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Liquid Chromatography-Tandem Mass Spectrometry(LC-MS/MS) Method for Determinationof Sirolimus in Human Plasma.

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Abstract:

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) method has proved to be a powerful research tool due to its sensitivity, high selectivity, and high throughput efficiency. Sirolimus was extracted from plasma by two-step extraction procedure using chloroform as extracting solvent.Determination of Sirolimus in human plasma method was developed and validated. In this study, ESI^+ sense was chosen as the ionization source. Signal intensity was high using ESI^+ source provided for the quantification of samples. Chromatographic separation was performed on phenomenax C-18 column (250x4.60mm 5microns).Mobile phase contains acetonitrile, water (80; 20 v/v) +0.1% acetic acid, flow rate 1 mL/min.The retention time of Sirolimus 8.4 min, the total run time10 min.Linearity correlation coefficients (r^2) curve was 0.997183.calibraction range 10-1000ng/mL.TheLLOQ of Sirolimus 0.10 ng/mL.Sirolimus, MRM (Multiple reaction monitoring) transition *m/z* 936.83-208.84 was selected to obtain maximum sensitivity. LC-MS/MS method have been successfully used in the pharmacokinetic analysis of Sirolimus in human plasma.

Keywords: Sirolimus; liquid chromatography-tandem mass spectrometry;human plasma; Chloroform; Liquid-liquid extraction

Introduction

Sirolimus (rapamycin, Rapamune®; **figure no : 1**′) is an immunosuppressant that inhibits cytokine-stimulated T-cell proliferation. Sirolimus acts by forming a complex with FK-binding protein-12 which in turn binds to mTOR kinase, a specific cell cycle regulatory protein, thereby inhibiting mTOR action. mTOR inhibition prevents cell cycle progression from G1 to S phase in T-cells and, thus, T-cell proliferation.Sirolimus is a macrocyclic lactone produced by Streptomyces hygroscopicus(Vezina et al., 1975and Sehgal et al., 1975), Studies in a variety of animal transplant models(Morris et al, 1992,Kahan et al., 1991,Stepkowski et al., 1991)and human clinical trials (Kahan et al.,1999, Groth et al., 1999, Mac Donald et al., 1999, Kahan et al., 1999) have shown that Sirolimus is a potent immunosuppressive agent. Sirolimus is metabolized in humans by hepatic and intestinal cytochrome P450 3A4 primarily leading to demethylated and hydroxylated metabolites (Stattler et al., 1992). When sirolimus is given in the presence of cytochrome P450 3A4 inducers or inhibitors or to patients with hepatic insufficiency, sirolimus blood concentrations may be affected and dose adjustments may be required. Therefore, therapeutic drug monitoring (TDM) of sirolimus concentration plays an important role in the selection of the optimum dose of sirolimus.

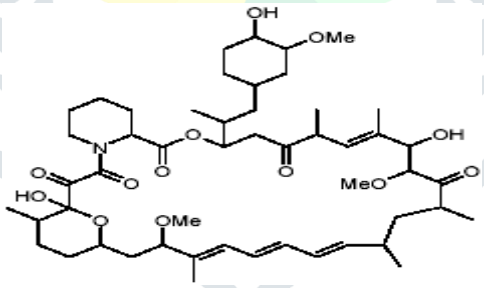


Figure no: 1 Sirolimus structure.

Sirolimus binds to the immnophilin FK506 binding protein and is sequesterd in red blood cells, leading to whole-blood/plasma ratios of 38 (Bierer et al., 1990, Zimmerman et al., 1997). Because of low sirolimus concentrations in plasma and limited stability in that matrix, whole blood is the matrix of choice for determining sirolimus concentrations (c_{min}) and sirolimus area under the concentration-time curve (Zimmerman et al., 1997, Ferron et al., 1998, Yatscoff et al., 1995, Kahan et al., 2000). A strong correlation between sirolimus c_{min} values provides a useful prediction of inadequate immunosuppression or potential

adverse events. When sirolimus is given with full-dose cyclosporine, the recommended therapeutic range for sirolimus whole-blood trough concentration is 5-15µg/L (Kahan et al., 2000). For an analytical method to be suitable for the TDM of sirolimus, it should be simple, sensitive (lower limit of quantification, <5 µg/L), and rapid (turn-around time, < 24h).

