Immunosuppressants DUAL KIT in whole blood/plasma by LC-MS – 500 TESTS – Code LC75315

(Tacrolimus, Sirolimus, Everolimus, Cyclosporine A, Mycophenolic Acid)

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.

Mycophenolic Acid (Mofetil or MPA) is a powerful immunosuppressant that reversible inhibits the enzyme Inosine-monophosphate dehydrogenase type 2 involved in the synthesis of guanosine. Since the mode of action of MPA differs from Cyclosporine, Tacrolimus and Sirolimus, it can be co-prescribed as part of a pharmacological panel.

MPA is metabolized by glucuronation to form an inactive metabolite (MPA-glucuronide) and also a minor metabolite (MPA-acyl-glucoronide).

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.

EUREKA srl – LAB DIVISION
VAT N° 01547310423
E-mail:info@eurekaone.com
www.eurekaone.com

eureka Lab Division

Head Quarter:
Via Enrico Fermi 25
60033 Chiaravalle (AN) ITALY
Tel. +39 071 7450790
Fax + 39 071 7496579

CE

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.

TECHNICAL FEATURES

Principle of the Method :

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

Recovery : 89 - 102 %

Sensitivity (LLOD) :

Tacrolimus	0,01 ng/ml
Sirolimus	0,01 ng/ml
Everolimus	0,01 ng/ml
Cyclosporine A	0,01 ng/ml
Mycophenolic Ac.	0,01 µg/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
Mycophenolic Ac.	0,05 – 80 µg/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
Mycophenolic Ac.	0,05 µg/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 erro %) 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%

Accuracy intra serie (relative error %) Mycophenolic Acid:

Cm	Cs
2 ug/ml	8 ug/ml
8,7%	2,2%

Accuracy inter serie (relative error %) Mycophenolic Acid:

Cm	Cs
2 ug/ml	8 ug/ml
11,0%	4,2%

Reproducibility intra serie (relative error %) Mycophenolic Acid:

C LLOQ	Cm	Cs
0,05 ug/ml	4 ug/ml	16 ug/ml
10,8%	4,9%	1,8%

Reproducibility inter serie (coefficient of variation %) Mycophenolic Acid:

C LLOQ	Cm	Cs
0,05 ug/ml	4 ug/ml	16 ug/ml
11,5%	5,1%	5,1%

Coefficient of correlation R2 + DS :

Tacrolimus	0,9951 ± 0,0026
Sirolimus	0,9939 ± 0,0026
Everolimus	0,9964 ± 0,0005
Cyclosporine A	0,9941 ± 0,0060
Mycophenolic Ac.	0,9954 ± 0,0026

Therapeutic Range:

Tacrolimus	1 – 50 ng/ml
Sirolimus	1 – 50 ng/ml
Everolimus	1 – 50 ng/ml
Cyclosporine A	10 – 2000 ng/ml
Mycophenolic Ac.	2 – 10 ug/ml

Components of the kit:

All the reagents are stable 3 years at 2 – 8 °C except the Reagent B and B1 that must be stored at –20 °C.

Reagent A – Deproteinization Sol, **1 x 150 ml**

Reagent B – Internal Standard, **1 x 2000 µl** — **Stable 6 months at –20 °C**

Reagent B1 – Internal Standard Sol. For Mycophenolic Ac., **1 x 500 µl** — **Stable 6 months at –20 °C**

Reagent C – Lysing Sol, **1 x 55 ml**

Reagent D – Diluting Solution, **1 x 250 ml**

Calibrator on whole blood – Level 0, 6 x 1 ml — **Code LC75016 (packed separately – see data sheet)**

Calibrator on whole blood – Level 1, 6 x 1 ml — **Code LC75016 (packed separately – see data sheet)**

Calibrator on whole blood – Level 2, 6 x 1 ml — **Code LC75016 (packed separately – see data sheet)**

Calibrator on whole blood – Level 3, 6 x 1 ml — **Code LC75016 (packed separately – see data sheet)**

Calibrator on whole blood – Level 4, 6 x 1 ml — **Code LC75016 (packed separately – see data sheet)**

Calibrator on whole blood – Level 5, 6 x 1 ml — **Code LC75016 (packed separately – see data sheet)**

Calibrator on whole blood – Level 6, 6 x 1 ml — **Code LC75016 (packed separately – see data sheet)**

Calibrator in plasma – Level 0, 6 x 1 ml — **Code LC75316 (packed separately – see data sheet)**

Calibrator in plasma – Level 1, 6 x 1 ml — **Code LC75316 (packed separately – see data sheet)**

Calibrator in plasma – Level 2, 6 x 1 ml — **Code LC75316 (packed separately – see data sheet)**

Calibrator in plasma – Level 3, 6 x 1 ml — **Code LC75316 (packed separately – see data sheet)**

Calibrator in plasma – Level 4, 6 x 1 ml — **Code LC75316 (packed separately – see data sheet)**

