High-Throughput Analysis of Immunosuppressive Drugs from Whole Blood by LC-MS/MS

By Shun-Hsin Liang and Frances Carroll

Abstract

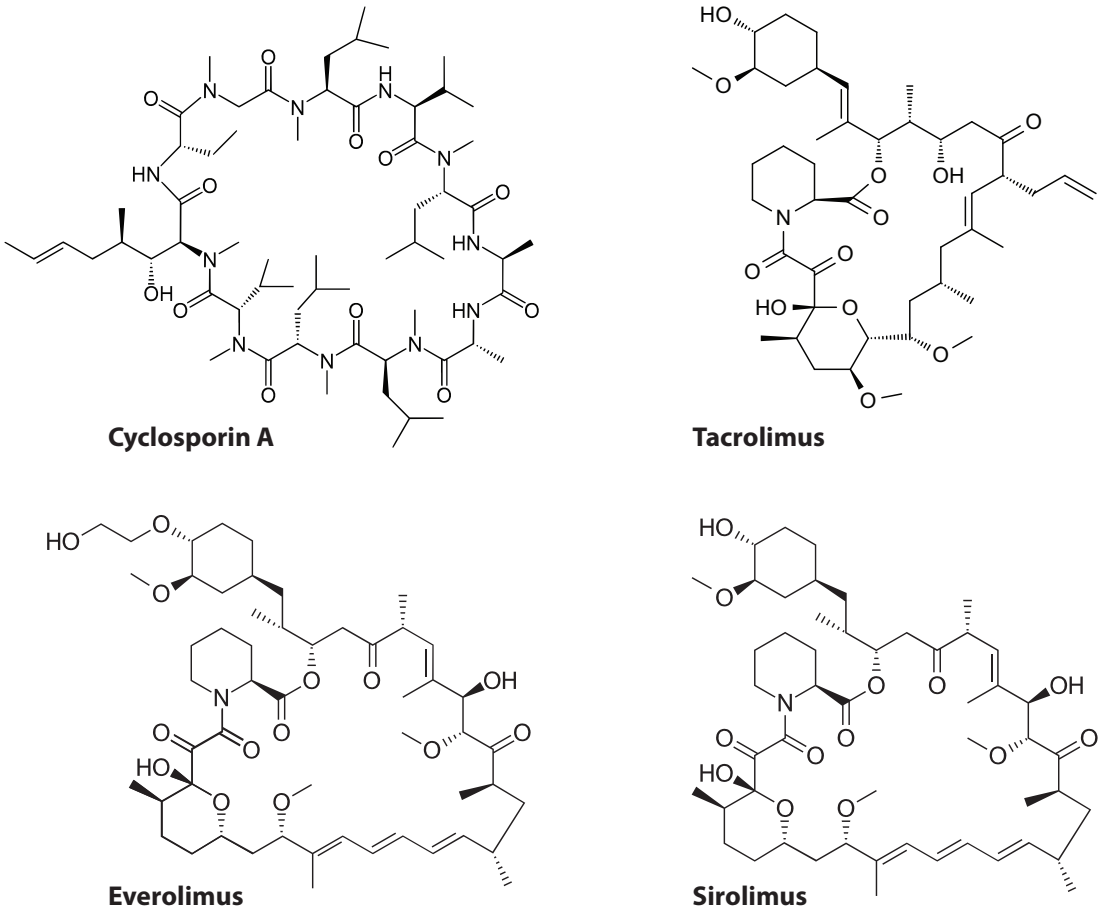
The success of organ transplant therapy depends in large part on the accurate and timely analysis of immunosuppressive drugs. In this study, we developed a fast, accurate method for the analysis of cyclosporin A, tacrolimus, sirolimus, and everolimus in whole blood. The method pairs a single precipitation step with LC-MS/MS analysis using a Raptor Biphenyl column. A fast, 3-minute analysis time was obtained with no interference from matrix components. Excellent results were obtained for linearity, robustness, accuracy, and precision, demonstrating that the method is suitable for high-throughput therapeutic drug monitoring.

