

Immunosuppressants in whole blood by LC-MS – Code LC75010

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

ASPEC PROCEDURE

INTRODUCTION

The immunosuppressive drugs are used for the 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 Mycophenolic Acid, Cyclosporine A, Tacrolimus, Sirolimus and Everolimus.

Mycophenolic Acid (Mofetil or MPA) is a potent immunosuppressant that reversibly 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, may be co-prescribed medication as part of a panel.

The MPA is metabolized by glucuronidation to form an inactive metabolite (MPA-glucuronide) and a minor metabolite (acyl-MPA-glucuronide).

Cyclosporine A is the active response that influences the 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, exerts its function primarily by blocking the transcription of cytokine genes in T lymphocytes.

Even **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 interactions to the contrary, including one with grapefruit which increases the drug concentration in blood plasma.

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

Sirolimus and **everolimus** are collecting 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 hyperlipidemia refractory to treatment, 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° 004	Immunosuppressants by LC/MS – Aspec Procedure	March 2012
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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 :

Mycophenolic Acid	0,1 mg/l
Tacrolimus	0,1 ng/ml
Sirolimus	0,1 ng/ml
Everolimus	0,1 ng/ml
Cyclosporine A	1,0 ng/ml

CV% :

Mycophenolic Acid	5.38%
Tacrolimus	5.92%
Sirolimus	5.50%
Everolimus	5.85%
Cyclosporine A	5.08%

Dynamic Range of Method :

Mycophenolic Acid	1 – 50.000 ng/ml
Tacrolimus	0,5 – 5.000 ng/ml
Sirolimus	0,5 – 5.000 ng/ml
Everolimus	0,5 – 5.000 ng/ml
Cyclosporine A	1 – 50.000 ng/ml

Therapeutic Range:

Mycophenolic Acid	4.000 – 15.000 ng/ml
Tacrolimus	1 – 100 ng/ml
Sirolimus	1 – 100 ng/ml
Everolimus	1 – 100 ng/ml
Cyclosporine A	10 – 1000 ng/ml

Components of the kit :

All the reagents are stable 3 years at 2 – 8 °C.

Reagent A – Deproteinization Sol. + Internal Standards, **1 x 30 ml**

Reagent B – Internal Standard, **4 x 10 ml**

To reconstitute with 10 ml of Reagent A at the moment to use. This solution so reconstitute is stable 3 months at – 20 °C. See Warnings

Reagent C1 – Calibrator on whole blood – Level 1, **1 x 1 ml**

See Warnings

Reagent C2 – Calibrator on whole blood – Level 2, **1 x 1 ml**

See Warnings

Reagent C3 – Calibrator on whole blood – Level 3, **1 x 1 ml**

See Warnings

Reagent C4 – Calibrator on whole blood – Level 4, **1 x 1 ml**

See Warnings

Reagent C5 – Calibrator on whole blood – Level 5, **1 x 1 ml**

Reagent M1 – Mobile Phase M1, **2 x 500 ml**

Reagent M2 – Mobile Phase M2, **1 x 500 ml**

Clean-up Columns, 100 pcs

Minimum Instrumental equipment required :

LC-MS Instrument triple quadrupole (mode MRM)
Divert Valve at 6 ways
ESI (+)Interface
Binary Pump
Loop 100 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 (ASPEC Procedure):

STEP 1 : Sample Preparation

Dispense in eppendorf vials:

	Calibrator	Sample	Controls
Reagent C – Calibrator lyophil.	100 µl		
Sample		100 µl	
Controls			100 µl
Reagent B – Internal Standard (previously reconstituted with Reagent A)	300 µl	300 µl	300 µl

STEP 2 : Load all deproteinized sample in the Clean-up columns

STEP 3 : Collect the eluate

INJECTION :

- Inject 100 µl of solution in LC system

Immunosuppressants in whole blood - Warnings

REAGENT C1-C2-C3-C4-C5 : CALIBRATOR LYOPHIL. (5 x 1 ml) Lot.

LEVELS	1	2	3	4	5
Mycophenolic Acid (mg/L)	0				
Tacrolimus (ng/mL)	0				
Sirolimus (ng/mL)	0				
Everolimus (ng/mL)	0				
Cyclosporine A (ng/mL)	0				
Use and Reconstitution: Blood Calibrators are used for calibration of the LC system. This lyophilised calibrator has to be prepared like a patient sample. Add exactly 1 ml of H ₂ O HPLC grade and mix for 20 min until all material is dissolved. Stir gently before sampling to ensure homogeneity.					
Storage and stability: 36 months if stored at 2 – 8 °C. After reconstitution 7 days if stored at 2 – 8 °C and 1 months at – 20 °C. Don't use after expiry date.					
Packaging: 5 x 1 ml					
Warning: The calibrator derives from human blood, so it could be potentially infected. It must be handled with care.					