Calibrator in plasma – Level 5, 6 x 1 ml — **Code LC75316 (packed separately – see data sheet)**

Calibrator in plasma – Level 6, 6 x 1 ml — **Code LC75316 (packed separately – see data sheet)**

Reagent M1 – Mobile Phase M1, **5 x 500 ml**

Reagent M2 – Mobile Phase M2, **5 x 500 ml**

Minimum Instrumental equipment required :	LC-MS Instrument triple quadrupole (mode MRM) Divert Valve at 6 ways ESI (+) Interface Binary Pump Operational Computer
Whole Blood Collection Procedure :	Test tube with anticoagulant EDTA Stable 4 weeks at 2 – 8 °C or 4 months at – 20 °C. For the analysis of the Mycophenolic Ac.: take 3 ml of venous blood into a tube without gel for serum and with heparin as an anticoagulant for plasma. Centrifuge at 4000 rpm for 5 minutes. Separate the whey and store it at - 20 ° C. Stable 4 weeks.

ANALYTICAL PROCEDURE:

STEP 1 : Sample Preparation

Dispense 100 µl of **Reagent B -** Internal Standards in a tube of 10 ml, 400 ul of Methanol and 500 ul 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
	Vortex for 10 sec.		
Reagent C – Lysing Solution	100 µl	100 µl	100 µl
	Vortex for 10 sec.		
Reagent A – Deproteinization Solution	300 µl	300 µl	300 µl

Vortex for 20 sec.

STEP 3 : Centrifuge at 14.000 rpm for 10 minutes

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

INJECTION :

- Inject 2-10 µl of solution in LC system

ANALYTICAL PROCEDURE for Mycophenolic Acid:

STEP 1 : Sample Preparation

Dispense 100 µl of **Reagent B1 -** Internal Standards for Mycophenolic Acid in a tube of 10 ml, 400 ul of Methanol and 500 ul 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 for Mycophenolic Acid (diluted as in STEP 1)	20 µl	20 µl	20 µl
	Vortex for 10 sec.		
Reagent C – Lysing Solution	100 µl	100 µl	100 µl
	Vortex for 10 sec.		
Reagent A – Deproteinization Solution	300 µl	300 µl	300 µl

Vortex for 20 sec.

STEP 3 : Centrifuge at 14.000 rpm for 10 minutes

STEP 4 : Take the surnatant and put in a glass vial with reduced volume, dilute 20 µl of surnatant in 480 µl of **Reagent D -** Diluting Solution

N.B.: at this step, the sample is stable 48 h at 2-8°C

INJECTION :

- Inject 2 µl of solution in LC system

Immunosuppressants in whole blood/plasma - Warnings

COLUMN CONDITIONING

Install a new analytical column RRHD Eclipse Plus C18 (2,1 mm x 50 mm – 1,8 um) thermostated at 60°C after the valve. Disconnect the detector and flux a solution of H_2O : 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 H_2O : 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 um
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 Spry 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
Mycophenolic Acid	321,0	207,0	29
$^{2}H_{4}$ Cyclosporine A	1.223,7	1.206,9	14
$^{13}C_{2}{}^{2}H_{4}$ Everolimus	981,3	914,5	14
$^{13}C^{2}H_{2}$ Tacrolimus	824	771	20
$^{13}C^{2}H_{3}$ Sirolimus	935,4	864,6	14
Mycophenolic Acid-D3	324,2	210,0	29

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 B with: **$^{13}C^{2}H_2$ Tacrolimus, $^{13}C_2{}^{2}H_4$ Everolimus, $^{13}C^{2}H_3$ Sirolimus, $^{2}H_4$ Cyclosporine A.** ***Store at – 20 °C.*** Solution B1 with: **Mycophenolic Acid-D3.** ***Store at -20 °C.***	
Immunosuppressants:	Internal Standard for calibration:
TACROLIMUS	**$^{13}C^{2}H_2$ Tacrolimus**
EVEROLIMUS	**$^{13}C_2{}^{2}H_4$ Everolimus**
SIROLIMUS	**$^{13}C^{2}H_3$ Sirolimus**
CYCLOSPORINE A	**$^{2}H_4$ Cyclosporine A**
MYCOPHENOLIC ACID	**Mycophenolic Acid-D3**

ACCESSORIES AND CONSUMABLES

CODE	***DESCRIPTION***	***PACKAGING***
LC75016	Calibrator in whole blood for Immunosuppressants	7 x 2 x 1 ml
LC75019	Control in whole blood for Immunosuppressants – Levels 1, 2 and 3	3 x 5 x 1 ml
LC75316	Calibrator in plasma for Mycophenolic Acid	7 x 2 x 1 ml
LC75319	Control in plasma for Mycophenolic Acid – Levels 1, 2 and 3	3 x 5 x 1 ml
SK75310	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

eureka Lab Division

Immunosuppressants in whole blood/plasma

(Reference Chromatograms/Spectrum by LC-MS)

