

Immunosuppressants in whole blood by LC-MS – Code LC75110
(Tacrolimus, Sirolimus, Everolimus, Cyclosporine A)

INTRODUCTION

Immunosuppressive drugs are used to control of severe allergic reactions, autoimmune diseases and diseases related to transplantation. Some drugs have a widespread effect on the immune system, while others act on a specific target. The main immunosuppressive drugs used are Cyclosporine A, Tacrolimus, Sirolimus and Everolimus.

Cyclosporine A has an active response that influences individual immunity. Like the others, this immunosuppressant is the natural product of certain fungi, and is the main therapeutic agent used to stop the rejection of an allogeneic. It functions primarily by blocking the transcription of cytokine genes in T lymphocytes.

Tacrolimus (also known as FK-506) is an immunosuppressive drug used especially in organ transplantation to reduce the activity of the patient's immune system and consequently the risk of rejection.

It has features similar to Cyclosporine, but is much more powerful at the same volume. Like Cyclosporine has a broad spectrum of negative interactions, including one with grapefruit which increases the drug concentration in blood plasma.

It is also used in topical preparations for the treatment of severe dermatitis.

Sirolimus and **everolimus** are gaining more and more support in the management of renal transplant as they represent an ideal strategy to reduce the use of calcineurin inhibitors in patients at low immunologic risk.

These drugs appear to be associated with a lower risk of hypertension and glucose intolerance. Although characterized by a lower nephrotoxicity than calcineurin inhibitors, they could enhance the renal toxicity of Cyclosporine and therefore require constant monitoring of renal function. The use of Sirolimus and Everolimus should be avoided perisurgical because their administration may cause significant delay in wound healing.

The budget risk / benefit of continuous therapy with these drugs in patients who develop an refractory hyperlipidemia, should be carefully reassessed.

Therapeutic drug monitoring of these medications is essential because of the risk of toxic effects such as anemia, leukopenia and thrombocytopenia.

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This product fulfills all the requirements of Directive 98/79/EC on in vitro diagnostic medical devices (IVD). The declaration of conformity is available upon request.

Release N° 005	Immunosuppressants in whole blood by LC/MS	May 2018
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TECHNICAL FEATURES

Principle of the Method :

This method determines the Immunosuppressants in whole blood after a simple deproteinization and reading in LC-MS.

Recovery : > 98 %

Sensitivity :

Tacrolimus	0,01 ng/ml
Sirolimus	0,01 ng/ml
Everolimus	0,01 ng/ml
Cyclosporine A	1,0 ng/ml

Minimum concentration analyzable (LLOQ) :

Tacrolimus	0,05 ng/ml
Sirolimus	0,05 ng/ml
Everolimus	0,05 ng/ml
Cyclosporine A	0,05 ng/ml

Linearity :

Tacrolimus	0,05 - 100 ng/ml
Sirolimus	0,05 - 100 ng/ml
Everolimus	0,05 - 100 ng/ml
Cyclosporine A	0,05 – 1.500 ng/ml

Accuracy intra serie (relative error %) Tacrolimus:

Cm	Cs
2,5 ng/ml	12,5 ng/ml
4,2%	5,1%

Accuracy inter serie (relative error %) Tacrolimus:

Cm	Cs
2,5 ng/ml	12,5 ng/ml
8,6%	9,9%

Reproducibility intra serie (relative error %) Tacrolimus:

C LLOQ	Cm	Cs
0,05 ng/ml	1,25 ng/ml	25 ng/ml
1,6%	4,6%	4,4%

Reproducibility inter serie (coefficient of variation %) Tacrolimus:

C LLOQ	Cm	Cs
0,05 ng/ml	1,25 ng/ml	25 ng/ml
16,9%	7,1%	3,7%

Accuracy intra serie (relative error %) Sirolimus:

Cm	Cs
2,5 ng/ml	12,5 ng/ml
5,3%	11,3%

Accuracy inter serie (relative error %) Sirolimus:

Cm	Cs
2,5 ng/ml	12,5 ng/ml
7,2%	7,1%

Reproducibility intra serie (relative error %) Sirolimus:

C LLOQ	Cm	Cs
0,05 ng/ml	1,25 ng/ml	25 ng/ml
9,7%	8,1%	1,9%

Reproducibility inter serie (coefficient of variation %) Sirolimus:

C LLOQ	Cm	Cs
0,05 ng/ml	1,25 ng/ml	25 ng/ml
8,4%	7,0%	5,6%

Accuracy intra serie (relative error %) Everolimus:

