Rapid Analysis of Cyclosporine A, Everolimus, Sirolimus, and Tacrolimus Drugs in Whole Blood Using an Agilent Triple Quadrupole LC/MS/MS System with Automated Online Sample Cleanup

Application Note

Clinical Research

Authors

Linda Côté, Siji Joseph, Sreelakshmy Menon, and Kevin McCann
Agilent Technologies, Inc.

Abstract

A highly sensitive and specific analytical method has been developed for quantitation of a panel of Cyclosporin A (CsA), Everolimus (Eve), Sirolimus (Sir), and Tacrolimus (Tac). This method has a run time of 2 minutes and is suitable for the simultaneous quantification of all four analytes in whole blood.

Agilent Technologies

Introduction

This application note describes the development of an analytical method for the sensitive and accurate determination of four drugs – Cyclosporin A (CsA), Everolimus (Eve), Sirolimus (Sir), and Tacrolimus (Tac) – in whole blood using an Agilent 1260 LC system coupled to an Agilent 6460 or 6470 Triple Quadrupole Mass Spectrometer with Agilent JetStream technology. Using tandem mass spectrometry (MS/MS) and multiple reaction monitoring (MRM), the method is linear from 1.95 ng/mL to 2,000 ng/mL for CsA and from 0.10 ng/mL to 100 ng/mL for Eve, Sir, and Tac.

Experimental

Reagents and standards

For development of the analytical method, all unlabeled and labeled standards were purchased from Cerilliant with the exception of Cyclosporin A-d4 and unlabeled Everolimus, which were purchased from Toronto Research Chemicals and Sigma-Aldrich, respectively. All standards were stored at –20 °C. Deuterated and analog internal standards (ISTD) were used to ensure accurate quantitation. The list of analytes and corresponding internal standards are given in Table 1. All other LC/MS grade solvents and reagents were purchased from Sigma-Aldrich and Honeywell. Disease free certified whole blood was purchased from a local blood bank.

Additionally, commercially available calibrators from ChromSystems and Quality Controls (QC) from BioRad were used to evaluate accuracy and precision of this method (Table 9).

To determine linearity, a high-level of each standard was spiked into whole blood – 2,000 ng/mL of CsA and 100 ng/mL each of Eve, Sir, and Tac. Serial two-fold dilutions with whole blood were used to achieve the remaining concentrations. Analyte concentrations for linearity are listed in Table 2.

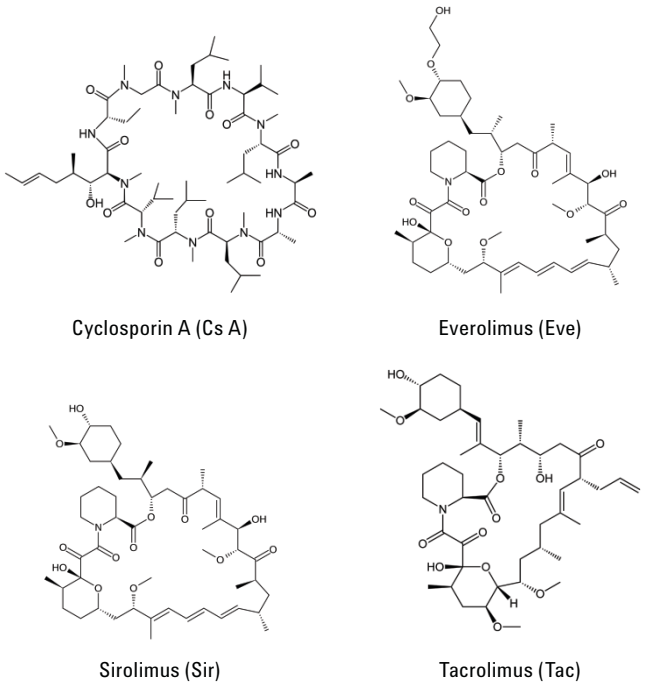

Figure 1. Structures for CsA, Eve, Sir, and Tac.

