Evaluation of Benchtop Quadrupole Orbitrap Ultra-High-Resolution Mass Spectrometer in Rapid Quantitative Analysis of Immunosuppressant Drugs in Blood Samples

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Overview

Purpose: To evaluate a benchtop quadrupole Orbitrap mass spectrometer for rapid quantitative analysis of immunosuppressant drugs in human blood samples for clinical research.

Methods: Blood samples were processed by protein precipitation, and analytes were chromatographically separated with a 3-minute LC gradient method. The mass spectrometer collected high resolution MS/MS spectra for each analyte, and the most abundant fragment was used for quantitation. The method was evaluated by obtaining limits of quantitation, intra- and inter-assay precisions, and investigating matrix effects. Cross-correlation between this method and a method from a collaborator's research laboratory was determined.

Results: We developed a fast and cost efficient method for analysis of Immunosuppressant drugs in blood. The method performance met clinical research laboratory criteria. Implementation of high resolution mass spectrometers in clinical research laboratories has advantages over conventionally used triple quadrupole instruments because of their versatile application range: screening, quantitation, and structure elucidation.

Introduction

Clinical research labs commonly use selective and cost-efficient LC-MS techniques for the analysis of immunosuppressant drugs. Usually, the quantitative method is developed on a triple quadrupole mass spectrometer. Here, we evaluated a high-throughput method implemented on a Thermo Scientific™ Q Exactive Focus™ hybrid quadrupole-Orbitrap mass spectrometer for improved selectivity when compared to the conventional LC-MS platform.

Methods

Calibrators

Calibrators containing cyclosporin A, everolimus, sirolimus, and tacrolimus in whole blood were purchased from Chromsystems Instruments & Chemicals GmbH (TABLE 1).

TABLE 1. Concentrations of calibrators.

Analyte	Cal 1	Cal 2	Cal 3	Cal 4	Cal 5	Cal 6
	Concentration (ng/mL)					
Tacrolimus	2.35	5.96	11.9	17.6	24.6	42.2
Sirolimus	2.27	6.12	11.9	18.4	27.9	46.1
Everolimus	2.27	5.94	11.7	18.1	25.1	45.4
Cyclosporin A	26.1	123	294	485	764	942

Sample Preparation

•100 µL of blood + 400 µL of precipitation solvent containing 150 µL of 0.1 M $ZnSO_4$ in water and 250 µL of internal standard (10 ng/mL ascomycin and 250 ng/mL cyclosporin D) in methanol

•Vortex, store at approx. 4 °C for 10 min

•Vortex, centrifuge for 10 min at 12,000 rpm

•Transfer supernatant into HPLC injection vial

•Inject 20 µL into LC-MS system

Liquid Chromatography

LC system
Pump: Thermo Scientific™ Dionex™ UltiMate™ 3000
Autosampler: Thermo Scientific™ OAS-3X00TXRS

Mobile phase A: 0.1% formic acid, 10 mM ammonium formate in water

Mobile phase B: 0.1% formic acid, 10 mM ammonium formate in methanol

Mobile phase C: acetonitrile/isopropyl alcohol/acetone 45:45:10 v/v/v

Column: Thermo Scientific™ Javelin™ guard column, 5 µm, 10 x 2.1 mm

Column temparature: 75 °C

LC gradient (TABLE 2).

Mass Spectrometry

Thermo Scientific™ Q Exactive™ Focus Orbitrap mass spectrometer with HESI ionization source

Data acquisition method: parallel reaction monitoring (PRM) experiment collecting MS/MS spectra for each analyte (TABLE 3)

Precursor isolation width: 1 amu

Resolution: 17.5K

Data Analysis

The most abundant fragment from MS/MS spectrum was selected for quantification (TABLE 3, FIGURE 1). Thermo Scientific™ TraceFinder™ software version 3.2 was used for data acquisition and data processing.

TABLE 2. HPLC gradient method.

