Analysis of Everolimus in Mitra VAMS Blood Samples for Clinical Research by Turbulent Flow LC-MS/MS and FAIMS-MS/MS

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ABSTRACT

Purpose: Evaluation of the suitability of turbulent flow-LC-MS/MS and turbulent flow-FAIMS-MS/MS for detection and quantitation of the immunosuppressant drug everolimus in blood microsamples collected using volumetric absorptive microsampling (VAMS) devices.

Methods: Neoteryx™ Mitra™ tips were used to collect 10 µL samples of blood spiked with varying levels of everolimus. Two extraction methods were used to prepare the analyte samples for TurboFlow™ LC-MS/MS and TurboFlow™ FAIMS-MS/MS analyses.

Results: TurboFlow LC-MS/MS and TurboFlow FAIMS-MS/MS analyses had comparable results with detection limits ranging from 2-7 ng/mL .

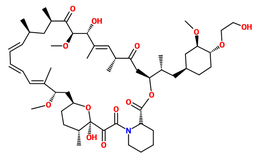
INTRODUCTION

Immunosuppressant drugs are routinely monitored in human blood for clinical research.[1,2] Volumetric absorptive microsampling (VAMS) is emerging as a less-invasive alternative to venipuncture to collect specimens for such purposes.[3] It allows for the absorption of a fixed volume of blood independent of hematocrit levels and has been commercialized by Neoteryx, LLC as the Mitra microsampling device.

Turbulent flow chromatography, implemented using Thermo Scientific™ TurboFlow™ columns, uses size exclusion and stationary phase chemistry to separate small molecules from biological matrices such as plasma, simplifying sample preparation workflows and reducing the amount of work needed to be done at the lab bench prior to LC-MS analysis. It can be included as a preparatory step in an LC-MS/MS workflow enabling "dilute and shoot" analysis of biofluids. Quantitation of immunosuppresant drugs in whole blood using TurboFlow-LC-MS/MS has previously been demonstrated.[4]

In pursuit of a simple workflow for quantitation of immunosuppressant drugs in Mitra samples, we evaluate the suitability of TurboFlow-LC-MS/MS for quantitation of everolimus in Mitra VAMS blood samples. Additionally, noting that the use of small sample volumes together with cleanup by TurboFlow chromatography will reduce ion suppression making FAIMS (field asymmetric ion mobility spectrometry) a viable alternative to HPLC for separation from MS/MS interferences, we investigate the suitability of TurboFlow-FAIMS-MS/MS for quantitation of everolimus in Mitra VAMS blood samples.

Figure 1. Chemical Diagram of Everolimus



Formula: $C_{53}H_{83}NO_{14}$

MW: 958.2 g/mol

MATERIALS AND METHODS

Sample Preparation

EDTA blood samples were spiked with everolimus (Sigma-Aldrich) to a total volume of 500 µL and equilibrated for two hours.10 µL samples were absorbed onto Mitra tips (Neoteryx, LLC) by placing the tip into contact with the full sample volume, and allowed to dry for at least 5 hours. Two extraction and sample preparation techniques were employed (Figure 2) and had the following differences: preparation method A included a methanol precipitation step and a rest at -20˚C, while preparation method B excluded these steps. In both methods, internal standard (everolimus-d4, Sigma Aldrich) was spiked into the extraction solvent and the Mitra tips containing the blood samples were ultrasonicated, vortexed, and centrifuged, collecting the supernatant for analysis.

Figure 2. Offline sample extraction and preparation methods for TurboFlow LC-MS/MS and TurboFlow FAIMS-MS/MS analyses.

