Rapid Quantitative Analysis of Immunosuppressant Drug Panels in Blood and Plasma by LC-MS/MS

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Introduction

Two highly sensitive and specific research methods have been developed for the quantitation of a panel of up to five common immunosuppressant drugs – Cyclosporine A (CsA), Everolimus (Eve), Sirolimus (Sir), Tacrolimus (Tac) and Mycophenolic Acid (MPA). The first is a rapid, 2-minute method suitable for the reliable quantification of CsA, Eve, Sir and Tac. The second method contains a longer gradient, critical to the accurate analysis of Mycophenolic Acid (MPA).

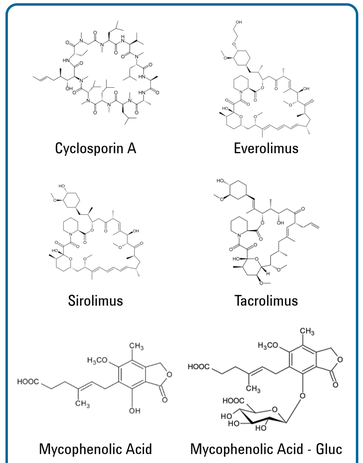

Figure 1. Immunosuppressive drug structures

When analyzing MPA, it is important to achieve chromatographic separation between MPA and its glucuronide (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.

Due to the distribution of these drugs in blood, MPA is typically measured in plasma while the remaining immunosuppressive drugs must be measured in whole blood. Despite this difference in sample type, a single sample preparation and hardware configuration has been developed for the analysis of all five drugs.

Experimental

Sample Preparation

100µl of plasma or whole blood is precipitated with precipitating reagent ($ZnSO_4$:methanol) containing internal standards. After vortexing and centrifugation, the clear supernatant is separated and injected onto the LC-MS/MS for analysis.

Analyte	Internal Standard
Cyclosporin A	Cyclosporin D
Everolimus	Ascomycin
Sirolimus	Ascomycin
Tacrolimus	Ascomycin
Mycophenolic Acid	Mycophenolic Acid-d_3

Table 1. Internal standard assignment

LC Method

Both LC-MS/MS methods were developed using common instrumentation and reagents to provide the greatest flexibility and efficiency.

Agilent 1290 and 1260 HPLC binary pumps (1 each), well plate sampler with thermostat, temperature-controlled column compartment, 2 position/6 ports switching valve

Parameter	Value
Trapping Column	Zorbax Eclipse Plus C18, 2.1x12.5mm, 5µm
Analytical Column	Poroshell 120 EC-C18, 3x50mm, 2.7µm
Injection Volume	Up to 40 µl
Autosampler Temp	4°C
Needle Wash	Flush port for 10 to 60 seconds
Mobile Phase A	NH_4 Acetate + Formic Acid in Water
Mobile Phase B	NH_4 Acetate + Formic Acid in Methanol

Table 2. LC Parameters

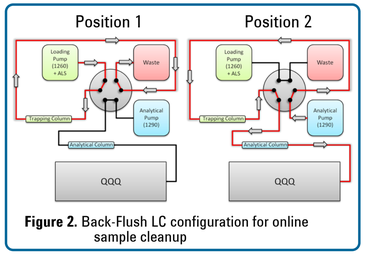

Figure 2. Back-Flush LC configuration for online sample cleanup

Experimental

MS Method

Agilent 6460 triple quadrupole mass spectrometer with JetStream technology

Ion mode:	AJS ESI+
Gas temperature:	225 °C
Drying gas (nitrogen):	9 L/min
Nebulizer gas (nitrogen):	35 psi
Sheath gas (nitrogen):	325 °C
Sheath flow:	12 L/min
Capillary voltage:	4000V
Nozzle voltage:	300V
Q1/Q3 Resolution:	0.7 unit
Dwell time:	10 msec
Delta EMV:	0 to 200V

Compound	Prec Ion	Prod Ion	Dwell	Frag (V)	CE (V)
Cyclosporine D	1233.9	1216.9	10	175	12
Cyclosporine A	1219.9	1202.8	10	170	12
Everolimus	975.6	908.5	10	185	12
Sirolimus	931.6	864.5	10	170	12
Tacrolimus	821.5	768.4	10	170	16
Ascomycin	809.5	756.4	10	175	16
Mycophenolic Acid Gluc	514.2	207.0	10	95	36
Mycophenolic Acid D_3	324.2	210.1	10	80	16
Mycophenolic Acid	321.1	207.0	10	80	16

Table 2. MRM Parameters

Results and Discussion

A rapid, 2-minute method has been developed for the determination of four of the five common immunosuppressant drugs – Cyclosporin A, Everolimus, Sirolimus and Tacrolimus. All four of these drugs are measured in whole blood, so simultaneous determination is possible. The fifth drug – Mycophenolic Acid – requires a longer gradient for accurate quantitation. All five drugs can be measured using the 4-minute method but MPA is traditionally measured in plasma rather than whole blood. This means MPA typically will not be measured in combination with another immunosuppressant due to differences in sample type. However, identical instrumentation and reagents were used to develop both methods, meaning it is possible to quickly switch between methods, even within the same worklist. Both methods utilizes a back-flushing liquid chromatography configuration for online sample cleanup to improve quantitation. Samples are injected onto a trapping column where the immunosuppressants are retained and washed. The wash is sent to waste, reducing the amount of matrix sent to the mass spectrometer. Shortly before the analytes elute off of the first column, a valve is switched and the analytes are eluted onto an analytical column where further chromatography is performed.

