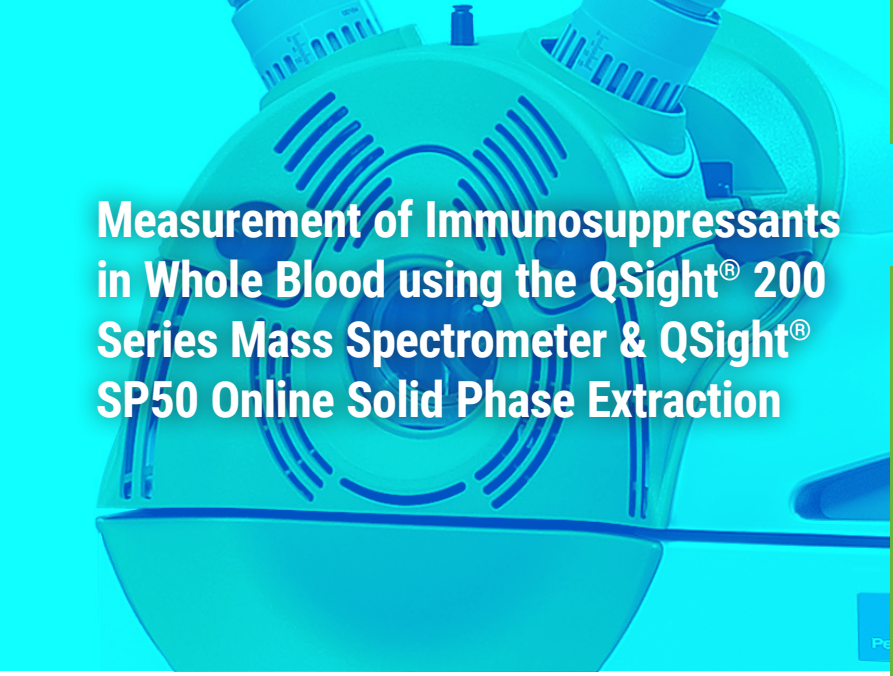

Measurement of Immunosuppressants in Whole Blood using the QSight® 200 Series Mass Spectrometer & QSight® SP50 Online Solid Phase Extraction

APPLICATION NOTE

QSight® 200 Series
Mass Spectrometer

AUTHORS

Andreu Fabregat Rossell
PerkinElmer, Inc. Madrid, Spain

Roberto Bozic, Lorenzo Bacci, Aristide Ganci
PerkinElmer, Inc. Milan, Italy

Elisa Bassotti
Eureka srl - Lab Division, Chiaravalle, Italy

INTRODUCTION

Immunosuppressants are drugs used to suppress or prevent human immune response. Some drugs have a widespread effect on the immune system, while others act on a specific target. The main immunosuppressive drugs used in the clinical domain are Cyclosporine A, Tacrolimus, Sirolimus and Everolimus (1).

Cyclosporin is a calcineurin inhibitor. It is a fungal peptide composed of 11 amino acids. Cyclosporin acts by binding to the cytosolic protein cyclophilin (an immunophilin) of immunocompetent lymphocytes, especially T-lymphocytes. The drug also inhibits lymphokine production and interleukin release, leading to reduce function of T-cells (2).

Tacrolimus is a fungal product. It is a macrolide lactone and acts by inhibiting calcineurin. It binds to an immunophilin, followed by the binding of the complex to calcineurin and the inhibition of its phosphate activity (2).

Sirolimus is a macrolide lactone and it is a structural analogue of tacrolimus. However, it acts differently. Tacrolimus affects the signal transduction of the T lymphocyte activation. It binds to the same receptor as tacrolimus, however the produced complex does not inhibit calcineurin but another protein (2).

Finally, **Everolimus** is a derivative of sirolimus and works similarly as an inhibitor of the T lumphocyte activation (2).

