Quantification of Sirolimus and Everolimus in Whole Blood by a Rapid LC-MS/MS Method for Therapeutic Drug Monitoring

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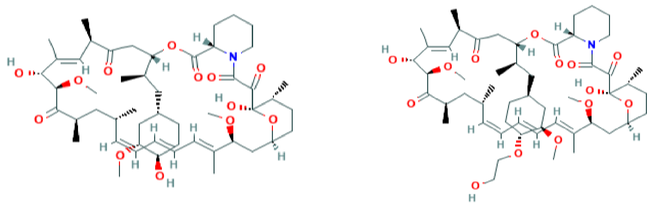
BACKGROUND

Sirolimus and its derivative, everolimus, are widely used today as immunosuppressive agents in transplantation medicine. Therapeutic drug monitoring is recommended for maintenance of immunosuppression due to the large inter- and intra-individual variability in their pharmacokinetic characteristics, as well as the narrow therapeutic windows of these drugs. Therefore, a fast, simple, and sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay was developed and validated for quantification of these two immunosuppressants.

Chemical Structures



Sirolimus **Everolimus**

Materials and Sample Extraction Method

Materials:

- ClinCal® Whole Blood Calibrator set for Immunosuppressants (RECIPE Chemicals + Instruments GmbH, Germany)
- Tri-Level Immunosuppressants Levels 1 and 2 (UTAK, Valencia, CA, USA)
- Sirolimus-d3 and everolimus-d4 solutions (Cerilliant, MilliporeSigma, Darmstadt, Germany)
- Interference Mix Kit (Cerilliant, MilliporeSigma, Darmstadt, Germany)
- Zinc sulfate, Ammonium formate and Formic acid (MilliporeSigma, Darmstadt, Germany)

Sample Extraction:

1. Mix 100 µL of sample with 500 µL of internal standard precipitation solution (1:4 (v/v) aqueous 0.1M $ZnSO_4$: acetonitrile) containing the internal standards
2. Vortex mixture for 10 seconds at high speed
3. Incubate at room temperature for 10 minutes
4. Centrifuge sample at 13,000 rpm for 10 minutes.
5. Transfer supernatant into an injection vial

LC Method

Chromatographic Conditions

HPLC: Thermo Scientific Transcend II LX-2

Column: Restek Raptor Biphenyl, 50x2.1 mm, 1.8 µm

Column temperature: 70 °C

Mobile Phase A: 10 mM Ammonium formate + 0.1% Formic acid in water

Mobile Phase B: 10 mM Ammonium formate + 0.1% Formic acid in Methanol

Flow rate: 0.5 mL/min

Method duration: 3.5 minutes

Injection volume: 25 µL

Time (min)	Gradient	%B
0.00	Step	30
0.50	Step	70
1.00	Step	100
2.50	Step	30

MS/MS Method

MS Parameters

Mass spectrometer: Thermo Scientific TSQ Quantis Triple Quad

Ionization method and Polarity: Heated ESI, Positive mode

Spray voltage: 4000 V

Sheath gas: 50

Aux gas: 5

Sweep gas: 1

Vaporizer temperature: 350 °C

Ion transfer tube temperature: 325 °C

Collision gas pressure: 1.5 mTorr

Cycle time: 0.5 seconds

MS Detection

Analyte	Transitions
Sirolimus	931.6>864.6/882.6
Sirolimus-d3	935.6>865.6/883.6
Everolimus	975.7>908.6/926.6
Everolimus-d4	979.7>912.6/930.6

RESULTS

- **Quantitative Matrix Effect Study**

Ion enhancement was observed for everolimus and sirolimus, but was compensated for by their internal standards. The response ratio of post-spiked patient samples to neat solution samples (n=5) ranged from 91.3% to 114.5%.

Table 1: Representative data of 3 levels of a spiked sample across the analytical measuring range

Patient 1	Set A (Neat Sample)-Mean (injection in triplicates)		Set B (Post-spiked Sample)-Mean (Injection in triplicates)		Matrix Effect
	Area	*CV*	Area	*CV*	ME%= B / A
Sirolimus					
Low Level	24433	*8.0%*	38851	*13.2%*	*159.0%*
Middle level	80520	*6.6%*	121022	*12.8%*	*150.3%*
High Level	148552	*11.0%*	251908	*2.4%*	*169.6%*
Sirolimus D3 (IS)					
Low Level	77670	*11.7%*	123766	*9.2%*	*159.3%*
Middle level	79317	*11.9%*	113330	*16.7%*	*142.9%*
High Level	84391	*7.4%*	134238	*2.0%*	*159.1%*
Response Ratio=Sirolimus/Sirolimus D3					
	Response Ratio	*CV*	Response Ratio	*CV*	ME%= B / A
Low Level	0.32	*4.6%*	0.31	*4.5%*	99.3%
Middle level	1.02	*6.2%*	1.07	*4.2%*	105.1%
High Level	1.76	*6.3%*	1.88	*3.7%*	106.7%

