Rapid Analysis of Mycophenolic Acid in Human Plasma Using an Agilent Triple Quadrupole LC/MS/MS System with Automated Online Sample Cleanup

Application Note

Clinical Research

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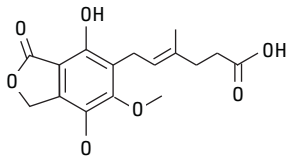
Abstract

A sensitive and specific research method has been developed for the accurate analysis of mycophenolic acid (MPA). This method has a run time of 4 minutes and is suitable for the reliable quantitation of MPA in presence of its glucuronide form (MPA-G).

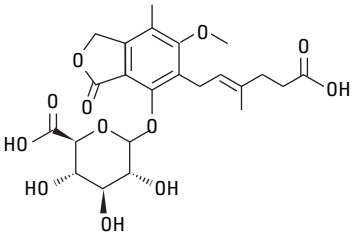
Agilent Technologies

Introduction

This application note describes the development of a method for the sensitive and accurate determination of MPA in plasma using an Agilent 1260 Infinity LC system coupled to an Agilent 6460 Triple Quadrupole Mass Spectrometer with Agilent JetStream technology. When analyzing MPA, it is important to achieve chromatographic separation between MPA and MPA-G. Without proper separation, in-source fragmentation of MPA-G can result in the loss of the glucuronide and falsely elevate quantitation of MPA. Using tandem mass spectrometry (MS/MS) and multiple reaction monitoring (MRM), the method is linear from 0.1 µg/mL to 25 µg/mL for MPA.



MPA



MPA-G

Figure 1. Structures for MPA and MPA-G.

Experimental

Reagents and standards

For method development, all unlabeled and labeled standards were purchased from Cerillant and stored at –20 °C. Deuterated MPA was used as internal standard (ISTD) to ensure accurate quantitation. Commercially available calibrators and quality controls (QC) from ChromSystems were used to evaluate accuracy and precision of this method. All other LC/MS grade solvents, reagents, and plasma were purchased from Aldrich India and Honeywell.

The highest concentration calibrator was prepared with 25 µg/mL of MPA and 125 µg/mL of MPA-G in human plasma. Serial two-fold dilutions with plasma were used to achieve the remaining concentrations, which cover biologically relevant concentrations. Analyte concentrations for linearity are listed in Table 1.

Table 1. Linearity levels used in this study.

Calibrator	MPA (µg/mL)	MPA-G (µg/mL)
9	25.00	125.00
8	12.50	62.50
7	6.25	31.25
6	3.13	15.63
5	1.56	7.81
4	0.78	3.91
3	0.39	1.95
2	0.20	0.98
1	0.10	0.49

Sample preparation

All calibrators, QCs, and samples were prepared using a simple protein precipitation procedure:

1. Mix 100 µL of plasma with 200 µL of precipitating reagent (1:4 ratio of 0.4 M zinc sulphate: methanol) containing internal standard.
2. Vortex for 30 seconds.
3. Centrifuge at 10,000 rpm for 4 minutes.
4. Transfer supernatant to autosampler vials or 96-well plate and analyze by LC/MS/MS.

LC configuration and conditions

An Agilent 1260 Infinity LC system was used for this analysis. The system consisted of:

- Agilent 1260 Infinity Binary Pump (×2)
- Agilent 1260 Infinity Thermostatted Column Compartment with 2-Position/6-Port column switching valve
- Agilent 1260 Thermostatted Autosampler

An inline filter (p/n 5067-1551) between the needle seat and the injector valve of the autosampler is also recommended to improve instrument robustness.

LC parameters are listed in Tables 2, 3, 4, and 5.

Automated on-line sample cleanup

The HPLC used for this method was configured for automated sample cleanup using two binary pumps (Figure 2). Samples were loaded onto a trapping column where the analytes were retained and washed by the first pump. The wash was sent to waste, reducing the amount of matrix introduced into the mass spectrometer. Shortly before the analytes elute off of the trapping column, a valve was switched and the analytes were eluted onto an analytical column where further chromatography was performed using the second binary pump.

Table 2. LC conditions.