Cm	Cs
2,5 ng/ml	12,5 ng/ml
6,6%	6,0%

Accuracy inter serie (relative error %) Everolimus:

Cm	Cs
2,5 ng/ml	12,5 ng/ml
8,2%	6,8%

Reproducibility intra serie (relative error %) Everolimus:

C LLOQ	Cm	Cs
0,05 ng/ml	1,25 ng/ml	25 ng/ml
11,7%	4,0%	2,1%

Reproducibility inter serie (coefficient of variation %) Everolimus:

C LLOQ	Cm	Cs
0,05 ng/ml	1,25 ng/ml	25 ng/ml
8,5%	6,6%	3,5%

Accuracy intra serie (relative error %) Cyclosporine A:

Cm	Cs
60 ng/ml	300 ng/ml
3,4%	10,1%

Accuracy inter serie (relative error %) Cyclosporine A:

Cm	Cs
60 ng/ml	300 ng/ml
12,0%	13,9%

Reproducibility intra serie (relative error %) Cyclosporine A:

C LLOQ	Cm	Cs
0,05 ng/ml	25 ng/ml	500 ng/ml
9,9%	3,7%	2,2%

Reproducibility inter serie (coefficient of variation %) Cyclosporine A:

C LLOQ	Cm	Cs
0,05 ng/ml	25 ng/ml	500 ng/ml
15,8%	7,3%	2,9%

Coefficient of correlation R2 + DS :

Tacrolimus	$0,9951 \pm 0,0026$
Sirolimus	$0,9939 \pm 0,0026$
Everolimus	$0,9964 \pm 0,0005$
Cyclosporine A	$0,9941 \pm 0,0060$

Therapeutic Range:

Tacrolimus	1 – 50 ng/ml
Sirolimus	1 – 50 ng/ml
Everolimus	1 – 50 ng/ml
Cyclosporine A	10 – 2000 ng/ml

Components of the kit:

All the reagents are stable 3 years at 2 – 8 °C except the Reagent A and B that must be stored at –20 °C.

Reagent A – Deproteinization Sol, 1 x 30 ml

Reagent B – Internal Standard, 1 x 400 µl

Stable 6 months at –20 °C

Reagent C – Lysing Sol, 1 x 10 ml

Calibrator on whole blood – Level 0, 1 x 1 ml

Code LC75016 (packed separately – see data sheet)

Calibrator on whole blood – Level 1, 1 x 1 ml

Code LC75016 (packed separately – see data sheet)

Calibrator on whole blood – Level 2, 1 x 1 ml

Code LC75016 (packed separately – see data sheet)

Calibrator on whole blood – Level 3, 1 x 1 ml

Code LC75016 (packed separately – see data sheet)

Calibrator on whole blood – Level 4, 1 x 1 ml

Code LC75016 (packed separately – see data sheet)

Calibrator on whole blood – Level 5, 1 x 1 ml

Code LC75016 (packed separately – see data sheet)

Calibrator on whole blood – Level 6, 1 x 1 ml

Code LC75016 (packed separately – see data sheet)

Reagent M1 – Mobile Phase M1, 2 x 500 ml

Reagent M2 – Mobile Phase M2, 1 x 500 ml

Minimum Instrumental equipment required :

LC-MS Instrument triple quadrupole (mode MRM)
Divert Valve at 6 ways
ESI (+)Interface
Binary Pump
Loop 50 µl
Operational Computer

Whole Blood Collection Procedure :

Test tube with anticoagulant EDTA
Stable 4 weeks at 2 – 8 °C or 4 months at – 20 °C.

ANALYTICAL PROCEDURE:

STEP 1 : Sample Preparation

Dispense 100 µl of **Reagent B - Internal Standards** in each bottle of 10 ml, 400 µl of Methanol and 500 µl of Water HPLC grade.

Vortex for 5 sec.

N.B.: 20 µl of diluted sample standard is required, then prepare an appropriate volume of solution.

STEP 2 : Dispense in eppendorf vials:

	Calibrator	Sample	Control
Calibrator	50 µl		
Sample		50 µl	
Control			50 µl
Internal Standard (diluted as in STEP 1)	20 µl	20 µl	20 µl
	<u>Vortex for 10 sec.</u>		
Reagent C – Lysing Solution	100 µl	100 µl	100 µl
	<u>Vortex for 10 sec.</u>		
Reagent A – Deproteinization Solution	300 µl	300 µl	300 µl
	<u>Vortex for 10 sec.</u>		

Vortex for 20 sec.

