A Robustness Study for the Agilent 6470 LC-MS/MS Mass Spectrometer

Application Note

Clinical Research

Authors

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Abstract

Long-term robustness studies were conducted on the Agilent 6470 LC-MS/MS mass spectrometer. Four common immunosuppressants in whole blood were used as a model system based on a high-throughput 2-minute method. We have demonstrated consistently less than 10 % peak area %RSD for over 14,000 injections following a simple protein precipitation sample preparation. The analytes were quantified at 0.1 ng/mL–2 mg/mL.

Introduction

This Application Note describes a robustness study based on a previously described high-throughput 2-minute analytical method for the sensitive and accurate determination of four immunosuppressant drugs: Cyclosporin A (CsA), Everolimus (Eve), Sirolimus (Sir), and Tacrolimus (Tac). Whole blood samples were analyzed using an Agilent 1260 LC system coupled to an Agilent 6470 Triple Quadrupole Mass Spectrometer with Agilent JetStream technology[1].

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Experimental

Reagents and standards

Analytes and corresponding isotopically labeled internal standards were acquired from Cerilliant, Toronto Research Chemicals and Sigma-Aldrich. Deuterated and analog internal standards (ISTD) were used as previously described[1] to ensure accurate quantitation. The list of analytes and commonly used 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.

For the calibration samples used during the robustness study, 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 are listed in Table 2.

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 the supernatant to autosampler vials, and analyze by LC-MS/MS.

LC configuration and conditions

An Agilent 1260 Infinity LC system was used for this robustness study. 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 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

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 1). 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 a robustness test of the Agilent 6470 Triple Quadrupole Mass Spectrometer equipped with JetStream technology. Unique MRM transitions ensured specificity in the quantitation of each analyte. Internal standards (ISTD) were used for 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.

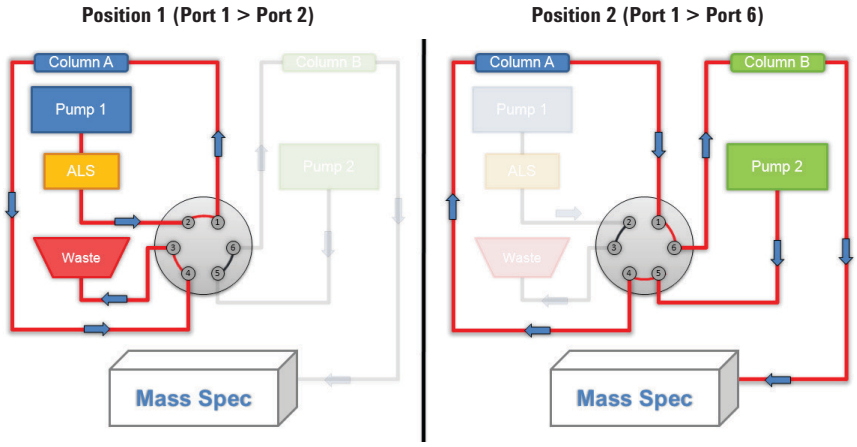

Figure 1. Valve diagram for backflushing liquid chromatography configuration for online sample cleanup using 2-position/6-port valve and two binary pumps.

Table 7. Conditions for an Agilent 6470 Triple Quadrupole Mass Spectrometer equipped with an Agilent JetStream 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 and associated parameters.

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

Agilent 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. Figure 2 shows representative extracted MRM chromatograms for the analytes.

Robustness study setup

Long-term robustness studies were conducted on an Agilent 6470 Triple Quadrupole Mass Spectrometer for over 14 days with four operators. 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 (one blank and 11 calibrators) and were run on a dedicated pair of trapping and analytical columns. These data served as a baseline measurement between stress-test sets to ensure that quantitation remained accurate and consistent throughout the experiment. Each stress-test set contained 1,079 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, and CVs were calculated from the stress test sets, until the coefficient of variation (CV) exceeded 10 % for the peak area of one or more analytes.