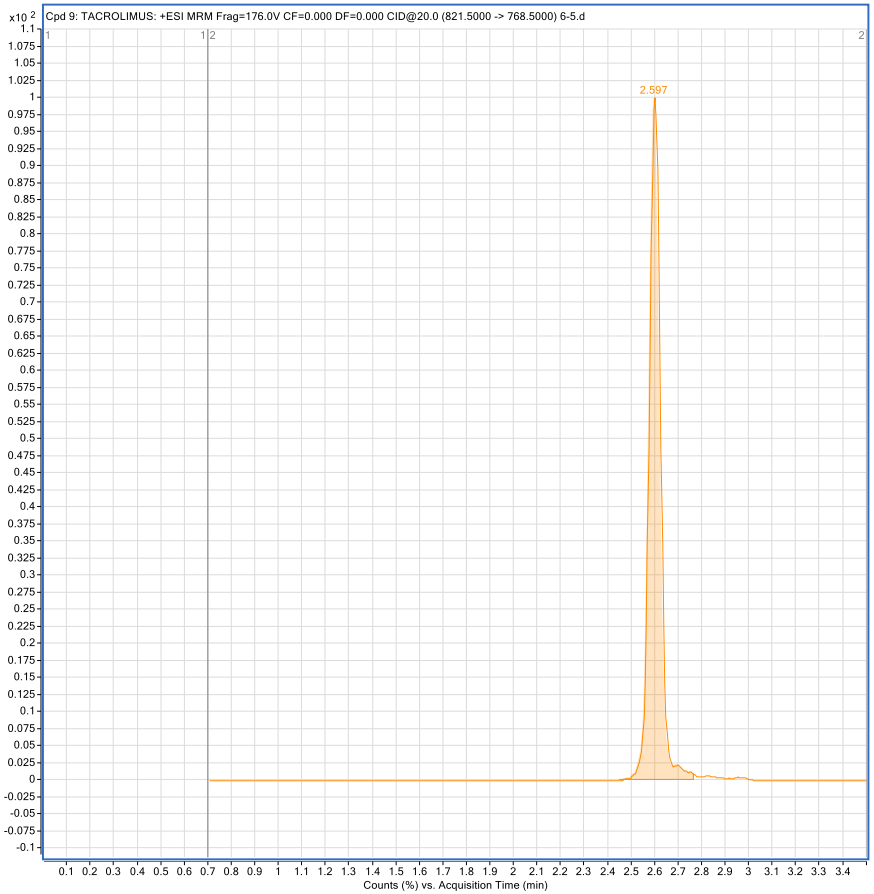

Fig. 1 :	***Tacrolimus***

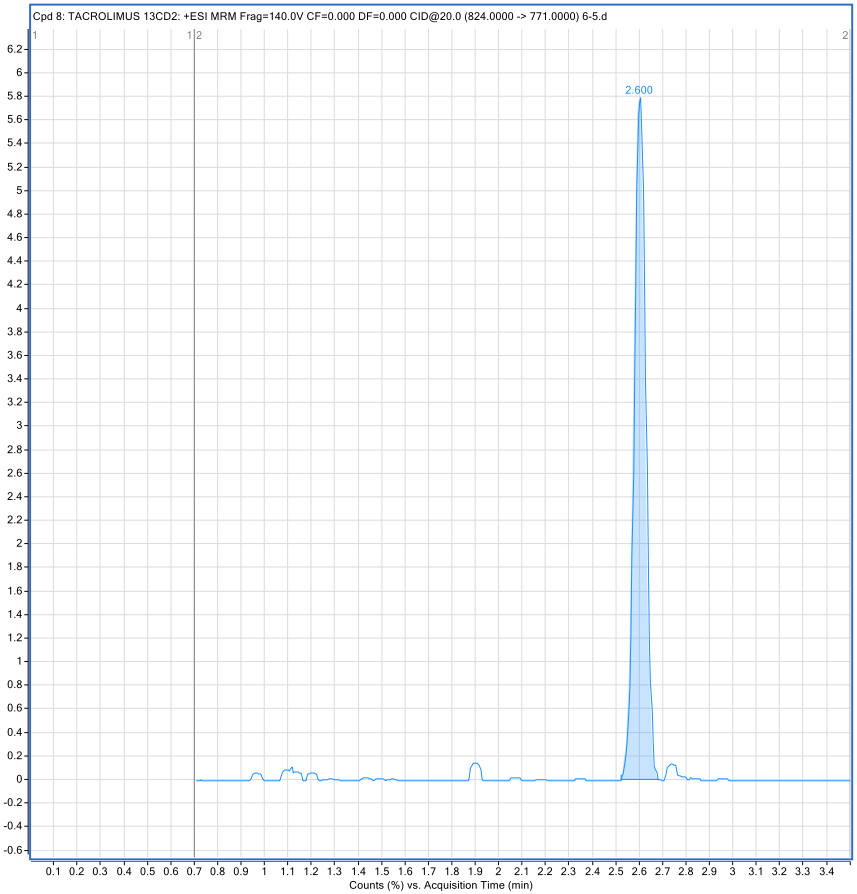

Fig. 2 :	***Internal Standard Tacrolims***

Immunosuppressants in whole blood/plasma

(Reference Chromatograms/Spectrum by LC-MS)