Several HPLC assays using ultraviolet (UV) detection (HPLC-UV) have been reported for the determination of sirolimus concentrations in whole blood (Yatscoff ea al., 1992, Napoil et al., 1943, Sevensson et al., 1997, Holt et al., 2000, Maleki et al., 2000). The assays are complicated by interfering peaks in the chromatograms, requiring tedious extraction procedures and long run times to resolve the peaks. The goal of the present study was to develop a simple (LC/MS/MS) assay for sirolimus that was precise and accurate at low concentrations and was capable of a high throughput. To accomplish this, it was necessary to eliminate interfering peaks at the sirolimus retention time and to decrease the run time by eliminating late-eluting peaks. Here we describe a simple and rapid LC/MS/MS method for the determination of sirolimu concentrations in human whole blood that meets these requirements. The validation was performed in accordance with regulatory guidelines (Shah et al., 1992).The aim of this study to establish a high accuracy, rapid and sensitive LC/MS/MS method to determine the concentration of Sirolimus in human plasma for pharmacokinetic analysis.

Materials and Methods

Chemical and reagents:

Sirolimus was obtained from Wyeth-Ayerst Analytical R&D.The purity of the compound was 99.9% as compared with standard.Acetonitrile, acetic acid of HPLC grade all from Merck. All other reagents were of analytical grade. Blank human plasma was obtained from healthy volunteers. Ultra pure water obtained from Milli-Q water purification system (S G waters, UK).

Preparation of standard samples:

Stock solution of Sirolimus was prepared by dissolving 2mg of Sirolimus in 2ml of Acetonitrile to give final concentration mg/mL. Standard solutions were obtained by diluting this solution with Acetonitrile to give final concentrations over the range of 10 -1000 ng/mL for preparation of the standard curve.

Sample preparation:

Plasma samples were obtained from healthy volunteers. A 500 µL aliquot of plasma was placed into a test tube, 50 µL standard solution of Sirolimus was added. The tube was vortex-mixed for 1min and kept at room temperature for 5min. After addition of 1ml of chloroform the tubes were vortex mixed for 1min and centrifuged for 3min at 2000g. The chloroform layer is completely removed and transferred to a clean test tube and evaporated to dryness under nitrogen, the residue was reconstituted with mobile phase. These samples are ready to analysis

Phosphate buffer saline (PBS) pH 7.2:

Phosphate buffer saline (PBS) ph 7.2 was prepared and filtered with 0.2µm pore size filter paper to avoid particulate matter.

Sample extraction:

Aliquots of 500µL of PBS ph 7.2 samples were collected. Added 2 mL of chloroform and votexd for 2 min.A ring was formed between aqueous and organic solvent.chloroform was collected from test tube dried under nitrogen. This evaporated sample was reconstituted with 1 ml of previously prepared mobile phase. Now the samples are ready to analysis.

Liquid chromatography and Mass spectrometric conditions:

LCMS/MS, Quattro micro API, triple quadrupole. Mass Lynx software, version 4.1. Consisted series of 2695 separation module and PDA (2996) detector all from Waters (Milford, MA, USA). Separation was achieved using phenomenax C-18 column (250x4.60 mm-5microns). The mobile phase contains 0.1% Acetic acid, (80:20, v/v) acetonitrile; water was prepared and degassed. Chromatographic separations were performed at 38°C. The flow rate was set to 1mL/min. Micro mass triple quadrupole mass spectrometer with an ESI^+ source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive ion mode and set up in multiple reaction monitoring (MRM).Desolvation Gas flow300(L/Hr).Gas cell pirani pressure 2.67 e-3(mbar).The capillary temperature was 3.25(KiloVolt), Cone 36.75 (Volt) for Sirolimus. Based on the full scan mass spectra of the analyte the most abundant ions were selected and mass spectrometer was set to the monitor the transitions of the precursors to the product ions as *m/z* 936.83 for Sirolimus.