COLUMN CONDITIONING

Install a new analytical column PURSUIT XRs C18 2 mm x 30 mm) thermostatate at 60 °C. Disconnect the detector and flux 30 ml of H₂O : Methanol (20 : 80 v/v) at the flow of 300 µl / min. **Don't recycle the washing solutions.** Condition further on the column at the flow of 300 µl / min and discharge the first 30 ml.

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.

The system works in SPE ONLINE (Monolithic Column RP C18 2,5 cm x 4,6mm)

COLUMN CLEANING

Disconnect the detector. Flux 30 ml of water and discharge. Flux a solution of H₂O : Methanol or Acetonitrile (20 : 80 v/v) for 30 minutes. Discharge.

AUTOSAMPLER SYRINGE WASHING

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

OPERATIONAL COMPUTER PARAMETERS

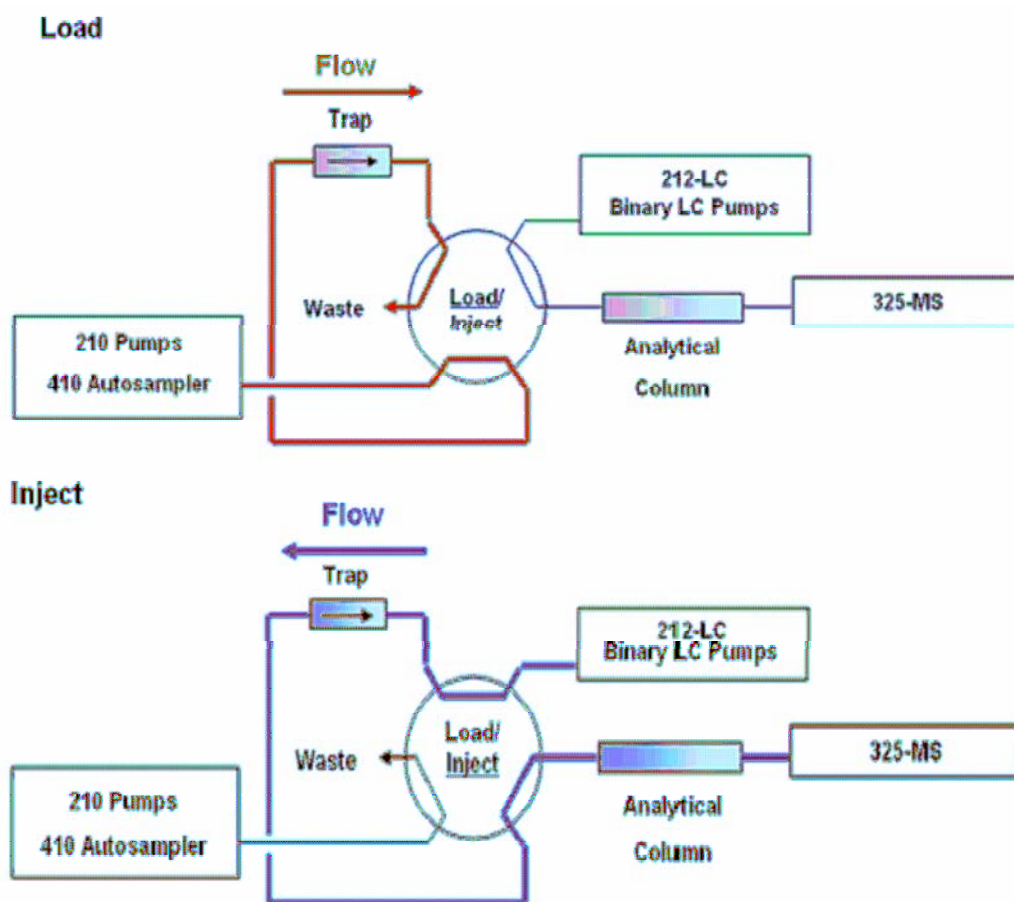
IN CONFORMITY WITH THE SPECIFICATION OF COMPUTER SOFTWARE

LC PARAMETERS

Recommended Flow SPE online	500 µl/min
Recommended Flow analytical column	400 µl/min

ANALYTE	PARENT ION
Mycophenolic Acid	321.0 (H ⁺)
Tacrolimus	826.4 (Na ⁺) 821.4 (NH ₄ ⁺)
Sirolimus	936.4 (Na ⁺) 931.4 (NH ₄ ⁺)
Everolimus	980.4 (Na ⁺) 975.4 (NH ₄ ⁺)
Cyclosporine A	1224.7 (Na ⁺) 1219.7 (NH ₄ ⁺)