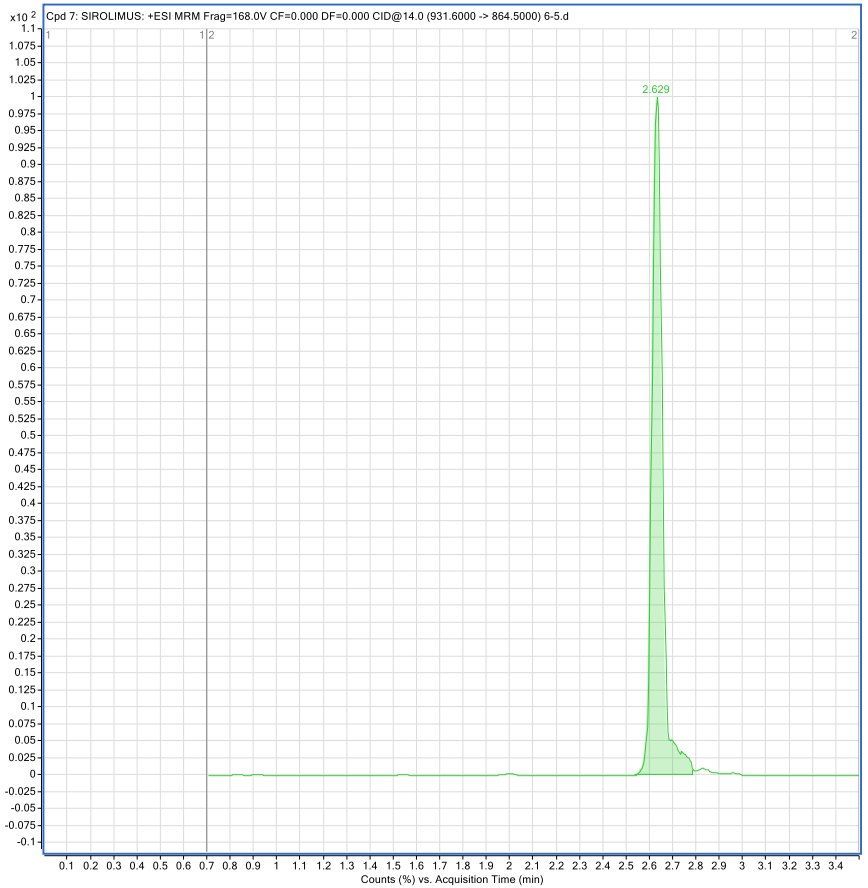

Fig. 3 :	***Sirolimus***

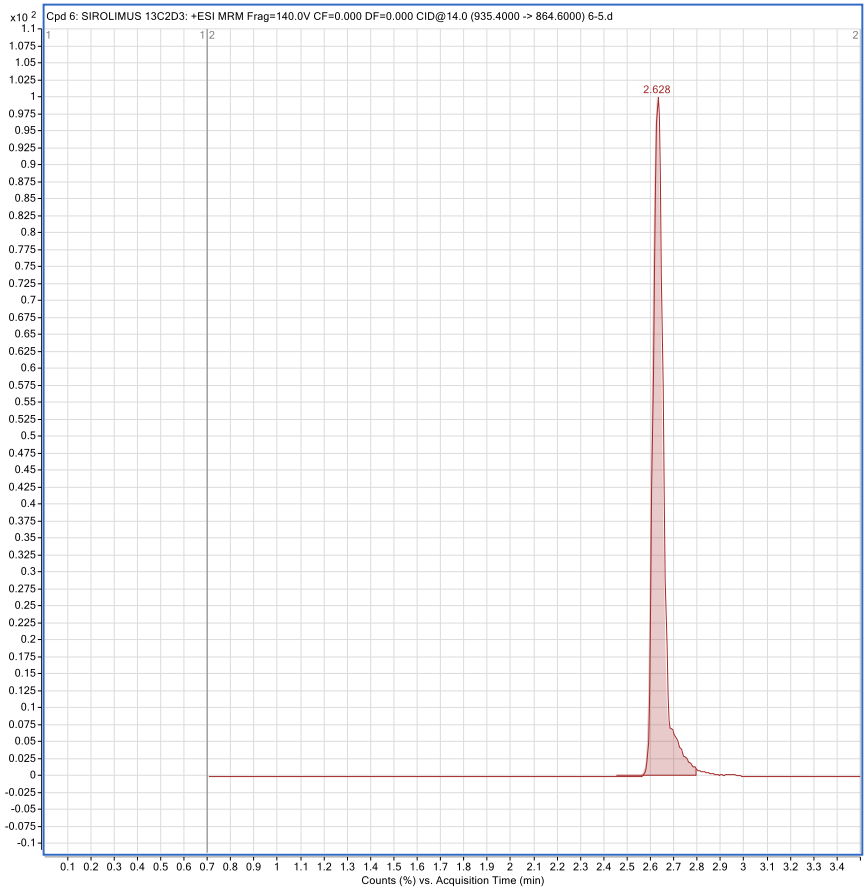

Fig. 4 :	***Internal Standard Sirolimus***

Immunosuppressants in whole blood/plasma

(Reference Chromatograms/Spectrum by LC-MS)