Time (min)	Flow rate (mL/min)	%A	%B	%C
0.00	0.5	70	30	0
0.25	0.5	70	30	0
0.50	0.5	0	100	0
1.80	0.5	0	100	0
1.81	0.5	0	0	100
2.00	0.5	0	0	100
2.01	1.5	0	0	100
2.29	1.5	0	0	100
2.30	1.5	0	100	0
2.31	1.5	70	30	0
3.00	1.5	70	30	0

TABLE 3. Mass spectrometer scan parameters and quantification fragments

Analyte	Precursor Mass [*m/z*]	Quantitation Fragment [*m/z*]	Collision Energy (eV)	Polarity
Cyclosporin D (IS)	1233.9	1216.8633	24	Positive
Cyclosporin A	1219.9	1202.8478	20	Positive
Everolimus	975.6	908.5494	20	Positive
Sirolimus	931.6	864.5242	22	Positive
Tacrolimus	821.5	768.4666	22	Positive
Ascomycin (IS)	809.5	756.4670	24	Positive

Method Performance Evaluation

Qualtiy Control (QC) samples containing cyclosporin A, everolimus, sirolimus, and tacrolimus in whole blood were purchased from Chromsystems Instruments & Chemicals GmbH (TABLE 4).

TABLE 4. QC sample concentrations.

Analyte	QC1	QC2	QC3	QC4
	Concentration (ng/mL)			
Tacrolimus	2.6	7.3	16.7	34.2
Sirolimus	2.9	10.1	20.4	38.5
Everolimus	2.3	4.4	8.5	28.8
Cyclosporin A	53.0	276	514	1111

Limit of quantitation (LOQ) were defined as the lowest concentrations that had back-calculated values within 20% of nominal and RSD for 5 replicates within 20%. FIGURE 1 shows chromatograms of the lowest calibration standards.

Method accuracy was evaluated by obtaining % difference from nominal for quality control samples.

Method precision was evaluated by analyzing five replicates of each QC sample in three different days (TABLE 5 and TABLE 6).

System robustness and reproducibility were evaluated by analyzing five replicate injections of each calibration standard containing cyclosporin A, everolimus, sirolimus, and tacrolimus (TABLE 7).

Matrix effects were evaluated by spiking 10 ng/mL of everolimus, sirolimus, tacrolimus, and 60 ng/mL of cyclosporin A into whole blood samples from five different donors in two replicates, and to three solvent blanks. Each sample was injected twice. Relative and absolute % recoveries were calculated against data collected for spiked solvent (TABLE 8). A total of 97 donor blood samples containing tacrolimus and cyclosporin A provided by collaborator lab were analyzed. Correlation between data generated with collaborator clinical research method using triple quadrupole mass spectrometer and method on Q Exactive Focus was calculated (FIGURE 4)

Results

FIGURE 1. MS/MS spectra for analytes and internal standards.

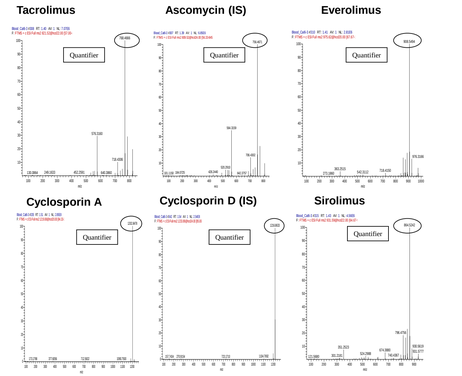
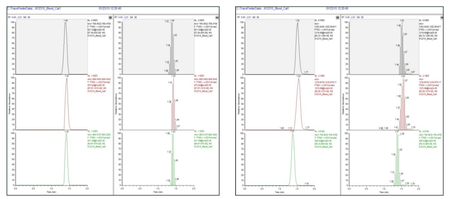


FIGURE 2. Chromatogram of lowest calibration standard (LOQ) reconstructed with a mass accuracy of 5 ppm



From top to bottom: tacrolimus, everolimus, sirolimus

From top to bottom: CsA, CsD, ascomycin

TABLE 5. Intra-assay precision and accuracy. Five replicates of QC samples containing cyclosporin A, tacrolimus, sirolimus, and everolimus were processed and analyzed in three batches.