STEP 3 : Centrifuge at 14.000 rpm for 10 minutes

STEP 4 : Take the supernatant and put in a glass vial

INJECTION :

- Inject 10 µl of solution in LC system

Immunosuppressants in whole blood - Warnings

COLUMN CONDITIONING

Install a new analytical column RRHD Eclipse Plus C18 (2,1 mm x 50 mm – 1,8 µm) thermostated at 60°C after the valve. Disconnect the detector and flux a solution of H₂O : Mobile Phase M2 (5 : 95 v/v) at the flow of 500 µl / min. **Don't recycle the washing solutions.** Condition the column with a solution of Mobile Phase M1 : Mobile Phase M2 (5 : 95 v/v) set flow 500 µl / minute, with the valve in position B, for 10 minutes. Then, with the valve in the position A, flux with 100% of Mobile Phase M1 for 5 minutes. Then, make 4 injections of mobile phase before analytical session.

It's NOT possible to make analysis at recycling phase.

If room temperature is > 20 °C store the Mobile Phase at 2-8 °C between an analytical session and another.

COLUMN CLEANING

Disconnect the detector. Flux a solution of H₂O : Mobile Phase M2 (5 : 95 v/v) at the flow of 500 µl / min for 15 minutes. Discharge. Store the column in the same solution.

ENRICHMENT COLUMN

Monodimensional

TF25403012101 Enrichment Column Hypersil GOLD PFP Guard 10 x 2.1 mm, 3 µm

TF84200 Drop in Holder 2 mm

Bidimensional

ZRE917950210 Enrichment Column Ultra PFPP 10 x 4.0 mm (SPE ON-LINE/Bidimensional Modality)

ZRE25021 Holder for 10 mm Guard Trident Guard System

Install enrichment column before valve and condition as analytical column.

AUTOSAMPLER SYRINGE WASHING

Wash the syringe and the needle, before every analytical session, with Methanol.

PARAMETERS SET ON TRIPLE QUADRUPOLE AGILENT 6460

Parameters ESI 6460

Gas Temperature 250 °C
Gas Flow 5 L/min
Nebulizer 60 psi
Sheath Gas Heater 300 °C
Sheath Gas Flow 12 L/min
Capillary 4000 V

PARAMETERS SET ON TRIPLE QUADRUPOLE SCIEX 4500

Parameters ESI + 4500

Curtain Gas (CUR): 25
Collision Gas (CAD): 10
Ion Spray Voltage: (IS): 5500 V
Temperature (TEM): 550°C
Ion Source Gas 1 (GS1): 60
Ion Source Gas 2 (GS2): 60

Fragmentations (optimized by TRIPLE QUADRUPOLE AGILENT 6460)

Analyte	Q1	Q3	COLLISION ENERGY
Tacrolimus	821,5	768,5	20
Sirolimus	931,6	864,5	14
Everolimus	975,6	908,6	12
Cyclosporine A	1.219,9	1.202,9	14
² H ₄ Cyclosporine A	1.223,7	1.206,9	14
¹³ C ₂ ² H ₄ Everolimus	981,3	914,5	14
¹³ C ₂ ² H ₂ Tacrolimus	824	771	20
¹³ C ₂ ² H ₃ Sirolimus	935,4	864,6	14

FLOW SET

GRADIENT			
Time (min)	% M1 (PUMP A)	% M2 (PUMP B)	Flow (µl/min)
0.00	100	0	500
0.50	100	0	500
SWITCHING VALVE			
0.51	100	0	600
1.50	5	95	600
3.00	5	95	600
SWITCHING VALVE			
3.01	100	0	500
3.50	100	0	500

REAGENT B – INTERNAL STANDARD SOLUTION:

Solution with: ¹³ C ₂ ² H ₂ Tacrolimus, ¹³ C ₂ ² H ₄ Everolimus, ¹³ C ₂ ² H ₃ Sirolimus, ² H ₄ Cyclosporine A. Store at – 20 °C.	
Immunosuppressants:	Internal Standard for calibration:
TACROLIMUS	¹³ C ₂ ² H ₂ Tacrolimus
EVEROLIMUS	¹³ C ₂ ² H ₄ Everolimus
SIROLIMUS	¹³ C ₂ ² H ₃ Sirolimus
CYCLOSPORINE A	² H ₄ Cyclosporine A

