Application Note

Clinical Research

Ultrafast Quantitation of Immunosuppressant Drugs in Whole Blood by Agilent 6475 LC/MS

Author

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Abstract

An ultrafast, 2.5-minute quantitation method using liquid chromatography/mass spectrometry (LC/MS) for four immunosuppressant drugs was established using an Agilent 6475 triple quadrupole LC/MS with the RECIPE therapeutic drug monitoring (TDM) kit. This application note emphasizes validation of an ultrafast measurement of tacrolimus, sirolimus, everolimus, and cyclosporine A in whole blood with outstanding sensitivity and interday reliability.

Introduction

TDM is essential for ensuring efficacy and minimizing the adverse effects of drug delivery. The need for immunosuppressant drugs for organ and bone marrow transplant patients has been growing annually. Administration of immunosuppressants is strictly monitored as many side effects, including nephrotoxicity and neurotoxicity, must be considered. LC/MS is considered the gold standard for measurement of immunosuppressants due to results showing remarkable sensitivity and specificity.

The RECIPE ClinMass TDM kit system for immunosuppressants in whole blood (Munich, Germany) was used on the 6475 triple quadrupole LC/MS for analysis of tacrolimus, sirolimus, everolimus, and cyclosporine A. Validation of the RECIPE TDM kit was implemented to evaluate sensitivity, linearity, and interday repeatability across three consecutive days (n = 3). Sample preparation is straightforward without the need for complicated cleanup or filtration.

Experimental

Reagents and chemicals

Mobile phases, sample preparation diluent (precipitant), whole blood calibration standard set, whole blood quality control (QC) samples, internal standards, prefilter column, and analytical column were acquired from the RECIPE ClinMass TDM kit system (MS9000A, MS99200) (Munich, Germany).

Sample preparation

The lyophilized calibration standard set was made up of certified human blood and comprised six levels and a blank. Five concentration levels of QC samples were used to evaluate the analytical method. Calibration and QC samples were prepared using the following sample preparation protocol (Figure 1).

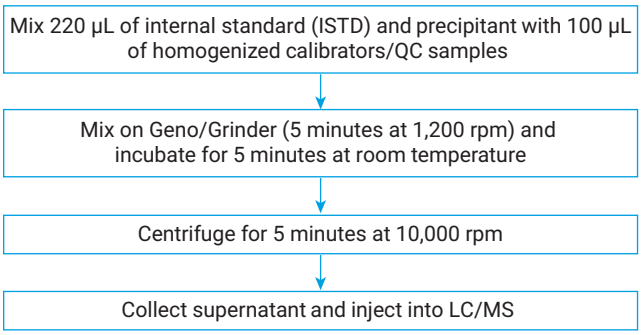

Figure 1. Sample preparation protocol.

Instrumentation

Table 1. Instrumentation.

Module	Part Number
Liquid Chromatography System	
Agilent 1290 Infinity II Binary Pump	G7120A
Agilent 1290 Infinity II Multisampler	G7167B
Agilent 1290 Infinity II Multicolumn Thermostat	G7116B
Mass Spectrometry System	
Agilent 6475 Triple Quadrupole LC/MS	G6475AA
Agilent Jet Stream (AJS) Source	

Method

Multiple reaction monitoring (MRM) transitions for analytes and ISTDs were optimized using Optimizer in the Agilent MassHunter Acquisition software to ensure selectivity of the quantitation method. Data were processed using the Agilent MassHunter Qualitative and Quantitative Analysis software version B.12.

Table 2. Agilent 1290 Infinity II parameters.

Parameter	Description
Column	RECIPE prefilter and analytical column (MS9032 and MS9030)
Column Temperature	70 °C
Mobile Phase	RECIPE mobile phase A (MS9007) RECIPE mobile phase B (MS9008)
Flow Rate	0.9 mL/min
Gradient Program	Time (min) %B 0 10 0.7 10 0.8 95 1.7 95 1.8 10 1.9 10
Stop Time	1.9 min
Post Time	0.6 min
Injection Volume	20 µL

Table 3. Agilent 6475 triple quadrupole LC/MS and source parameters.

Parameter	Description
Drying Gas Temperature	250 °C
Drying Gas Flow	9 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	10 L/min
Nebulizer	35 psi
Capillary Voltage	3,000 V (+)
Nozzle Voltage	500 V (+)
Measurement Mode and Polarity	MRM, positive

Table 4. Timetable.