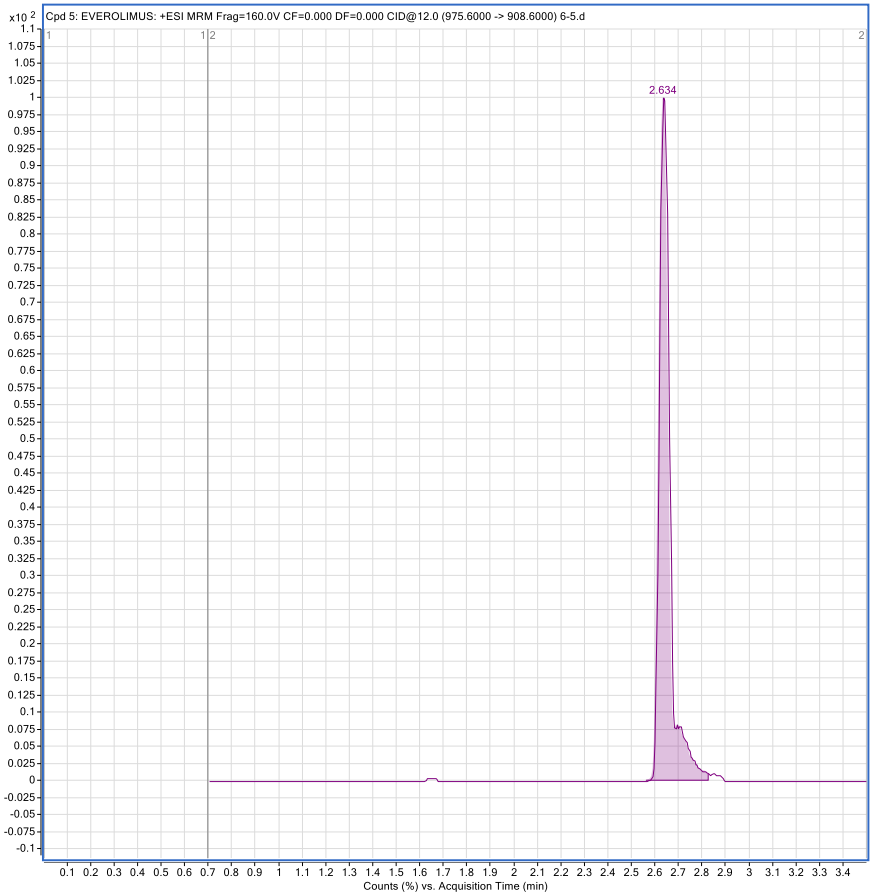

Fig. 5 :	***Everolimus***

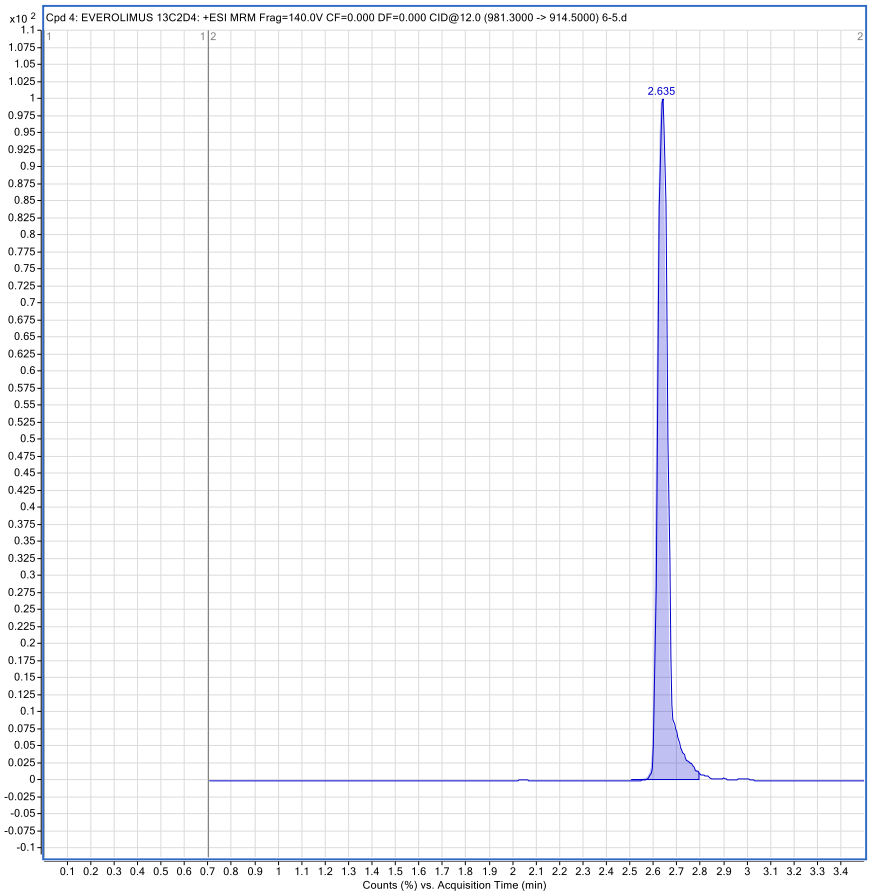

Fig. 6 :	***Internal Standard Everolimus***

Immunosuppressants in whole blood/plasma

(Reference Chromatograms/Spectrum by LC-MS)

