Rapid Quantitation of five Immunosuppresants from body fluids

Siji Joseph [(1)], Sreelakshmy Menon [(1)], Linda Côté [(2)] and Kevin McCann[(3)]
[1];Agilent Technologies, Bangalore, India
[2];Agilent Technologies, Montréal, QC Canada
[3];Agilent Technologies, Santa Clara, CA USA

HPLC 2013
Poster number: BIOM25_MO

Agilent Technologies

Introduction

Two highly sensitive and specific research methods have been developed for 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 method with a run time of two minutes is suitable for the reliable quantification of CsA, Eve, Sir and Tac. The second method contains a longer gradient, required for the accurate analysis of Mycophenolic Acid (MPA). 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 other immunosuppressive drugs are measured in whole blood. Despite this difference, a single sample preparation and hardware configuration has been developed for the analysis of all five drugs.

Experimental

Procedure

Standards were purchased from Cerillant (Stored at –20°C). Calibration standard solutions in whole blood/plasma were prepared by a two fold serial dilution mode which covers biologically relevant concentrations and linearity curves were constructed. Quality Control (QC) samples from Chromsystems were used for recovery studies. Specific MRM transitions assured the specificity of each analytes. Internal standards (ISTD) were used for relative quantification and thus reduced the error due to loss of analytes during sample preparations. The list of analytes and corresponding internal standards are given in table 1. Linearity levels and QC samples were prepared by protein crash from a 100 µL blood/plasma using precipitation reagent followed by centrifugation. 1:4 v/v 0.4M ZnSO4:methanol with ISTDs was used as precipitating reagent. The details of protein precipitation procedure is given in Figure 1.

Table 1: List of analytes and corresponding ISTD

Analyte	Internal Standard	Method
Cyclosporin A	Cyclosporin D	2 Min
Everolimus	Ascomycin	
Sirolimus and Tacrolimus	Ascomycin	
MPA/MPA-G	Mycophenolic Acid-d3	4 Min

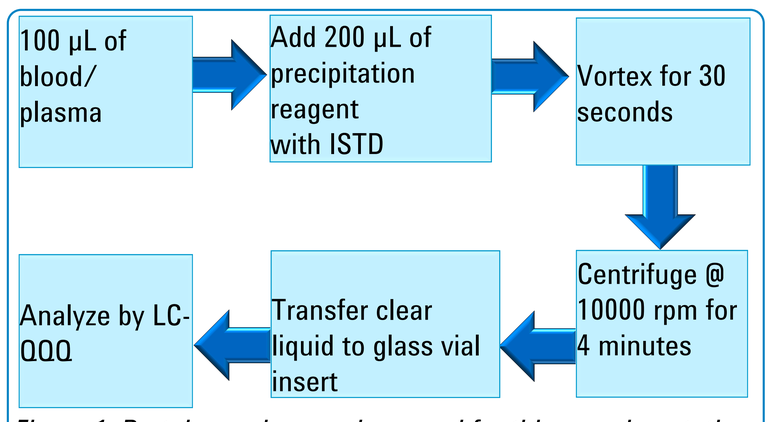

Figure 1: Protein crash procedure used for this experimentation

Instrumentation

An Agilent 1290 Infinity LC system with an additional 1260 Infinity Binary pump, coupled with a 6400 Series Triple Quadrupole Mass Spectrometer was used for the analysis. A back-flushing liquid chromatography configuration for online sample cleanup using 2-position/6-port valve has been implemented to reduce the contamination of the mass spectrometer by matrix compounds.

Table 2: LC Configuration and Parameters

Loading pump (1)	Agilent 1260 Infinity Binary
Analytical Pump (2)	Agilent 1290 Infinity Binary
Column thermostat	Agilent 1290 series with 2 Pos/6 port valve-head at 60°C
Column A	Zorbax Eclipse Plus C18, 2.1x12.5mm, 5µm
Column B	Poroshell 120 EC-C18, 3x50mm, 2.7µm
Injection volume	2min method: 40 µL 4min method: 2µL
Needle wash	Flush port using 1:1:1:1 Methanol: Acetonitrile: Isopropyl alcohol:H_2O + 0.1% FA (10 s for 2min method and 60 s for 4min method)
Mobile phase A	10mM NH4 acetate + 0.2% Formic acid in water
Mobile phase B	10mM NH4 acetate + 0.2% Formic acid in Methanol
Injector Temp	5°C

Experimental

Table 3: LC Gradient and Valve Timings for 2 min method

Loading (pump 1) Gradient			Loading (pump 2) Gradient			Valve Timings	
Time	Flow (mL/min)	%B	Time	Flow (mL/min)	%B	Time	Position
0	0.1	50	0	0.5	95	0	1
0.01	2.5	50	1.3	0.5	95	0.5	2
1.5	2.5	50	1.35	1	95	1.65	1
1.8	0.1	50	1.55	1	95		
2	0.1	50	1.65	0.5	95		
			2	0.5	95		