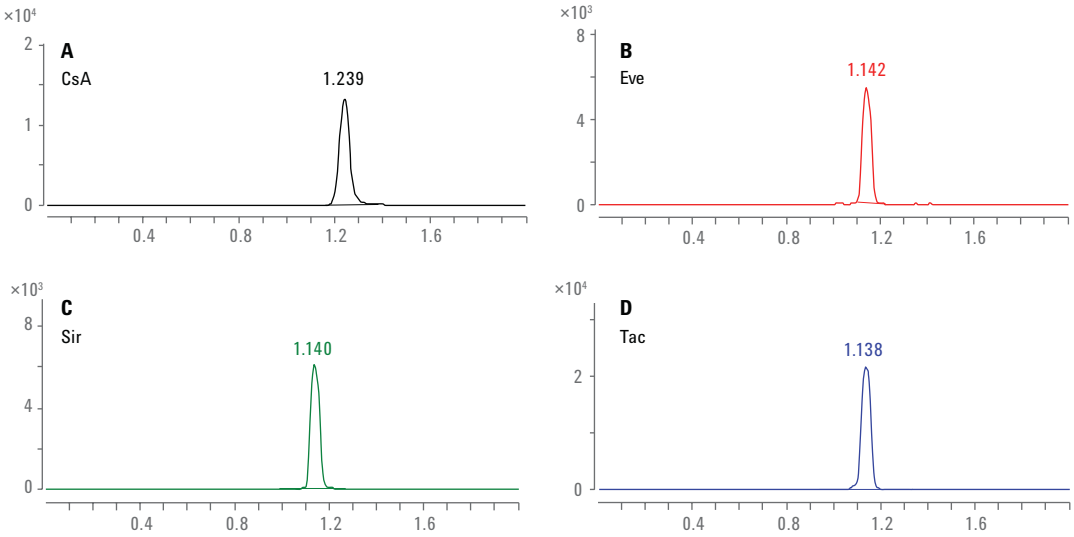

Figure 2. Chromatograms of quantifier MRM transitions for Cyclosporin A (A), Everolimus (B), Sirolimus (C), and Tacrolimus (D).

Results and Discussion

The 6470 Triple Quadrupole Mass Spectrometer ran for 13 batches, a total of 14,495 injections, before exceeding the 10 % threshold set for this experiment, showing very low peak area variation (Figure 3). These 13 alternating batches of calibration sets and stress-test sets equates to a total of 14,495 injections. As a precaution, the trapping column was changed before each stress-test set (or every 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, having completed over 8,500 injections – no significant decrease in column performance was observed to that point.

It is important to note that even once the peak area variation exceeded the threshold that was set, quantitation remained consistent. Figure 4 shows the calibration curve for all four analytes on the 6470 Triple Quadrupole Mass Spectrometer before the experiment was conducted, while Figure 5 shows the same calibration curves after the experiment was complete. Excellent linearity was observed in the calibration set for all analytes, with R^2 values >0.997 before the robustness testing, and R^2 values of >0.994 after the robustness testing, with over 14,000 injections performed during the robustness testing. While peak area variation did increase, internal standard correction allowed for continuous, accurate quantitation.

Figure 3. Peak area variation on the Agilent 6470 Triple Quadrupole Mass Spectrometer for Cyclosporin A (A), Everolimus (B), Sirolimus (C), and Tacrolimus (D).