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. Their structures are shown in Figure 1. 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 to minimize toxic side effects. Therefore, a fast and accurate measurement of drug concentration is critical to assist clinicians with timely and proper treatment of patients.

Several commercial products are available for the simultaneous analysis of cyclosporin A, tacrolimus, sirolimus, and everolimus in whole blood. However, most of these commercial kits require an additional in-line trap column or SPE sample cleanup prior to chromatographic analysis. Here, we reduced sample preparation time and effort by pairing a single precipitation step with a Raptor Biphenyl analytical column. This analytical column was chosen because it provides suitable retention and selectivity for these compounds so that a short analysis time (3 minutes) can be achieved without encountering matrix interference. By combining a simple sample preparation step and a fast chromatographic elution, a high-throughput analysis was established for the simultaneous analysis of four immunosuppressive drugs in human whole blood.

Figure 1: Analyte Structures



Experimental

Calibration Standards and Blood Control Samples

Human whole blood was fortified with four immunosuppressive drugs 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 general therapeutic ranges are 25–400 ng/mL for cyclosporin A, 5–20 ng/mL for tacrolimus and sirolimus, and 3–8 ng/mL for everolimus. Accordingly, the calibration standard ranges were evaluated from 10 to 1,000 ng/mL for cyclosporin A and from 1 to 100 ng/mL for tacrolimus, sirolimus, and everolimus. Three QC levels were prepared: 15, 150, and 800 ng/mL for cyclosporin A and 5, 15, and 80 ng/mL for tacrolimus, sirolimus, and everolimus.

Sample Preparation

Each blood sample (100 µL) was mixed with 200 µL of precipitation solution [1:4 (v/v) 0.2 M $ZnSO_4$:methanol] containing 50 ng/mL of cyclosporin D and 5 ng/mL of ascomycin. The mixture was vortexed for 20 seconds at 3,000 rpm and then centrifuged for 10 minutes at 4,300 rpm. 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.

LC-MS/MS Analysis

Analysis of immunosuppressive drugs (cyclosporin A, tacrolimus, sirolimus, and everolimus) was conducted using a Waters Xevo TQ-S mass spectrometer with an ACQUITY UPLC under the conditions shown below. The analyte transitions that were monitored are presented in Table I.

Analytical column:	Raptor Biphenyl, 2.7 µm, 50 mm x 2.1 mm (cat.# 9309A52)	
Guard column:	Raptor Biphenyl EXP, 2.7 µm, 5 mm x 2.1 mm (cat.# 9309A0252)	
Mobile phase A:	0.05% Formic acid, 5 mM 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 I: Analyte Transitions

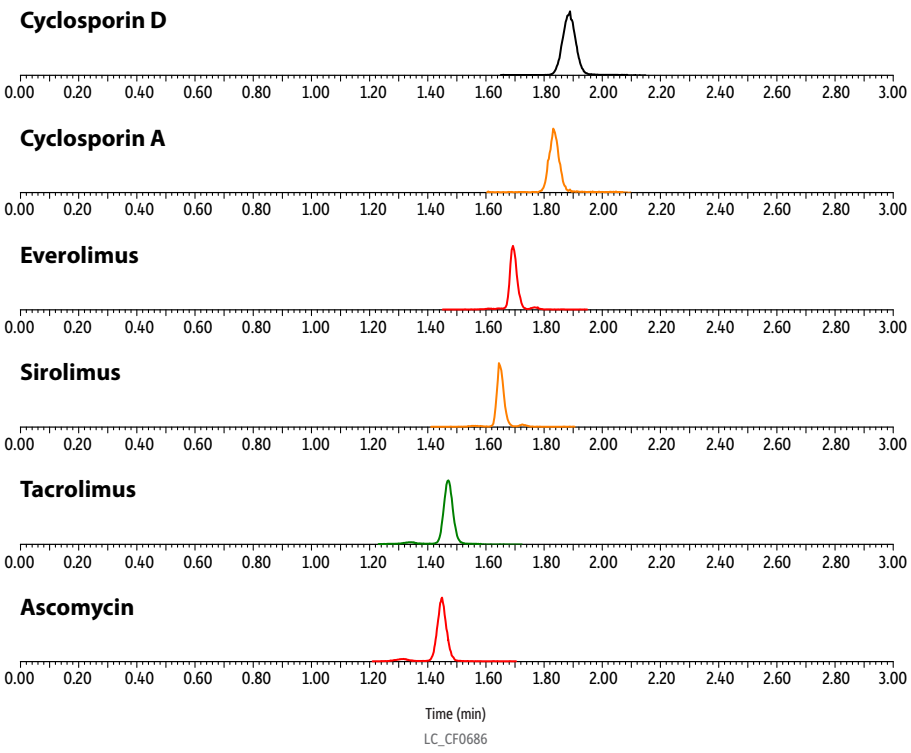
Analyte	Precursor Ion	Product Ion Quantifier	Product Ion Qualifier
Cyclosporin A	1219.83	1202.87	1184.58
Everolimus	975.68	908.62	926.57
Sirolimus	931.63	864.57	882.58
Tacrolimus	821.61	768.51	786.52
Cyclosporin D	1233.91	1216.88	—
Ascomycin	809.53	756.50	—