Table 4: LC Gradient and Valve Timings for 4 min method

Loading (pump 1) Gradient			Loading (pump 2) Gradient			Valve Timings	
Time	Flow (mL/min)	%B	Time	Flow (mL/min)	%B	Time	Position
0	0.1	20	0	0.5	50	0	1
0.01	2	20	1.9	0.5	95	0.5	2
2	2	20	1.95	1	95	2.4	1
2.4	2	20	2.4	1.2	95		
2.65	0.1	20	2.41	2	95		
			3.5	2	95		

The HPLC was configured for automated sample cleanup that includes two binary pumps (Figure 2). Samples were loaded onto a trapping column where the analytes were retained and washed by the first pump (position1). The wash was sent to waste, reducing the amount of matrix introduced into the mass spectrometer. Shortly before the analytes elute from the trapping column, the valve was switched to position 2 (back–flush) and the analytes were eluted onto an analytical column where further chromatography was performed using the second binary pump.

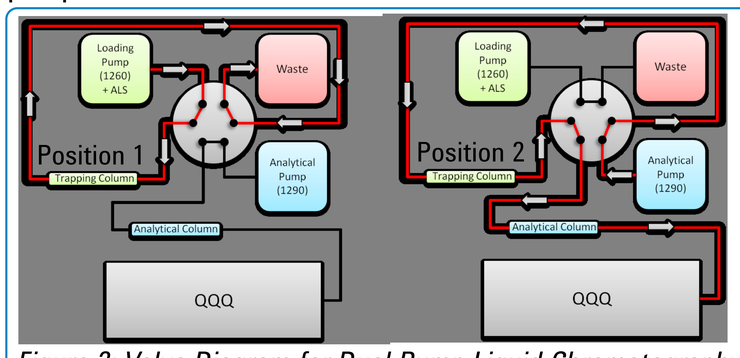

Figure 2: Valve Diagram for Dual Pump Liquid Chromatography

Table 5: MS/MS conditions using Agilent 6460 QqQ LC/MS equipped with Agilent JetStream ESI source.

Ion mode	Positive
Drying gas temperature	225°C
Drying gas flow	9L/min
Nebulizer pressure	35 psi
Sheath gas temperature	325 °C
Sheath gas flow	12 L/min
Capillary voltage	4,000 V
ΔEMV	200 V (0v for MPA)
Nozzle voltage	300v
Q1/Q3 resolution	0.7 unit

Table 6: Quantifier MRM Transitions monitored

Compound	Precursor	Product	Dwell (msec)	Frag. (v)	CE (v)
Cyclosporine D	1233.9	1216.9	10	175	12
Cyclosporine A	1219.9	1202.8	10	175	12
Everolimus D4	979.6	912.5	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
MPA	321.1	207.0	10	80	16
MPA-G	514.2	207.0	10	95	36
MPA_D3	324.2	210.1	10	80	16

Results and Discussion

Representative chromatogram for 2min and 4 min methods are given in figure 3 and 4. The excellent reproducibility of the retention time guaranteed the repeatability of the method. Extracted Ion Chromatograms (EICs) of MRM transitions were employed for quantitation. Excellent separation was observed between MPA and MPA-G in 4 min method. The significant abundance of analytes at lower linearity concentrations assured the remarkable sensitivity of method.

Results and Discussion

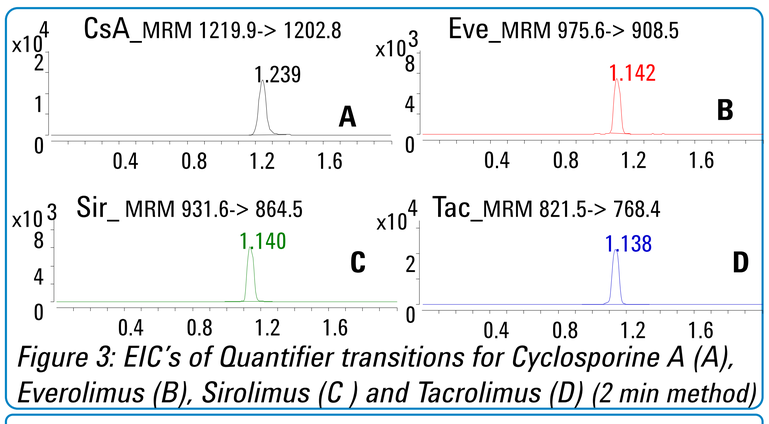

Figure 3: EIC's of Quantifier transitions for Cyclosporine A (A), Everolimus (B), Sirolimus (C) and Tacrolimus (D) (2 min method)