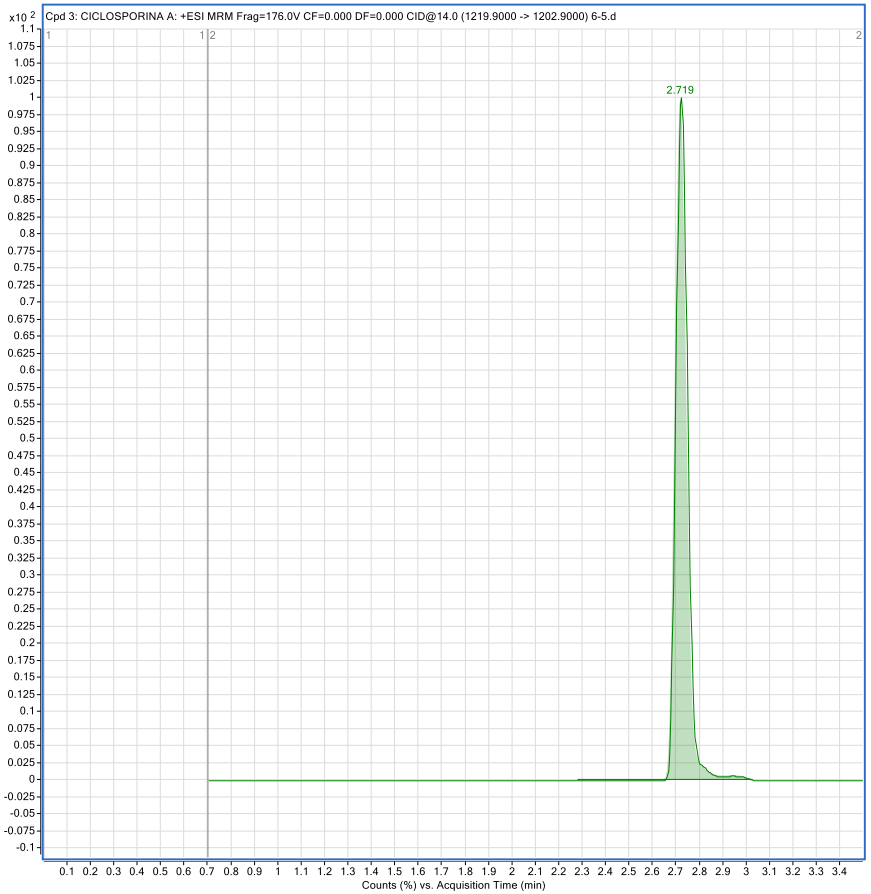

Fig. 7 :	***Cyclosporine A***

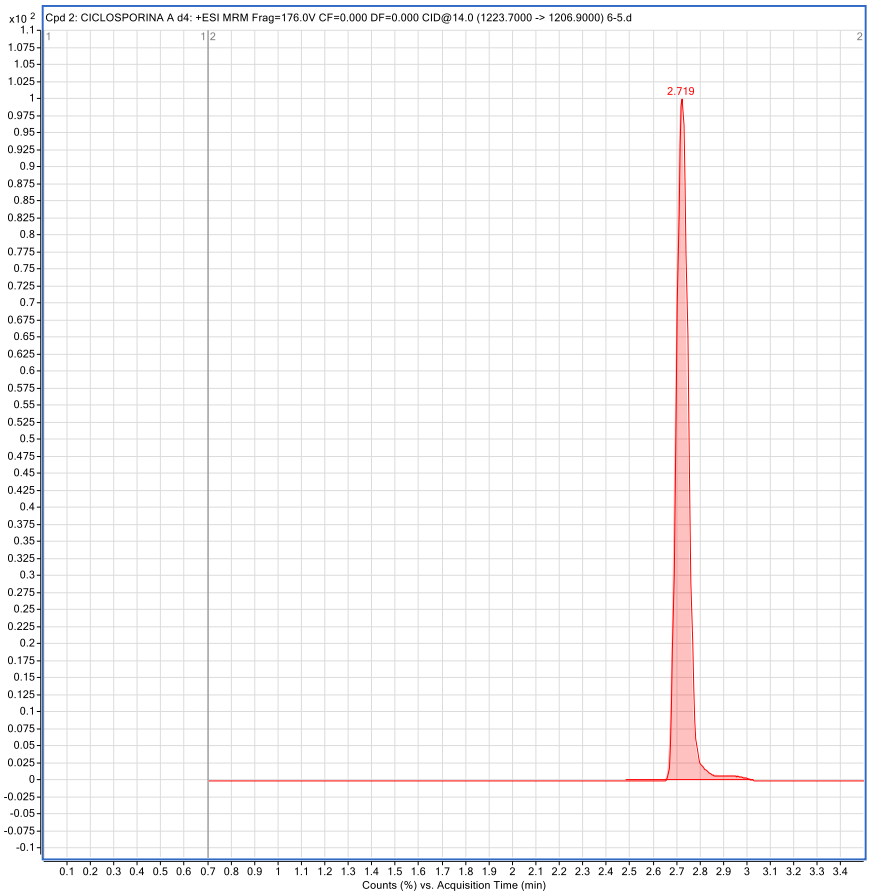

Fig. 8 :	***Internal Standard Cyclosporine A***

Immunosuppressants in whole blood/plasma

