TECHNICAL NOTE 66028

Quantification of mycophenolic acid in human plasma by LC-HRAM mass spectrometry for clinical research

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Application benefits

- Increased accuracy of method through implementation of a comprehensive ClinMass® kit for sample preparation
- High-resolution mass spectrometry for improved selectivity
- Robust, sensitive hardware enables increased confidence in data

Goal

Implementation of an analytical method for the quantification of mycophenolic acid in human plasma on a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer.

Introduction

Therapeutic drug monitoring (TDM) research involving mycophenolic acid (MPA), an immunosuppressive agent commonly used for organ transplant and autoimmune support, can be effectively performed using liquid chromatography coupled to high-resolution mass spectrometry (LC-MS). Alternative technologies, such as immunoassays, often produce results influenced by cross-reactivity, thus prompting laboratories to adopt accurate, sensitive, and selective analytical methods, such as LC-MS. Availability of convenient and easy-to-use drug-specific kits for TDM research further enable the accuracy and preference of this method.

High-resolution mass spectrometry delivers high selectivity and sensitivity even in full scan (Full MS) mode. The additional use of fragmentation in Parallel Reaction Monitoring (PRM) mode provides enhanced specificity to the analytical method.

In this report, two acquisition approaches, Full MS and PRM, were applied for the quantification of mycophenolic acid in human plasma. Plasma samples were extracted by offline internal standard addition and protein precipitation. Extracted samples were injected onto a Thermo Scientific™ Vanquish™ Duo UHPLC system. Detection was performed on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with heated electrospray ionization (HESI), either by Full MS or by PRM. Method performance was evaluated using the ClinMass® TDM Platform with the ClinMass Add-On Set for Mycophenolic Acid in Serum/Plasma from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response within the calibration range, lower limit of quantification (LLOQ), carryover, accuracy, trueness of measurements, and intra- and inter-assay precision. The ability of this method to chromatographically resolve mycophenolic acid from its glucuronide was also investigated.

Experimental

Target analytes

The target analyte was mycophenolic acid with d_3-mycophenolic acid being used as the internal standard.

Sample preparation

Four calibrators (MS99113 batch #1059), including a blank, from RECIPE covering a concentration range of 0.391–7.4 mg/L were used together with two controls (MS99183 batch #1417). Samples of 50 µL of plasma were protein precipitated using 100 µL of precipitating solution containing the internal standard. Precipitated samples were vortex-mixed and centrifuged. Then, 50 µL of the supernatant were transferred to a clean plate or vial and diluted with 450 µL of Diluting Solution D (MS9022) prior to injection.

Liquid chromatography

A Vanquish Duo UHPLC system with binary Flex pumps, a dual-channel instrument configured for both LC-only and online solid-phase extraction applications (Figure 1), was used for chromatographic separation. The LC-only channel was used in this case, utilizing mobile phases and an analytical column provided by RECIPE. Details of the analytical method are reported in Table 1. Total runtime was 1.9 minutes.

