

Analysis of Cyclosporine, Everolimus, Sirolimus, and Tacrolimus in Whole Blood for Clinical Research

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Abstract

This application note describes a clinical research method using protein precipitation of whole blood with internal standards. Chromatographic elution was completed within 1.8 minutes using a Waters ACQUITY UPLC HSS C₁₈ SB Column on a Waters ACQUITY UPLC I-Class System followed by detection on a Xevo TQD Mass Spectrometer

Benefits

- Analytical selectivity afforded by chromatography and highly selective mass detection
- Simple and inexpensive sample preparation using low sample volumes
- Fast analytical run time

Introduction

Cyclosporine, everolimus, sirolimus, and tacrolimus are commonly used, powerful immunosuppressive agents, with a variety of active mechanisms. High inter and intra-individual variability is known due to a variety of factors,¹ therefore, an accurate, analytically sensitive quantitative method may play a role in researching the pharmacokinetic and pharmacodynamic effects of administration of cyclosporine, everolimus, sirolimus, and tacrolimus.

Here we describe a clinical research method using protein precipitation of whole blood with internal standards. Chromatographic elution was completed within 1.8 minutes using a Waters ACQUITY UPLC HSS C₁₈ SB Column on a Waters ACQUITY UPLC I-Class System followed by detection on a Xevo TQD Mass Spectrometer (Figure 1).



Figure 1. The ACQUITY UPLC I-Class System and Xevo TQD Mass Spectrometer.

Experimental

Sample preparation

Whole blood calibrators and quality control materials containing cyclosporine, everolimus, sirolimus, and tacrolimus were used. Internal standards ascomycin (Cerilliant, USA), $^2\text{H}_{12}$ -cyclosporine (Alsachim, France), $^2\text{H}_4$ -everolimus (Cerilliant, USA) and $^2\text{H}_3$ -sirolimus (IsoSciences, USA) were also sourced. The calibration range was 25–1500 ng/mL for cyclosporine and 1–30 ng/mL everolimus, sirolimus, and tacrolimus. QC

concentrations were 150, 400, and 900 ng/mL for cyclosporine and 2, 8, and 22 ng/mL for everolimus, sirolimus, and tacrolimus.

Sample extraction

To 50 µL of sample, add 200 µL 0.1 M ZnSO_{4(aq)}, vortex mix, add 500 µL of internal standard (ISTD) containing 2 ng/mL ascomycin and ²H₄-everolimus, 25 ng/mL ²H₁₂-cyclosporine and 4 ng/mL ²H₃-sirolimus were added and vortex mix. Tubes were centrifuged for 2 minutes at 16,100 g and supernatant analyzed. Each analyte was analyzed individually.

LC conditions

System:	ACQUITY UPLC I-Class (FTN)
Needle:	30 µL
Column:	ACQUITY UPLC HSS C ₁₈ SB Column, 1.8 µm, 2.1 x 30 mm (p/n 186004117)
Mobile phase A:	Water + 2 mM ammonium acetate + 0.1% formic acid
Mobile phase B:	Methanol + 2 mM ammonium acetate + 0.1% formic acid
Needle wash solvent:	80% aqueous methanol + 0.1% formic acid
Purge solvent:	Mobile phase A
Seal wash:	20% aqueous methanol
Column temp.:	55 °C (pre-column heater active)
Injection volume:	20 µL
Flow rate:	0.40 mL/min (equilibrate at 0.6 mL/min from 1.2

to 1.8 min)

Run time: 1.8 minutes (2.2 minutes injection-to-injection)

Gradient:

Time (min)	% Mobile phase A	% Mobile phase B	Curve
Initial	50	50	Initial
0.2	50	50	1
0.6	0	100	6
1.2	50	50	11

Table 1. Chromatographic elution timetable.

MS conditions

System:	Xevo TQD
Resolution:	MS1 (0.7 FWHM) MS2 (1.2 FWHM)
Acquisition mode:	Multiple Reaction Monitoring (MRM) (See Table 2 for details)
Polarity:	ESI+
Capillary:	1.0 kV
Source temp.:	120 °C
Desolvation temp.:	400 °C
Dwell time:	0.05 seconds

Inter-scan delay: 0.02 seconds

Inter-channel delay: 0.01 seconds

Data management

MassLynx v4.1 with TargetLynx Application Manager

Method conditions

Analyte	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	Cone voltage (V)	Collision energy (eV)
Cyclosporine(Quantifier)	1219.8	1202.8	34	22
Cyclosporine (Qualifier)	1219.8	1184.8	34	34
² H ₁₂ -Cyclosporine (Internal standard)	1231.8	1214.8	34	22
Everolimus (Quantifier)	975.6	908.6	32	18
Everolimus (Qualifier)	975.6	926.6	32	14
² H ₄ -Everolimus (Internal standard)	979.6	912.6	32	18
Sirolimus (Quantifier)	931.6	864.5	32	16
Sirolimus (Qualifier)	931.6	882.5	32	12
² H ₃ -Sirolimus (Internal standard)	934.6	864.5	32	16
Tacrolimus (Quantifier)	821.5	768.5	40	22
Tacrolimus (Qualifier)	821.5	786.5	40	18
Ascomycin (Internal standard)	809.5	756.5	40	20

Table 2. Guideline MRM parameters for analytes and internal standards used in this study.