(Reference Chromatograms/Spectrum by LC-MS)

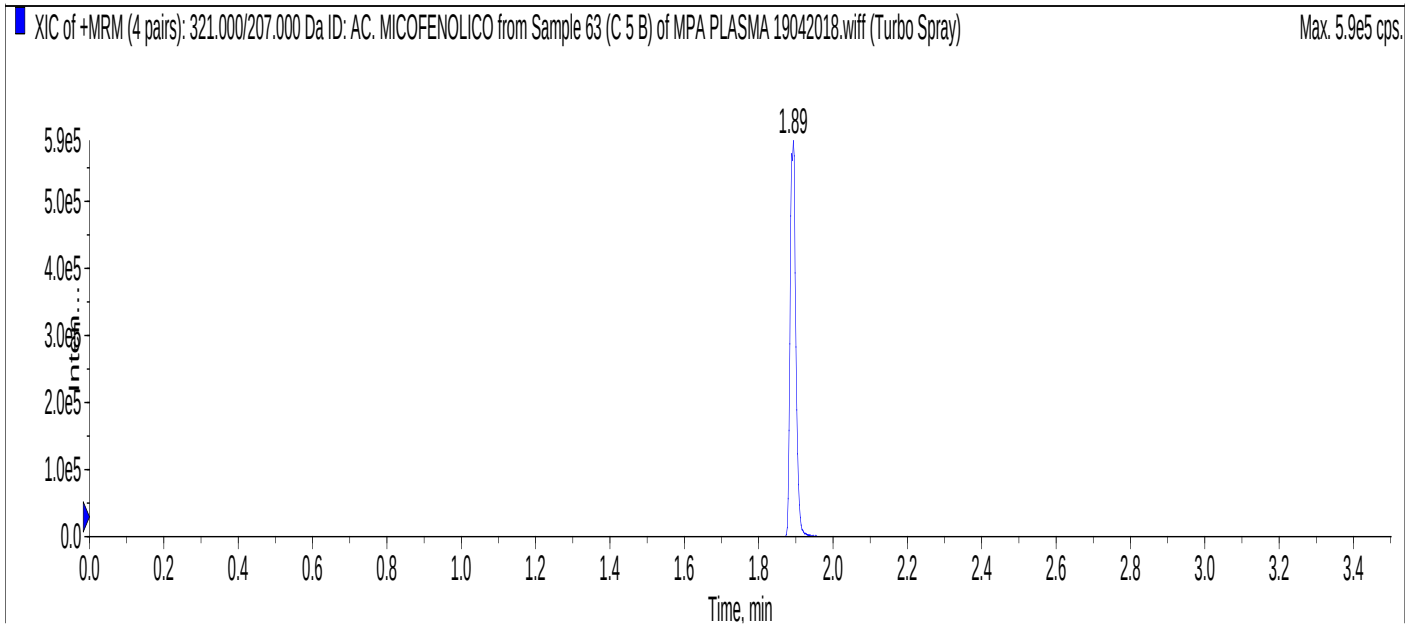

Fig. 9 :	***Calibrator in plasma***
	R.T. 1.89 Mycophenolic Acid 16 µg/ml

Immunosuppressants in whole blood/plasma

(Reference Chromatograms/Spectrum by LC-MS)

