



Rapid and Sensitive HPLC Method for the Determination of Sirolimus with Ketoconazole as Internal Standard and Its Further Applications

A Anil Kumar^{1*}, P Srinivas¹, K Spandana², N. Rama³, J Vidya Sagar¹

¹Bio Analytical, Research and Development Division, Relisys Medical Devices Ltd (RMDL), Hyderabad-501 510, Andhra Pradesh, India

²Sri Siddhartha Pharmacy College, Nuzvid, Krishna District, Andhra Pradesh, India

³Clinical Research, Research and Development Division, Relisys Medical Devices Ltd (RMDL), Hyderabad-501 510, Andhra Pradesh, India

ABSTRACT

Sirolimus and Ketoconazole are used in organ transplantation regimen and potential metabolic interactions of these drugs were reported when administered concomitantly. An analytical method based on high-performance liquid chromatography (HPLC) with photo diode array (PDA) detection was developed for quantification of sirolimus using ketoconazole as internal standard. Extraction was performed using dichloromethane under nitrogen atmosphere and the separation of sirolimus and ketoconazole was accomplished by reverse phase chromatography. The mobile phase consists of a combination of methanol, water and glacial acetic acid at 90:10:0.1% ratios run isocratically through a C18 (250mm × 4.6mm, 5µm) reverse phase analytical column. The PDA detection was done at 278nm with analytical run time less than 6 min. The average mean recovery was found to be 98.3% for 1, 3, 5, 10µg/ml concentrations. The assay exhibited good linear relationship. LLOQ was 10ng/ml with 0.84% and 1.28% of accuracy and precision over the concentration range of 0.1-10µg/ml. The method can be successfully applied for estimation of sirolimus from *in-vitro* elution studies of sirolimus eluting stents, simultaneous estimation of ketoconazole and sirolimus in therapeutic drug monitoring and other pharmacokinetic studies.

Keywords: HPLC, Ketoconazole, Sirolimus, Therapeutic drug monitoring.

INTRODUCTION

Sirolimus (Rapamycin), a natural 31-membered macrocyclic lactone produced by *Streptomyces hygroscopicus*. Sirolimus (SRL) is a potent immunosuppressive agent used to prevent rejection in organ transplantation especially useful in kidney transplants approved by the Food and Drug Administration. [1] It can down-regulate cytokine dependent kinase mTOR (mammalian target of rapamycin), thus inducing cell-cycle arrest in the late G1 phase. [2-3] It is well known that sirolimus is only effective when bound to sirolimus binding protein (FKBP), present on smooth muscle cells. [4] SRL inhibits the proliferation of both rat and human smooth muscle cells *in vitro* and reduces intimal thickening in models of vascular injury. [5-6] It is the most preferred drug for inhibiting restenosis in cardiac stenting and used as drug eluting stent (DES) in coronary artery interventions.

***Corresponding author: Mr. A Anil Kumar**, Bio Analytical, Research and Development Division, Relisys Medical Devices Ltd (RMDL), Hyderabad-501 510, Andhra Pradesh, India; **Tel.:** +91-40-23493349; **Fax:** +91-40-23493301; **E-mail:** annampellianil@yahoo.com

SRL suffers from poor oral bioavailability due, in large part, to extensive presystemic metabolism by cytochrome P450 3A (CYP3A). Studies in humans have shown a relationship between trough blood SRL concentrations and immunosuppressive efficacy and toxicity of the drug. [7-9] This suggests that Therapeutic Drug Monitoring (TDM) of SRL concentrations is advisable to optimize the drug dosing regimen. Since the drug has a narrow therapeutic index, it is important to develop a sensitive and rapid method to quantify SRL in biological matrices.

Ketoconazole (KTZ) is an antifungal agent used along with SRL in organ transplantation regimens to prevent fungal infections. KTZ inhibits cytochrome P450 3A4, leading to a 10-fold increase in SRL blood levels. Clinical drug interactions have demonstrated that SRL levels are significantly increased when administered with KTZ, an established CYP3A inhibitor. [10-11]

SRL possesses a high molecular weight (MW; 913.6 Da) It is sparingly soluble in water, aliphatic hydrocarbons, and diethyl ether, whereas it is soluble in alcohols, halogenated hydrocarbons, and dimethyl sulfoxide. [12-15] Sirolimus is unstable in solution, degrades in plasma, low- and neutral-pH

buffers at 37°C, with a half-life of <10h. The structure of SRL was shown in Fig. 1.