Preparation Method A	Preparation Method B
10 µL blood sample picked up with Mitra tip	10 µL blood sample picked up with Mitra tip
Sample dried for 5-16 hour	Sample dried for 5-16 hour
Ultrasonication in 60:40 water:methanol (100 µL) with internal standard for 30 minutes	Ultrasonication in 60:40 water:methanol (300 µL) with internal standard for 30 minutes
Addition of 200 µL of methanol	Sample vortexed for 30 minutes
Sample vortexed for 15 minutes	Sample centrifuged for 15 minutes
Sample ultrasonicated for 15 minutes	Supernatant transferred and centrifuged for another 5 minutes
Sample vortexed for 15 minutes	Supernatant transferred to sampling vial for TurboFlow™ MS/MS analysis
Sample centrifuged for 5 minutes	
Supernatant cooled at -20 ºC for 10 minutes and centrifuged for 5 minutes	
Supernatant transferred to sampling vial for TurboFlow™ MS/MS analysis	

Test Methods

Turbulent flow-LC-MS/MS Analysis: 30 µL of extracted sample were injected onto a Thermo Scientific™ TurboFlow™ Cyclone-P™ column (0.5 x 50 mm) at 1.5 mL/min flow rate with 70:30 water:methanol solvent containing 10 mM ammonium formate and 0.05% formic acid (Figure 3). The analyte was then transferred at a flow rate of 150 µL/min and focused onto a Thermo Scientific™ Accucore™ C8, 2.6 µm, 3.0 x 30 mm HPLC column. The analyte was eluted off the analytical column with a ramp gradient at 500 µL/min. The 975.6→908.5 Da (CE=16 V) and 975.6→ 926.4 Da (CE=12 V) SRM transitions were used to detect the analyte on a Thermo Scientific™ TSQ Altis™ mass spectrometer.

Figure 3. Screen shot of the gradient setup for the Transcend™ II system with Thermo Scientific™ Aria™ operating software

Step	Start	Sec	Flow	Grad	%A	%B	%C	Tee	Loop
1	0.00	25	1.50	Step	70.0	30.0	-	═══	out
2	0.42	5	0.15	Step	70.0	30.0	-	═══	out
3	0.50	60	0.15	Step	70.0	30.0	-	┴	in
4	1.50	15	2.00	Step	-	-	100.0	═══	in
5	1.75	15	2.00	Step	-	-	100.0	═══	in
6	2.00	15	1.50	Step	100.0	-	-	═══	out
7	2.25	15	2.00	Step	-	-	100.0	═══	in
8	2.50	15	1.50	Step	100.0	-	-	═══	out
9	2.75	15	1.00	Step	-	100.0	-	═══	in
10	3.00	15	1.00	Step	-	100.0	-	═══	out
11	3.25	15	1.00	Step	-	100.0	-	═══	in
12	3.50	30	1.00	Step	-	100.0	-	═══	in
13	4.00	60	1.50	Step	70.0	30.0	-	═══	out

Solvents

A: water + 10 mM ammonium formate + 0.05% formic acid.

B: methanol + 10 mM ammonium formate + 0.05% formic acid.

C: 45% acetonitrile + 45% isopropanol + 10% acetone

FAIMS-MS/MS Optimization: with a syringe pump, neat everolimus was teed into an 150 µL/min flow of 0:100 water:methanol solvent containing 10 mM ammonium formate and 0.05% formic acid and delivered to the FAIMS device using a HESI ion source. The inner and outer electrodes of the FAIMS device (prototype) were set to 100 ºC. Compensation voltage was scanned using the Tune UI™ CV Scan Tool to find the CV optimal for transmission of the 975.6→908.5 Da (CE=16 V) and 975.6→926.4 (CE=12 V) Da SRM transitions.

Turbulent flow-FAIMS-MS/MS analysis: 30 µL of extracted sample were injected onto a TurboFlow Cyclone-P column (0.5 x 50 mm) at 1.5 mL/min flow rate with 70:30 water:methanol solvent containing 10 mM ammonium formate and 0.05% formic acid with a Thermo Scientific™ Vanquish™ HPLC pump and autosampler. The analyte was eluted by reverse flow from the TurboFlow column isocratically with 0:100 water:methanol solvent containing 10 mM ammonium formate and 0.05% formic acid at 150 µL/min. With FAIMS compensation voltage set to 25.8 V and the inner and outer electrodes set to 100 ºC, 975.6→908.5 Da (CE=16 V) and 975.6→926.4 Da (CE=12 V) SRM transitions were used to detect the analyte on a Thermo Scientific™ TSQ Altis™ mass spectrometer.