Start Time (min)	Type	Description
0	Diverter	To Waste
1	Diverter	To MS
1.8	Diverter	To Waste

Table 5. Optimized MRM transitions.

Compound	Precursor (*m/z*)	Product (*m/z*)	Fragmentor (V)	CE (V)	Polarity
Tacrolimus	821.5	768.3	155	18	Positive
	821.5	576.2	155	26	Positive
$^{13}Cd_2$-Tacrolimus	824.5	771.4	155	22	Positive
	824.5	579.3	155	26	Positive
Sirolimus	931.5	882.3	140	10	Positive
	931.5	864.3	140	14	Positive
$^{13}Cd_3$-Sirolimus	935.6	882.4	170	10	Positive
	935.6	864.4	170	14	Positive
Everolimus	975.6	926.4	160	10	Positive
	975.6	908.4	160	18	Positive
$^{13}C_2d_4$-Everolimus	981.6	932.5	160	10	Positive
	981.6	914.5	160	18	Positive
Cyclosporine A	1,219.7	1,202.5	190	10	Positive
	1,219.7	1,184.5	190	38	Positive
d_{12}-Cyclosporine A	1,232	1,214.6	190	10	Positive
	1,232	1,196.6	190	38	Positive

Results and discussion

Chromatograms of quantifier MRM transitions at the lowest calibration standard for tacrolimus (0.39 µg/L), sirolimus (0.43 µg/L), everolimus (0.38 µg/L), and cyclosporin A (6.78 µg/L) are shown in Figure 2. Due to the ultrafast run time, four immunosuppressant drugs are eluted in a close retention time (RT) window. Superior selectivity of MRM nullifies the effect of cross talk between the analytes and the matrix interference.

Calibration and QC samples were measured six times (n = 6) and repeated in three consecutive days (n = 3) to establish interday validation. Accuracy and precision of calibration samples were evaluated. Accuracy was determined as the ratio of measured over preset concentrations of whole blood control samples.

Calibration sets exhibited outstanding linearity for all immunosuppressants with $R^2 > 0.996$ across three days using the six concentration levels (Figure 3). The concentration range for the calibration curve was as follows:

- **Tacrolimus**: 0.39 to 13.4 µg/L
- **Sirolimus**: 0.43 to 14.3 µg/L
- **Everolimus**: 0.38 to 13.9 µg/L
- **Cyclosporine A**: 6.78 to 395.3 µg/L

QC samples were set with a wide dynamic range. The highest level of QC samples was out of calibration range. Despite this result, the linearity of the calibration curves was still maintained.

Interday accuracy and precision were evaluated based on five concentration levels of QC samples. The observed accuracy and precision for each level of QC samples are listed in Table 6. Precision obtained over three consecutive days was less than 6.5% and accuracy of QC concentrations ranged between 93 to 113%. No significant carryover was observed for the four immunosuppressant drugs during the entire run.