ACCESSORIES AND CONSUMABLES

CODE	DESCRIPTION	PACKAGING
LC75016	Calibrator in whole blood for Immunosuppressants	7 x 2 x 1 ml
LC75017	Control in whole blood for Immunosuppressants – Level 1	5 x 1 ml
LC75018	Control in whole blood for Immunosuppressants – Level 2	5 x 1 ml
LC75019	Control in whole blood for Immunosuppressants – Levels1 and 2	2 x 5 x 1 ml
SK75210	Starter kit for Immunosuppressants	1 Pc
For UHPLC systems		
Z959757902	RRHD Eclipse Plus C18 (50 x 2,1 mm - 1,8 um) Analytical Column	1 Pc
For HPLC systems		
TF17126032130	ACCUCORE C 18 (30 x 2,1 mm, 2,6 um) Analytical Column	1 Pc
Monodimensional		
TF25403012101	Prefilters Hypersil GOLD PFP Guard (10 x 2,1 mm, 3 um)	1 Pc
TF84200	2mm DROP-IN HOLDER	1 Pc
Bidimensional		
ZRE917950210	Ultra PFPP 10 x 4,0 mm (SPE ON-LINE/Bidimensional Modality)	1 x 3 Pcs
ZRE25021	Holder for 10 mm Guard Trident Guard System	1 Pc
Z51843550	Clear glass vials with reduced volume from 1,5 ml to 15 ul	1 x 100 Pcs
Z51820717	Caps for Clear glass vials with reduced volume from 1,5 ml to 15 ul	1 x 100 Pcs



Immunosuppressants in whole blood

(Reference Chromatograms/Spectrum by LC-MS)

