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A Rapid Quantitative Analysis of Five Immunosuppressant Drugs in Blood by LC-MS/MS for Clinical Research

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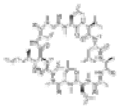
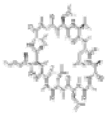
Introduction

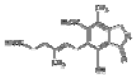
Cyclosporin A (CsA), everolimus (Eve), mycophenolic acid (MPA), sirolimus (Sir) and tacrolimus (Tac) are five common immunosuppressive drugs. MPA concentrations are typically measured in plasma since it is not found in any other blood fraction. However, CsA, Eve, Sir and Tac are found distributed throughout blood fractions and must be measured in whole blood.



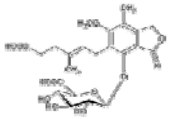
Cyclosporin A



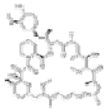
Cyclosporin D



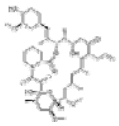
Mycophenolic Acid



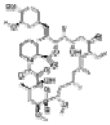
Mycophenolic Acid - Gluc



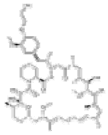
Sirolimus



Tacrolimus



Ascomycin



Everolimus

Figure 1. Immunosuppressive drugs and internal standard structures

A selective, rapid and convenient LC-MS/MS method for determining the concentration of multiple immunosuppressive drugs in blood is a powerful tool for clinical research. The proposed method allows for the analysis of five drugs utilizing the same hardware and reagents, regardless of whether starting with whole blood or plasma samples. Matrix-stripping liquid chromatography has been implemented to increase sensitivity and reproducibility without the addition of any off-line sample preparation.

Experimental

Sample Preparation
If a sample is to be analyzed for MPA, whole blood is centrifuged at 10,000 rpm for 10 minutes and plasma is separated. CsA, Eve, Sir and Tac are all analyzed in whole blood. 100µl of plasma or whole blood is precipitated with 200µl of precipitating reagent (1:4 v/v 0.4M $ZnSO_4$:methanol) containing internal standards. After vortexing for 30 seconds, the sample is centrifuged at 10,000 rpm for 4 minutes. The clear supernatant is separated and injected onto the LC-MS/MS for analysis.

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

Columns
Trapping: Zorbax Eclipse Plus C18, 2.1x12.5mm, 5µm
Analytical: Poroshell 120 EC-C18, 3x50mm, 2.7µm

Column Temperature: 60 ºC
Injection Volume: 40 µL (2 µL for MPA)
Autosampler Temperature: 4 ºC
Needle Wash: 1:1:1:1 MeOH:ACN:IPA:H_2O + 0.1% FA
10 seconds (60 seconds for MPA)

Switching Valve
0.0 minutes – Position 1
0.5 minutes – Position 2
2.4 minutes – Position 1

Mobile Phase
A: 10 mM NH_4 Acetate + 0.2% Formic Acid in Water
B: 10 mM NH_4 Acetate + 0.2% Formic Acid in Methanol

Gradient (followed by 30 second post time)

Loading Pump (1260)

Time	Flow	% Solvent 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
3.50	0.1	20

Analytical Pump (1290)

Time	Flow	% Solvent 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

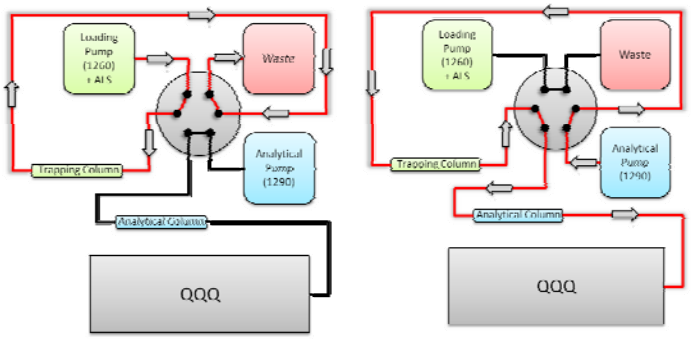


Figure 2. Back-Flush Matrix-Stripping LC Diagram

Experimental

MS Method

Agilent 6460 triple quadrupole mass spectrometer with JetStream technology

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

During method development, MPA was evaluated in positive and negative mode and found to perform better in positive mode, eliminating the need of +/- switching.

