Application Note

Clinical Research

Simultaneous Analysis of Tacrolimus, Sirolimus, Everolimus, and Cyclosporine A in Whole Blood Using an Agilent Ultivo Triple Quadrupole LC/MS

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Abstract

Immunosuppressants are a class of drugs that suppress or reduce the strength of the body's immune system and are administered to prevent the rejection of transplanted organs or tissues and the treatment of diverse autoimmune disorders. According to many clinical research studies, liquid chromatography/mass spectrometry (LC/MS) methods for the analysis of tacrolimus, sirolimus, everolimus, and cyclosporine A have been proven superior because of their increased sensitivity and selectivity.

This application note describes an analytical method for the sensitive and accurate determination of four immunosuppressant drugs in whole blood using an Agilent Ultivo triple quadrupole LC/MS with an Agilent Jet Stream ionization source.

Introduction

The JASEM immunosuppressive drugs analysis method has been implemented on the Ultivo triple quadrupole LC/MS for quantitation of cyclosporine A, everolimus, sirolimus, and tacrolimus. This method has a run time of 7.5 minutes and is suitable for the simultaneous quantification of all four analytes in whole blood in a "dilute and shoot" manner.

Experimental

Reagents and chemicals

Mobile phases, sample preparation reagents, whole blood calibration standards, internal quality control samples, internal standards, and analytical column were from the ready-to-use JASEM Immunosuppressive Drugs Kit.

Sample preparation

Calibration standards for tacrolimus, sirolimus, everolimus, and cyclosporine A were prepared from the certified lyophilized calibrator set. These lyophilized calibrators are based in human blood and are available as a calibrator set with seven levels, including a blank. All calibrators, QCs, and samples were prepared using the following sample preparation procedure.

1. Aliquot 500 µL of whole blood sample into a glass centrifuge tube and add 25 µL of internal standard; vortex for 5 seconds.
2. Add 975 µL of JASEM Reagent-1 and vortex for 15 seconds.
3. Centrifuge at 3,000 rpm for 5 minutes.
4. Decant the clear supernatant into an HPLC vial for analysis.

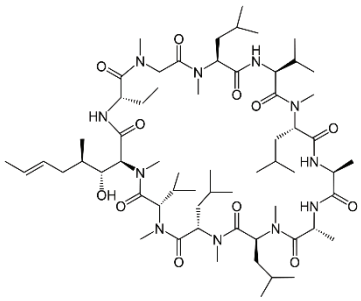
Instrumentation

Liquid chromatography system

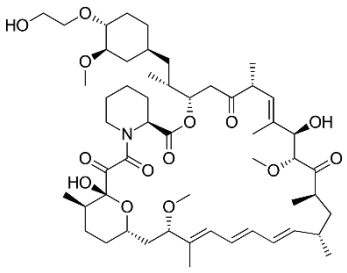
- Agilent 1260 Infinity II Binary Pump (G7112B)
- Agilent 1290 Infinity II Vialsampler (G7129B)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)

Mass spectrometry system

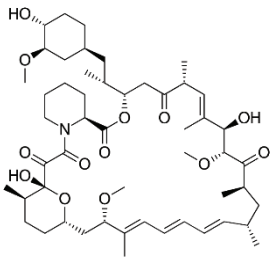
- Agilent Ultivo triple quadrupole LC/MS (G6465B)
- Agilent Jet Stream (AJS) ionization source



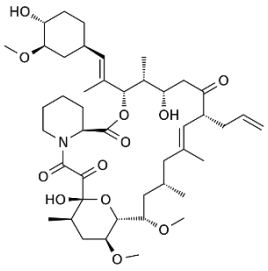
Cyclosporine A
$C_{62}H_{111}N_{11}O_{12}$
MW: 1202.6 g/mol



Everolimus
$C_{53}H_{83}NO_{14}$
MW: 958.22 g/mol



Sirolimus
$C_{51}H_{79}NO_{13}$
MW: 914.17 g/mol



Tacrolimus
$C_{44}H_{69}NO_{12}$
MW: 804.018 g/mol

Figure 1. Chemical structures, elemental compositions, and molecular weights of the respective immonosuppressants.