Divert Valve



SPE ONLINE	
Time (min)	Valve Position
0-1	Load
1-4	Inject
4-5	Load

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

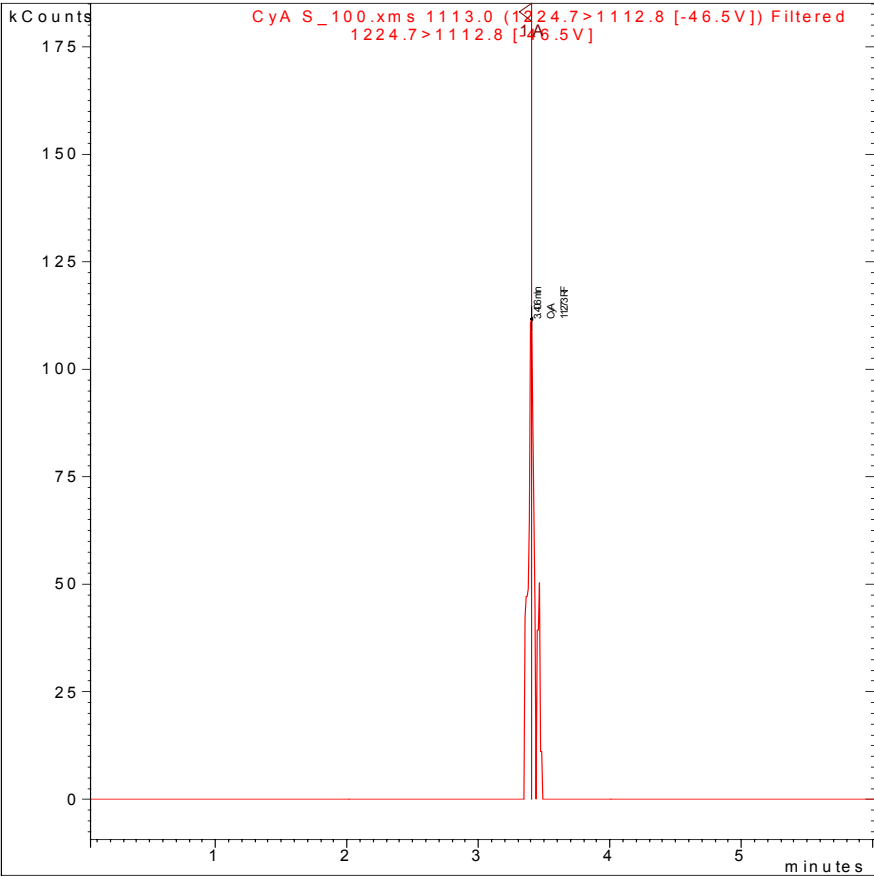


Fig. 1 :	Whole Blood Sample