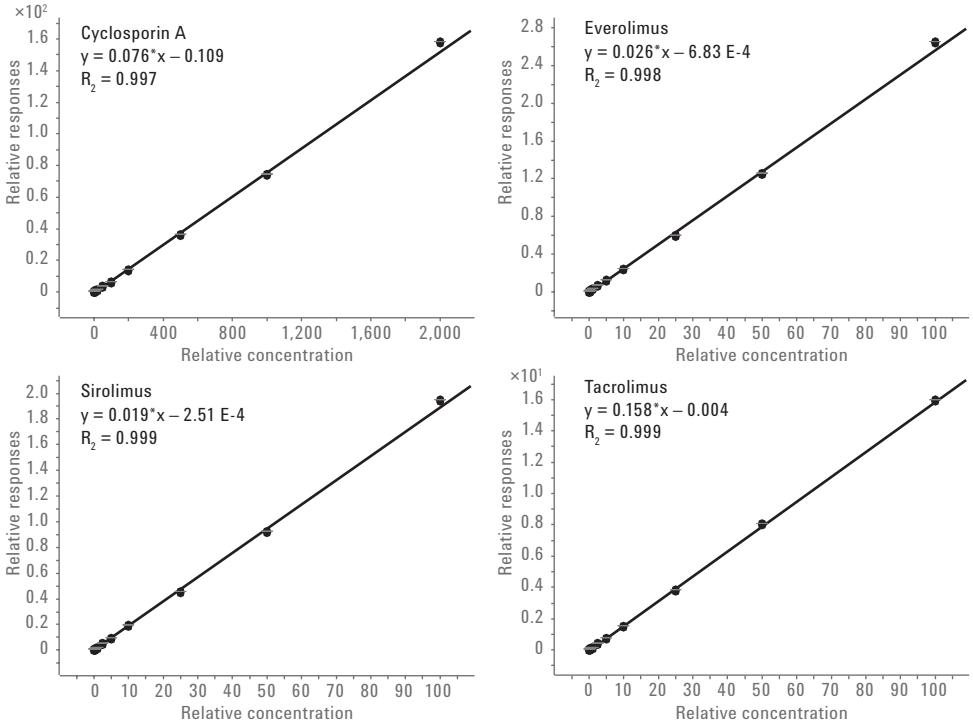

Figure 4. Calibration curves for analytes before robustness testing show Cyclosporin A, Everolimus, Sirolimus, and Tacrolimus 11 levels, triplicate injections (type: linear, origin: ignore, weight: 1/x)

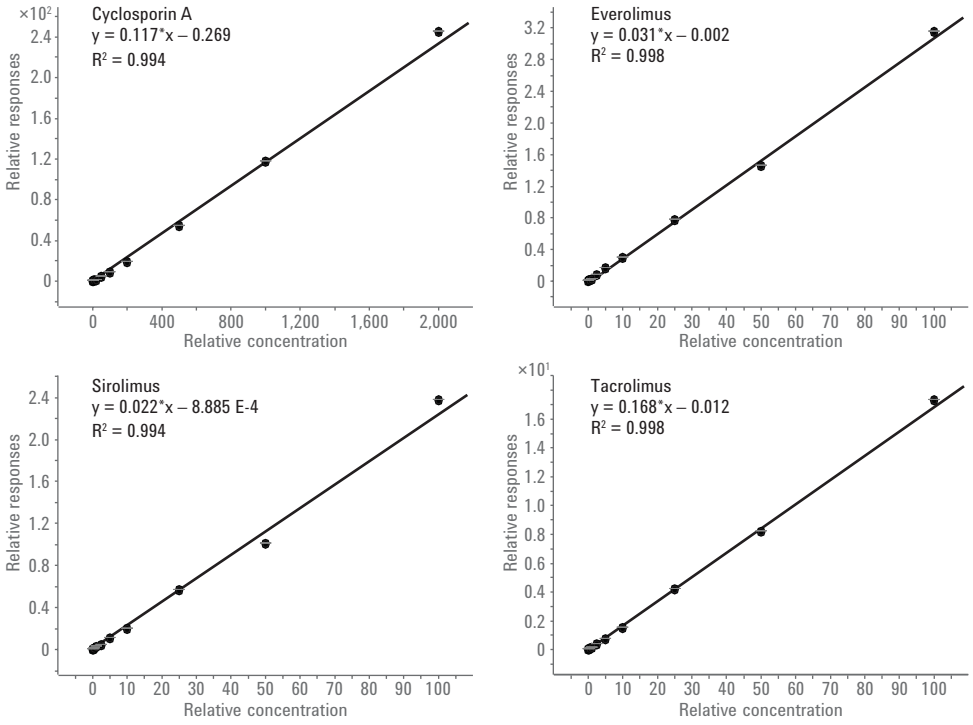

Figure 5. Calibration curves for analytes after robustness testing show Cyclosporin A, Everolimus, Sirolimus, and Tacrolimus 11 levels, triplicate injections (type: linear, origin: ignore, weight: 1/x)

Conclusion

Based on a high-throughput, 2 minute analytical method for the quantitation of Cyclosporin A, Everolimus, Sirolimus, and Tacrolimus in whole blood samples, a robustness study for the Agilent 6470 Triple Quadrupole Mass Spectrometer has been conducted. The system was able to maintain low peak area variation for a long period of time, not exceeding the 10 % threshold until batch 13 for over 14,000 injections. Even then, internal standard correction maintained quantitation fidelity.

Reference

1. 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. *Agilent Technologies Application Note*, publication number 5991-3344EN.

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