These drugs have a narrow therapeutic index as well as significant toxicities. Both sub- and supratherapeutic levels of these agents may cause significant morbidity and mortality. For this matter, a method that can accurately detect and quantify them is of outmost importance.

PerkinElmer®
For the Better

These drugs have a narrow therapeutic index as well as significant toxicities. Both sub- and supratherapeutic levels of these agents may cause significant morbidity and mortality. For this matter, a method that can accurately detect and quantify them is of outmost importance.

Here, we present a method for the detection of immunosuppressants in whole blood by LC-MS/MS using Qsight® 200 series mass spectrometer together with QSight® SP50 online solid phase extraction and a commercial kit. This LC-MS/MS method provides a fast, sensitive, accurate and reproducible solution for the determination and quantification of Tacrolimus, Sirolimus, Everolimus and Cyclosporine A (Fig 1).

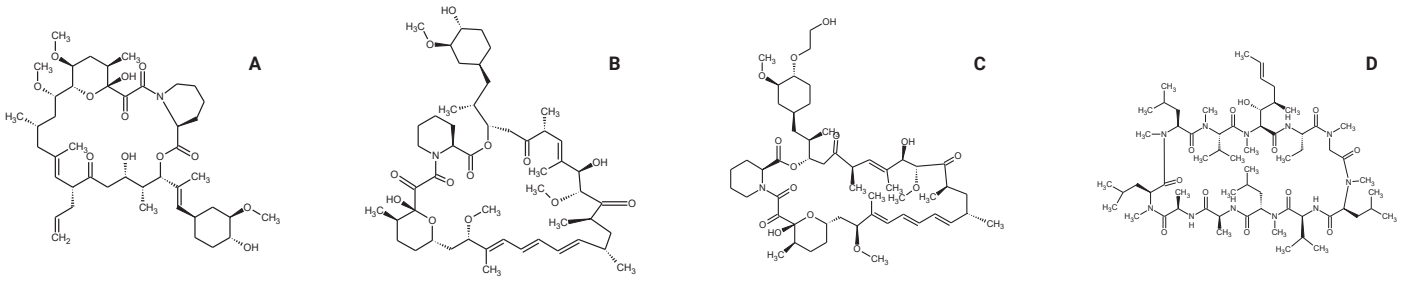

Figure 1: *Chemical structure of a) Tacrolimus, b) Sirolimus, c) Everolimus and d) Cyclosporin*

Experimental

Chemicals and Materials

The LC75110 Eureka kit for determination of immunosuppressants in whole blood by LC-MS/MS (Eureka Lab division, Ancona, Italy) consisted of quality control (QC) samples, calibrators, lysing solution, deproteinization solution, diluting solutions, stable isotope labeled internal standard, mobile phase A and B.

Sample Preparation

The samples were prepared according to the instructions provided by the kit manufacturer. Briefly, 50 µL of blood sample (calibrators or QCs) was mixed with 20 µL for Internal standard solution (previously prepared) and vortex for 10 seconds. After this, 100 µL of lysing solution was added to the mix and vortex for 10 seconds. Finally, 300 µL of deproteinization solution were added and everything was mixed for 20 seconds. The solution was finally centrifuge at 14.000 rmp for 10 minutes and 20 µL of the supernatant were injected into LC-MS/MS for the analysis.

Mass Spectrometry Conditions

The LC-MS/MS analysis was performed using a QSight® 220 mass spectrometer equipped with ESI source operating in positive ion mode. Table 1 outlines the MS instrumental source parameter settings. The optimized MRM transition parameters for analytes involved in this assay are shown in Table 2.