Results and Discussion

Figure 2 shows an example chromatogram of calibrator 1 (25 ng/mL cyclosporine and 1 ng/mL everolimus, sirolimus, and tacrolimus).

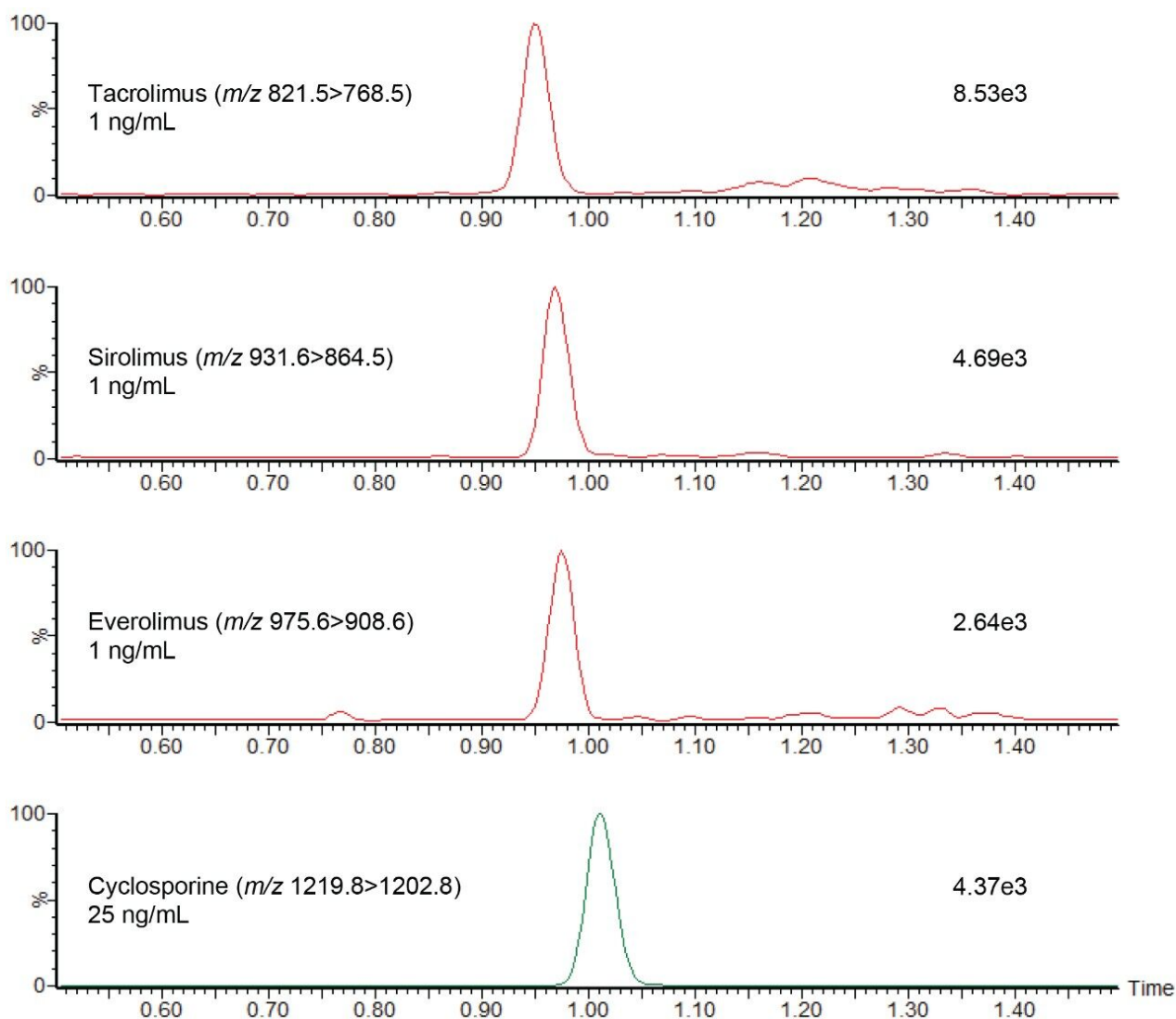


Figure 2. Chromatogram showing the analysis of 25 ng/mL cyclosporine and 1 ng/mL everolimus, sirolimus, and tacrolimus using the ACQUITY UPLC I-Class/Xevo TQD IVD System.