Method Validation: The method was validated for selectivity, accuracy, precision, recovery, calibration curve range and reproducibility according to the FDA guidelines for validation of bio analytical method (FDA, 2001). The selectivity was investigated by preparing and analyzing four individual human blank plasma samples set LLOQ.

Accuracy and precision were assessed by determining QC samples at three concentration levels (five samples each concentration) on three different validation days. The precisions were

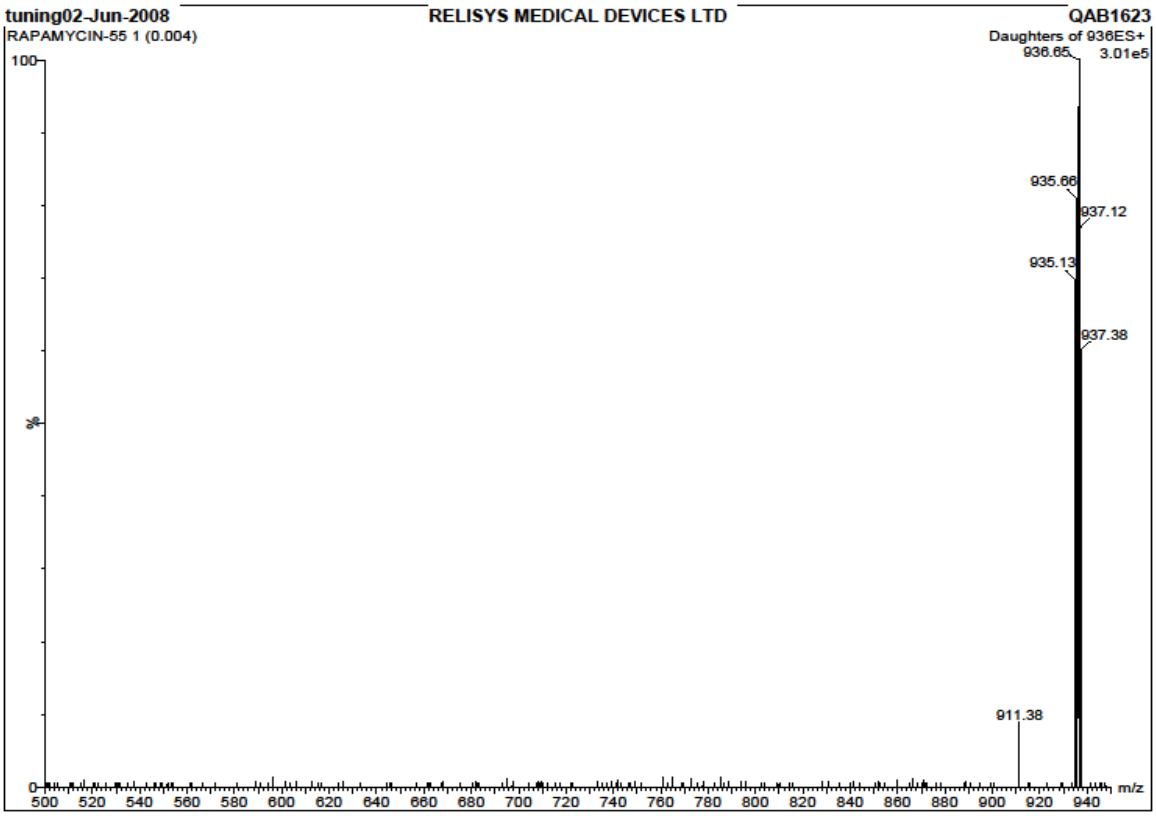


Figure no: 2. Sirolimus parent molecule spectrum

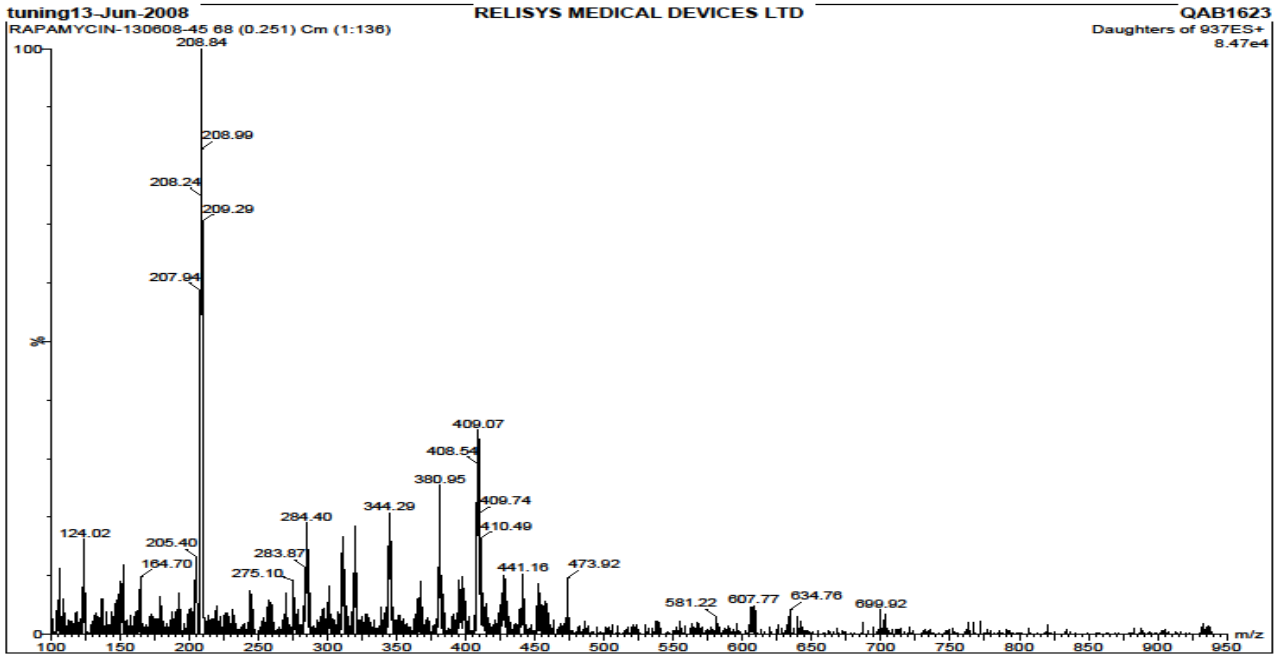


Figure no: 3. Product ions spectrum of Sirolimus.

determined as the RSD (%) and the accuracies were expressed as a percentage of the nominal concentration. The criteria used to assess the suitability of precision and accuracy was as follows: the RSD should not exceed 15% and the accuracy should be within 80-120%. Furthermore, the recovery (extraction efficiency) of analytic from human plasma was determined by comparing the areas of spiked plasma samples before and after liquid extraction that represent 100% recovery.

Results:

The Sirolimus parent molecule contains adduct formation; Sodium (Na^+ 22) was formed with parent molecule. Signal intensity was high in Sirolimus using ESI^+ source. ESI^+, Sirolimus formed predominantly protonated molecules $[M+H]^+$ of *m/*z 936.83 in full scan spectra **(Figure no. 2).** The most abundant ion in the product ion mass spectrum was at 208.84 for Sirolimus is represented in **figure no. 3.** To determined Sirolimus using MRM mode, full scan and product ion spectra of the analytic work investigated. The MRM method transition of *m/z* 936.83-208.84 for Sirolimus was selected to obtain maximum sensitivity. The U V detection of Sirolimus was at 278(277.78) nm **(Figure no. 4).**

Present study, a simple liquid- liquid extraction procedure was used. Extraction was carried out with different organic solvents like methanol, dichloromethane, diethyal ether, acetonitrile, chloroform. It was found that all solvents gave high extraction efficiency for Sirolimus. Extraction efficiency was increased when liquid-liquid

extraction was carried out with chloroform as extractive organic solvent. Among all, chloroform was rapid evaporation and 100 % extraction organic solvent for Sirolimus.

Method validation.