Table 1. List of analytes and corresponding ISTD.

Analyte	Internal standard
Cyclosporin A	Cyclosporin A-d4
Everolimus	Everolimus-d4
Sirolimus and Tacrolimus	Ascomycin

Table 2. Linearity levels used in this study.

Calibrator	CsA (ng/mL)	Eve, Sir, Tac (ng/mL)
11	2,000	100
10	1,000	50
9	500	25
8	250	12.50
7	125	6.25
6	62.50	3.13
5	31.25	1.56
4	15.63	0.78
3	7.81	0.39
2	3.91	0.20
1	1.95	0.10

Sample preparation

All calibrators, QCs, and samples were prepared using a simple protein precipitation procedure:

1. Mix 100 µL of whole blood with 200 µL of precipitating reagent (1:4 ratio of 0.4 M zinc sulphate:methanol) containing internal standard.
2. Vortex for 30 seconds.
3. Centrifuge at 10,000 rpm for 4 minutes.
4. Transfer supernatant to autosampler vials and analyze by LC/MS/MS.

LC configuration and conditions

An Agilent 1260 Infinity LC system was used for this analysis. The system consisted of:

- Agilent 1260 Infinity Binary Pump (×2)
- Agilent 1260 Infinity Thermostatted Column Compartment with 2-Position/6-Port column switching valve
- Agilent 1260 Thermostatted Autosampler

An inline filter (p/n 5067-1551) between the needle seat and the injector valve of the autosampler is also recommended to improve instrument robustness.

LC conditions are listed in Tables 3, 4, 5, and 6.

Table 3. LC conditions.

Parameter	Value
Columns	**Trapping:** Agilent ZORBAX Eclipse Plus C18, 2.1 × 12.5 mm, 5 µm (p/n 821125-936) **Analytical:** Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm (p/n 699975-302)
Column temperature	60 °C
Injection volume	40 µL
Needle wash	1:1:1:1 methanol:acetonitrile:isopropyl alcohol:water + 0.1 % FA for 10 seconds
Injector temperature	4 °C
Run time	2 minutes
Buffer A	10 mM ammonium acetate + 0.2 % formic acid in water
Buffer B	10 mM ammonium acetate + 0.2 % formic acid in methanol

Table 4. Loading gradient (Pump 1).

Time	Flow (mL/min)	%B
0.00	0.1	50
0.01	2.5	50
1.50	2.5	50
1.80	0.1	50
2.00	0.1	50

Table 5. Analytical gradient (Pump 2).

Time	Flow (mL/min)	%B
0.00	0.5	95
1.30	0.5	95
1.35	1.0	95
1.55	1.0	95
1.65	0.5	95
2.00	0.5	95

Table 6. Valve timing.

Time	Position
0.00	1
0.50	2
1.65	1

Automated online sample cleanup

The HPLC used for this method was configured for automated sample cleanup using two binary pumps (Figure 2). Samples were loaded onto a trapping column where the analytes were retained and washed by the first pump. The wash was sent to waste, reducing the amount of matrix introduced into the mass spectrometer. Shortly before the analytes eluted off of the trapping column, a valve was switched and the analytes were eluted onto an analytical column where further chromatography was performed using the second binary pump.

MS conditions

This method was developed for and tested on the Agilent 6460 and 6470 Triple Quadrupole Mass Spectrometers. Both of these instruments are equipped with JetStream technology. Unique MRM transitions ensured specificity in the quantitation of each analyte. Internal standards (ISTD) were used for relative quantification and thus reduced the error due to any loss of analytes during sample preparation or variation in the sample matrix. MS conditions and MRM transitions are listed in Tables 7 and 8. Note that source conditions and MRM transition parameters are the same for the Agilent 6460 and 6470 Triple Quadrupole Mass Spectrometers.