Table 1: *MS source conditions.*

ESI Voltage (V)	4750
HSID Temp (°C)	200
Nebulizer Gas Setting	250
Drying Gas Setting	150
Source Temp. (°C)	240

Table 2: *Optimized MRM parameters.*

Compound	Type	Precursor (m/z)	Fragment (m/z)	CCL1	CCL2	CCL3	CCL4	PF	CC	EV	ID	Scan time (ms)
Tacrolimus	Quant	821.3	768.3	-22	-95	-28	-46	-19	-27	20	-146	25
	Quant IS	824.3	771.3	-22	-95	-28	-46	-19	-27	20	-146	25
Sirolimus	Quant	931.3	864.3	-28	-81	-24	-40	-21	-34	22	-164	25
	Quant IS	935.3	864.3	-28	-81	-24	-40	-21	-24	22	-164	25
Everolimus	Quant	975.4	908.4	-37	-73	-25	-46	-22	-24	29	-160	25
	Quant IS*	981.4	914.4	-37	-73	-25	-46	-22	-24	29	-160	25
Cyclosporine A	Quant	1219.5	1202.6	-47	-103	-28	-28	-26	-28	11	-188	25
	Quant IS*	1223.6	1206.7	-47	-103	-28	-28	-26	-28	11	-188	25

LC Conditions

Online SPE purification of the sample and chromatographic separation were accomplished with a QSight® SP50 Online SPE System.

Online SPE is accomplished through two six-port valves on the autosampler and a high-pressure dispenser (HPD). Per Figure 2, valve A is dedicated to SPE, while valve B allows for the flexible switching from direct injection to online SPE mode.

The system was configured with a 10 µL stainless steel needle, 50 µL sample loop, 250 µl needle and 2 mL buffer tubing. Conditioning and equilibration solvents are delivered via the HPD and are directed to waste upon passing through the SPE cartridge (table 3). The sample is then aspirated into the sample loop using the autosampler syringe and subsequently transferred via a load solvent from the loop to the SPE cartridge. Analytes are then eluted off the SPE cartridge and onto the analytical column using the LC gradient. There is no separate SPE elution step needed, as the focused analytes on the SPE cartridge are eluted right onto the analytical column, as part of the chromatographic run.

After sample elution a software-controlled diverter valve was set to waste position between 0 to 0.6 min, while the LC flow was switched to enter the mass spectrometer position from minute 0.6 to 1.4, before being switched back to the initial (table 4).

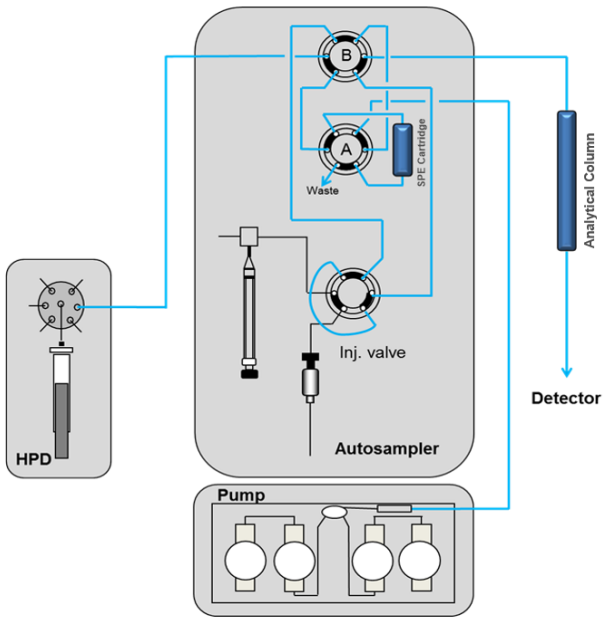

Figure 2: *Schematic of QSight SP-50 Automated Sample Handler*

The SPE and LC method parameters are shown in Tables 3 and 4 respectively. This method was performed with a SPE online column PFP propyl column (10 x 40 mm), a C18 analytical column (2.1 x 50 mm, 1.8 µm) and an in-line guard cartridge (10 mm), all included in the kit.