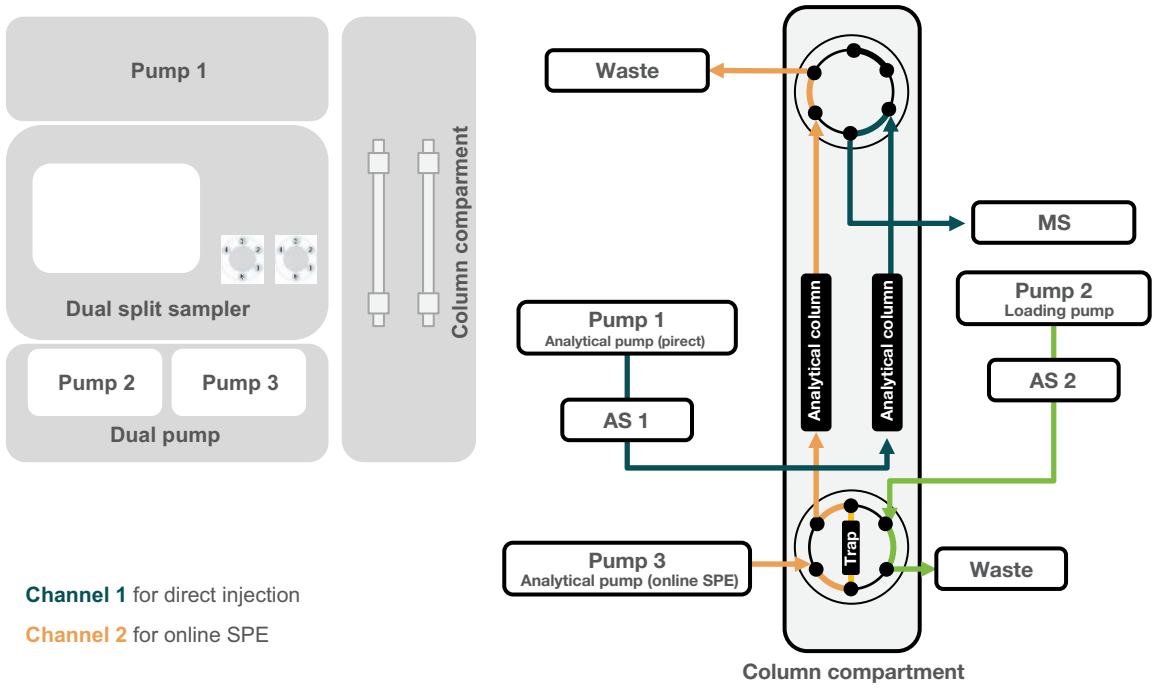

Figure 1. Schematic representation of the Vanquish Duo UHPLC system setup

Table 1. LC method description

Gradient profile			
Time (min)	**Flow rate (mL/min)**	**%A**	**%B**
0.00	0.5	83	17
0.01	0.5	83	17
0.30	0.5	20	80
0.55	0.5	20	80
0.60	0.5	83	17
1.90	0.5	83	17
Other parameters			
Injection volume		20 µL	
Column temperature		40 °C	

Mass spectrometry

Analyte and internal standard were detected by both Full Scan and PRM mode on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with a HESI source operated in positive ion mode. In Full Scan mode, the precursor/parent ion is extracted from the data using a window of 5 ppm, and the resulting peak is used for quantitation. In PRM mode, the precursor ion is isolated by the quadrupole and fragmented in the HCD cell. Quantitation is performed using one of the specific resulting fragments. A summary of the MS conditions is reported in Table 2.

Table 2. MS settings

Ion source parameters	
Source type	Heated electrospray source ionization (HESI)
Spray voltage - Positive (V)	3500
Sheath gas (Arb)	50
Aux gas (Arb)	15
Sweep gas (Arb)	0
Ion transfer tube temperature (°C)	250
Vaporizer temp (°C)	400
S-lens RF level	60
Full Scan settings	
Orbitrap resolution (@ *m/z* 200)	70,000
Scan range *(m/z)*	50–750
AGC target	1e6
Maximum IT (ms)	200
PRM Scan settings	
Orbitrap resolution (@ *m/z* 200)	17,500
Isolation window *(m/z)*	2.0
AGC target	2e5
Maximum IT (ms)	20
Collision energy (CE)	20

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration range, LLOQ, carryover, accuracy, trueness of measurements, and intra- and inter-assay precision for both analytes.

LLOQ was determined by diluting the lowest calibrator 2-, 5-, 10-, and 20-fold using blank matrix from the kit. A full set of calibrators (four levels), diluted calibrators (four levels), and controls (two levels) were extracted in replicates of five (n=5), injected in a single batch and all used for the linear interpolation. The LLOQ was set as the lowest level that could be determined with a CV <20%.

Carryover was calculated in terms of percentage ratio between peak area in the highest calibrator and a blank sample injected just after it.

Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations using the quality control samples provided by RECIPE at two different levels prepared and analyzed in replicates of five on three different days.

Trueness of measurement was also evaluated as percentage bias using certified external quality controls (INSTAND 602, 2019, Probes 21 and 22) prepared and analyzed in replicates of five on a single day.

Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at two different levels in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at two levels in replicates of five prepared and analyzed on three different days).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 5.1 software.

Results and discussion

A linear response with 1/× weighting was used not only in the calibration range covered by the calibrators but also down to an LLOQ of 0.078 mg/L for both acquisition modes. The percentage bias between nominal and back-calculated concentration was consistently within ±15% for all the calibrators (±20% for the lowest calibrator). Representative chromatograms for the LLOQ for analyte and internal standard using both approaches are depicted in Figure 2. Representative calibration curves in the concentration range covered by the kit (three calibrators) are shown in Figure 3.