Patient 1	Set A (Neat Sample)-Mean (injection in triplicates)		Set B (Post-spiked Sample)-Mean (Injection in triplicates)		Matrix Effect
	Area	*CV*	Area	*CV*	ME%= B / A
Everolimus					
Low Level	17289	*16.6%*	28308	*7.9%*	163.7%
Middle level	61122	*10.8%*	90402	*8.3%*	147.9%
High Level	116192	*10.8%*	188769	*0.4%*	162.5%
Everolimus D4 (IS)					
Low Level	96375	*13.5%*	148534	*5.4%*	154.1%
Middle level	94243	*12.9%*	134234	*12.6%*	142.4%
High Level	102012	*7.4%*	162151	*2.3%*	159.0%
Response Ratio=Everolimus/Everolimus D4					
	Response Ratio	*CV*	Response Ratio	*CV*	ME%= B / A
Low Level	0.18	*3.1%*	0.19	*2.5%*	106.3%
Middle level	0.65	*3.4%*	0.68	*4.2%*	104.0%
High Level	1.14	*3.5%*	1.16	*2.1%*	102.4%

- **Interference Study**

No interferences were observed from icterus, lipemia, and over 100 exogenous compounds.

RESULTS (cont.)

- **Analytical Measuring Range**: 1.0 – 50.0 ng/mL
- **Analytical Recovery**: 97.1 - 114.5% (sirolimus)

  93.9 - 101.6% (everolimus)
- **Precision**: Sirolimus (top) and everolimus (bottom) at three concentrations across the analytical measuring range

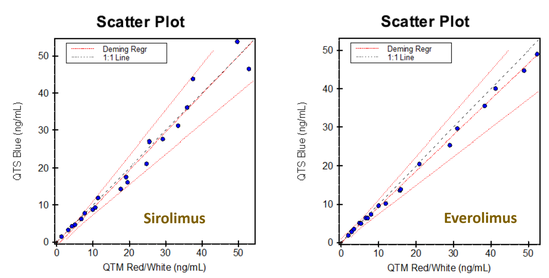
Sirolimus	Low Level	Mid Level	High Level
N=	30	30	30
Grand Mean=	4.3	9.5	14.4
Total %CV	*4.2*	*4.7*	*4.3*
Total SD	0.2	0.5	0.6
Within Run SD	0.2	0.3	0.5
Within Run %CV	*4.2*	*3.5*	*3.3*

Everolimus	Low Level	Mid Level	High Level
N=	30	30	30
Grand Mean=	4.3	9.8	15.4
Total %CV	*5.0*	*4.3*	*2.9*
Total SD	0.2	0.4	0.5
Within Run SD	0.2	0.4	0.4
Within Run %CV	*4.5*	*4.3*	*2.7*

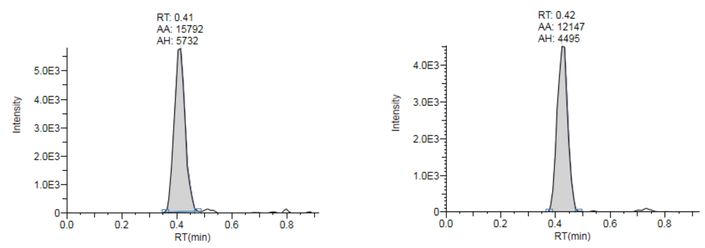
- **Method Comparison**:

  sirolimus: Bias = -3.09%, Slope = 1.018, R = 0.9849

  everolimus: Bias = -6.83%, Slope = 0.993, R = 0.9988



- **Analytical Sensitivity**: Sirolimus (left) and everolimus (right) at the LLOQ (1 ng/mL)



- **Carryover**: No carryover was observed at twice the ULOQ (50 ng/mL)
- **Dilution Integrity**: Up to 10x with RECIPE ClinCal® Level 0 with recovery of 87.5 -112.5%
- **Stability**:
  - Unextracted:

    Room Temperature: 7 days

    2 to 8 °C: 14 days

    <-20 °C: 8 weeks
  - Extracted:

    2 to 8 °C: 7 days

CONCLUSIONS

This LC-MS/MS method for sirolimus and everolimus in whole blood is accurate and efficient. It requires a simple one-step protein precipitation for sample preparation, thus enabling a short turnaround time.