Results and Discussion

Chromatographic Performance

A fast 3-minute chromatographic analysis of all four immunosuppressive drugs (Figure 2) was achieved with direct injection of the supernatant without any further in- or off-line sample treatment. No matrix interference was observed for all four analytes and internal standards (Figure 3) with the established sample preparation and chromatographic methods. The Raptor Biphenyl column provided more retention than a C18 phase and less retention than a fluorophenyl phase, resulting in optimum retention and selectivity for cyclosporin A, tacrolimus, sirolimus, and everolimus.

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



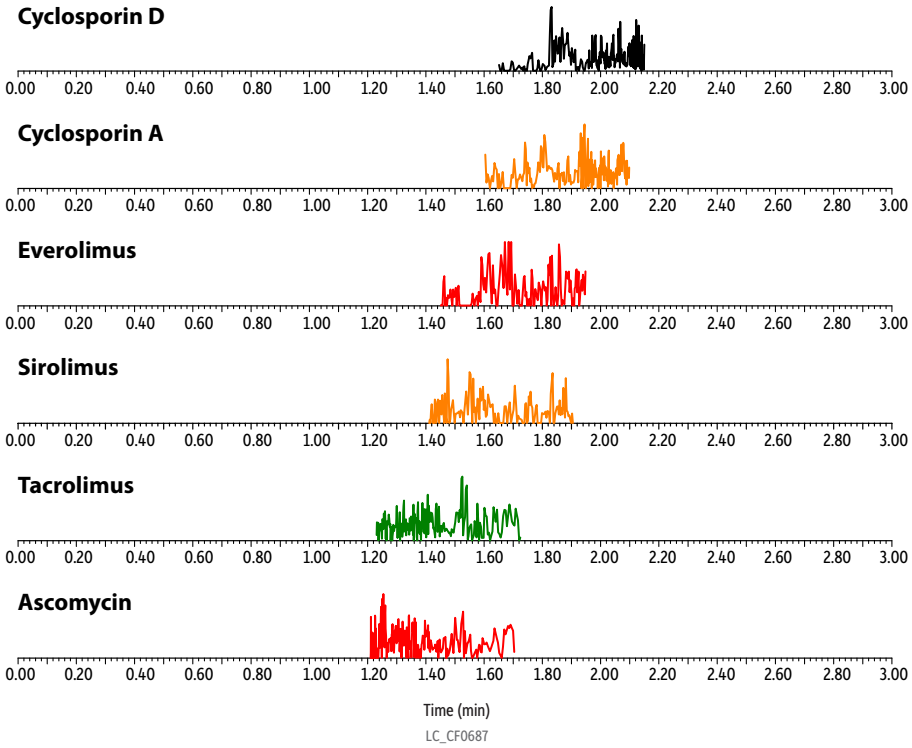
Peaks	t_R (min)	Conc. (ng/mL)	Precursor Ion	Product Ion
1. Ascomycin	1.45	10	809.53	756.50
2. Tacrolimus	1.47	10	821.61	768.51
3. Sirolimus	1.64	10	931.63	864.57
4. Everolimus	1.69	10	975.68	908.62
5. Cyclosporin A	1.83	10	1219.83	1202.87
6. Cyclosporin D	1.89	100	1233.91	1216.88