Method

Table 1 summarizes the liquid chromatographic method conditions. Table 2 summarizes the Ultivo triple quadrupole LC/MS parameters and AJS source parameters. Analysis was carried out using positive ionization and multiple reaction monitoring (MRM). Data were evaluated using the Agilent MassHunter Quantitative Analysis software B.09. Unique MRM transitions were selected, ensuring specificity of quantitation for each analyte; the details of these transitions are listed in Table 3. Internal standards (IS) were used for relative quantification, thus reducing the error due to loss of analytes during sample preparation or variation in the sample matrix.

Table 1. Agilent 1260 Infinity II LC parameters.

Parameter	Value
Column	JASEM Immunosuppressants Analytical Column
Column Temperature	60 °C
Observed Column Backpressure Range	80 to 150 Bar
Injection Volume	20 µL
Mobile Phase	A) JASEM Mobile Phase A for Immunosuppressants B) JASEM Mobile Phase B for Immunosuppressants
Flow Rate	0.9 mL/min
Gradient	Time (min) %B 0.0 0 2.0 0 2.1 29 3.0 29 3.1 93 5.5 93 5.6 0 7.5 0
Stop-Time	7.5 min
Post-Time	1.0 min

Table 2. Agilent Ultivo triple quadrupole LC/MS and source parameters.

Parameter	Value
Drying Gas Temperature	350 °C
Drying Gas Flow	11 L/min
Sheath Gas Temperature	400 °C
Sheath Gas Flow	11 L/min
Nebulizer Pressure	40 psi
Capillary Voltage	3,500 V(+)
Nozzle Voltage	0 V(+)
Cycle Time	511 ms

Table 3. Optimized transitions.

Compound Name	Precursor (*m/z*)	Product (*m/z*)	RT (min)	RT Window (min)	Fragmentor (V)	CE (V)	Polarity
Tacrolimus	825.9	615.7	2.97	1.0	190	30	Positive
Sirolimus	936.9	408.9	3.39	1.0	240	56	Positive
Everolimus	980.9	388.9	3.53	1.0	260	56	Positive
Cyclosporine A	1224.8	1112.7	4.06	1.0	340	75	Positive
Ascomycin-IS	813.9	603.8	2.91	1.0	200	30	Positive
Everolimus-IS	984.9	392.9	3.53	1.0	260	56	Positive
Cyclosporine D-IS	1238.1	1125.9	4.13	1.0	330	66	Positive

Table 4. Timetable.

Start Time (min)	Type	Value
0	Diverter	To Waste
1.4	Diverter	To MS
5.0	Diverter	To Waste

Results and discussion

Chromatograms of quantifier MRM transitions for tacrolimus (1.30 µg/L), sirolimus (1.51 µg/L), everolimus (1.45 µg/L), and cyclosporine A (26.30 µg/L) are shown in Figure 2.

Excellent linearity was observed for all analytes, with R^2 values >0.995 using the seven concentration levels tested. Cyclosporine A gives quadratic calibration curve due to its wide therapeutic range. The concentration ranges for the calibration curve were:

- Tacrolimus: 0 to 44.0 µg/L
- Sirolimus: 0 to 49.9 µg/L
- Everolimus: 0 to 48.0 µg/L
- Cyclosporine A: 0 to 1,288 µg/L

The calibration curves are shown in Figure 3.

JASEM internal quality control (QC) levels were used to evaluate the accuracy and repeatability of the method. Validation data was acquired over three days. The observed accuracies for each level of QC are listed in Table 5. RSD values obtained from repeatability data were less than 5% and recovery values were between 98 to 103%.

LOQs were determined as 0.16 µg/L for tacrolimus, 1.54 µg/L for sirolimus, 1.46 µg/L for everolimus, and 1.22 µg/L for cyclosporine A. No significant carryover was measured for any of the immunosuppressive drugs.