	R.T. 3.40 CYCLOSPORINE A 100 ng/ml

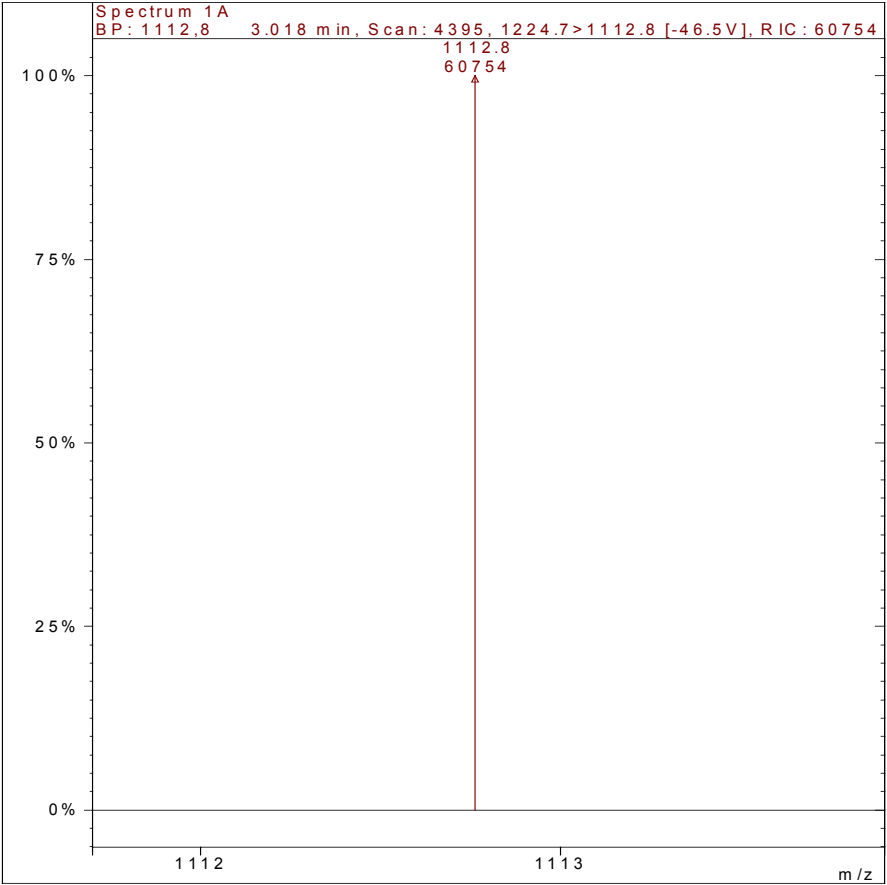


Fig. 2 :	Massa Spectrum of Cyclosporine A
	Transition 1224.7 > 1112.8

Immunosuppressants in whole blood

(Reference Chromatograms/Spectrum by LC-MS)