Selectivity.LC/MS/MS method was demonstrated high specificity only ions derived from the analytes of interest were monitored. The retention time of Sirolimus was 8.4 min **(Figure no. 5).**The slope, the intercept and correlation coefficient (r) for each standard curve from analytical run was determined automatically by mass lynx software.

Linearity and lower limit of quantification. The slop, the intercept and the correlation coefficient (r) for each standard curve from each analytical run were determined automatically.The representative standard curve for Sirolimus was 0.298484* x + -0.51036.

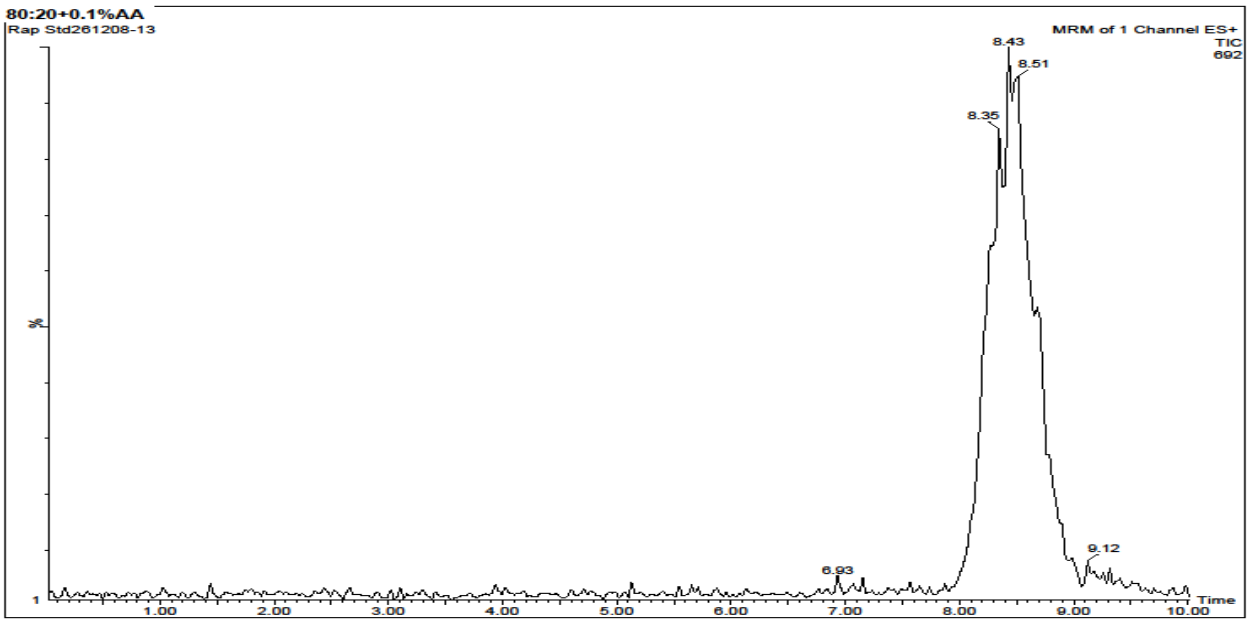


Figure no: 4 Retention time (RT) of Sirolimus.

Table 1. Intra – batch and inter – batch precision and accuracy data for assays of Sirolimus in human plasma

Nominal Concentration of Sirolimus (ng/ml)	Precision		Accuracy
	Mean ± SD	RSD (%)	Mean relative error (%)
Inter-batch (n=5)			
0.5	0.5 ± 0.0	5.0	9.6
6.0	5.8 ± 0.8	12.9	-3.1
45.0	42.6 ± 3.0	7.1	-5.4
Intra batch (n=3)			
0.5	0.5 ± 0.1	10.2	3.0
6.0	6.3 ± 0.3	4.6	5.9
45.0	46.3 ± 1.7	3.7	3.0

RSD = Relative Standard Deviation.

Mean relative error = (Overall mean assayed concentration – added concentration) / (added concentration) x 100.

The mean squared correlation coefficient (r^2) for calibration standard curve was 0.997183**(Figure no. 6).** Sirolimus gave linear response as a function of the concentrations ranges showed excellent linearity over 10-1000ng/mL.

