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

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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.

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Introduction

This application note describes the development of an analytical method for the sensitive and accurate determination of four immunosuppressive 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 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.

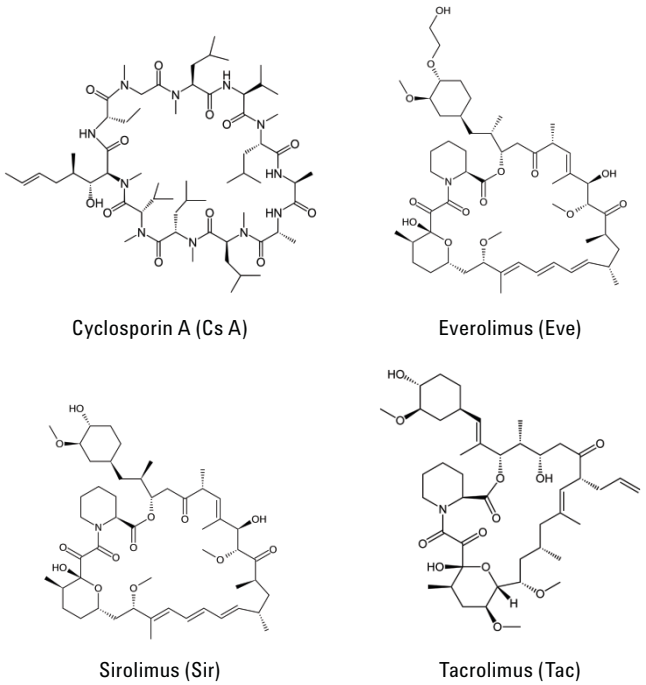

Figure 1. Structures for CsA, Eve, Sir, and Tac.

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

An Agilent 6460 Triple Quadrupole Mass Spectrometer with JetStream technology was used for this analysis. 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.

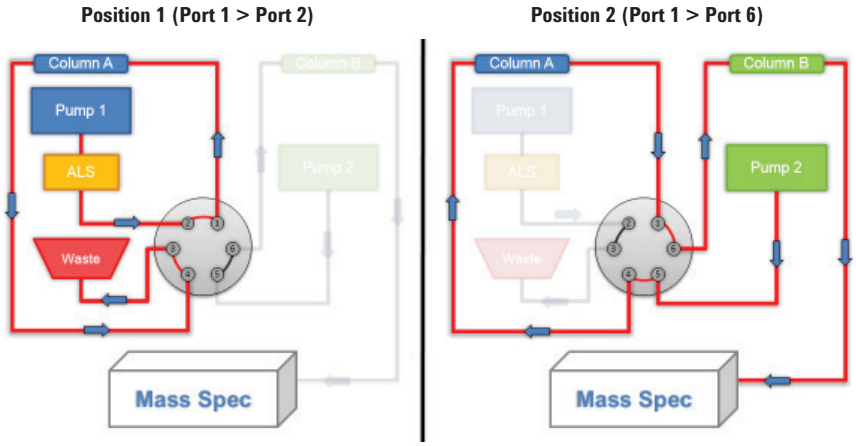

Figure 2. Valve diagram for backflushing liquid chromatography configuration for online sample cleanup using 2-position/6-port valve and two binary pumps.

Table 7. Conditions for an Agilent 6460 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.04.01 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, 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.