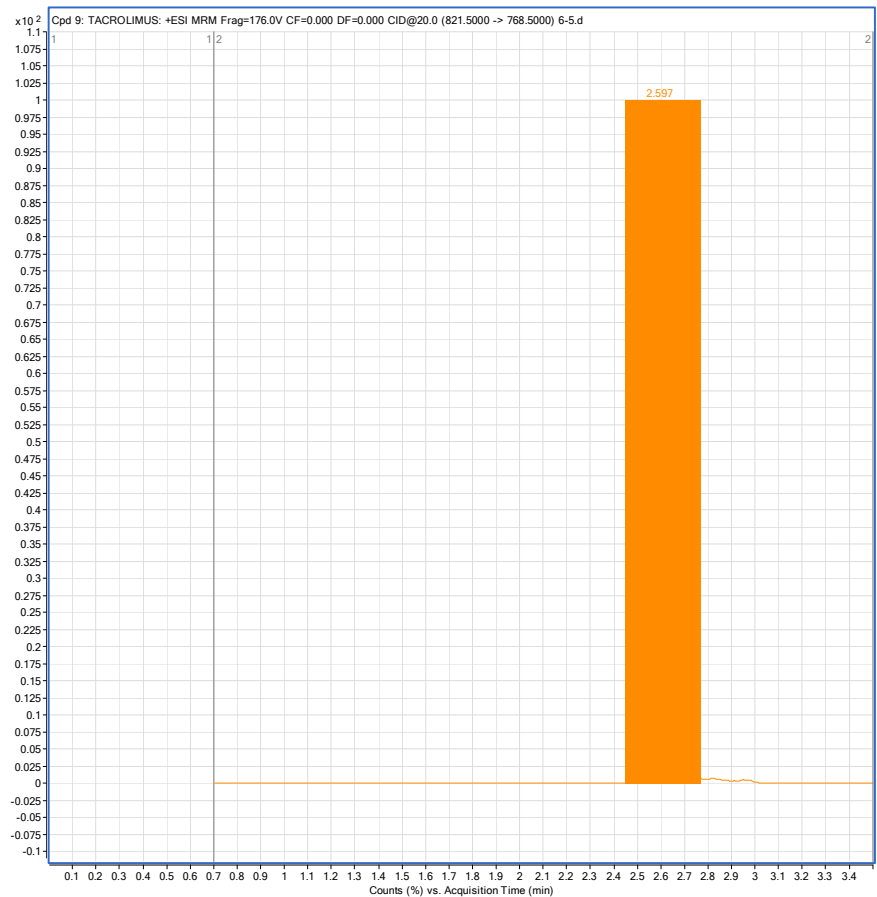


Fig. 1 : Tacrolimus

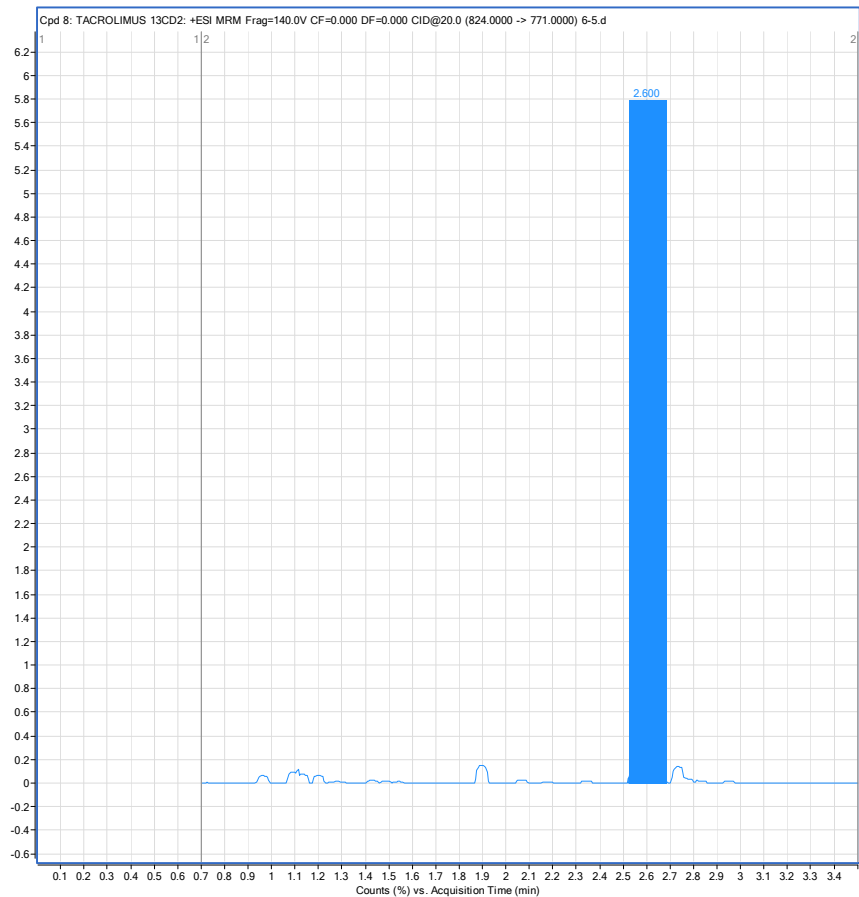


Fig. 2 : Internal Standard Tacrolims

Immunosuppressants in whole blood
(Reference Chromatograms/Spectrum by LC-MS)