The lower limit of quantification(LLOQ) for sirolimus was 0.10 ng/mL.The analyte response at these concentration levels was > 20 times the base line noise.The recession and accuracy at these concentration levels were acceptable, with < 9.6% of the CVs.

Precision and accuracy.

The intra-batch inter-batch precision and accuracy data for Sirolimus was summarized in **Table 1.** All values of accuracy and precision were within recommended limits (FDA, 2001).The intra-batch precision was 3.7-10.2%, and the inter-batch precision was 5.1-12.9%. The mean inter-batch error was between-5.4-9.6%.

Recovery.

Table 2 shows the recovery (extraction efficiency) for Sirolimus from human plasma following chloroform extraction**.** Recovery of Sirolimus from human plasma extraction recoveries were 100.0 %, and were similar at all analyte concentractions, which indicated that the extraction efficiency for Sirolimus using chloroform was satisfactory.

Table 2. The recovery (extraction efficiency) for Sirolimus in human plasma (n=5)

Nominal concentration of Sirolimus (ng/ml)	Recovery (mean ± SD, %)	RSD (%)
0.5	88.9 ± 8.9	10.0
6.0	100.0 ± 4.6	4.6
45.0	96.4 ± 3.5	3.6

Stability.

The stability of Sirolimus in human plasma under different storage conditions is observed.No degradation products were detected under selected MS conditions.Sirolimus in human plasma can be stored at 35°C around 90 days.

Discussion:

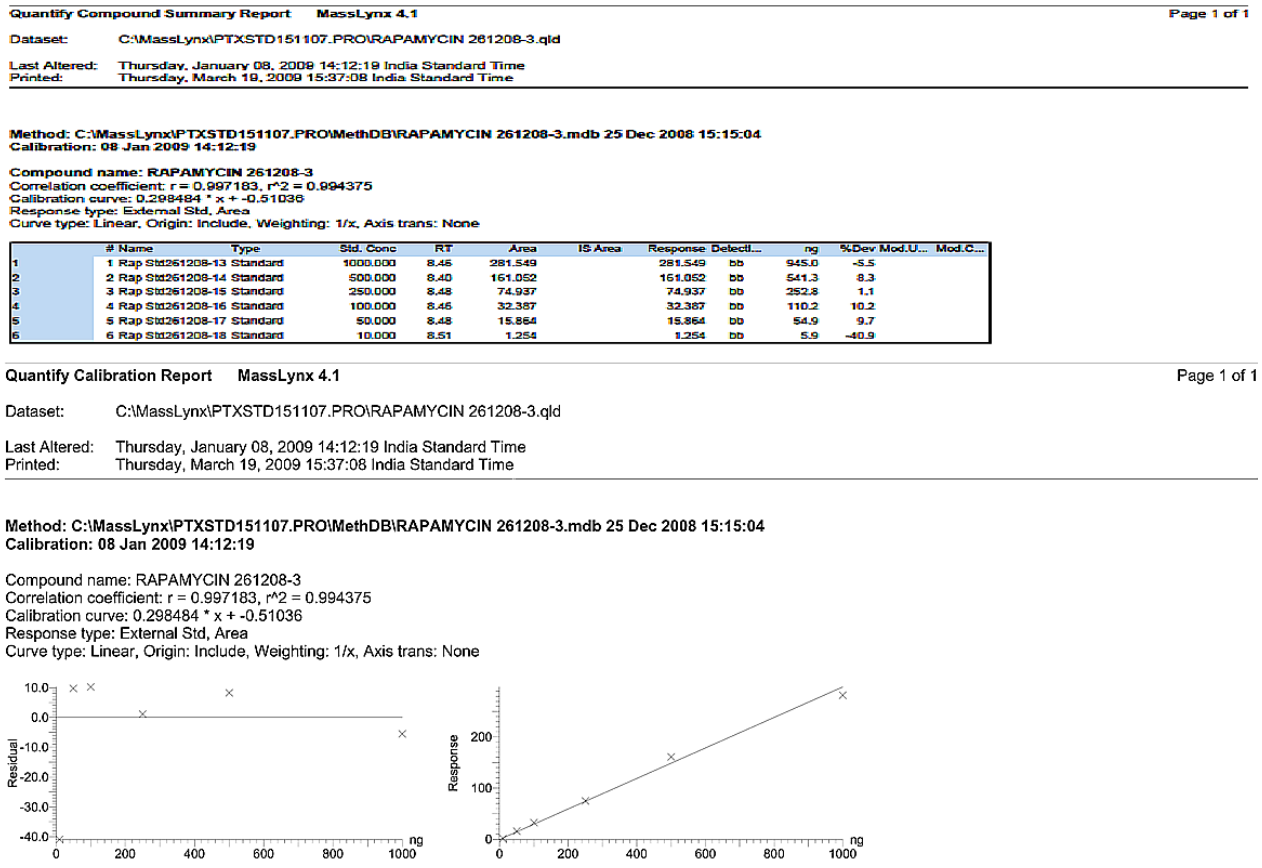
In this study, ESI^+ was chosen as the ionization source. Signal intensity was high using ESI^+ source provided for the quantitation of samples. Sirolimus formed predominantly protonated molecules $[M+H]^+$ of *m/z* 936.83