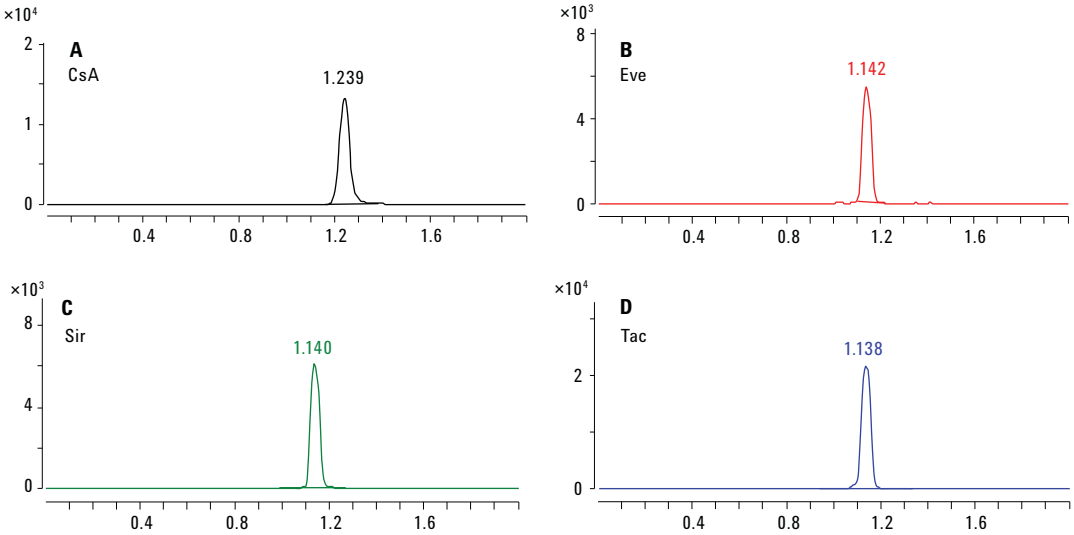

Figure 3. Chromatograms of quantifier MRM transitions for Cyclosporin A (A), Everolimus (B), Sirolimus (C), and Tacrolimus (D).

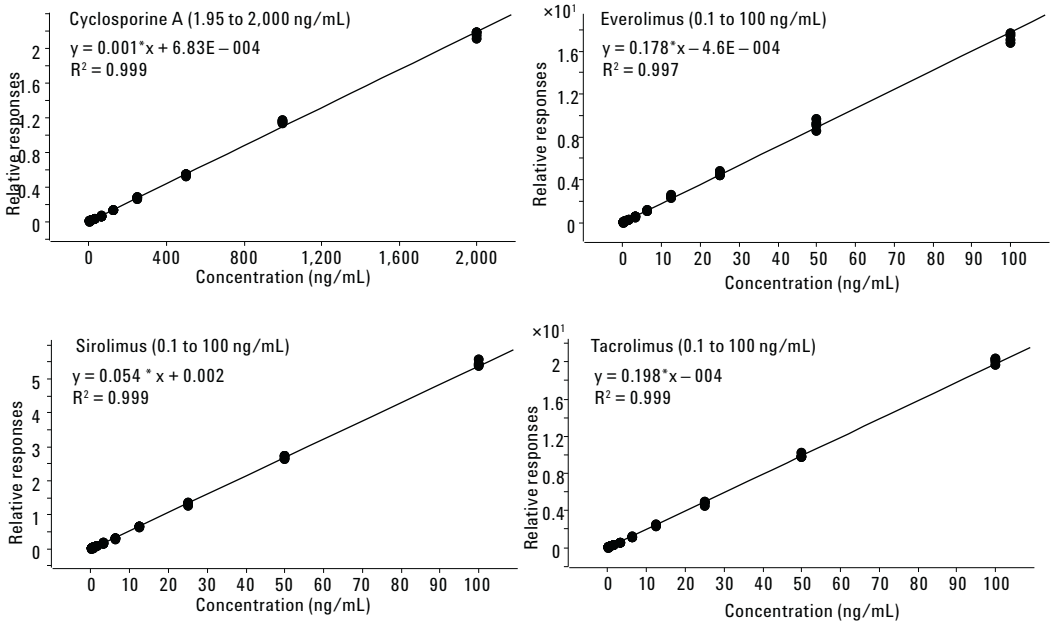

Figure 4. Calibration curves of Cyclosporin A (A), Everolimus (B), Sirolimus (C), and Tacrolimus (D) 11 levels, 44 points, (type: linear, origin: ignore, weight: 1/x).

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

Conclusion

A high throughput, 2 minute analytical method for the quantitation of immunosuppressive Cyclosporin A, Everolimus, Sirolimus, and Tacrolimus has been developed using an Agilent 6460 Triple Quadrupole LC/MS/MS. 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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