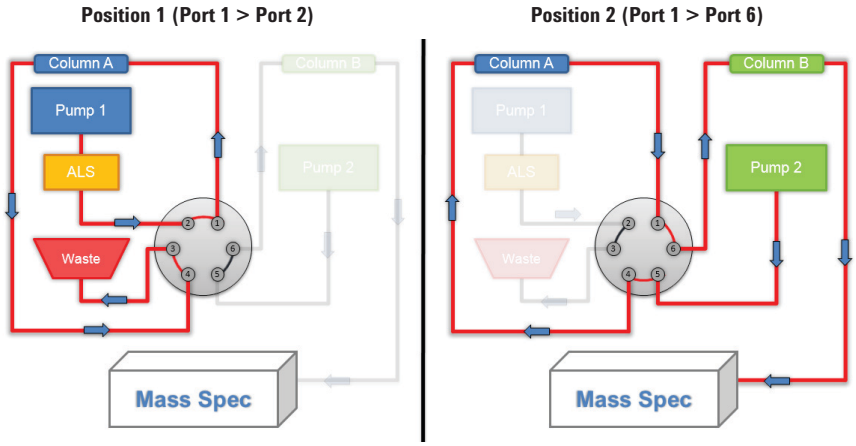

Figure 2. Valve diagram for backflushing liquid chromatography configuration for online sample cleanup using 2-position/6-port valve and two binary pumps.

Table 7. Conditions for an Agilent 6460/6470 Triple Quadrupole Mass Spectrometer equipped with an Agilent Jet Stream source.

Parameter	Value
Ion mode	Positive
Drying gas temperature	225 °C
Drying gas flow	9 L/min
Nebulizer pressure	35 psi
Sheath gas temperature	325 °C
Sheath gas flow	12 L/min
Capillary voltage	4,000 V
DEMV	200 V
Nozzle voltage	300 V
Q1/Q3 resolution	0.7 unit

Table 8. MRM transitions monitored.

Compound	Precursor	Product	Dwell (msec)	Frag. (V)	CE (V)	CAV
Cyclosporin A-d4	1,223.9	1,206.8	10	170	12	4
Cyclosporin A	1,219.9	1,202.8	10	175	12	4
Everolimus-d4	979.6	912.5	10	170	12	4
Everolimus	975.6	908.5	10	185	12	4
Sirolimus	931.6	864.5	10	170	12	4
Tacrolimus	821.5	768.4	10	170	16	4
Ascomycin	809.5	756.4	10	175	16	4

Data analysis

MassHunter Quantitative Software B.07.00 was used for data analysis. Calibration curves were constructed for all analytes using MRM peak area ratios to a known concentration of the internal standard. For the linearity regression of the calibration curves, a weighing factor of 1/x was used. Interday injections were performed with QC samples to assess recovery and reproducibility. Representative extracted MRM chromatograms for the analytes are given in Figure 3.

Results and Discussion

Excellent linearity was observed for all analytes on both instruments, with R^2 values > 0.995 including all 11 concentration levels tested (Figure 4). Consistent retention times for each analyte guaranteed the reproducibility of the method. Sufficient analyte response at low linearity levels assures accurate quantitation down to the lowest concentrations tested.

Interday injections were performed with commercially available calibrators (ChromSystems) and QC samples