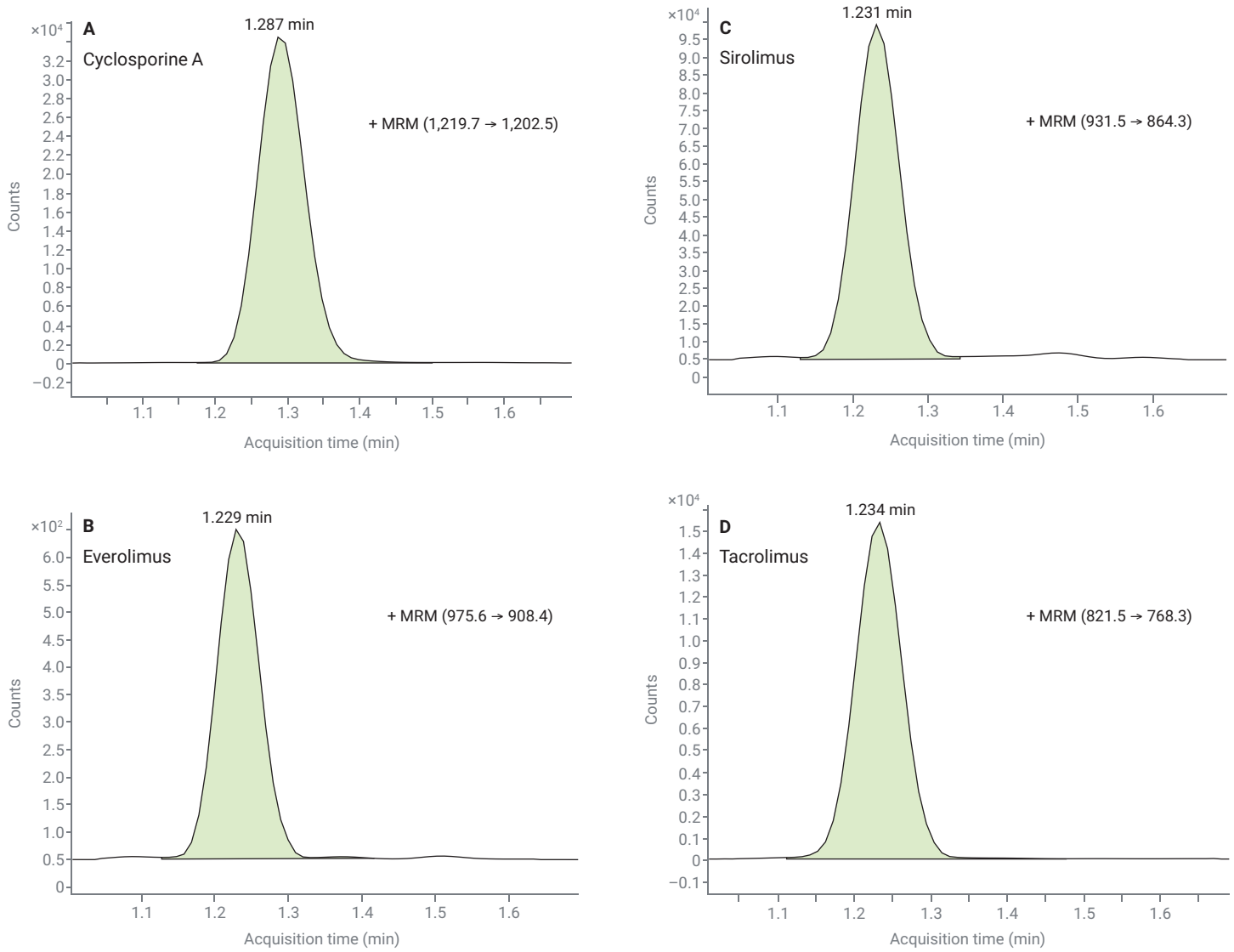

Figure 2. Chromatograms of the quantifier ion for immunosuppressants at the lowest calibration level.