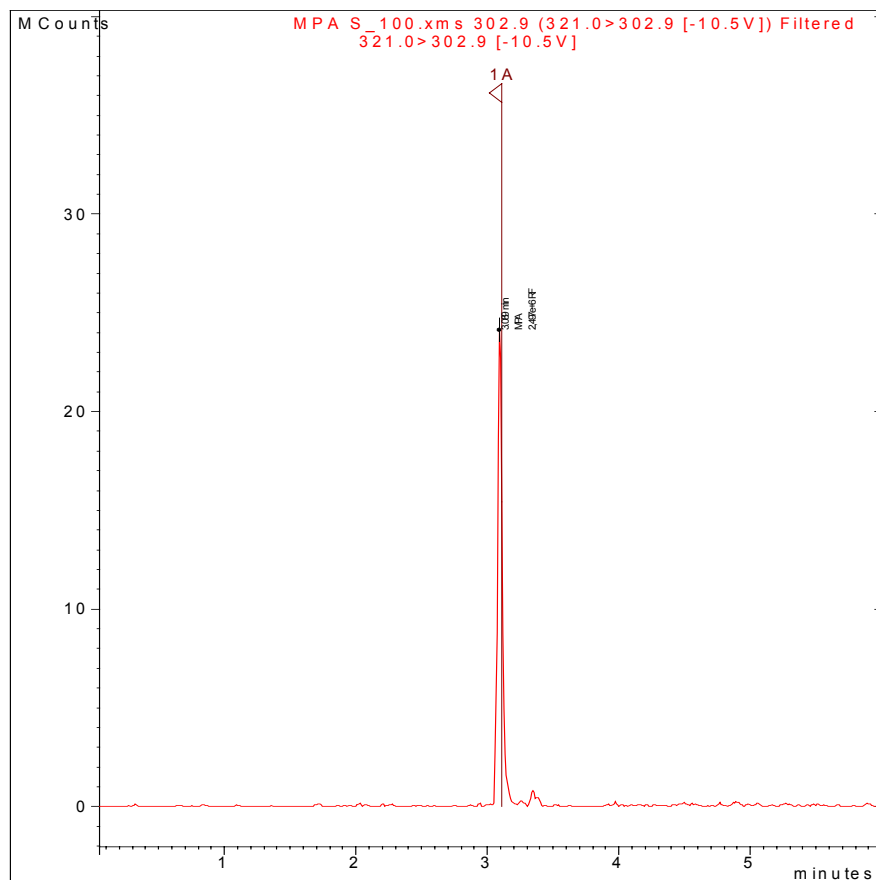


Fig. 3 :	Whole Blood Sample
	R.T. 3.09 MYCOPHENOLIC ACID 100 ng/ml

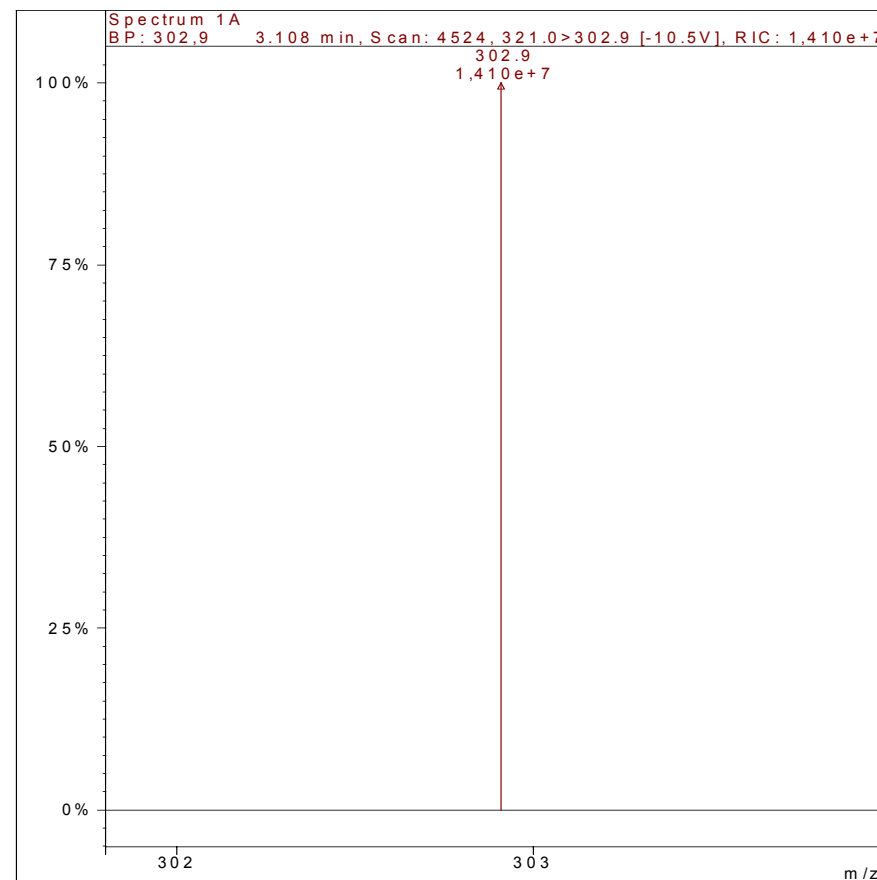


Fig. 4 :	Massa Spectrum of Mycophenolic Acid
	Transition 321.0 > 302.9

Immunosuppressants in whole blood

(Reference Chromatograms/Spectrum by LC-MS)

