Analysis of Immunosuppressive Drugs From Whole Blood by LC-MS/MS

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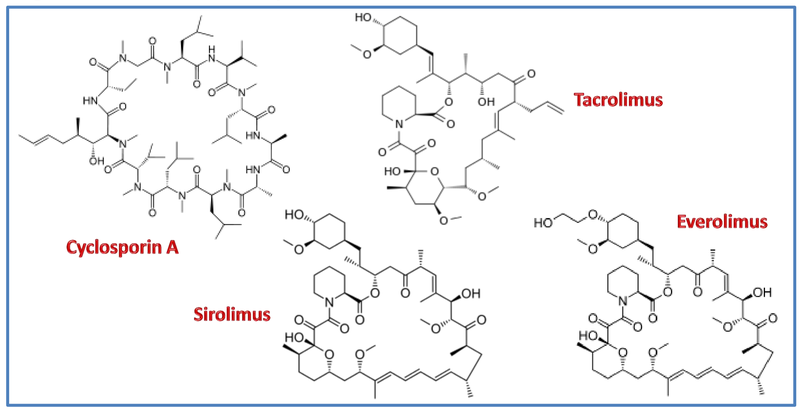
Abstract & Introduction

Immunosuppressive drugs are used to suppress the body's immune response and are typically administered to prevent the rejection of transplanted organs or tissues. Cyclosporin A, tacrolimus, sirolimus, and everolimus are four of the most commonly used drugs in the therapy of organ transplantation. Cyclosporin A and tacrolimus are classified as calcineurin inhibitors, and sirolimus and everolimus are grouped as mTOR inhibitors. These two classes of drugs can be used in combination for synergistic blocking of T cell activation and proliferation. Due to their pharmacokinetic variabilities and narrow therapeutic indexes, time-sensitive and highly accurate therapeutic drug monitoring is necessary, not only to prevent rejection but also minimize toxic side effects. Therefore, a fast and accurate measurement of drug concentration is critical to assist the clinicians for timely and proper treatment of patients. By combining a simple sample preparation step and a fast chromatographic elution with a Raptor Biphenyl column, a high-throughput analysis was established for simultaneous measurement of these four drugs in human whole blood.

Figure 1: Structure of Analytes



Methods

Table 1: Analytical Conditions for Waters Xevo™ TQ-S with Acquity UPLC®

Analytical Column	Raptor Biphenyl 2.7µm, 50 mm x 2.1 mm (Restek Part No. 9309A52)	
Guard Column	Raptor Biphenyl EXP Guard Colum Cartridge 2.7µm, 5 mm x 2.1 mm (PN 9309A0252)	
Mobile Phase A	0.05% formic acid, 5mM ammonium formate in water	
Mobile Phase B	Methanol	
Gradient	**Time (min)**	**%B**
	0.00	60
	2.00	100
	2.01	60
	3.00	60
Flow Rate	0.5 mL/min	
Injection Volume	5 µL	
Column Temp.	70°C	
Ion Mode	Positive ESI	

Table 2: Analyte Transitions

Analyte	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier
Cyclosporin A	1219.83	1202.87	1184.58
Everolimus	975.68	908.62	926.57
Sirolimus	931.63	864.57	882.58
Tacrolimus	821.61	768.51	786.52
Cyclosporin D	1233.91	1216.88	-
Ascomycin	809.53	756.50	-

Calibration Standards and Blood Control Samples

Human whole blood was fortified with 4 analytes to prepare the calibration standards and QC samples. For quantitation, cyclosporin D was used as the internal standard for cyclosporin A and ascomycin was used as the internal standard for tacrolimus, sirolimus, and everolimus. The concentration of calibration standards ranged from 10 to 1000 ng/mL for cyclosporin A and 1 to 100 ng/mL for tacrolimus, sirolimus, and everolimus. Three QC levels were prepared at 15, 150, and 800 ng/mL for cyclosporin A; 5, 15, and 80 ng/mL for tacrolimus, sirolimus, and everolimus

Sample Preparation

The blood sample (100 µl) was mixed with 200 µl of precipitation solution (1:4 v/v 0.2M $ZnSO_4$:methanol) containing 50 ng/mL of cyclosporin D and 5ng/mL of ascomycin. The mixture was vortexed for 20 seconds at 3000rpm and then centrifuged for 10 minutes at 4300rpm. The supernatant was directly injected (5 µl) onto a Raptor Biphenyl 2.7 µm, 50 mm x 2.1 mm column equipped with a Raptor Biphenyl EXP® 2.7 µm, 5 mm x 2.1 mm guard column for analysis.