Column Raptor Biphenyl (cat.# 9309A52)
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Guard Column: Raptor Biphenyl EXP guard column cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9309A0252)
Temp.: 70 °C
Inj. Vol.: 5 µL
Mobile Phase
A: 0.05% Formic acid, 5 mM ammonium formate in water
B: Methanol

Time (min)	Flow (mL/min)	%A	%B
0.00	0.5	40	60
2.00	0.5	0	100
2.01	0.5	40	60
3.00	0.5	40	60

Max Pressure: 305 bar
Detector MS/MS
Ion Mode: ESI+
Mode: MRM
Instrument UHPLC
Notes Sample Preparation Procedure: Human whole blood was fortified with 4 immunosuppressive drugs at 10 ng/mL. 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 blood sample (100 µL) was mixed with 200 µL of precipitation solution (1:4 v/v 0.2 M $ZnSO_4$:methanol) containing 50 ng/mL of cyclosporin D and 5 ng/mL of ascomycin. The mixture was vortexed for 20 seconds at 3,000 rpm and then centrifuged for 10 minutes at 4,300 rpm. The supernatant was directly injected (5 µL) for analysis.

Figure 3: Blank Human Whole Blood



Peaks	Conc. (ng/mL)	Precursor Ion	Product Ion
1. Ascomycin	0	809.53	756.50
2. Tacrolimus	0	821.61	768.51
3. Sirolimus	0	931.63	864.57
4. Everolimus	0	975.68	908.62
5. Cyclosporin A	0	1219.83	1202.87
6. Cyclosporin D	0	1233.91	1216.88

Column Raptor Biphenyl (cat.# 9309A52)
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Guard Column: Raptor Biphenyl EXP guard column cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9309A0252)
Temp.: 70 °C
Inj. Vol.: 5 µL
Mobile Phase
A: 0.05% Formic acid, 5 mM ammonium formate in water
B: Methanol

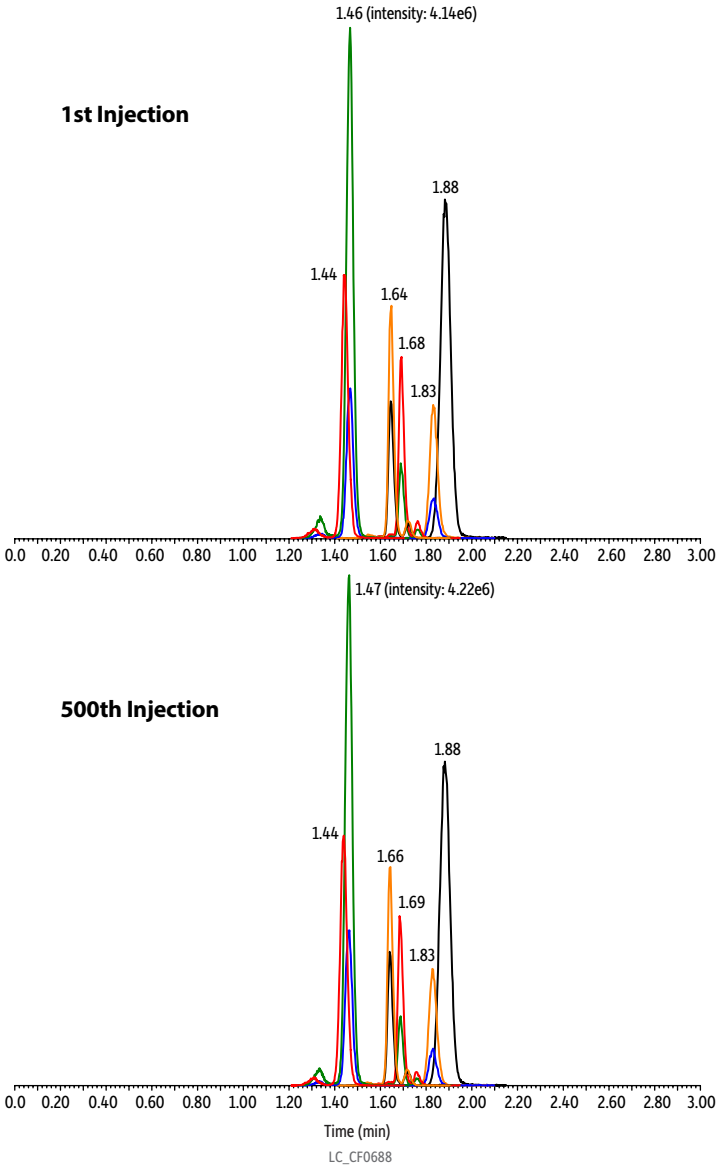
Time (min)	Flow (mL/min)	%A	%B
0.00	0.5	40	60
2.00	0.5	0	100
2.01	0.5	40	60
3.00	0.5	40	60