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 1. MRM Parameters

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

Table 2. Internal standard assignment

Results and Discussion

This method utilizes matrix-stripping liquid chromatography, which allows for cleaner throughput to the mass spectrometer by performing online sample cleanup. Samples are injected onto a trapping column where the immunosuppressants are retained and washed, reducing the amount of matrix sent to the mass spectrometer. After 30 seconds, a valve is switched and analytes are eluted onto an analytical column where further chromatography is performed.

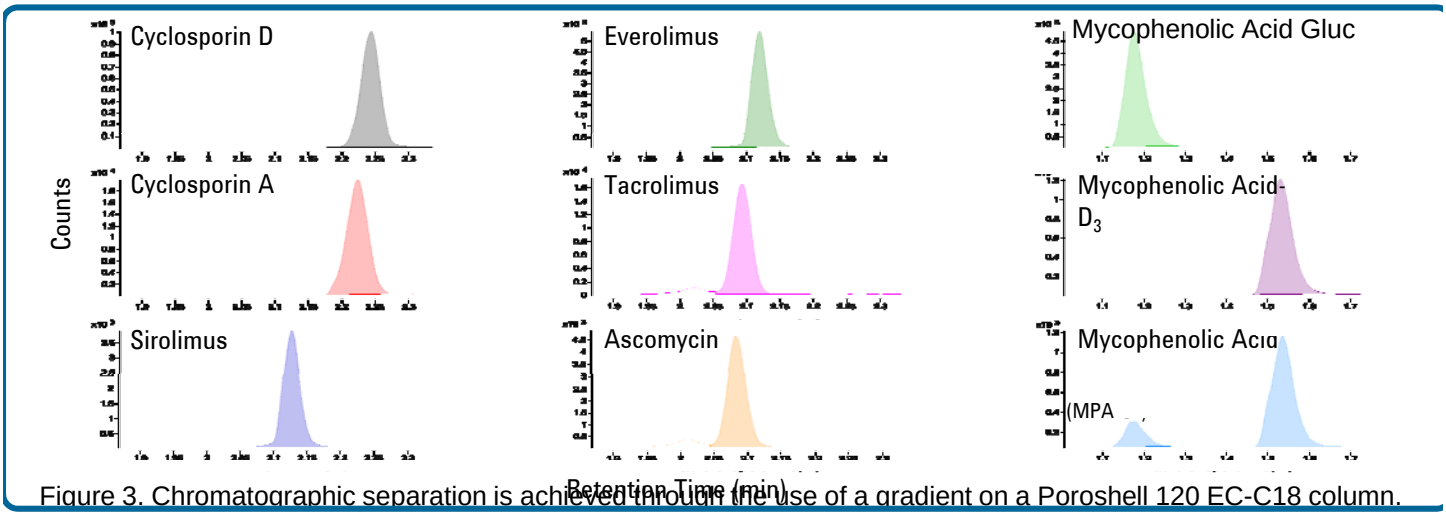


Figure 3. Chromatographic separation is achieved through the use of a gradient on a Poroshell 120 EC-C18 column.