Chromatograms

Figure 2: Fortified Human Whole Blood at 10 ng/mL

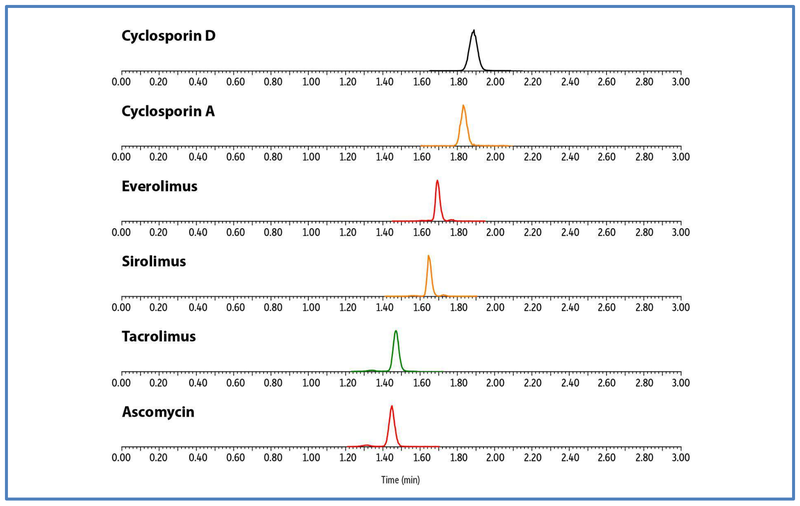


Figure 3: Blank Human Whole Blood

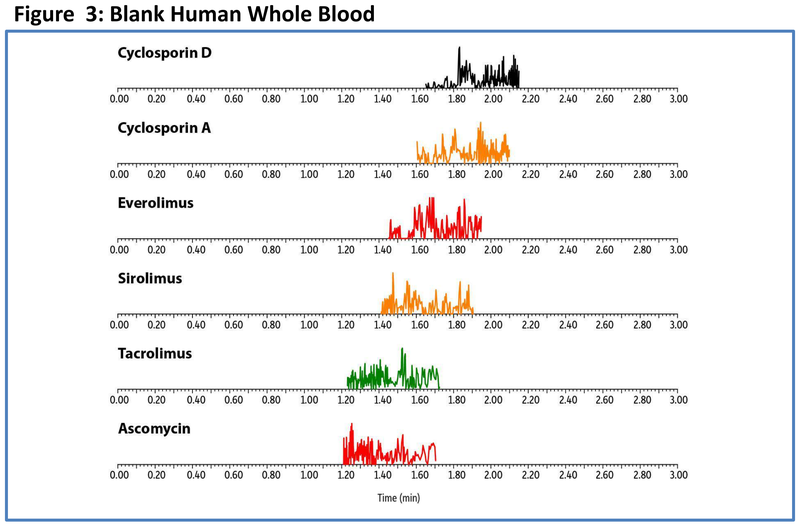
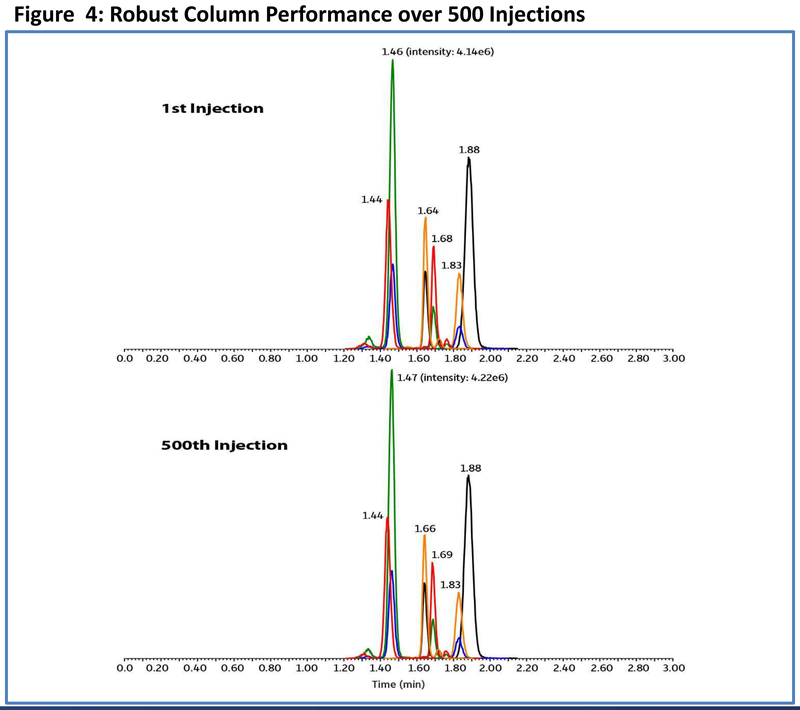