Analyte	QC1	QC2	QC3	QC4
	%RSD			
Tacrolimus	4.12 – 5.01	3.68 – 5.64	2.65 – 4.19	3.88 – 5.12
Sirolimus	2.27 – 5.49	3.40 – 4.48	3.21 – 6.06	4.28 – 4.96
Everolimus	3.50 – 4.59	2.04 – 4.29	3.24 – 5.67	2.12 – 5.25
Cyclosporin A	2.21 – 4.21	2.76 – 4.88	3.86 – 6.85	4.58 – 5.08
Analyte	**Day-1**	**Day-2**	**Day-3**	
	Accuracy (%Diff)			
Tacrolimus	7.73% to -14.7%	4.23% to -11.2%	8.77% to -9.38%	
Sirolimus	5.91% to -8.76%	9.41% to -9.48%	9.58% to -13.3%	
Everolimus	8.34% to -6.49%	7.24% to -9.17%	10.2% to -6.62%	
Cyclosporin A	8.82% to -16.8%	10.5% to -12.0%	11.7% to -13.5%	

FIGURE 3. Calibration curves and lowest calibration standard peaks.

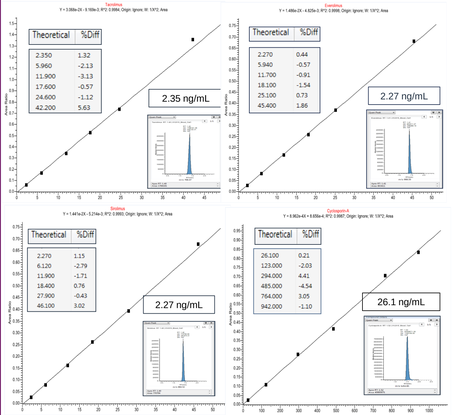


TABLE 6. Inter-assay precision and accuracy. Five replicates of QC samples were processed and analyzed in three batches.

Analyte	QC1	QC2	QC3	QC4
	%RSD			
Tacrolimus	4.65	4.55	3.77	4.21
Sirolimus	4.16	3.71	4.22	4.24
Everolimus	5.92	4.47	4.46	3.88
Cyclosporin A	3.46	3.5	5.11	4.59
Analyte	**QC1**	**QC2**	**QC3**	**QC4**
	%Recovery			
Tacrolimus	101	94.2	95.2	99.8
Sirolimus	99.9	98.2	99.1	102
Everolimus	96.6	96.3	101	102
Cyclosporin A	101	94.4	94.7	104

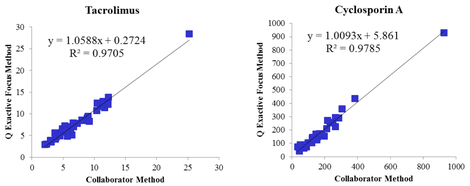
TABLE 7. System robustness and reproducibility. Five replicate injections of each calibration standard were performed to demonstrate the system robustness and reproducibility.

Analyte	Std-1	Std-2	Std-3	Std-4	Std-5	Std-6
	%RSD					
Tacrolimus	3.65	3.03	3.05	3.50	3.06	4.39
Sirolimus	10.3	4.28	3.23	2.46	1.64	3.42
Everolimus	5.57	1.98	2.34	4.78	3.58	4.20
Cyclosporin A	3.93	2.81	0.97	1.17	3.82	1.83
	Mean %Recovery					
Tacrolimus	102	96.2	101	96.2	101	104
Sirolimus	101	96.3	106	95.3	103	98.2
Everolimus	100	96.9	107	95.5	103	97.6
Cyclosporin A	99.8	101	102	96.6	101	99.9

TABLE 8. Matrix effects.

Analyte	Blood-1	Blood-2	Blood-3	Blood-4	Blood-5
	Absolute %Recovery				
Tacrolimus	96.9	107	109	107	108
Sirolimus	94.3	93.2	103	103	104
Everolimus	104	98.6	112	109	108
Cyclosporin A	90.8	91.5	101	101	82.0
	Relative (IS ratio) %Recovery				
Tacrolimus	85.1	92.9	97.0	94.8	93.7
Sirolimus	82.8	80.9	92.1	90.8	90.3
Everolimus	91.6	85.5	99.9	96.2	94.0
Cyclosporin A	98.5	95.9	96.3	101	85.5

FIGURE 4. Methods cross-correlation. Samples from collaborator lab that contain cyclosporin A and tacrolimus.



Conclusion

- We demonstrated a fast, cost-efficient, highly specific method for the analysis of immunosupressant drugs on a high=resolution Orbitrap mass spectrometer.
- Implementation of a high-resolution instrument resulted in chromatograms containing just analyte peak, which significantly improves accuracy of data processing and reduces data review time.
- Method meets clinical research requirements for analysis of immunosuppressant drugs in whole blood samples.

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