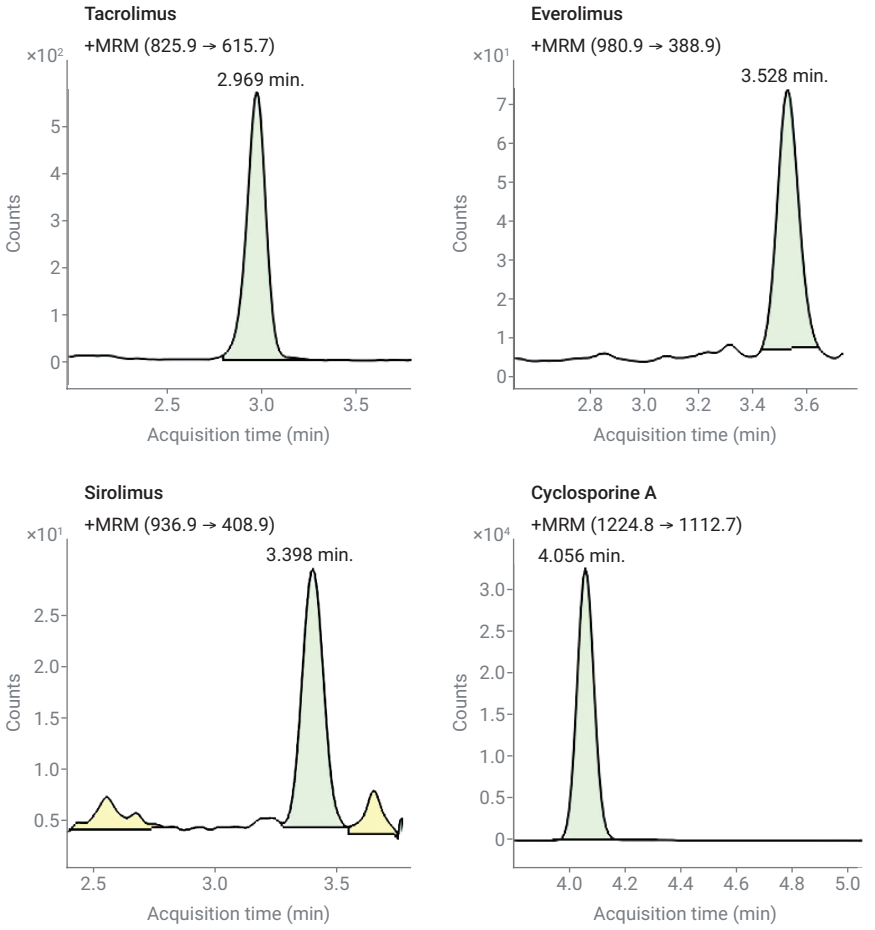


Figure 2. Chromatograms of quantifier MRM transitions for tacrolimus, everolimus, sirolimus, and cyclosporine A.

Table 5. Observed accuracies for three levels of QC of tacrolimus, sirolimus, everolimus, and cyclosporine A.

Analyte	Target (µg/L)	Mean (µg/L)	Accuracy (%)	RSD (%)
Tacrolimus	3.46 7.08 14.6	3.55 7.06 14.39	102 99 98	2.02 1.70 0.76
Sirolimus	3.69 11.9 20.3	3.72 11.75 19.79	101 99 98	1.09 0.64 2.01
Everolimus	3.68 11.5 19.3	3.66 11.52 19.22	99 100 99	1.09 1.02 0.78
Cyclosporine A	61.03 125.5 237.0	58.88 122.01 234.60	103 102 99	3.21 2.71 1.49

Conclusion

A 7.5-minute, high-throughput analytical method for the quantification of cyclosporin A, everolimus, sirolimus, and tacrolimus has been successfully developed on the Ultivo LC/TQ. Using this method, reliable measurement of all four analytes in whole blood was demonstrated with excellent accuracy and precision. When used with the 1260 Infinity II LC and Ultivo triple quadrupole LC/MS, JASEM's reagent kits and ready-to-use method provides a whole workflow solution for customers looking to implement immunosuppressant measurements.