No system carryover was observed following analysis of whole blood samples with 3000 ng/mL of cyclosporine and 60 ng/mL of everolimus, sirolimus, and tacrolimus.

Analytical sensitivity investigations indicate the method would allow for precise quantification (<20% RSD, <15% bias) at 8 ng/mL for cyclosporine, 0.5 ng/mL for everolimus, 1 ng/mL for sirolimus, and 0.5 ng/mL for tacrolimus.

Total precision was determined by extracting and quantifying five replicates of four concentrations of whole blood pools over five separate days (n=25). Repeatability was assessed by analyzing five replicates at each

QC level. Table 3 presents results of these experiments, where total precision and repeatability at the four concentrations assessed (50, 350, 800, and 1200 ng/mL for cyclosporine, 3.5, 10, 22, and 28 ng/mL for everolimus, sirolimus, and tacrolimus) was $\leq 9.1\%$ RSD.

Compound	Total QC precision (% RSD)				QC repeatability (% RSD)			
	QC1	QC2	QC3	QC4	QC1	QC2	QC3	QC4
Cyclosporine	3.9	2.2	3.4	5.7	1.8	1.2	1.2	1.3
Everolimus	7.3	7.6	7.6	7.7	3.9	4.7	3.5	3.5
Sirolimus	8.5	8.3	8.3	9.1	6.1	5.0	4.9	3.2
Tacrolimus	5.9	6.3	6.3	5.7	2.6	2.3	1.6	1.8

Table 3. Total precision and repeatability for the analysis of cyclosporine, everolimus, sirolimus, and tacrolimus.

The method was shown to be linear over the range of 19.4–1662 ng/mL for cyclosporine, 1.0–30.1 ng/mL for everolimus, 0.7–30.1 ng/mL for sirolimus, and 0.7–34.8 ng/mL for tacrolimus when different ratios of high and low concentration pools were combined and analyzed.

A bias of less than 10% was achieved after 1:1 dilution (using blank whole blood) of high samples to be within the calibration range of the assay.

Matrix effects were evaluated as the peak area of extracted post spiked serum samples (n=6) taken as a percentage of extracted solvent samples spiked to equivalent concentrations. Calculation using analyte:internal standard response ratio indicated compensation for signal enhancement by the internal standard. Testing was carried out at 25 ng/mL for cyclosporine, 3 ng/mL for everolimus, 4 ng/mL for sirolimus, and 2 ng/mL for tacrolimus. Table 4 presents the results.

Compound	Analyte peak area		Response ratio	
	Matrix effect	Range	Matrix effect	Range
Cyclosporine	1.17	1.15–1.21	1.02	1.00–1.04
Everolimus	1.45	1.36–1.58	1.04	1.02–1.06
Sirolimus	1.39	1.24–1.49	1.03	0.98–1.11
Tacrolimus	1.55	1.40–1.65	1.02	1.00–1.07

Table 4. Matrix factor summary.

Potential interference from endogenous compounds (albumin, bilirubin, cholesterol, creatinine, triglycerides, uric acid, and vitamin B12) spiked at high concentrations was assessed by determining the recovery (n=3) from low and high pooled whole blood samples (100 and 1200 ng/mL cyclosporine, 4 and 20 ng/mL everolimus, sirolimus and tacrolimus. Recoveries ranged from 89.7–114.3% when assessing the effects of the presence of the other immunosuppressant drugs and internal standards. A substance was deemed to interfere if a recovery range of 85%–115% was exceeded.

NEQAS whole blood samples (n=33) were sourced and analyzed to assess method accuracy. All laboratory trimmed mean LC-MS concentrations spanned from 0–2119 ng/mL for cyclosporine, 0–38.6ng/mL for everolimus, 0–32.4 ng/mL for sirolimus, and 0–48.5 ng/mL for tacrolimus. Mean deviations of -0.2%, -11.9%, -10.7, and 1.6% respectively for cyclosporine, everolimus, sirolimus, and tacrolimus were obtained using this method.

Note: Only two laboratories returned NEQAS results for everolimus.

Conclusion

Using only 50 µL of sample with fast and inexpensive sample preparation, this analytical method provides sufficient analytical sensitivity to analyze low concentrations with excellent precision performance.

The method has been demonstrated as linear over the intended measuring intervals, with neither system carryover nor matrix effects and showed good agreement with an external quality assurance scheme.

References

1. Seger C et al., Assuring the Proper Analytical Performance of Measurement Procedures for Immunosuppressive Drug Concentrations in Clinical Practice: Recommendations of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology Immunosuppressive Drug Scientific Committee. *Ther Drug Monit.* 2016; 38: 170–189.

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