Figure 4: Robust Column Performance over 500 Injections



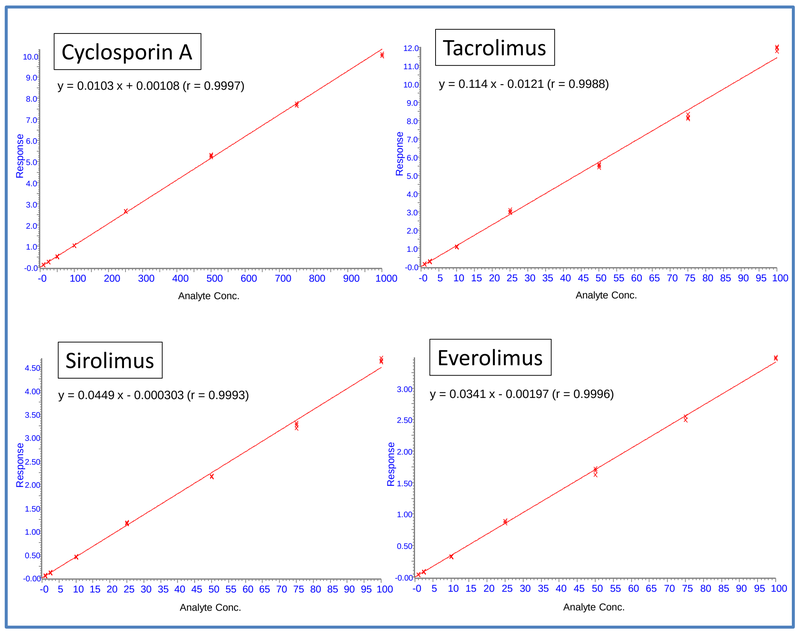
Results and Discussion

Chromatographic Performance: A fast 3-minute chromatographic analysis (Figure 2) was obtained with direct injection of supernatant without further in- or off-line sample treatment. No matrix interference was observed for all 4 analytes and internal standards (Figure 3) with the established sample preparation and chromatographic methods.

Method Robustness: Following 500 injections of a whole blood extract (25 ng/mL), all chromatographic peaks maintained the initial peak shape, retention time, and intensity (Figure 4). The maximum system pressure also remained at the same level indicating no column clogging had occurred.

Linearity: Using $1/x^2$ weighted linear regression for cyclosporin A and 1/x weighted linear regression for tacrolimus, sirolimus, and everolimus, all 4 compounds showed good linearity with r^2 values of 0.999 or greater, and the % deviations were <10% (Figure 5). The signal-to-noise values of the lowest standard samples were from 100 to 300 indicating that this method could be used for the detection of much lower concentrations if necessary.

Figure 5: Standard Curves



Accuracy & Precision: Precision and accuracy analyses were performed on three different days. The method accuracy was demonstrated with %recovery <10% of the nominal concentration for all QC levels. The %RSD was 0.2-4.0% and 1.2-5.4% for intra-day and inter-day, respectively, indicating good method precision (Table 3).

Table 3: Accuracy and Precision of QC Samples

Analyte	QC-1 5.0 ng/mL (Cyclosporin A: 15 ng/mL)			QC-2 15 ng/mL (Cyclosporin A: 150 ng/mL)			QC-3 80 ng/mL (Cyclosporin A: 800 ng/mL)		
	Avg. Conc. (ng/mL)	Avg. Accuracy	%RSD	Avg. Conc. (ng/mL)	Avg. Accuracy	%RSD	Avg. Conc. (ng/mL)	Avg. Accuracy	%RSD
Cyclosporin A	15.0	99.9	5.4	152.3	101.5	1.2	795.6	99.5	2.1
Tacrolimus	5.2	103.6	1.8	14.3	95.1	1.9	81.8	102.3	1.3
Sirolimus	5.2	103.9	4.3	14.4	96.2	2.7	83.0	103.7	2.1
Everolimus	5.1	101.1	2.0	14.2	94.8	2.1	80.8	101.0	2.8

Conclusions

It was demonstrated that the Raptor Biphenyl column is excellent for rapid and accurate analysis of cyclosporin A, tacrolimus, sirolimus, and everolimus in human whole blood. With a fast and simple sample preparation procedure and 3 minutes of chromatographic analysis time, the established method provides high-throughput therapeutic drug monitoring for these commonly used immunosuppressive drugs.