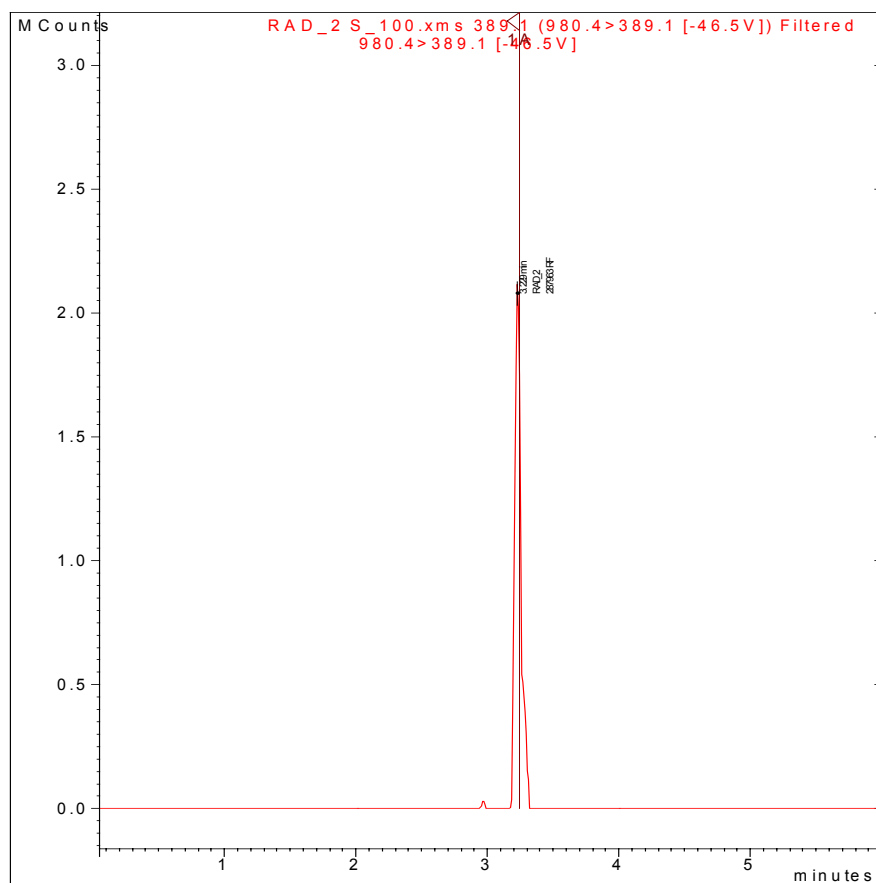


Fig. 5 :	Whole Blood Sample
	R.T. 3.23 EVEROLIMUS 100 ng/ml

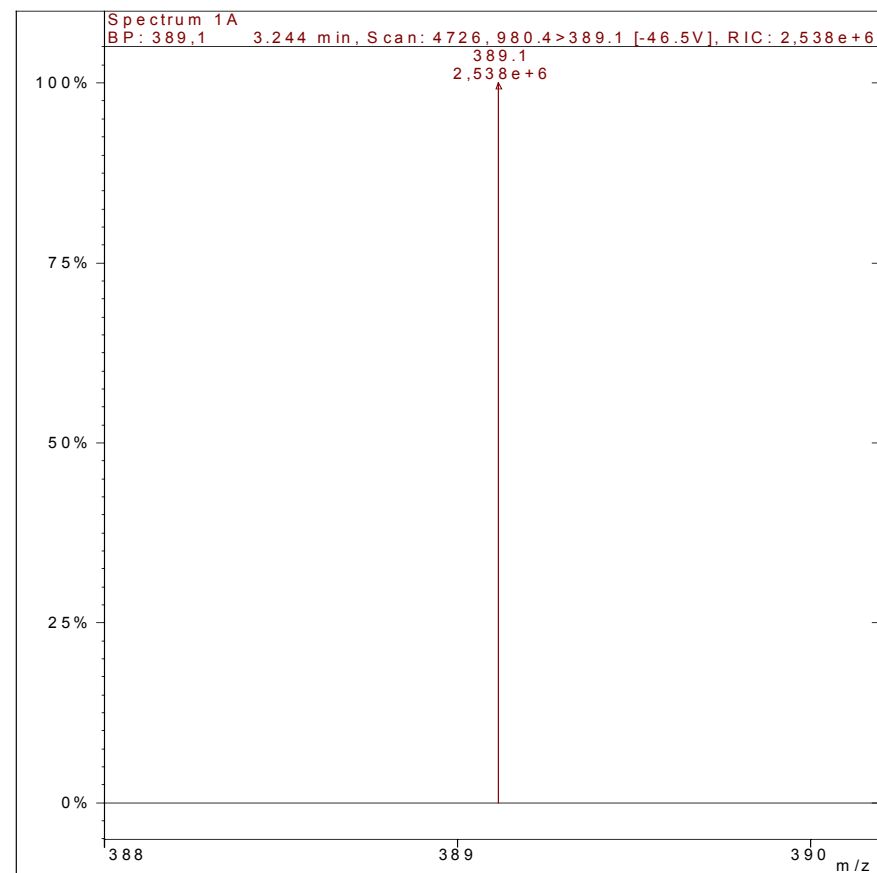


Fig. 6 :	Massa Spectrum of Everolimus
	Transition 980.4 > 389.1

Immunosuppressants in whole blood

(Reference Chromatograms/Spectrum by LC-MS)