in full scan spectra. The most abundant ion in the product ion mass spectrum was at 208.84.The MRM transition of *m/z* 936.83-208.84 for Sirolimus was selected to obtain maximum sensitivity.

For quantification of Sirolimus a new MRM method was created. Sirolimus the parent molecule was fragmented into the daughter ions through the collision energy. Argon was used as collision energy. A standard curve of Sirolimus in different range of concentrations 10, 50,100,250,500,1000ng/ml was prepared. The calibration curve displayed excellent linearity (r^2>0.997183) over the concentration range investigated.

In the present study, Liquid-Liquid extraction procedure was used. The extraction efficiency was increased when liquid-liquid extraction solvent as chloroform. The proposed chromatographic conditions of LCMS/MS analysis was carried out. Retention time of Sirolimus approximately 8.4 min. Optimization was achieved by monitoring varying reversed phase column, mobile systems, flow rate and wavelength.

In the present study the most important LCMS/MS technique for determination of Sirolimus in biological fluids were estimated. This technique is rapid and reliable and simple extraction method.



Quantify Compound Summary Report MassLynx 4.1 Page 1 of 1

Dataset: C:\MassLynx\PTXSTD151107.PRO\RAPAMYCIN 261208-3.qld

Last Altered: Thursday, January 08, 2009 14:12:19 India Standard Time
Printed: Thursday, March 19, 2009 15:37:08 India Standard Time

Method: C:\MassLynx\PTXSTD151107.PRO\MethDB\RAPAMYCIN 261208-3.mdb 25 Dec 2008 15:15:04
Calibration: 08 Jan 2009 14:12:19

Compound name: RAPAMYCIN 261208-3
Correlation coefficient: r = 0.997183, r^2 = 0.994375
Calibration curve: 0.298484 * x + -0.51036
Response type: External Std, Area
Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None

	#	Name	Type	Std. Conc	RT	Area	IS Area	Response	DetectI...	ng	%Dev	Mod.U...	Mod.C...
1	1	Rap Std261208-13	Standard	1000.000	8.45	281.549		281.549	bb	945.0	-5.5		
2	2	Rap Std261208-14	Standard	500.000	8.40	161.052		161.052	bb	541.3	8.3		
3	3	Rap Std261208-15	Standard	250.000	8.48	74.937		74.937	bb	252.8	1.1		
4	4	Rap Std261208-16	Standard	100.000	8.46	32.387		32.387	bb	110.2	10.2		
5	5	Rap Std261208-17	Standard	50.000	8.48	15.864		15.864	bb	54.9	9.7		
6	6	Rap Std261208-18	Standard	10.000	8.51	1.254		1.254	bb	5.9	-40.9		

Quantify Calibration Report MassLynx 4.1 Page 1 of 1

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Response type: External Std, Area
Curve type: Linear, Origin: Include, Weighting: 1/x, Axis trans: None

Figure no: 5. Standard calibration curve of Sirolimus.

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