Parameter	Value
Columns	**Trapping:** Agilent ZORBAX Eclipse Plus C18, 2.1 × 12.5 mm, 5 µm (p/n 821125-936) **Analytical:** Agilent Poroshell 120 EC-C18, 3 × 50 mm, 2.7 µm (p/n 699975-302)
Column temperature	60 °C
Injection volume	2 µL
Needle wash	1:1:1:1 methanol:acetonitrile:isopropyl alcohol:water + 0.1 % FA for 60 seconds
Injector temperature	4 °C
Run time	2 minutes
Buffer A	10 mM ammonium acetate + 0.2 % formic acid in water
Buffer B	10 mM ammonium acetate + 0.2 % formic acid in methanol

Table 3. Loading gradient (Pump 1).

Time	Flow (mL/min)	% B
0.00	0.1	20
0.01	2.0	20
2.00	2.0	20
2.40	2.0	20
2.65	0.1	20

Table 4. Analytical gradient (Pump 2).

Time	Flow (mL/min)	% B
0.00	0.5	50
1.90	0.5	95
1.95	1.0	95
2.40	1.2	95
2.41	2.0	95
3.50	2.0	95

Table 5. Valve timing.

Time	Position(s)
0.00	1
0.50	2
2.40	1

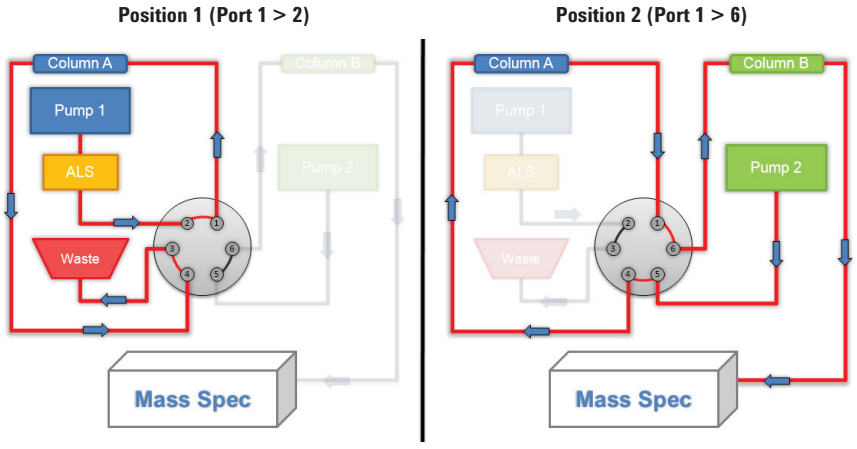


Figure 2. Valve diagram for back-flushing liquid chromatography configuration for online sample cleanup using a 2-position/6-port valve and two binary pumps.

MS conditions

An Agilent 6460 Triple Quadrupole Mass Spectrometer with JetStream technology was used for this analysis. Unique MRM transitions assured specificity in the quantitation of each analyte. MPA-d3 was used as internal standard (ISTD) for relative quantification and thus reduced the error due to any loss of analytes during sample preparation or variation in sample matrix. MS conditions and MRM transitions are listed in Tables 6 and 7.

Data analysis

MassHunter Quantitative Software B.04.01 was used for our data analysis. Calibration curves were constructed for MPA and MPA-G using MRM peak area ratios to a known concentration of the internal standards. For the Linearity regression of the calibration curves a weighing factor of 1/x was used. Intra- and inter-day injections were performed with the QC samples to assess accuracy and reproducibility. Representative extracted MRM chromatograms for the analytes are given in Figure 3. Note that two peaks are observed in the chromatogram for MPA. The peak at 1.126 minutes is from MPA-G that lost its glucuronide due to in-source fragmentation. This chromatographic separation is critical to the accurate quantitation of MPA.

Table 6. Conditions for the Agilent 6460 Triple Quadrupole Mass Spectrometer equipped with an Agilent JetStream source.

Parameter	Value
Ion mode	Positive
Drying gas temperature	225 °C
Drying gas flow	9 L/min
Nebulizer pressure	35 psi
Sheath gas temperature	325 °C
Sheath gas flow	12 L/min
Capillary voltage	4,000 V
DEMV	200 V
Nozzle voltage	300 V
Q1/Q3 resolution	0.7 unit

Table 7. MRM transitions monitored.