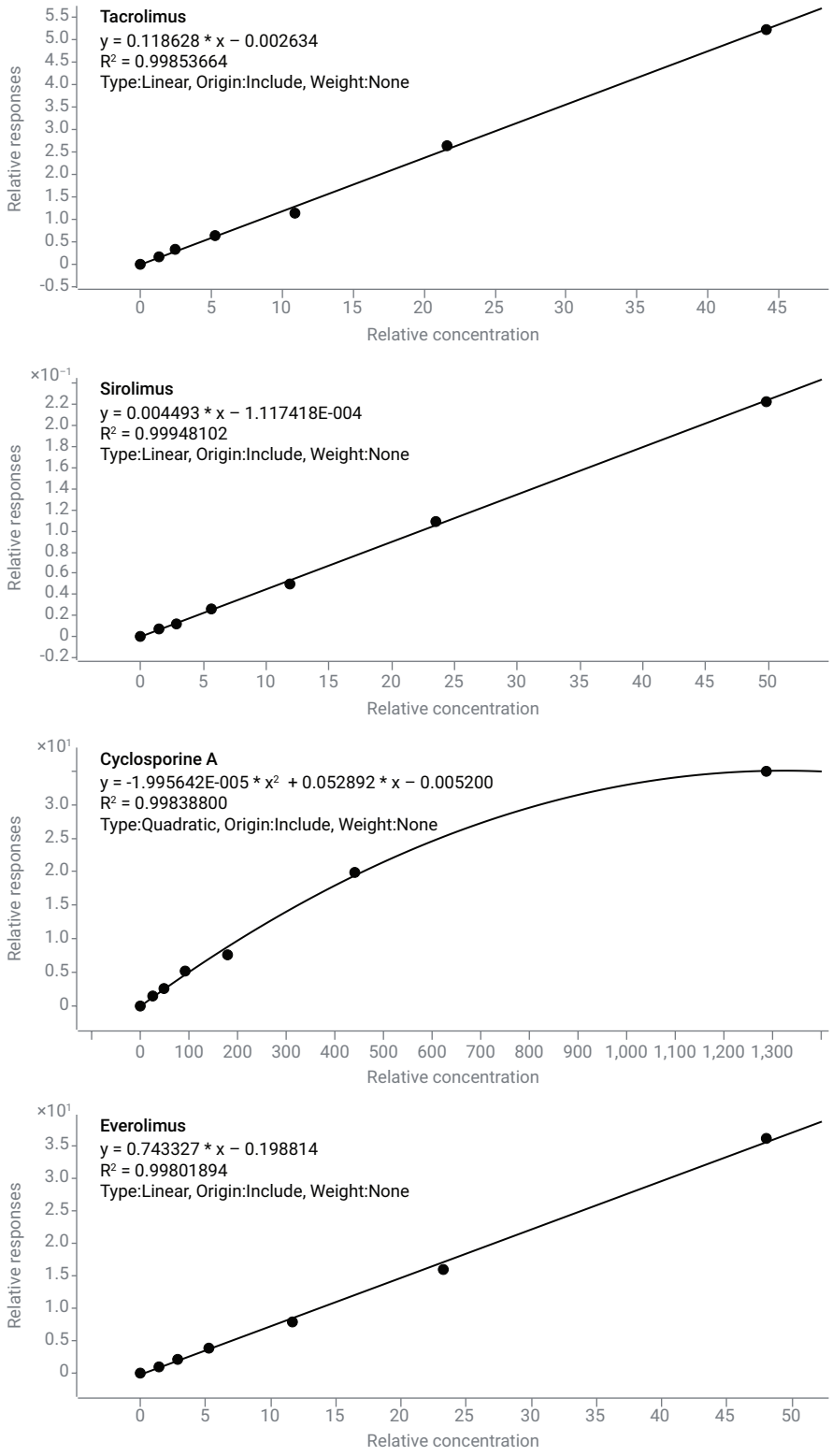


Figure 3. Calibration curves of tacrolimus, sirolimus, cyclosporine A, and everolimus.

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