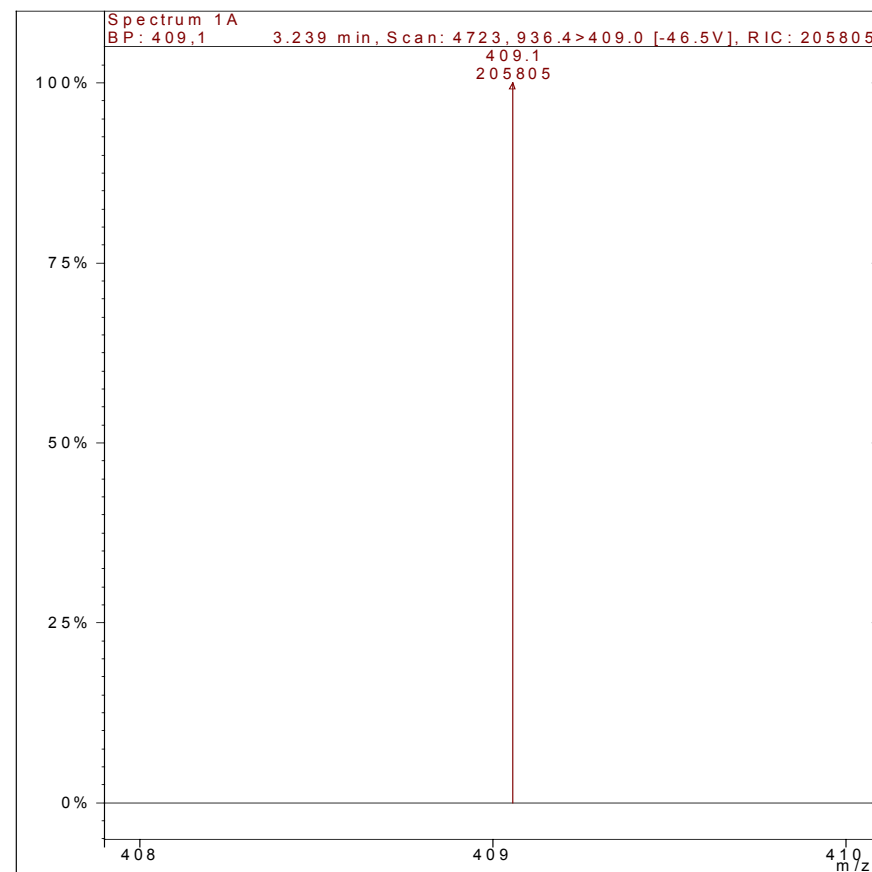
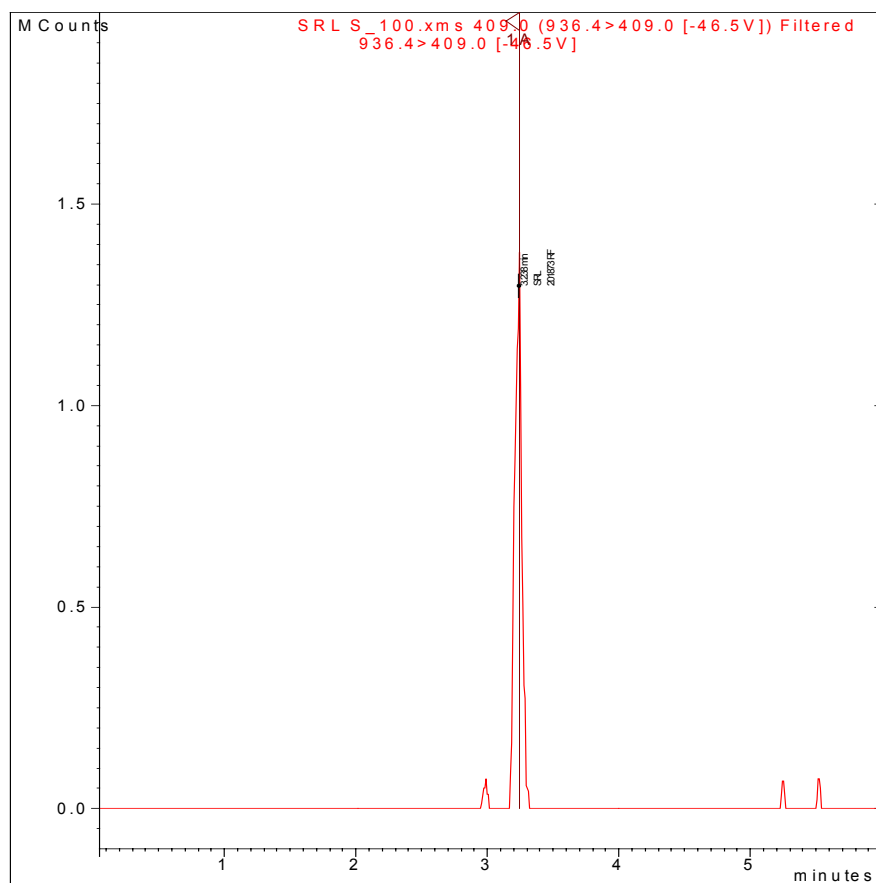


Fig. 7 :	Whole Blood Sample
	R.T. 3.24 SIROLIMUS 100 ng/ml

Fig. 8 :	Mass Spectrum of Sirolimus
	Transition 936.4 > 409.0

Immunosuppressants in whole blood

(Reference Chromatograms/Spectrum by LC-MS)