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

Results and Discussion

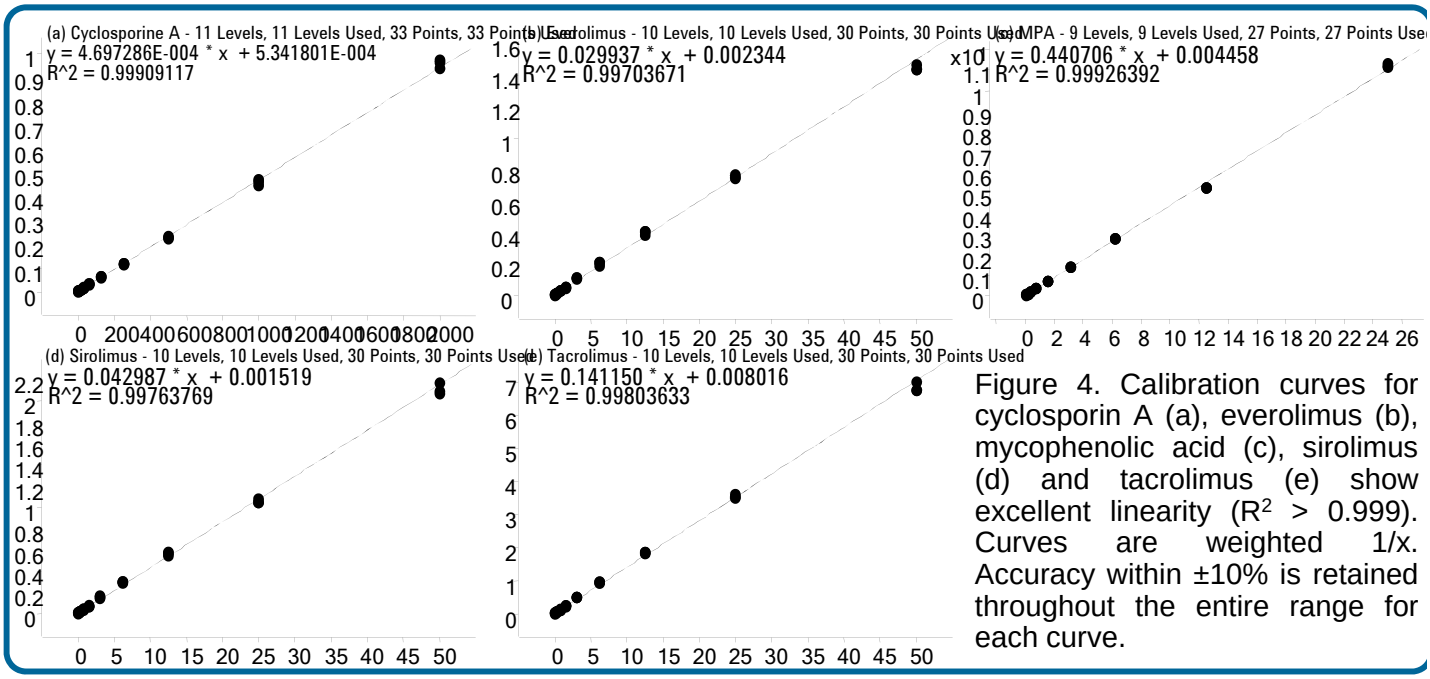


Figure 4. Calibration curves for cyclosporin A (a), everolimus (b), mycophenolic acid (c), sirolimus (d) and tacrolimus (e) show excellent linearity (R^2 > 0.999). Curves are weighted 1/x. Accuracy within ±10% is retained throughout the entire range for each curve.

All five immunosuppressive drugs show excellent linearity (R^2 > 0.999) and reproducibility. Both intraday and interday variations were less than 20% for all analytes at the lowest concentrations measured and less than 10% for all other concentrations. Signal to noise ratios and CVs indicate that LLOQs are lower than measured here for several analytes.

Compound	R^2	Level	Concentration (ng/ml)	Accuracy (%) n = 3	Intraday CV (%) n = 3	Interday CV (%) n = 6
Cyclosporin A	0.999	LLOQ	3.9	91.8	13.1	13.1
		MID	250.0	99.9	2.0	2.7
		ULOQ	2000.0	101.6	1.8	1.9
Everolimus	0.999	LLOQ	0.4	106.2	19.0	19.5
		MID	12.5	105.9	2.8	4.4
		ULOQ	50.0	96.5	1.3	1.7
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
Sirolimus	0.999	LLOQ	0.4	98.2	1.4	4.6
		MID	12.5	104.7	2.6	4.0
		ULOQ	50.0	98.0	2.6	1.9
Tacrolimus	0.999	LLOQ	0.2	94.2	10.9	11.6
		MID	12.5	104.7	0.8	3.0
		ULOQ	50.0	96.8	2.0	2.0

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

A specific, rapid and convenient method for quantifying five immunosuppressive drugs with excellent reproducibility and accuracy has been developed. Back-flushing, matrix-stripping liquid chromatography has been utilized to reduce the throughput of matrix to the mass spectrometer. This method utilizes the same hardware and reagents to quickly quantitate all five of the analytes allowing for the greatest flexibility while eliminating the need to maintain multiple configurations and solvents. Future work will include a faster method for the quantitation of cyclosporin A, everolimus, sirolimus and tacrolimus to increase throughput when MPA determination is not required.