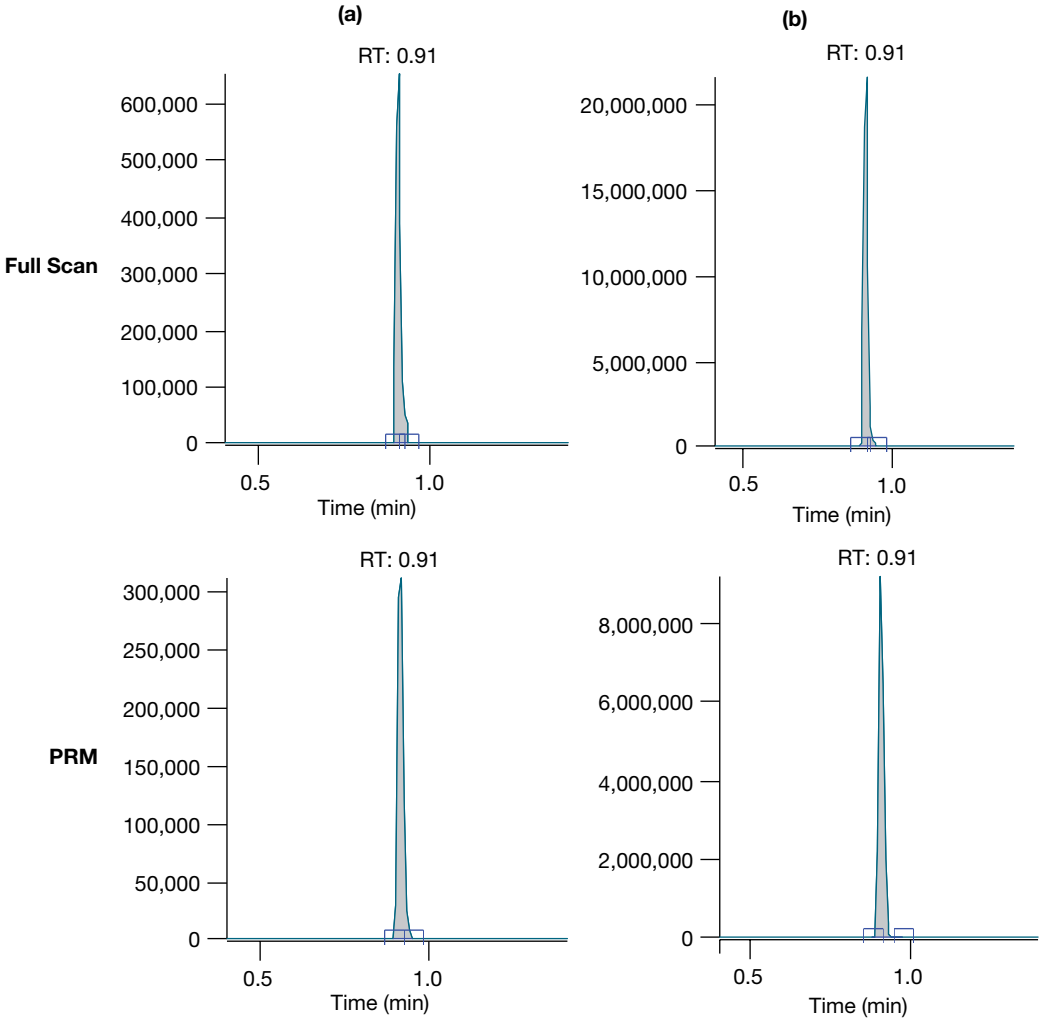

Figure 2. Representative chromatograms of the LLOQ (0.078 µg/mL) for (a) mycophenolic acid and (b) d_3-mycophenolic acid using full scan and PRM acquisition modes