Data Analysis

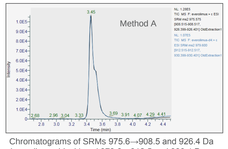
Thermo Scientific™ TraceFinder™ software was used for chromatographic peak detection and integration of the analyte and internal standard. Thermo Scientific™ FreeStyle™ was used to plot chromatograms. NumPy and Matplotlib were used to perform weighted least squares for calibration curve fits to the area ratio of the analyte to the internal standard, and to plot the calibration curves. Detection limits were obtained using the conventional method documented in CLSI EP17-A2..

RESULTS

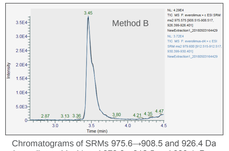
Recovery of everolimus from dried blood samples

To estimate the recovery of everolimus from the dried blood sample (500 ng/mL everolimus spiked in 10 µL of blood) on the Mitra tip for both preparation method A and B, internal standard (everolimus-d4) was spiked into the extraction solvent at the expected concentration of everolimus assuming 100% recovery from the dried blood sample and the ratio of the peak area of analyte to that of internal standard was evaluated (Figure 4).

Figure 4. Chromatograms of everolimus and internal standard from dried blood samples using preparation method A and B.



Chromatograms of SRMs 975.6→908.5 and 926.4 Da (everolimus, black) and 979.6→912.5 and 930.4 Da (internal standard, blue) using TurboFlow LC-MS/MS method and sample preparation method A



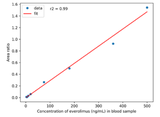
Chromatograms of SRMs 975.6→908.5 and 926.4 Da (everolimus, black) and 979.6→912.5 and 930.4 Da (internal standard, blue) using TurboFlow LC-MS/MS method and sample preparation method B

Across three Mitra tip samples, preparation method A had an average ratio of the peak area of analyte to that of internal standard of 1.3 with an 8% CV and preparation method B had an average ratio of 1.3 with a 10% CV, indicating efficient recovery of analyte from the Mitra tip in both preparation methods.

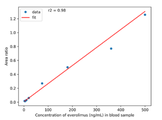
Quantitation of everolimus from dried blood samples using TurboFlow LC-MS/MS

Mitra tip samples prepared with 10 µL blood samples spiked with varying concentrations of everolimus were extracted using preparation method A and B. Internal standard was spiked into extraction solvent at a constant concentration level. Samples were analyzed using the TurboFlow LC-MS/MS method, and the peak area ratios of everolimus to internal standard were used to construct calibration curves (Figure 5).

Figure 5. Calibration curves of everoliums from TurboFlow LC-MS/MS analysis of Mitra tip dried blood samples extracted with preparation method A and B.



Calibration curve of everolimus using samples extracted with preparation method A



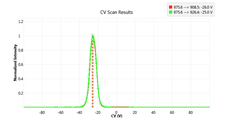
Calibration curve of everolimus using samples extracted with preparation method B

Calibration curves were fit with weighted least squares. Limits of detection (LOD) were determined for both preparation method A and B using the traditional method as documented in CLSI EP17-A2. Using TurboFlow LC-MS/MS, the LOD for preparation method A was determined to be 3.2 ng/mL in the 10 µL blood samples and the LOD for preparation method B was determined to be 7.4 ng/mL in the blood samples. The results show that the additional steps in preparation method A allowed clinically relevant concentrations of everolimus to be detected in small volumes of blood using Mitra microsampling and TurboFlow LC-MS/MS analysis.

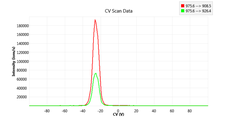
Quantitation of everolimus from dried blood samples using TurboFlow FAIMS-MS/MS

Compensation voltage scans were obtained with neat everolimus and automatically fit using the CV scan feature of the TSQ Altis Tune 3.1 instrument control software to determine the optimal compensation voltage (Figure 6). The FAIMS electrodes were set to this optimal compensation voltage during the analysis of Mitra tip samples with TurboFlow FAIMS-MS/MS.