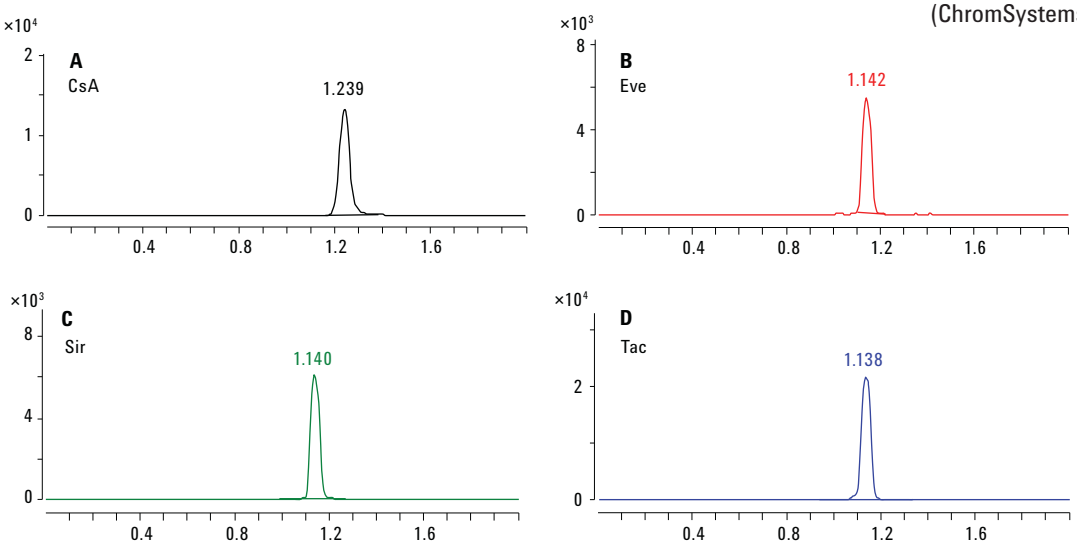

Figure 3. Chromatograms of quantifier MRM transitions for Cyclosporin A (A), Everolimus (B), Sirolimus (C), and Tacrolimus (D).

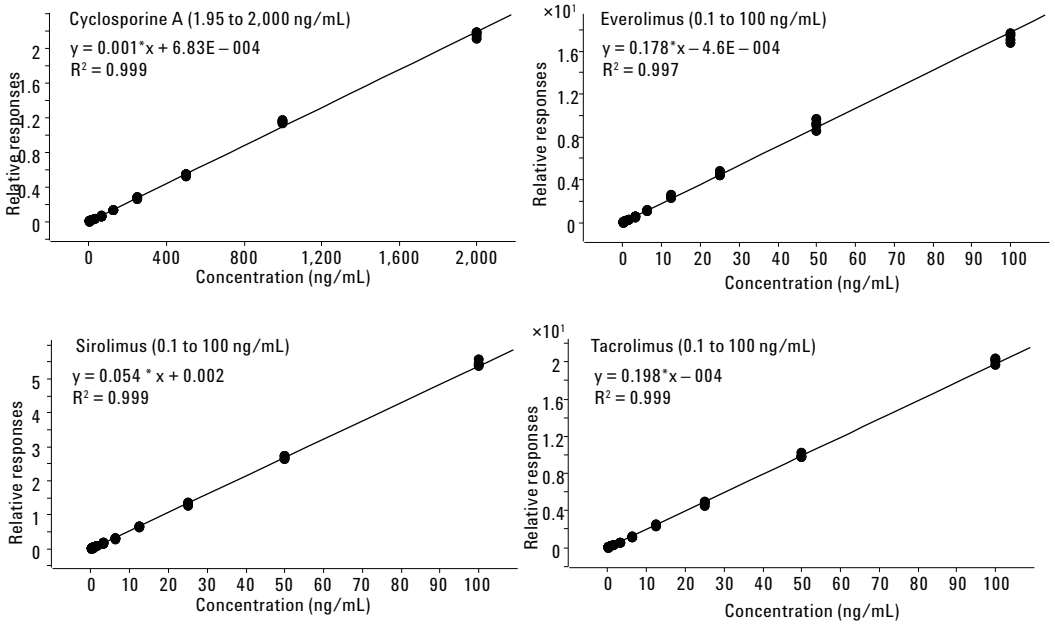

Figure 4. Calibration curves of Cyclosporin A (A), Everolimus (B), Sirolimus (C), and Tacrolimus (D) 11 levels, 44 points, (type: linear, origin: ignore, weight: 1/x).

(BioRad) to evaluate the accuracy and robustness of this method. Data was acquired over 14 days by four different operators. The observed accuracies for each level of QC are tabulated in Table 9.

Table 9. BioRad QC results.