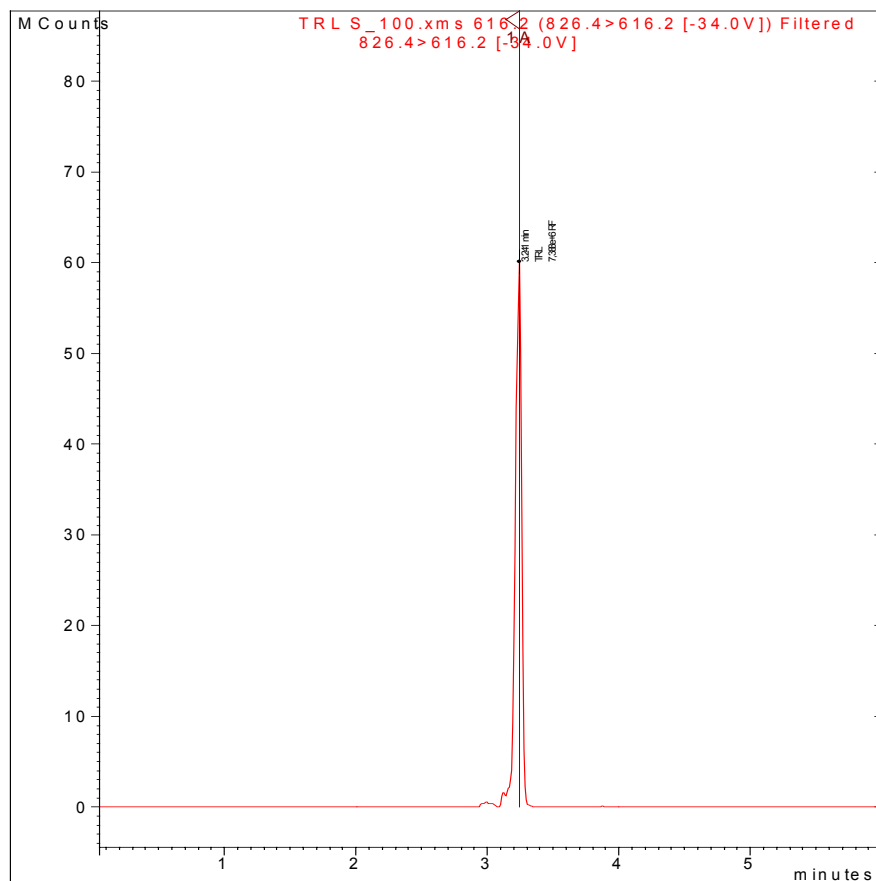


Fig. 9 :	Whole Blood Sample
	R.T. 3.24 TACROLIMUS 100 ng/ml

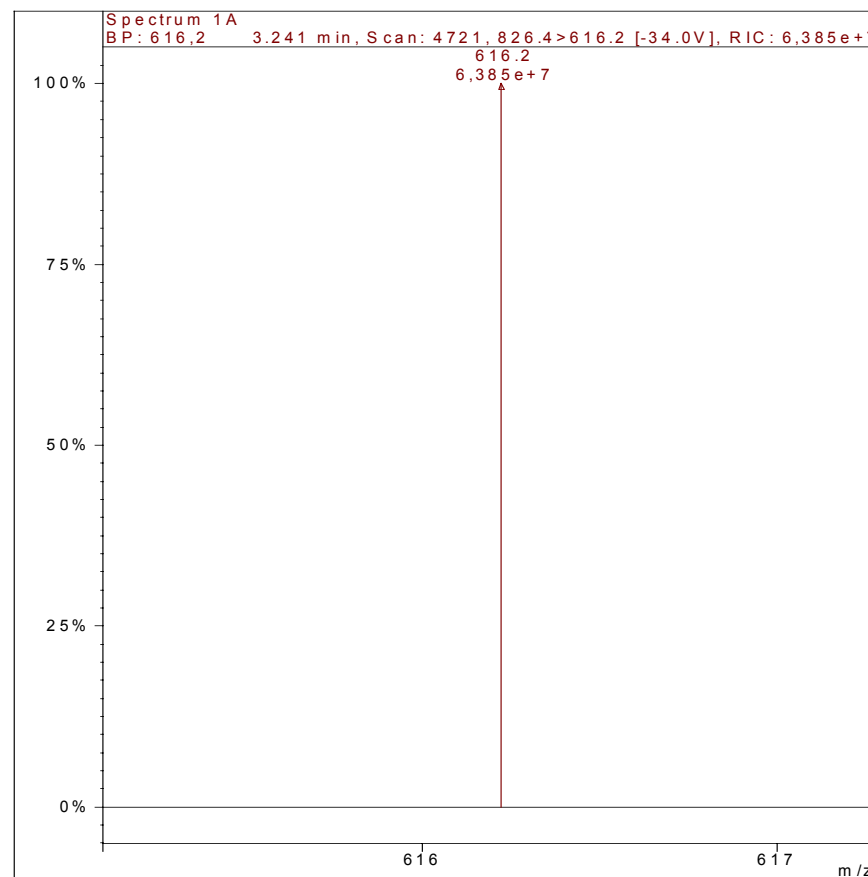


Fig. 10 :	Massa Spectrum of Tacrolimus
	Transition 826.4 > 616.2