Max Pressure: 305 bar
Detector MS/MS
Ion Mode: ESI+
Mode: MRM
Instrument UHPLC
Notes Sample Preparation Procedure: Human whole blood (100 µL) was mixed with 200 µL of precipitation solution (1:4 v/v 0.2 M $ZnSO_4$:methanol). The mixture was vortexed for 20 seconds at 3,000 rpm and then centrifuged for 10 minutes at 4,300 rpm. The supernatant was directly injected (5 µL) for analysis.

Method Robustness

Following 500 injections of a prepared whole blood standard (25 ng/mL), the chromatographic peaks of all four immunosuppressive drug analytes 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.

Figure 4: Robust Column Performance Over 500 Injections



Peaks	Conc. (ng/mL)	Precursor Ion	Product Ion
1. Ascomycin	10	809.53	756.50
2. Tacrolimus	25	821.61	768.51
3. Sirolimus	25	931.63	864.57
4. Everolimus	25	975.68	908.62
5. Cyclosporin A	25	1219.83	1202.87
6. Cyclosporin D	100	1233.91	1216.88

See chromatograms for retention times.

Column Raptor Biphenyl (cat.# 9309A52)
Dimensions: 50 mm x 2.1 mm ID
Particle Size: 2.7 µm
Pore Size: 90 Å
Guard Column: Raptor Biphenyl EXP guard column cartridge 5 mm, 2.1 mm ID, 2.7 µm (cat.# 9309A0252)
Temp.: 70 °C
Inj. Vol.: 5 µL
Mobile Phase
A: 0.05% Formic acid, 5 mM ammonium formate in water
B: Methanol

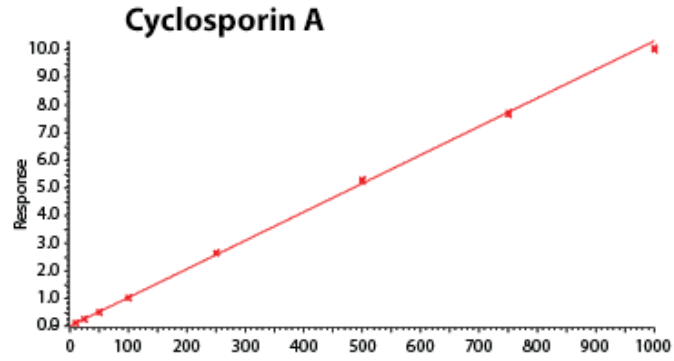
Time (min)	Flow (mL/min)	%A	%B
0.00	0.5	40	60
2.00	0.5	0	100
2.01	0.5	40	60
3.00	0.5	40	60

