Quantitative Evaluation of Immunosuppressant Drugs by High Resolution Accurate Mass Using Selected Ion Monitoring Mode

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Overview

Purpose: To evaluate high resolution accurate mass for the quantitation of Tacrolimus and Sirolimus utilizing Selected Ion Monitoring (SIM) to monitor the sodium adduct species for each target analyte.

Methods: Tacrolimus and Sirolimus were analyzed in the presence of human plasma sample matrix using Single Ion Monitoring (SIM) mode. Data analysis and data processing were performed using Thermo Scientific™ TraceFinder software.

Results: The lower limit of quantitation (LOQ) was determined to be 25 pg/mL for Sirolimus and 50 pg/mL for Tacrolimus.

INTRODUCTION

Sirolimus and Tacrolimus are large cyclic macromolecules commonly used for immunosuppression treatment after organ transplantation. These compounds are effective at low dosing levels and therefore require a sensitive and robust analytic method[1]. Analytical methods have been developed using LCMS/MS, but these compounds demonstrate intense sodium adduct formation for the precursor ion which does not produce intense fragmentation. The addition of ammonium formate is often added to the mobile phase solution to promote ammonium adduct formation which produces better fragmentation but does not eliminate the sodium adduct precursor which remains present at a much greater intensity[2]. Here we investigate quantitative performance using high resolution accurate mass LCMS to monitor the sodium adduct precursor ion of each target analyte without the need for MSMS fragmentation.

METHODS

Sample Preparation

Crashed human plasma stock solutions were prepared using an Acetonitrile (ACN) crash at a ratio of 3:1, ACN to plasma. The resulting solution was centrifuged at 10,000rpm for 10 minutes. The supernatant was removed and added to an equivalent volume of water to make the final crashed plasma stock solution containing approximately 35% ACN and 65% water. Stock solutions of Tacrolimus and Sirilomus at 1mg/mL were diluted in the crashed plasma stock to produce a concentration ranges from 25 pg/mL to 25,000pg/mL. All analyte stock analytes were obtained as certified standard solutions in methanol and obtained from Cerilliant Corporation, Round Rock, Texas.

Liquid Chromatography

Chromatographic separation was achieved using a Thermo Scientific™ Vanquish™ UHPLC System. Samples were injected at a 5uLvolume onto a 2.1 x 100mm, 1.5um Thermo Scientific™ Vanquish™ column. Gradient elution was accomplished with water + 0.1% formic acid (Mobile Phase A) and acetonitrile + 0.1% formic acid (Mobile Phase B), with a 3 minute gradient at a flow rate of 800uL/min (Table 1). Total run time including column equilibration was approximately 3.5minutes.

Mass Spectrometry

Target analytes were analyzed utilizing a Thermo Scientific™ Q Exactive™ Focus benchtop Orbitrap™ MS with heated electrospray ionization. Generic source conditions suitable for a 800uL/min LC flow rate were applied for all sample analyses. (Table 2) All data was acquired at a resolution setting of 70,000 (FWHM) at *m/z* 200, utilizing selected Ion monitoring (SIM) mode with an external mass calibration.

Data Analysis

All data was collected and processed utilizing Thermo Scientific™ TraceFinder™ software. Chromatographic integration was accomplished using a 5 ppm mass extraction window and method defined processing settings. No manual integration or smoothing was applied to any chromatographic or spectral data.

Time (min)	Flow rate (uL/min)	%A	%B
0	800	50	50
0.1	800	50	50
2.3	800	2	98
2.8	800	2	98
2.8	800	50	50
3.5	800	50	50

Table 1. LC gradient method utilized for sample analysis.

HESI Source Settings	Value
Spray Voltage (V)	4000
Vaporizer temperature (°C)	450
Capillary Temperature (°C)	350
Sheath Gas Pressure (Arb)	50
Aux Gas Pressure (Arb)	20
Ion Sweep Gas Pressure (Arb)	1

MS Scan Settings	Value
Scan Type	SIM
Resolution	70,000
AGC Target	2.00E+04
IT Fill Time (ms)	260
Isolation Window	10

Table 2. Mass Spectrometer settings utilized for sample analysis.

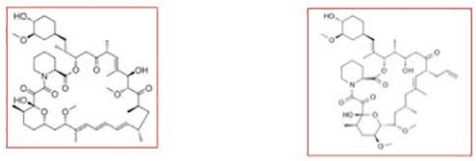

Figure 1. Chemical structure of Sirolimus (Right) and Tacrolimus (Left).

RESULTS

One of the primary tools for bioanalysis in the drug discovery environment is quantitative analysis using LCMS/MS. LCMS/MS provides elimination of matrix interferences through the monitoring analyte fragmentation enabling analyte quantitation at low concentration levels. While this technique provides sensitivity and robustness for the quantitative analysis of many compounds, some compounds do not necessarily fragment well providing potential challenges for low concentration level analysis. Here we examine high resolution accurate mass analysis for the quantitation of two immunosuppressant drug compounds which demonstrate sodium adduct formation, that do not fragment efficiently in a traditional LCMS/MS analysis. Selected ion monitoring at a resolution setting of 70,000 was utilized to monitor the sodium adduct of the target analytes without the need for compound fragmentation.