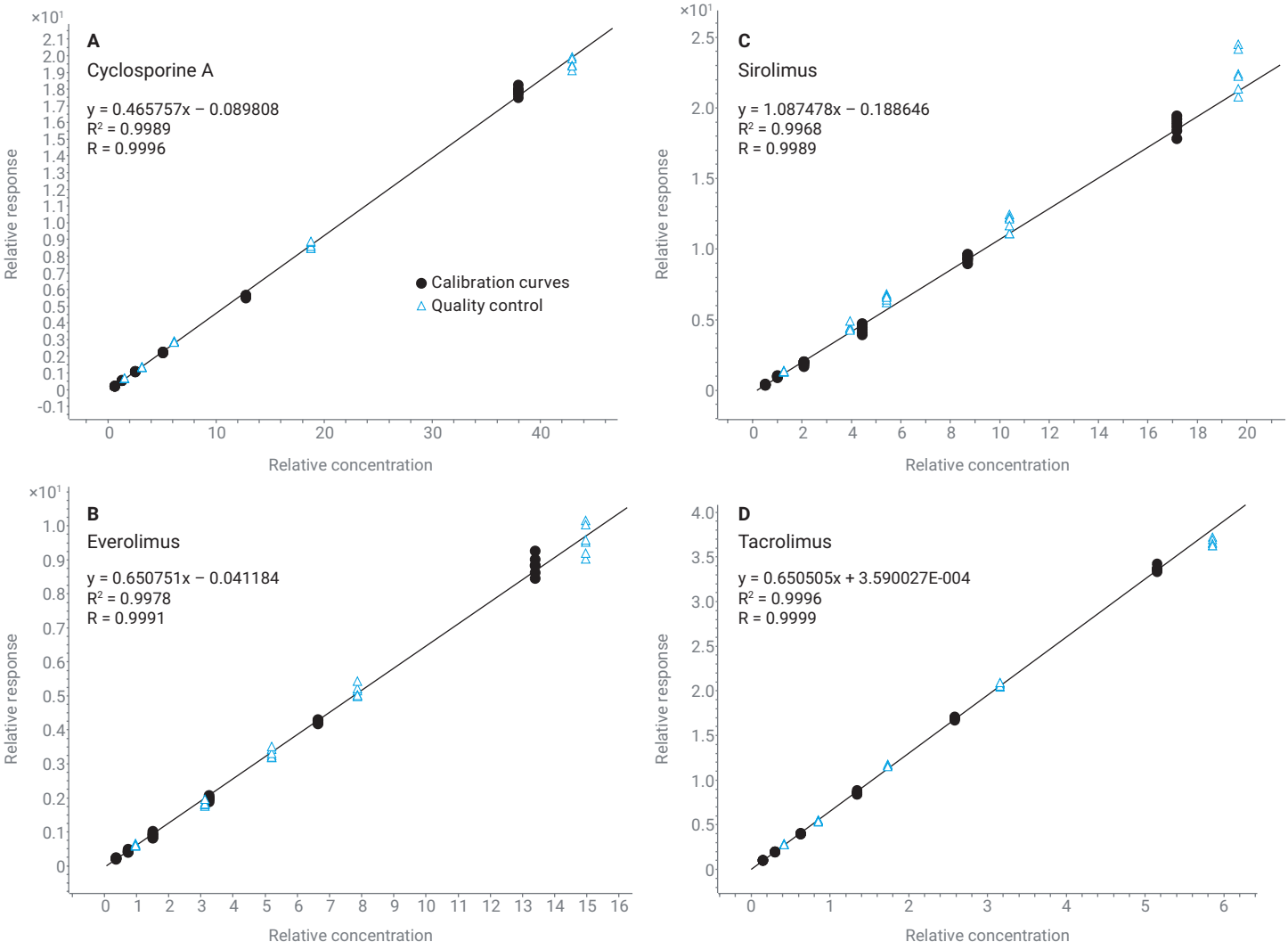

Figure 3. Calibration curves (●) and QC (△) sets of tacrolimus, sirolimus, everolimus, and cyclosporine A.

Table 6. Interday accuracy and precision of QC samples.

Compound	Level	Target (µg/L)	Acquired (mean, µg/L)	Accuracy (%)	RSD (%)
Tacrolimus	I	1.09	1.14	105.0	2.4
	II	2.22	2.21	99.5	3.2
	III	4.50	4.59	101.9	1.9
	IV	8.22	8.27	100.6	0.9
	V	15.22	15.27	100.4	3.4
Sirolimus	I	1.04	1.12	108.0	5.8
	II	3.28	3.57	108.9	4.8
	III	4.50	5.08	113.0	2.7
	IV	8.63	9.21	106.7	5.7
	V	16.31	17.76	108.9	5.5
Everolimus	I	1.00	1.05	105.4	5.6
	II	3.25	3.02	93.0	5.0
	III	5.41	5.32	98.4	4.9
	IV	8.19	8.27	101.0	3.6
	V	15.56	16.45	105.7	6.3
Cyclosporine A	I	15.93	17.24	108.2	1.8
	II	32.82	33.18	101.1	3.8
	III	63.75	65.82	103.3	1.2
	IV	195.63	196.14	100.3	1.9
	V	447.19	461.88	103.3	3.9

Conclusion

A rapid analysis of tacrolimus, sirolimus, everolimus, and cyclosporine A in whole blood was established on an Agilent 6475 triple quadrupole LC/MS. Together with straightforward sample preparation, the method demonstrates robust measurement of immunosuppressant drugs in biological samples. Ultrafast acquisition times allow the high-throughput analysis of more than 500 samples per day. By coupling with the ready-to-use RECIPE ClinMass TDM kit system, Agilent provides a comprehensive end-to-end solution for therapeutic drug monitoring (TDM) of immunosuppressants.

References

1. Yeung, P.; *et al.* Mass Spectrometry Quantitation of Immunosuppressive Drugs in Clinical Specimens Using Online Solid-Phase Extraction and Accurate-Mass Full Scan-Single Ion Monitoring. *J. Mass Spectrometry Adv. Clin. Lab.* **2023**, 99–104.
2. Gunay, G.; *et al.* Simultaneous Analysis of Tacrolimus, Sirolimus, Everolimus, and Cyclosporine A in Whole Blood Using an Agilent Ultivo Triple Quadrupole LC/MS. *Agilent Technologies application note*, publication number 5994-2395EN.

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RA45551.4609375

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Printed in the USA, October 1, 2024
5994-7829EN

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