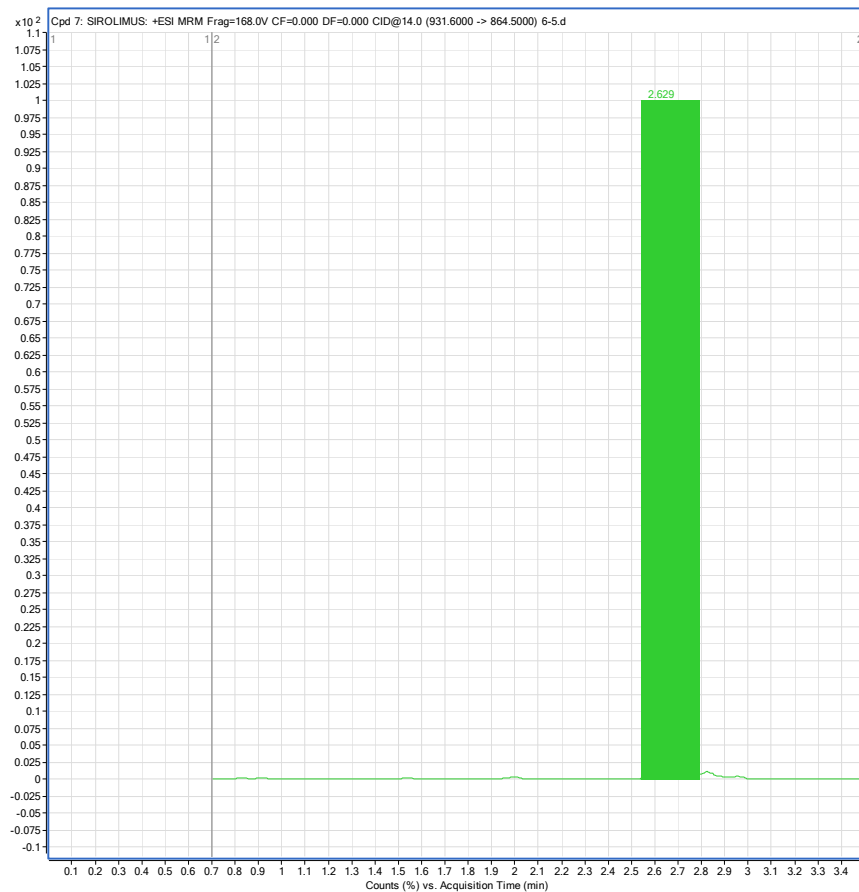


Fig. 3 : **Sirolimus**

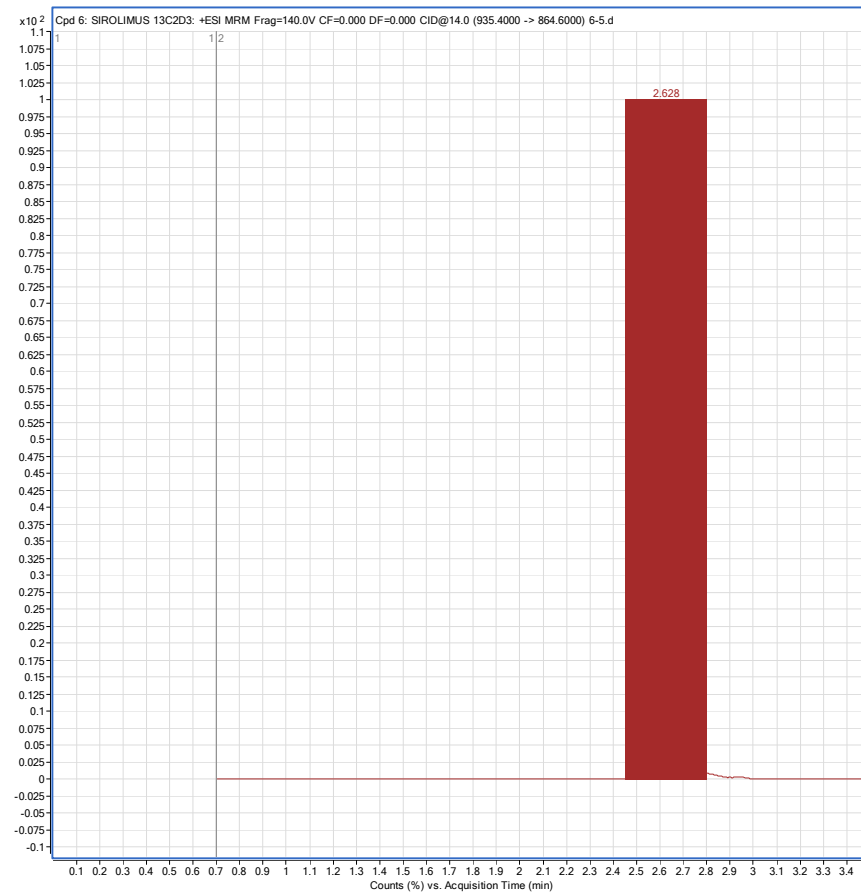


Fig. 4 : **Internal Standard Sirolimus**

Immunosuppressants in whole blood

(Reference Chromatograms/Spectrum by LC-MS)