Figure 6. Compensation voltage optimization of everolimus at 150 µL/min flow rate and 95% mobile phase B under H-ESI ion source conditions.



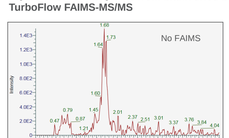
Normalization and fit of the compensation voltage scans for SRMs 975. →908.5 Da and 975.6→926.4 Da.



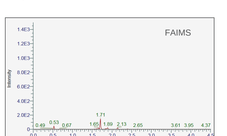
Compensation voltage scans for SRMs 975.6→908.5 Da and 975.6→926.4 Da.

Chromatograms for a blank Mitra dried blood sample extracted with preparation method B were obtained with TurboFlow MS/MS (no LC nor FAIMS) and with TurboFlow FAIMS-MS/MS (Figure 7). The method with FAIMS resulted in a blank with little background in the chromatogram of the SRM transitions of everolimus, while the method without FAIMS (and no LC) had significant chemical background.

Figure 7. Chromatograms from blank dried blood samples with TurboFlow MS/MS and TurboFlow FAIMS-MS/MS



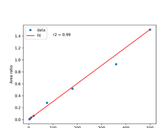
Chromatograms of SRMs 975.6→908.5 and 926.4 Da (everolimus) using TurboFlow MS/MS method and sample preparation method B



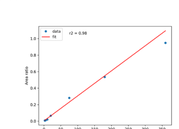
Chromatograms of SRMs 975.6→908.5 and 926.4 Da (everolimus) using TurboFlowFAIMS-MS/MS method and sample preparation method B

As in the TurboFlow LC-MS/MS analysis, analyte at varying concentration levels was extracted from the Mitra tip using preparation methods A and B. The peak area ratios of everolimus to internal standard were used to construct calibration curves (Figure 8).

Figure 8. Calibration curves of everoliums from TurboFlow FAIMS-MS/MS analysis of Mitra tip dried blood samples extracted with preparation method A and B.



Calibration curve of everolimus using samples extracted with preparation method A



Calibration curve of everolimus using samples extracted with preparation method B

As in the TurboFlow LC-MS/MS analysis, calibration curves were fit with weighted least squares, and limits of detection (LOD) were determined for both preparation method A and B using the conventional method as documented in CLSI EP17-A2. Using TurboFlow FAIMS-MS/MS, the LOD for preparation method A was determined to be 4.2 ng/mL in the 10 µL blood samples and the LOD for preparation method B was determined to be 2.3 ng/mL in the blood samples. The results show that both preparation methods were comparable when using TurboFlow FAIMS-MS/MS analysis and that the protein precipitation step in preparation method A did not improve the limits of detection for the FAIMS method.

Table 1. Limits of detection from TurboFlow LC-MS/MS and TurboFlow FAIMS-MS/MS analyses using sample preparation methods A and B.

Preparation Method	Analytical Method	Limit of Detection (ng/mL)
A	TurboFlow LC-MS/MS	3.2
B	TurboFlow LC-MS/MS	7.4
A	TurboFlow FAIMS-MS/MS	4.2
B	TurboFlow FAIMS-MS/MS	2.3

CONCLUSIONS

- Additional steps in preparation method A allowed clinically relevant concentrations (3-5 ng/mL) of everolimus to be detected in small volumes of blood using Mitra microsampling and TurboFlow LC-MS/MS analysis.
- The FAIMS device significantly reduced background noise and allowed for collection of calibration curves to be obtained and everolimus to be quantitated at clinically relevant concentrations without use of HPLC.
- Both preparation methods (A and B) were comparable when using TurboFlow FAIMS-MS/MS analysis; the additional steps in preparation method A did not improve the limits of detection for the FAIMS method.
- The limit of detection for TurboFlow FAIMS-MS/MS was comparable TurboFlow LC-MS/MS when using preparation method B as the extraction method while TurboFlow LC-MS/MS with preparation method A allowed for a lower limit of detection.
- Future work includes the use of more chemically selective TurboFlow columns and further optimization of the TurboFlow FAIMS-MS/MS method to improve limits of detection.

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