Table 3: *SPE Parameters*

SPE Cartridge	PFP propyl column (10X40 mm)
SPE Solvents	Phase B Phase A
SPE Program	Step 1: Wash/Conditioning — Flow (mL/min): 2.0 — Phase B (mL): 0.5 — Phase A (mL): - Step 2: Equilibration — Flow (mL/min): 2.0 — Phase B (mL): - — Phase A (mL): 0.75 Step 3: Loading — Flow (mL/min): 1.0 — Phase B (mL): - — Phase A (mL): 0.5 Elution time: 1.2 min.

Table 4: *LC Parameters used to separate Immunosuppressants*

Analytical column	C18 column (2.1 x 50 mm, 1.8 µm)
Guard cartridge	10 mm in-line
Mobile Phase	Mobile Phase A Mobile Phase B Solvent Program

Step	Time (min)	Flow (mL/min)	%A	%B
1	0	0.6	100	0
2	0.5	0.6	5	95
3	1.2	0.6	5	95
4	1.21	0.6	100	0

Diverter valve switched to waste between 0 and 0.6 min and between 0.6 and 1.4 to enter the mass spectrometer

Wash solvent	250 µL of Methanol
Oven Temperature	60 °C
Analysis Time	2.5 min sample to sample
Pressure	~5000 psi/345 bar

Results & Discussion

As can be observed in figure 3, the QSight® series 200 is able to reach the low-level calibrator for each compound with a good sensitivity offering a robust solution for the analysis of immunosuppressants.

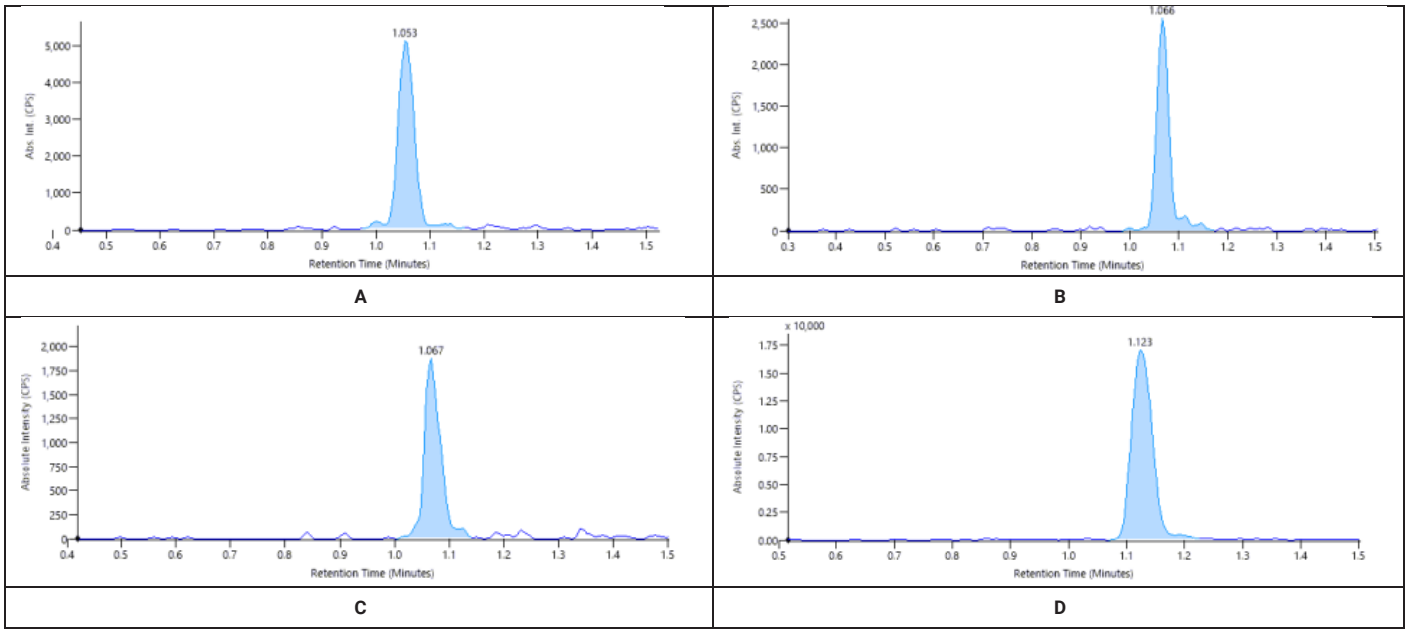

Figure 3: *Chromatograms for (a) Tacrolimus at 0.9 ng/mL, (b) Sirolimus at 1.7 ng/mL, (c) Everolimus at 1.4 ng/mL and (d) Cyclosporine A at 11 ng/mL*