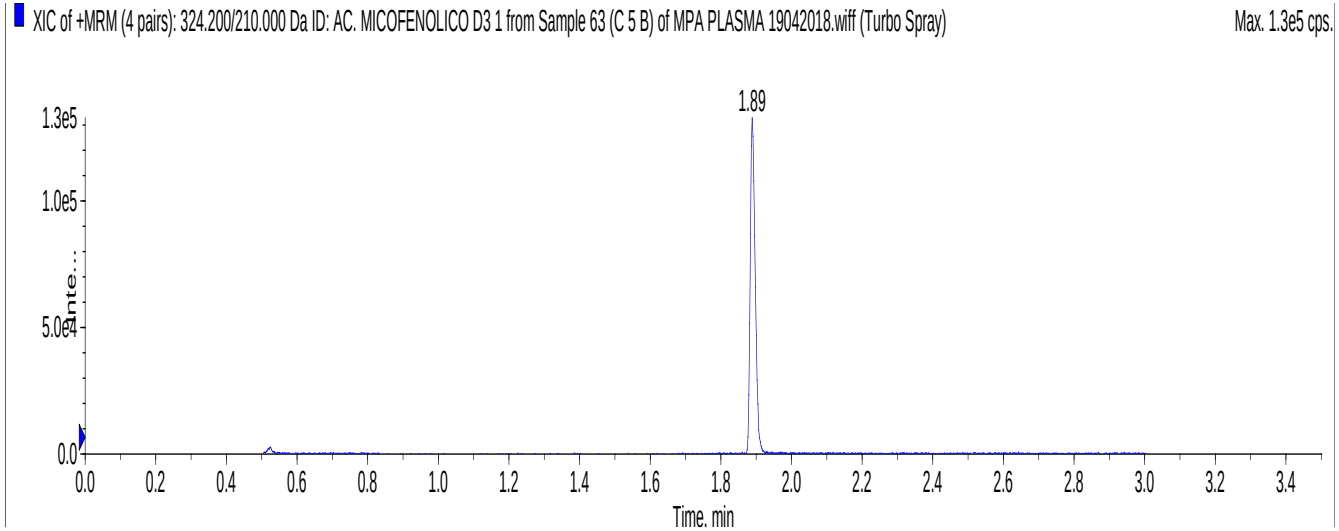

Fig. 10 :	***Calibrator in plasma***
	R.T. 1.89 Mycophenolic Acid-D3

Immunosuppressants in whole blood/plasma

(Reference Chromatograms/Spectrum by LC-MS)

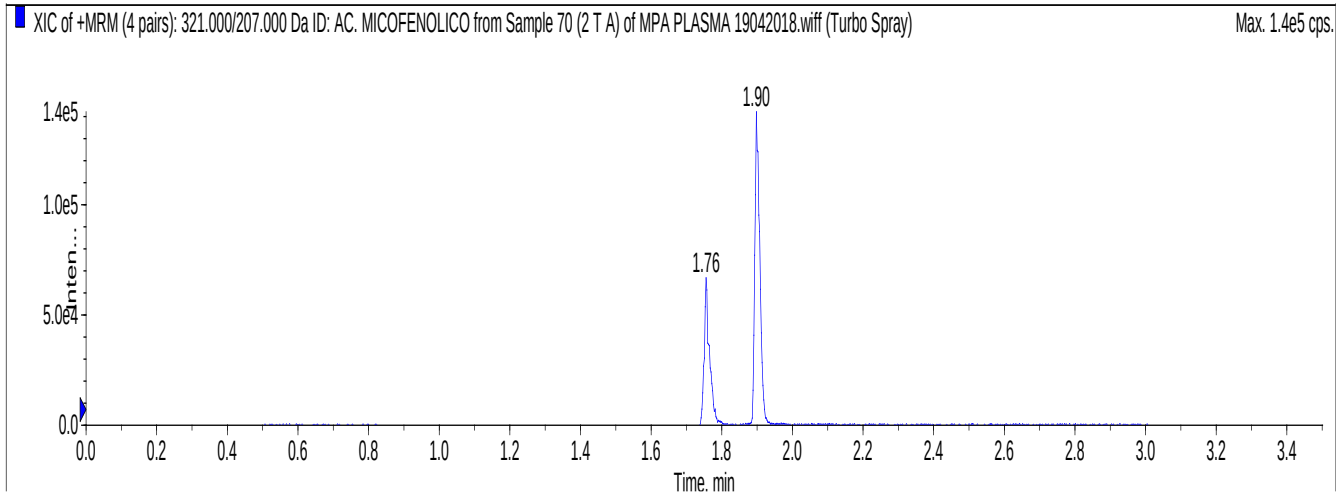

Fig. 11 :	***Sample in plasma***
	R.T. 1.90 Mycophenolic Acid 3,35 µg/ml