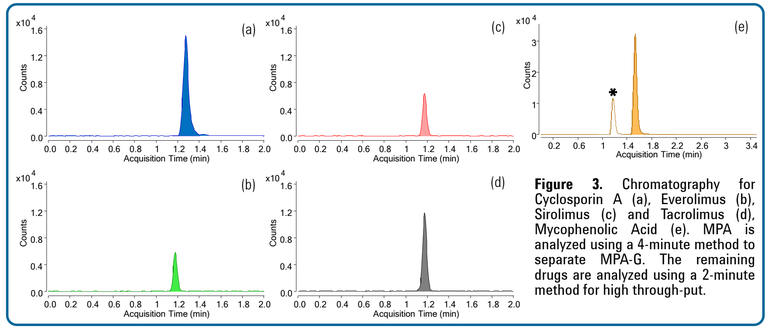

Figure 3. Chromatography for Cyclosporin A (a), Everolimus (b), Sirolimus (c) and Tacrolimus (d), Mycophenolic Acid (e). MPA is analyzed using a 4-minute method to separate MPA-G. The remaining drugs are analyzed using a 2-minute method for high through-put.

In the case of MPA, chromatography is particularly important due to the presence of mycophenolic acid glucuronide (MPA-G) in plasma. MPA-G is susceptible to in-source fragmentation where the glucuronide is easily lost. If these analytes are not separated by retention time (figure 3c), deglucuronidated MPA-G (MPA-G*) can falsely elevate the determination of mycophenolic acid concentrations.

Results and Discussion

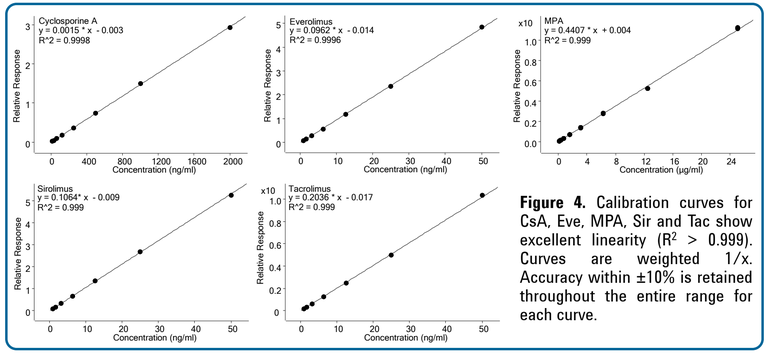

Figure 4. Calibration curves for CsA, Eve, MPA, Sir and Tac show excellent linearity (R^2 > 0.999). Curves are weighted 1/x. Accuracy within ±10% is retained throughout the entire range for each curve.

Compound	R^2	Level	Concentration (ng/ml)	Accuracy (%) n = 3	Intraday CV (%) n = 3	Interday CV (%) n = 6
Cyclosporin A	0.999	LLOQ	2.0	110.0	3.85	3.90
		MID	250.0	98.4	2.38	1.65
		ULOQ	2000.0	100.9	0.95	1.30
Everolimus	0.999	LLOQ	0.1	107.1	10.69	9.18
		MID	12.5	100.7	1.03	1.83
		ULOQ	50.0	99.3	1.44	1.22
Sirolimus	0.999	LLOQ	0.1	100.7	7.54	6.83
		MID	12.5	103.1	2.79	2.04
		ULOQ	50.0	98.3	1.65	1.74
Tacrolimus	0.999	LLOQ	0.1	101.2	7.34	5.84
		MID	12.5	99.7	1.16	1.16
		ULOQ	50.0	99.3	1.38	1.16
Mycophenolic Acid*	0.999	LLOQ	0.1	100.0	0.3	7.5
		MID	3.1	100.6	0.7	0.6
		ULOQ	25.0	101.7	0.6	0.7

Table 3. Summary of analyte performance for five immunosuppressive drugs
*MPA concentrations are in µg/ml concentrations

Conclusions

Two specific, rapid and convenient methods for quantifying five immunosuppressive drugs with excellent reproducibility and accuracy have been developed for clinical research. A back-flushing liquid chromatography configuration for online sample cleanup has been implemented to reduce the throughput of matrix to the mass spectrometer. These methods utilize the same hardware and reagents to allow fast quantitation of all five of the analytes. This approach allows for the greatest flexibility while eliminating the need to maintain multiple configurations and solvents. The two-minute method is used for the quantitation of cyclosporin A, everolimus, sirolimus and tacrolimus to increase throughput when MPA determination is not required. The four-minute method allows for sufficient chromatographic separation between MPA and its glucuronide to ensure accurate quantitation.