Compound	Target (ng/mL)	Mean (ng/mL)	Accuracy (%)	CV (%)
CsA	95.6	95.6	100.0	6.3
	187.0	197.6	105.7	4.9
	307.0	321.6	104.8	4.8
Sir	5.1	4.8	94.1	13.9
	8.5	8.6	101.2	11.5
	17.3	17.9	103.5	10.4
Tac	4.2	4.5	107.1	7.4
	7.6	7.7	101.3	6.6
	12.5	13.1	104.8	7.9

Robustness

Long-term robustness studies were conducted on both the Agilent 6460 and 6470 Triple Quadrupole Mass Spectrometers. Testing was performed by alternating between batches of a calibration set and a stress-test set. The calibration sets consisted of triplicate injections of a calibration curve and were run on a dedicated pair of trapping and analytical columns. This data served as a baseline measurement between stress-test sets to insure quantitation remained accurate and consistent throughout the experiment. Each stress-test set contained 1079 injections, where every 11th injection was spiked with a known concentration of analytes – 10 ng/mL of Everolimus, Sirolimus, and Tacrolimus, and 200 ng/mL of Cyclosporin A. All injections in the calibration and stress-test sets were whole blood samples prepared to the specifications above (see Sample Preparation). Alternating of batches continued until the coefficient of variation (CV) exceeded 10 % for the peak area of one or more analytes. Figure 5 displays the results.

