NA
Sirolimus	0.691/12.9	-10.1/7.56	-14.7/4.66	NA
Everolimus	-9.17/18.6	-10.9/9.49	-5.18/8.12	NA
Cyclosporine A	5.12/7.07	-3.20/4.94	1.71/3.98	8.10/3.78

**Concentration of (tacrolimus, sirolimus, everolimus)/cyclosporine A in ng/mL*

Method Comparison Samples

Donor samples previously analyzed with a validated method utilizing a triple-stage quadrupole mass spectrometer were reanalyzed on the Exactive Plus MS. 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 3 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.

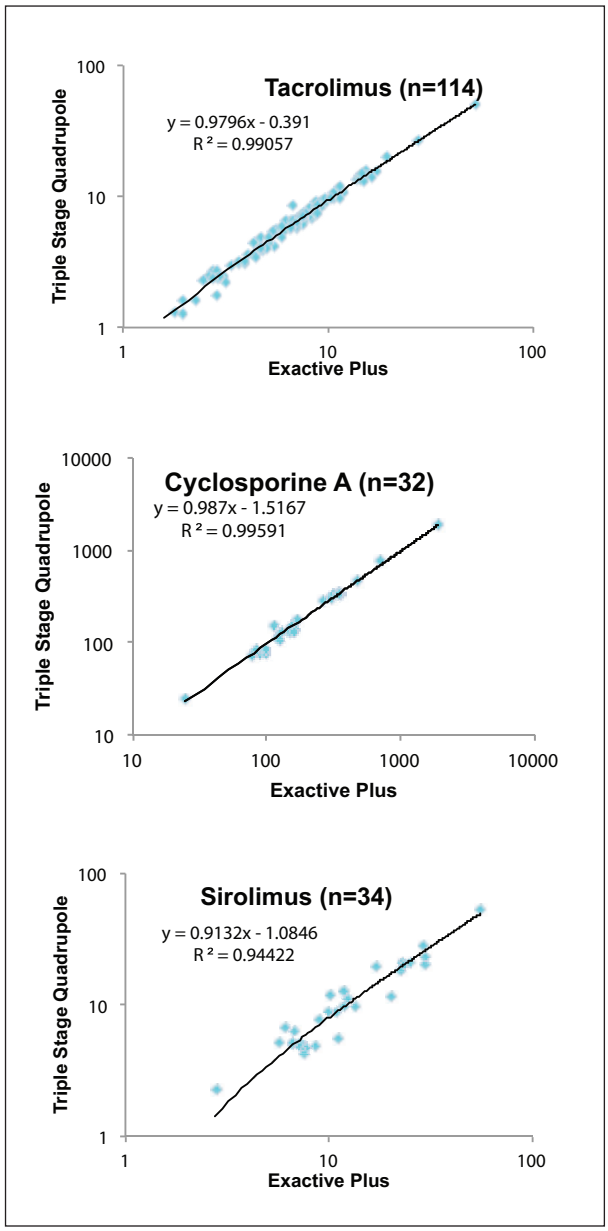

Figure 3. Correlation of results from Exactive Plus and triple-stage quadrupole instruments

Conclusion

- The method was easy to set up. No compound tuning was required.
- The method showed good linearity across the calibration ranges.
- Controls indicated good method precision and robustness.
- The Exactive Plus MS produced results comparable to a triple-stage quadrupole method, showing the suitability of Orbitrap technology for routine quantitation of whole blood samples by clinical research laboratories.

Acknowledgement

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Reference

1. Thermo Scientific ClinSpec Immunosuppressants Test product insert.

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