Compound	Precursor	Product	Dwell (msec)	Frag. (V)	CE (V)
Mycophenolic acid	321.1	207.0	10	80	16
Mycophenolic acid gluc	514.2	207.0	10	95	36
Mycophenolic acid-d3	324.2	210.1	10	80	16

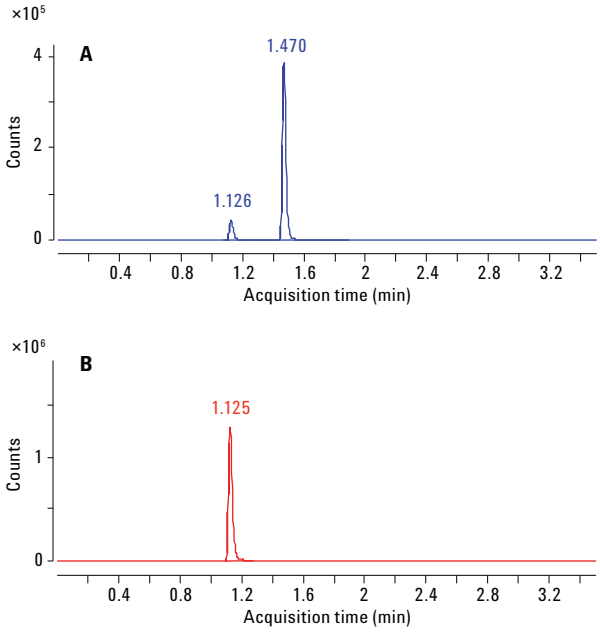


Figure 3. Chromatograms of quantifier MRM transitions for MPA (A) and MPA-G (B).

Results and Discussion

Excellent linearity was observed for both MPA and MPA-G with R^2 values > 0.993 including all nine concentration levels tested (Figure 4). The excellent reproducibility of the retention time for each analytes guaranteed the repeatability of the method. The significant response of analytes at low linearity levels assures accurate quantitation.

Intra- and inter-day injections were performed with commercially available calibrators and QC samples (ChromSystems) to evaluate the accuracy and robustness of this method. Data was acquired over eight days by two operators. The observed accuracies for each QC level of MPA are tabulated in Table 8.

Conclusion

A four minute research method for the quantitation of Mycophenolic Acid in the presence of its glucuronide was developed using an Agilent 6460 Triple Quadrupole LC/MS/MS. A simple protein precipitation followed by automated online sample cleanup minimized the matrix effect and ion suppression due to biological compounds present in plasma. Using this method, reliable and quick quantitation of MPA from plasma matrix was demonstrated. Excellent linearity of MPA has been confirmed over the desired ranges.

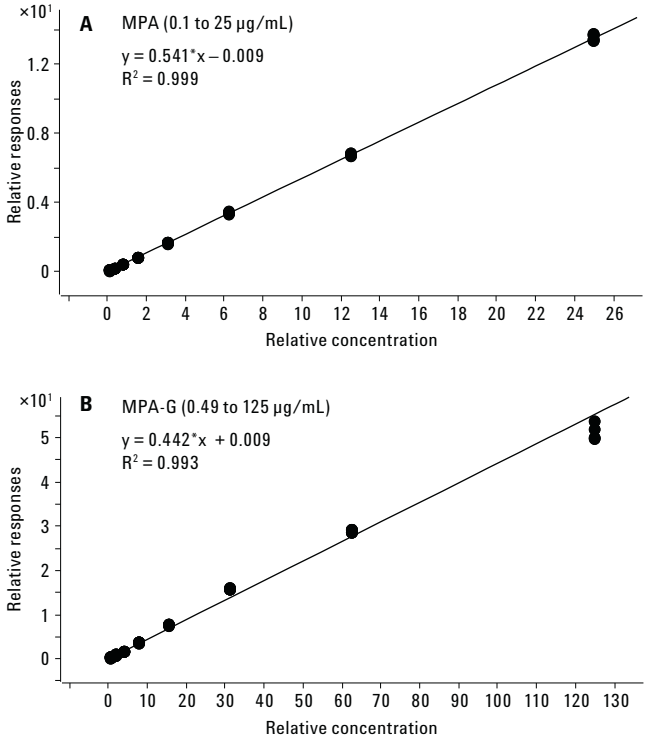


Figure 4. Calibration curve of MPA (A) and MPA-G (B) 9 levels, 36 points (type: linear, origin: ignore, weight: 1/x).

Table 8. Mycophenolic acid (MPA) QC results.

	Target (µg/mL)	Mean (µg/mL)	Accuracy (%)	CV (%)	RT RSD (%)
Inter-day (n = 8)	1.90	1.86	97.90	4.18	0.42
	5.62	5.80	103.2	4.47	0.49
Intra-day (n = 20)	1.90	1.86	98.10	1.90	0.00
	5.62	5.90	104.9	2.30	0.00

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