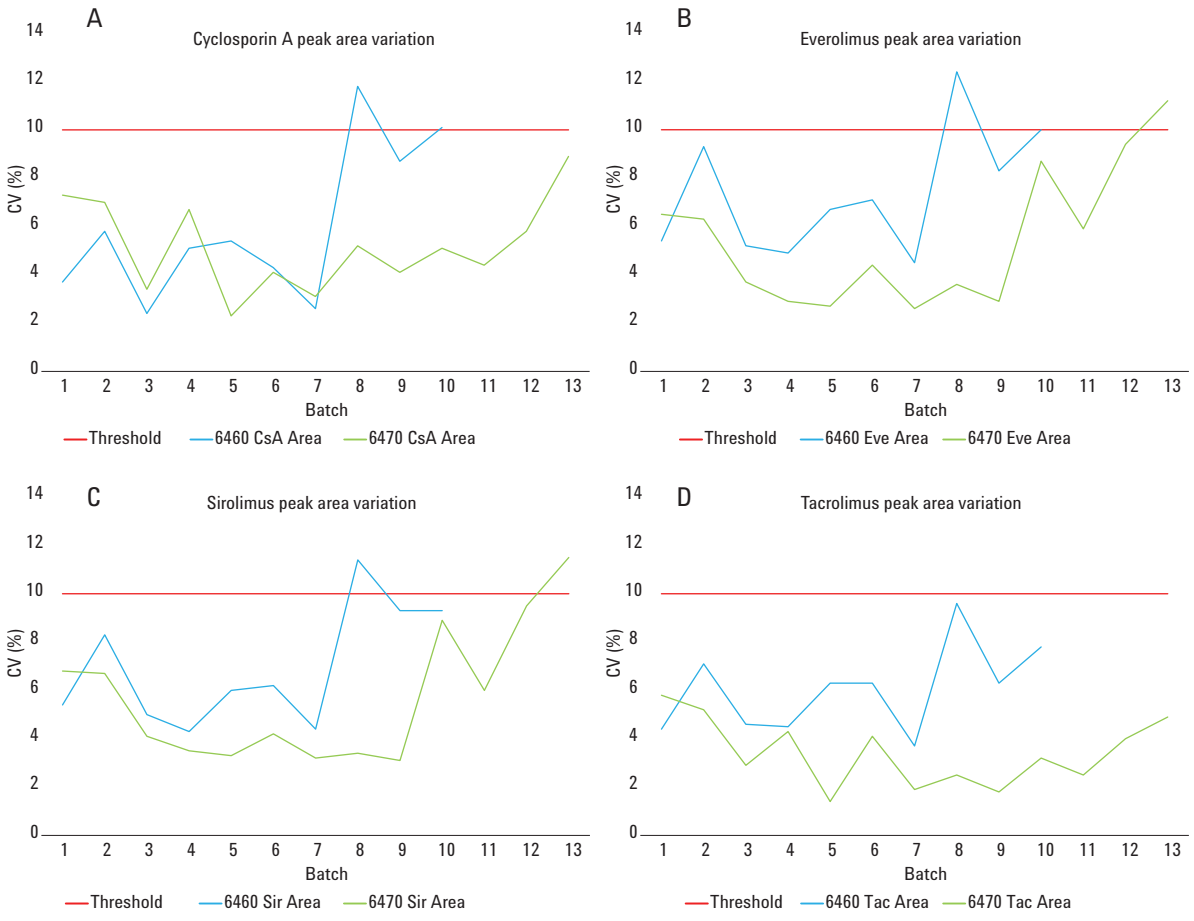

Figure 5. Peak area variation on the Agilent 6460 Triple Quadrupole Mass Spectrometer (blue) and the Agilent 6470 Triple Quadrupole Mass Spectrometer (green) for Cyclosporin A (A), Everolimus (B), Sirolimus (C), and Tacrolimus (D).

The 6460 Triple Quadrupole Mass Spectrometer ran for 10 batches before exceeding the 10 % threshold set for this experiment. Note that batch 8 did exceed the threshold for three analytes, but it was determined that the variation was due to an autosampler issue. After addressing the issue, the variation for all analytes fell below the threshold for the following batch. These 10 alternating batches of calibration sets and stress-test sets equates to a total of 11,150 injections. As a precaution, the trapping column was changed after each stress-test set (1,079 injections). However, there were no signs of decreased performance, suggesting the trapping column could last even longer. The analytical column was changed at batch 8 while troubleshooting the autosampler, having completed over 8,500 injections – no significant decrease in column performance was observed to that point.

In general, the 6470 Triple Quadrupole Mass Spectrometer showed even lower peak area variation than the 6460 Triple Quadrupole Mass Spectrometer. The 6470 Triple Quadrupole Mass Spectrometer was also able to maintain this low variation for a longer period of time, not exceeding the 10 % threshold until batch 13 – a total of 14,495 injections. To remain consistent with the experiment run on the 6460, the trapping column was changed before each stress-test set, and the analytical column was changed after batch 8.

It is important to note that even once the peak area variation exceeded the threshold that was set, quantitation remained consistent. Figure 6 shows the calibration curve for all four analytes on the 6470 Triple Quadrupole Mass Spectrometer before the experiment was conducted, while Figure 7 shows the same calibration curves after the experiment was complete. The 6460 Triple Quadrupole Mass Spectrometer displayed similar results. While peak area variation did increase, internal standard correction allows for continuous, accurate quantitation.