The linearity of the method was tested using 6 calibration points for each compound by triplicate, the linearity ranges can be seen in table 5. The results showed good results for linearity for all compounds with an R2>0.99 in all the analytes. Figure 4 shows the regression fit with 1/x weighting for all the compounds.

Moreover, results for accuracy and reproducibility were also satisfactory for all analytes as can be seen in table 5 with an accuracy between 92-107% and RSD precision always below 10%.

Table 5: *Linearity, accuracy and precision for immunosuppressants LC-MS/MS method.*

Compound	Linearity range (ng/mL)	Linearity (R^2)	Retention Time (RT)	Level	Concentration	Accuracy mean % (n=3)	Precision RSD % (n=3)
Tacrolimus	0.9-34.6	0.998	1.0	C1 C2 C3 C4 C5 C6	0.9 1.8 4.3 8.3 16.3 34.6	102 100 98 100 100 99	0.3 3.3 1.0 3.5 1.4 1.3
Sirolimus	1.7-49.9	0.998	1.0	C1 C2 C3 C4 C5 C6	1.7 3 6.7 12.5 23 49.9	104 95 100 95 101 100	0.7 3.6 6.1 2.5 2.3 4.8
Everolimus	1.4-39.6	0.999	1.0	C1 C2 C3 C4 C5 C6	1.4 2.4 6.7 9.4 21.1 39.6	102 101 92 103 104 98	5.4 2.4 2.5 2.3 2.7 2.6
Cyclosporine A	11-929.7	0.998	1.1	C1 C2 C3 C4 C5 C6	11 41.5 86.8 198.8 406.8 929.7	96 98 104 100 100 98	1.4 6.1 4.4 2.5 2.9 1.7