Assay performance and reproducibility were assessed using SIM scanning mode to monitor the sodium adduct of both Tacrolimus and Sirolimus. Calibration curves ranging from 25 pg/mL to 100 ng/mL were evaluated and each analyte concentration level was analyzed with replicates of n=6. Linearity and reproducibility were calculated across the working range of the curve. The limit of quantitation (LOQ) was defined as the lowest concentration level that is both within <20% difference of the linear fit and <20% RSD for each group of replicate concentration points. The overall assay LOQ was determined to be 25 pg/mL for Sirolimus and 50 pg/mL for Tacrolimus utilizing the SIM scanning mode with a 5 uL injection volume. The full results summary is listed Table 3.

Sirolimus				Tacrolimus			
Conc.Level (pg/mL)	Mean Calc Amt	Replicate % CV	Avg. % Diff		Mean Calc Amt	Replicate % CV	Avg. % Diff
25	25.4	10.68%	5.3%		--	--	--
50	50.4	2.98%	2.3%		48.5	10.91%	7.9%
100	91.1	4.52%	8.9%		99.6	6.38%	4.2%
500	512	1.31%	2.5%		483	2.37%	3.3%
1000	1015	0.47%	1.6%		944	1.55%	5.5%
5000	5398	1.30%	8.0%		4930	1.81%	1.9%
10000	9999	5.38%	4.2%		10158	1.71%	1.8%
50000	51968	1.27%	3.9%		52677	1.33%	5.4%
100000	90441	2.54%	9.6%		102849	1.11%	2.9%

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Full Scan Mobile Phase Modifier Evaluation

Prior to chromatographic analysis, neat stock solutions at 500 ng/mL were teed with LC mobile phase at 500 uL/min and infused at 10 ul/min directly to the mass spectrometer to evaluate adduct formation and to determine the most suitable m/z for the chromatographic analysis. Sample infusion was performed using both 50:50 Water:ACN with 0.1 formic acid and 50:50 Water:ACN with 10 mM ammonium formate to evaluate the LC mobile phase modifier influence on analyte adduct formation. Full scan analysis demonstrated the presence of both the sodium adduct and the ammonium for both Tacrolimus and Sirolimus, but the M+H species was not detected in the full scan infusion for either analyte. (Figure 2 and 3) Additionally the infusion of the LC mobile phase containing the ammonium formate modifier did not significantly alter the adduct ratios and the sodium adduct remained the most intense analyte signal.

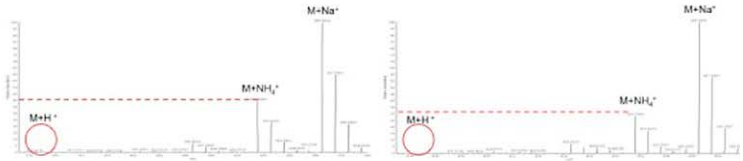


Figure 2. Sirolimus infusion with 50:50 Water:ACN with Formic Acid (Left) and 50:50 Water:ACN with 10mM Ammonium Formate (Right)

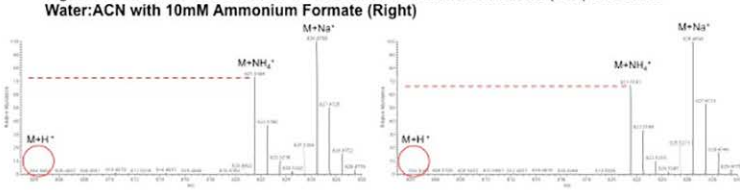


Figure 3. Sirolimus infusion 10mM Ammonium Formate (Left) FA (Right)

SIM Analysis

The LOQ for the 5 uL injection volume SIM analysis was determined to 25 pg/mL for Sirolimus and the signal response was determined to be linear from the LOQ to 100 ng/mL. (Figure 4) The LOQ was determined to 50 pg/mL for Tacrolimus and the signal response was determined to be linear from the LOQ to 100 ng/mL. (Figure 5)

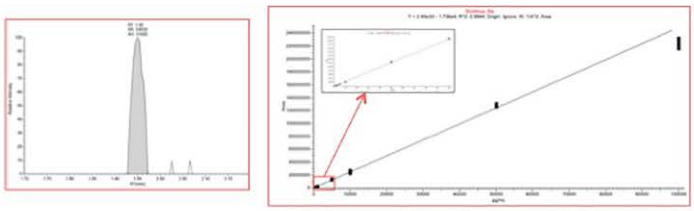


Figure 4. SIM Scan XIC for Sirolimus at 25 pg/mL (Left) and the linear response curve fit across the working range of the assay from 25 pg/mL to 100 ng/mL (Right).