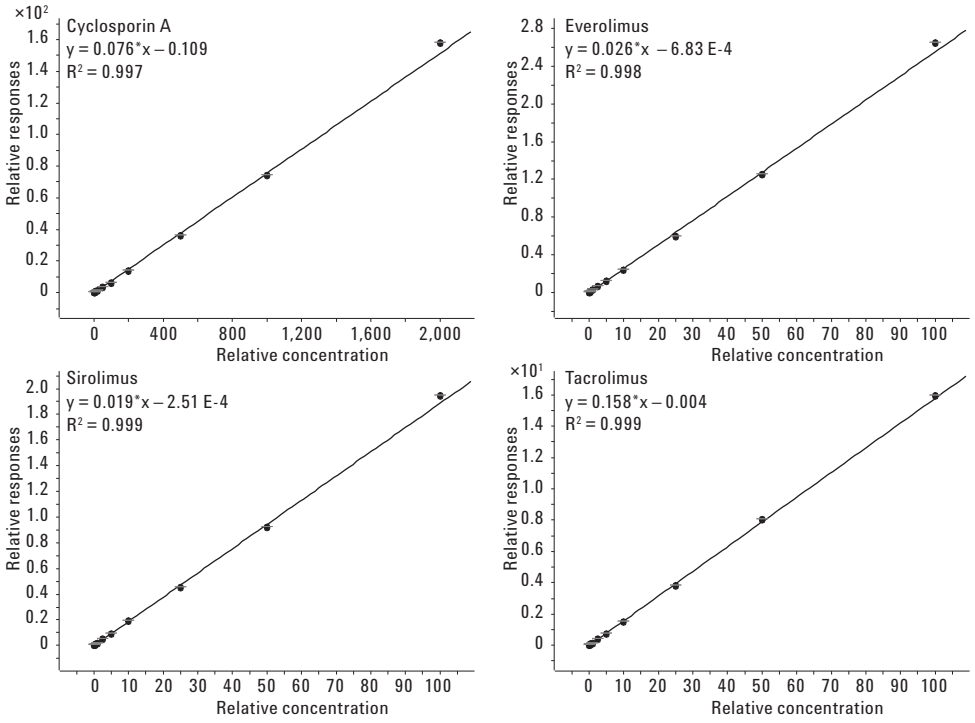

Figure 6. Calibration curves for analytes before robustness testing.

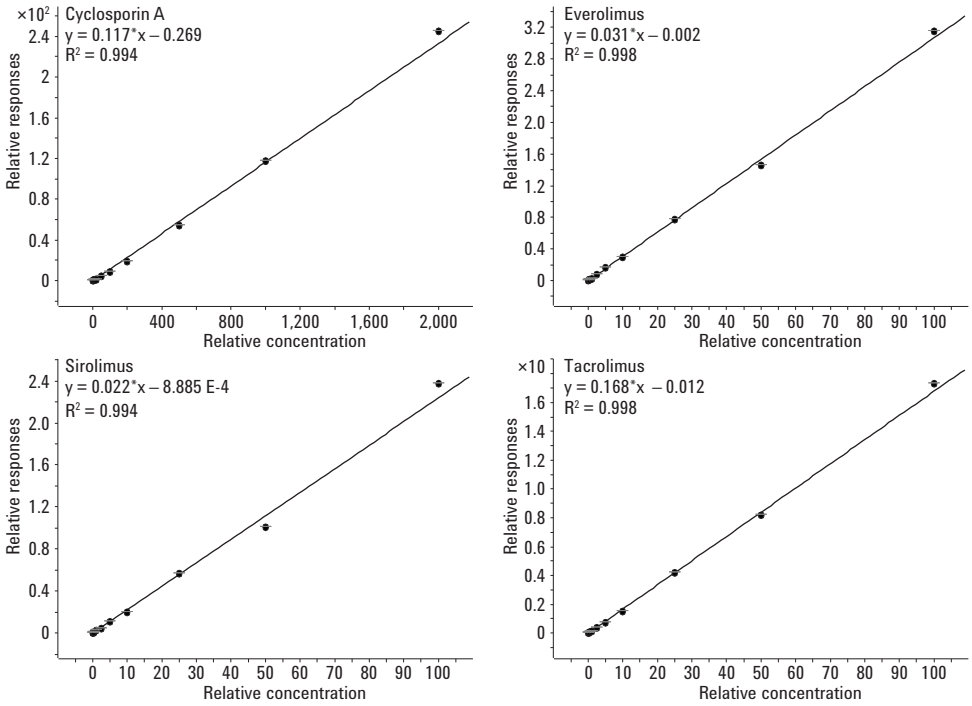

Figure 7. Calibration curves for analytes after robustness testing.

Conclusion

A high throughput, 2 minute analytical method for the quantitation of Cyclosporin A, Everolimus, Sirolimus, and Tacrolimus has been developed using an Agilent 6460 or 6470 Triple Quadrupole Mass Spectrometer. A simple protein precipitation followed by automated online sample cleanup minimized the matrix effect and ion suppression due to biological compounds present in blood. Using this method, reliable and quick quantitation of of all four analytes in whole blood matrix was demonstrated. Excellent linearity of all analytes has been confirmed over the desired ranges.

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