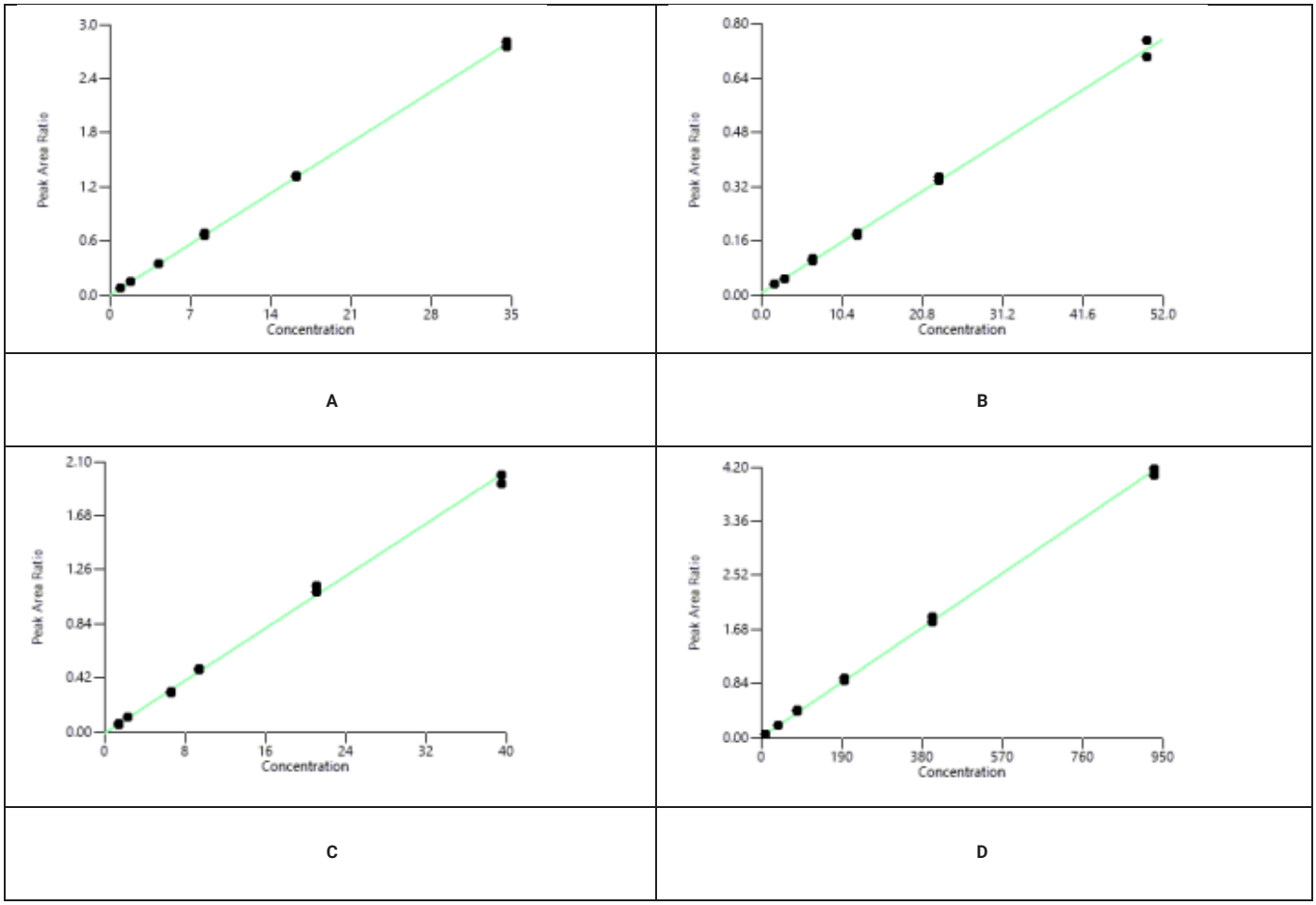

Figure 4: *Regression curve for a) Tacrolimus, b) Serolimus, c) Everolimus and d) Cyclosporine*

The applicability of the method was demonstrated by injecting different quality controls levels with known concentration of each compound (L1, L2 and L3) 5 times. As detailed in table 6, the quantification obtained for these quality controls was always with an accuracy between 90-108% demonstrating the good performance of the method with real samples.

Table 6: *Quality control accuracy results*

Compound	Level	Spiked amount (ng/mL)	Quantification (ng/mL)	Accuracy (%)
Tacrolimus	L1	2.8	2.7	98
	L2	12	12.4	104
	L3	25.4	25.5	100
Sirolimus	L1	4.4	3.9	90
	L2	16.5	16.3	99
	L3	33.5	34.7	104
Everolimus	L1	3.7	3.8	105
	L2	16.1	16.6	104
	L3	28.4	30.3	107
Cyclosporine A	L1	63.5	63.3	100
	L2	310.1	325.8	105
	L3	605.6	655	108

Conclusion

The QSight® 200 series UHPLC-MS/MS system equipped with SP50® online solid phase extraction offers an excellent tool for the determination and quantification of immunosuppressants in whole blood. This method in combination with the Eureka commercial kit provide a superb analytical performance for the measurement of this analytes in blood.

References

1. *Levy GL, Long-term immunosuppression and drug interactions. Live Transplantation 2001; 11:S53-S59*
2. *Armstrong SC, Cozza KL, Pimentel EA, Med-phys drug-drug interactions update. Psychosomatics 2004. 45:354-360*

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

PerkinElmer®

AG012207_25_AP PKI