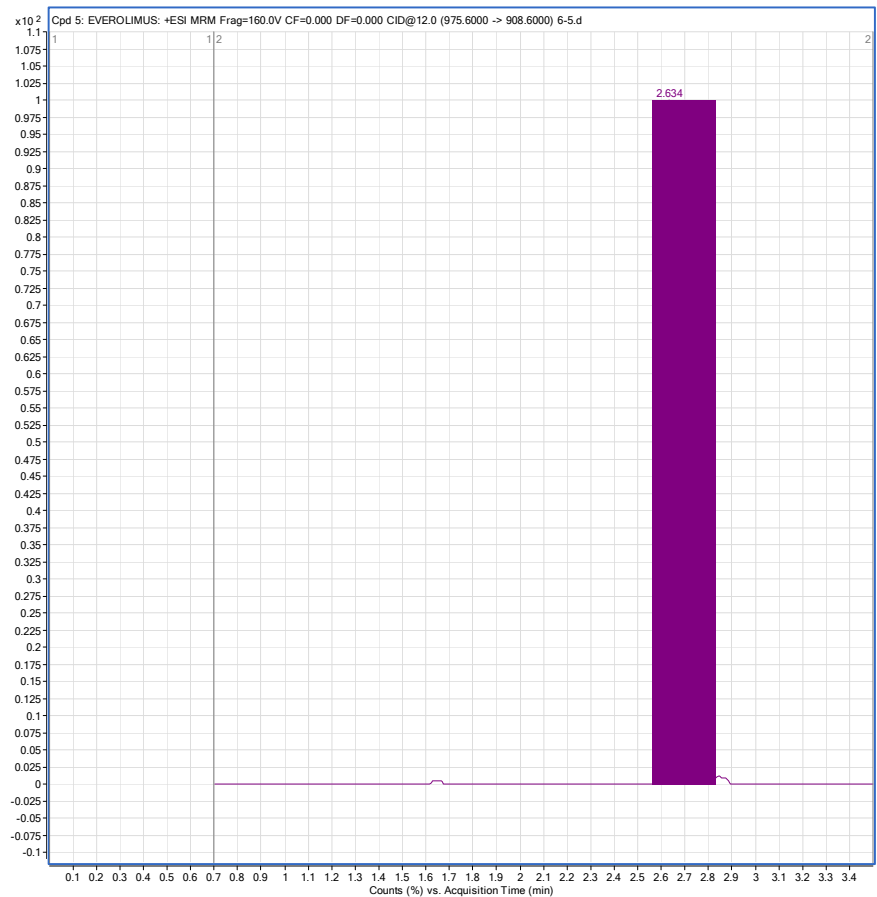


Fig. 5 : Everolimus

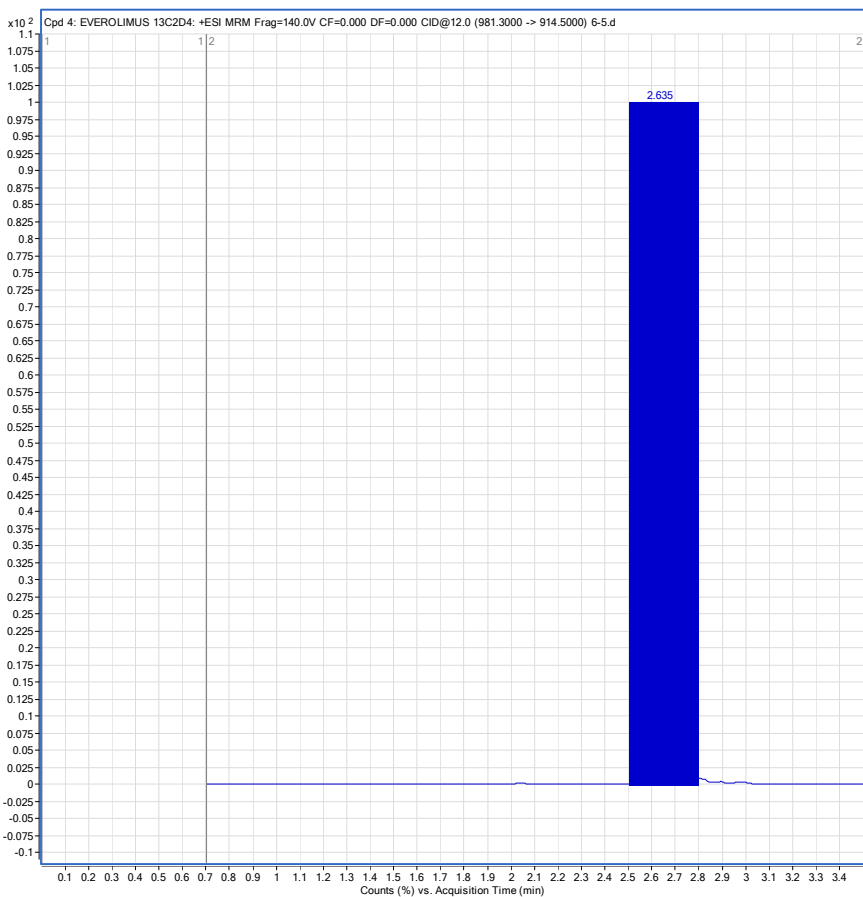


Fig. 6 : Internal Standard Everolimus

Immunosuppressants in whole blood

(Reference Chromatograms/Spectrum by LC-MS)