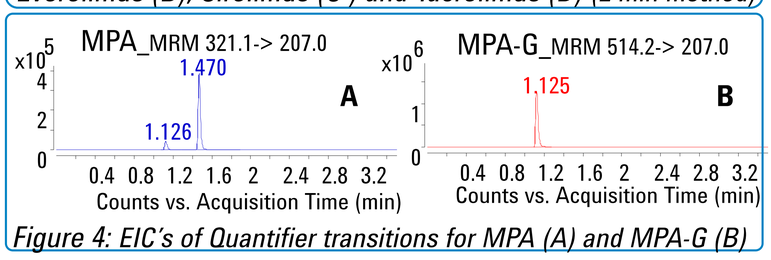

Figure 4: EIC's of Quantifier transitions for MPA (A) and MPA-G (B)

Calibration curves were constructed for all the analytes. Excellent linearity was obtained for all analytes, with R^2 values >0.99 (Figures 5 and 6). (*Type: Linear, Origin: Ignore, Weight:1/x)*

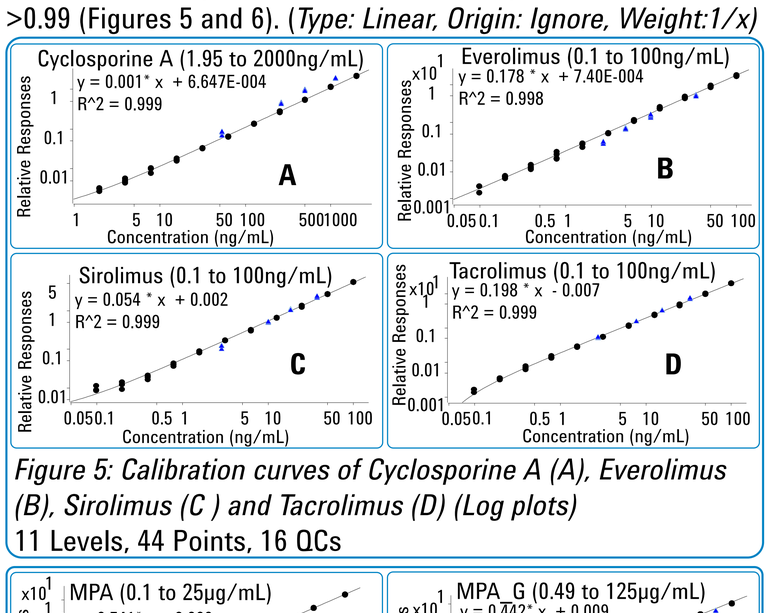

Figure 5: Calibration curves of Cyclosporine A (A), Everolimus (B), Sirolimus (C) and Tacrolimus (D) (Log plots)
11 Levels, 44 Points, 16 QCs

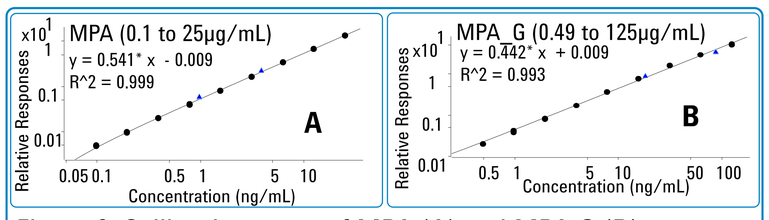

Figure 6: Calibration curve of MPA (A) and MPA-G (B)
9 Levels, 36 Points, 8 QCs

The observed RT RSD and accuracies for each level and also for QC's are tabulated in table 7. The recovery range was within 80-115%

Table 7: Representative accuracy and precision for analytes

Sample	RT RSD (%)	Accuracy (%)	CV (%)	RT RSD (%)	Accuracy (%)	CV (%)
	Cyclosporine A			Everolimus		
QC1	0.40	107.5	1.1	0.26	80.5	3.9
QC2	0.40	114.0	3.0	0.26	84.2	4.6
QC3	0.0	113.3	3.4	0.0	88.0	4.2
QC4	0.24	113.4	0.9	0.0	88.7	3.6
	Sirolimus			Tacrolimus		
QC1	0.2	81.3	4.2	0.0	102.0	2.5
QC2	0.0	94.4	2.7	0.0	105.4	1.2
QC3	0.0	108.6	3.8	0.0	107.7	3.9
QC4	0.0	109.1	4.1	0.26	109.3	3.0
	MPA			MPA-G		
QC1	0.12	114.4	1.15	0.21	113.0	1.18
QC2	0.10	111.0	1.17	0.10	89.3	2.40

Conclusions

- A high throughput two minute method for four immunosuppressive drugs was developed using an Agilent 6460 Triple Quadrupole LC/MS.
- This two-minute method was used for the quantitation of cyclosporine A, everolimus, sirolimus and tacrolimus (when MPA determination is not required).
- A four minutes method was developed to quantify mycophenolic acid, with excellent separation between MPA and its glucuronide.
- A simple protein crash extraction followed by automated online sample cleanup using a 2-position/ 6-port valve minimized the matrix effect and ion suppression due to biological compounds present in blood/plasma
- Excellent linearity of all analytes has been demonstrated over the biologically relevant range.
- Same instrument configuration was used for both methods and this approach allowed flexibility and eliminated the need to maintain multiple configurations and solvents.
- "No compound missed".