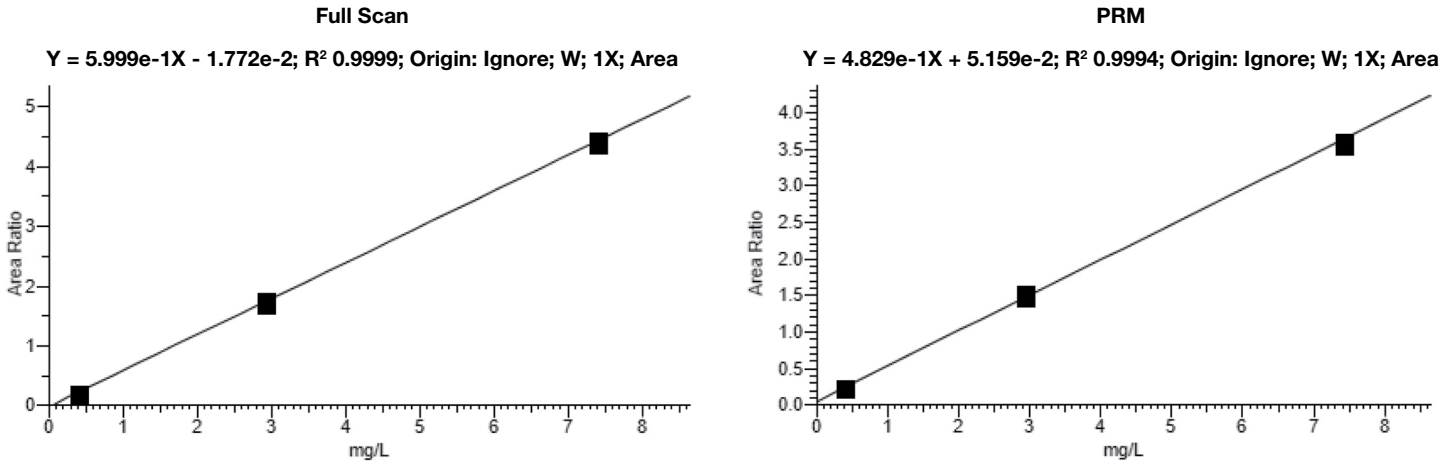

Figure 3. Representative calibration curves using Full Scan and PRM acquisition modes

The method was able to chromatographically resolve mycophenolic acid from its glucuronide. A representative chromatogram is reported in Figure 4.

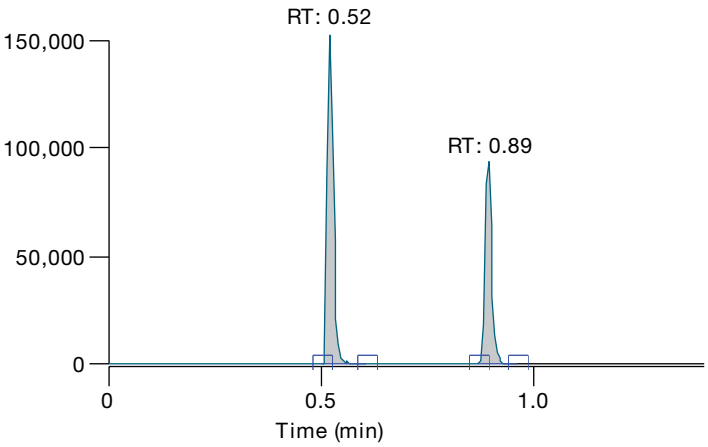

Figure 4. Representative chromatogram of the separation obtained between mycophenolic acid (RT=0.89 min) and its glucuronide (RT=0.52 min) in Full Scan acquisition mode

No carryover was observed, with no signal detected in the blank injected after the highest calibrator.

The data demonstrated outstanding accuracy of the method with the percentage bias between nominal and average back-calculated concentration for the used control samples ranging between -0.3% and 0.0% in Full MS mode and between 0.7% and 2.2% in PRM mode (Table 3).

Excellent results were obtained also from the trueness of measurements evaluation, with a percentage bias between -1.9% and 4.9% (Table 4).

Table 3. Analytical accuracy results for control MS99183 batch #1417

Acquisition mode	Control	Nominal concentration (µg/mL)	Average calculated concentration (µg/mL)	Bias (%)
Full Scan	Level I	0.491	0.491	0.0
	Level III	4.59	4.58	-0.3
PRM	Level I	0.491	0.495	0.7
	Level III	4.59	4.69	2.2

Table 4. Analytical accuracy results for controls Instand 602, 2019

Acquisition mode	Control	Nominal concentration (µg/mL)	Average calculated concentration (µg/mL)	Bias (%)
Full Scan	Probe 21	0.749	0.735	-1.9
	Probe 22	3.718	3.657	-1.6
PRM	Probe 21	0.749	0.770	2.9
	Probe 22	3.718	3.900	4.9

The %CV for intra-assay precision was always below 7.2% for both acquisition modes. The maximum %CV for inter-assay precision including both acquisition modes was 6.3%. Results for intra- and inter-assay precision reported in Table 5.

Table 5. Analytical intra- and inter-assay precision results for control MS99183 batch #1417

Acquisition mode	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3			
		Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)	Average calculated concentration (µg/L)	CV (%)
Full Scan	Level I	0.481	2.5	0.495	1.9	0.497	1.7	0.491	2.4
	Level III	4.649	2.5	4.532	1.6	4.545	0.9	4.575	2.0
PRM	Level I	0.505	7.2	0.505	4.1	0.474	3.8	0.495	5.8
	Level III	4.921	4.6	4.528	5.4	4.617	6.4	4.689	6.3

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Conclusions

A robust, reproducible, and sensitive liquid chromatography–high-resolution Orbitrap mass spectrometry method for clinical research for the quantification of mycophenolic acid in human plasma was implemented. The ClinMass TDM Platform with the ClinMass Add-On Set for Mycophenolic Acid in Serum/Plasma from RECIPE was used. The method was analytically validated on a Vanquish Duo UHPLC system connected to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with a HESI probe. Full scan and PRM experiments were used for data acquisition. The method described here offers quick and simple offline protein precipitation with concomitant internal standard addition. Both full scan and PRM approaches meet research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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