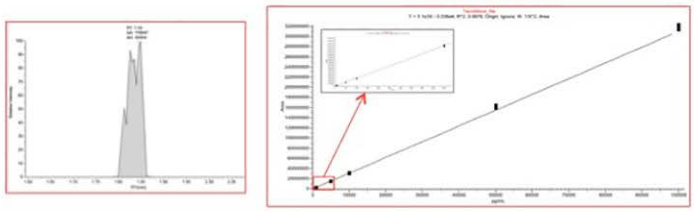


Figure 5. SIM Scan XIC for Tacrolimus at 50 pg/mL (Left) and the linear response curve fit across the working range of the assay from 50 pg/mL to 100 ng/mL (Right).

Resolution Evaluation

Assay sensitivity, robustness, and reliability is dependent on successfully differentiating between analyte signal and background interferences throughout the course of sample analysis. Here we evaluate the mass spectra of Sirolimus (Figure 6) and Tacrolimus (Figure 7) at their respective LOQ levels to confirm the mass resolution of each target from background interferences ions present in the sample matrix. At the resolution setting of 70,000 (FWHM) at *m/z* 200, both target analytes were fully adequately from all co-eluting interferences. As seen in the figure, although interferences are present at analyte elution, the target analyte m/z was baseline resolved from nearby interferences at the 70,000 resolution setting.

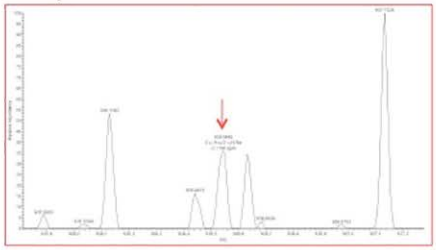
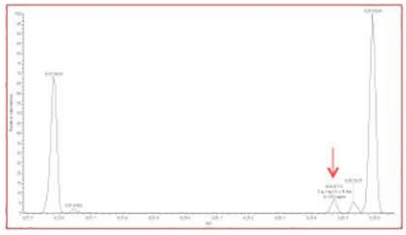


Figure 6. Spectra analysis of Sirolimus at 25 pg/mL in human plasma. All interferences present in the sample were demonstrated to be baseline resolved at the 70,000 resolution setting.

Figure 7. Spectra analysis of Tacrolimus at 50 pg/mL in human plasma. All interferences present in the sample were demonstrated to be baseline resolved at the 70,000 resolution setting.



TraceFinder Data Review

Data acquisition, data processing, and data review were performed using Thermo Scientific™ TraceFinder software. Data processing and review was simplified in the TraceFinder software package through the use of customizable data review layouts enabling fast and efficient review of large data sets. Multiple chromatograms can be easily viewed in user defined groups, individual chromatograms can be easily magnified, and calibration curves can be displayed and resized as needed all in a single display help simplify quantitative data review. (Figure 8)

Figure 8. TraceFinder Compound View display for Sirolimus. Sample replicates, magnified chromatogram, and calibration curve displayed in a single customizable layout.

Mass Accuracy

Here we examine the scan to scan mass accuracy for Tacrolimus and Sirolimus analysis at 100 pg/mL (Figure 9). The mass accuracy for each scan across the analyte peaks was demonstrated to be less than 3ppm for both analytes. The high level of mass accuracy from scan to scan allows the utilization of a narrow mass extraction window, providing robust and reproducible results at low analyte concentration levels while in the presence of a biological matrix. Evaluation of the scan to scan mass accuracy provides a potential method development and troubleshooting tool that is unique to HRAM and the Orbitrap mass analyzer.

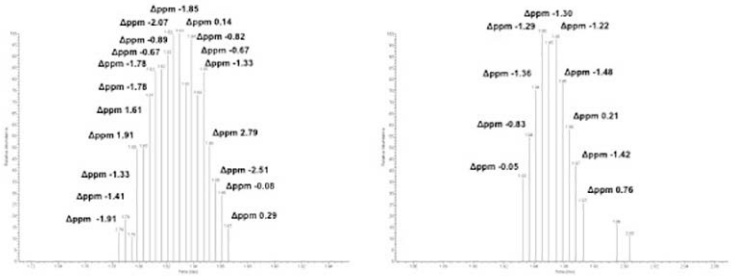


Figure 9. Scan to scan mass accuracy of Tacrolimus (Left) and Sirolimus (Right) for the 5 uL injection SIM Scan experiment at 100 pg/mL.

CONCLUSIONS

Assay LOQ was determined to be 25 pg/mL for Sirolimus and 50 pg/mL for Tacrolimus utilizing SIM analysis and a 5 uL sample injection volume.

Teed sample infusion demonstrated a minimal difference in adduct formation regardless of the LC mobile phase modifier, each resulting in sodium adduct formation as the most intense analyte ion.

The resolution setting of 70,000 (FWHM) at *m/z* 200, provided adequate mass resolution from sample interferences and a scan to scan mass accuracy of less than 3 ppm was observed.

REFERENCES

1. P. E. Morgan. et al., Ther. Drug Monit, 2014, 36, 358–365
2. R.A. Koster. et al., Talanta, 2013, 115, 47–54

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