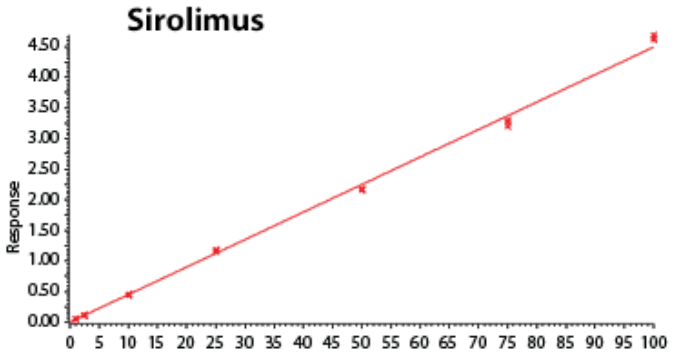
Max Pressure: 305 bar
Detector MS/MS
Ion Mode: ESI+
Mode: MRM
Instrument 57I
Notes Sample Preparation Procedure: Human whole blood was fortified with 4 analytes at 25 ng/mL. For quantitation, cyclosporin D was used as the internal standard for cyclosporin A and ascomycin was used as the internal standard for tacrolimus, sirolimus, and everolimus. The blood sample (2.5 mL) was mixed with 5 mL of precipitation solution (1:4 v/v 0.2 M $ZnSO_4$:methanol) containing 50 ng/mL of cyclosporin D and 5 ng/mL of ascomycin. The mixture was vortexed for 20 seconds at 3,000 rpm and then centrifuged for 10 minutes at 4,300 rpm. The supernatant was directly injected (5 µL) for analysis.

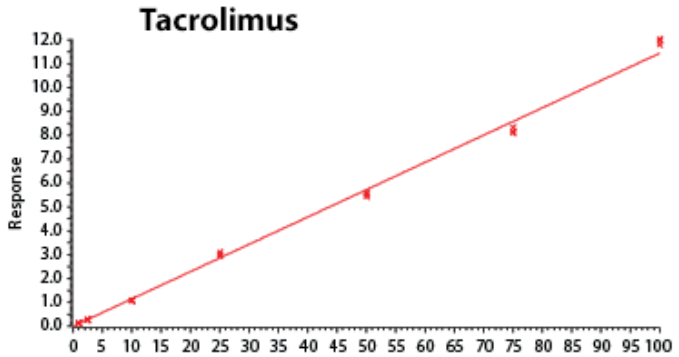
Linearity

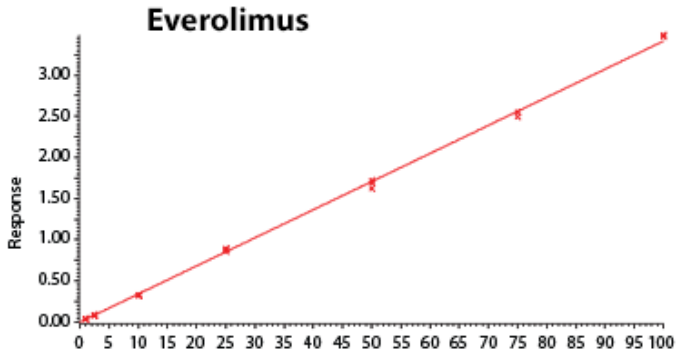
Using a $1/x^2$ weighted linear regression for cyclosporin A and a 1/x weighted linear regression for tacrolimus, sirolimus, and everolimus, all four immunosuppressive drug compounds showed very good linearity with r values of 0.999 or greater, and with deviations of <10% (Figure 5). The signal-to-noise 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 and Precision

Precision and accuracy analyses were performed on three different days. Method accuracy was demonstrated with recovery values that were within 10% of the nominal concentration for all QC levels. The %RSD across all analytes and fortification levels was 0.2–4.0% and 1.2–5.4% for intraday and interday evaluations respectively, indicating good method precision (Table II).

Table II: Accuracy and Precision of QC Samples

	QC Level 1 (5.0 ng/mL) (Cyclosporin A: 15 ng/mL)			QC Level 2 (15.0 ng/mL) (Cyclosporin A: 150 ng/mL)			QC Level 3 (80 ng/mL) (Cyclosporin A: 800 ng/mL)		
Analyte	Average Conc. (ng/mL)	Average Accuracy (%)	%RSD	Average Conc. (ng/mL)	Average Accuracy (%)	%RSD	Average Conc. (ng/mL)	Average 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

Conclusion

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

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