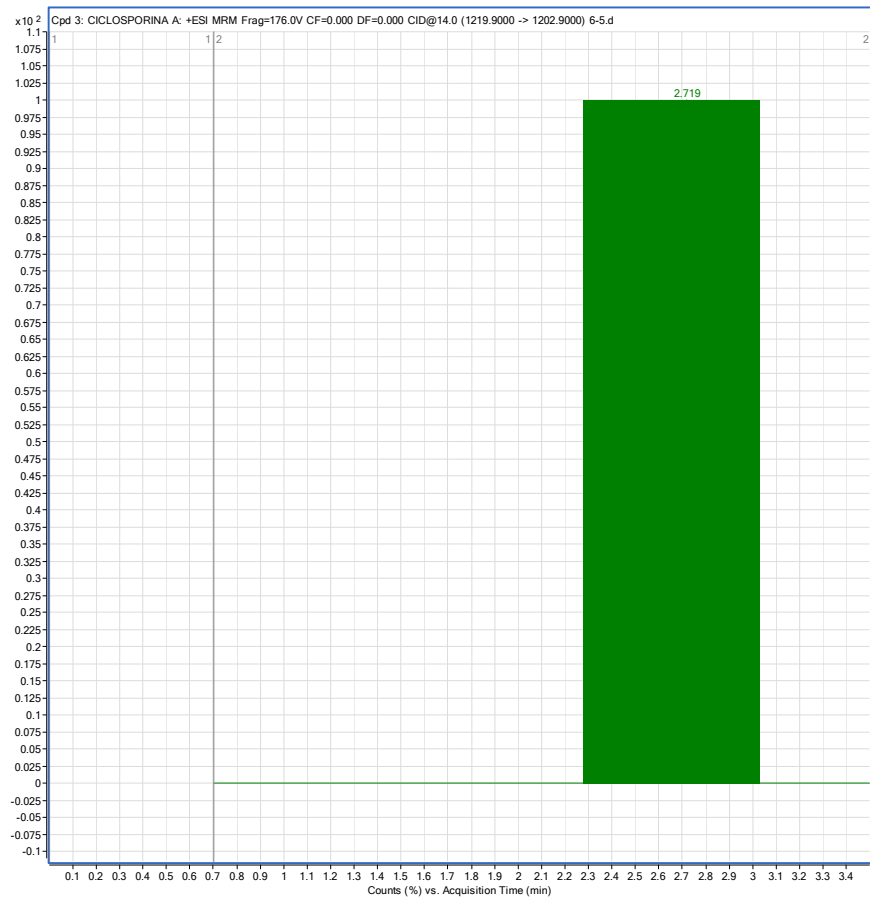


Fig. 7 : Cyclosporine A

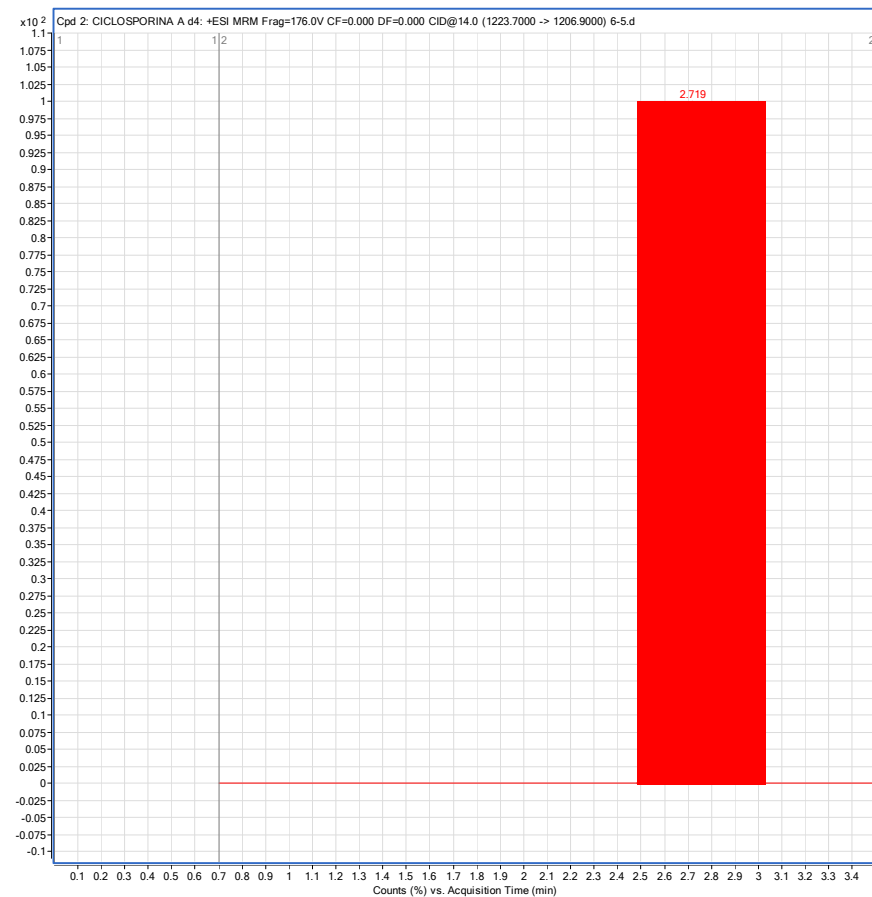


Fig. 8 : Internal Standard Cyclosporine A