The aim of the present study is to develop rapid and sensitive HPLC method for the determination of sirolimus with ketoconazole as internal standard. This method offers the advantage of simplicity with adequate sensitivity, selectivity, precision and accuracy. This analytical method can be used for estimation of sirolimus from *in-vitro* elution studies of sirolimus eluting stents, simultaneous estimation of ketoconazole and sirolimus in therapeutic drug monitoring and other pharmacokinetic studies.

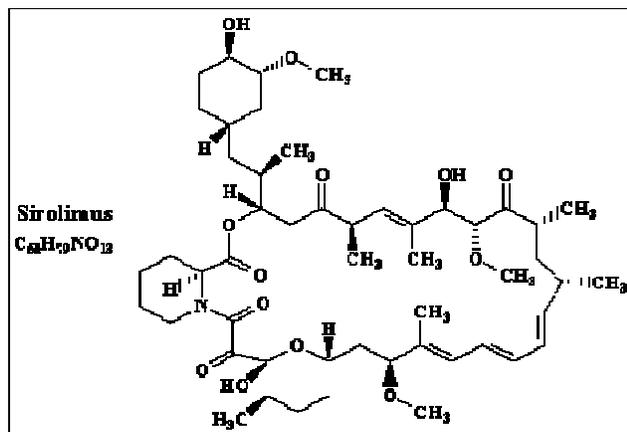


Fig. 1: Structure of sirolimus

MATERIALS AND METHODS

SRL was purchased from World (JIANGSU) Industry Co., LTD, Jiangsu, China, and KTZ was purchased from Glochem laboratories Hyderabad. Acetonitrile, Dichloromethane and Glacial acetic acid were purchased from Merck India. All the solvents used in the studies are of HPLC grade. Phosphate buffer saline (PBS) pH 7.4 was purchased from Himedia laboratories Pvt. Ltd, Mumbai. Double distilled Water for analytical purpose was obtained from Milli-Q R-O system (SG Analytical, India). Sirolimus eluting stents (SES) (Release - R) were from Relisys Medical Devices, Hyderabad, India.

Chromatographic conditions

The HPLC system consisted of a Shimadzu LC-20AT liquid chromatographic pump, Rheodyne injection port (Rheodyne, Cotati, CA, USA) with a 20 µl sample loop and SPD-M20A Photo diode array (PDA) detector (Shimadzu, Kyoto, Japan). Data collection, integration and calibration were accomplished using LC Solutions chromatography Data system.

The chromatographic separation of sirolimus was accomplished using 250 × 4.6 mm Waters Xterra MS C₁₈ - 5 µm reverse phase analytical column. The mobile phase consisted of methanol, water and glacial acetic acid in the ratios of 90:10:0.1% v/v. Before use, the mobile phase was filtered by passing it through a 0.45 µm filter and the filtrate is degassed by using bath sonicator. The mobile phase was pumped at an isocratic flow of 1 ml/min at room temperature. The PDA detection wave length was set at 278 nm. All separations were performed at ambient temperature.

Preparation of stock solution

Stock solution of SRL was prepared in methanol at 1 mg/ml and was kept at -20°C. This stock solution was diluted with methanol to obtain the concentrations required for

preparation of standard working solutions. SRL working solutions were prepared in the range of 0.1-10 µg/ml. Stock solution of KTZ was prepared in methanol at 1 mg/ml and was kept at -20°C. This stock solution was diluted with methanol to obtain the concentrations required for preparation of standard working solutions. 10 µg/ml of KTZ solution was added to each sample. Samples for the determination of recovery, precision and accuracy were prepared by spiking quality control (QC) standard SRL concentrations (1, 3, 5, 10 µg/ml) and stored at -20°C.

Extraction procedure

SRL *in-vitro* samples were obtained from release studies of SRL eluting stents where PBS is used as the dissolution media. Blood samples were obtained from patients on SRL for kidney transplantation and also from patients co administered with KTZ. Plasma separated from the blood samples and stored at -4°C till analysis. SRL/ KTZ were extracted from plasma / PBS by adding equal volume of dichloromethane. The samples were vortexed for 3 min, supernatant separated and evaporated at room temperature under nitrogen atmosphere. The evaporated samples are reconstituted with 1 ml of mobile phase and spiked.

Assay validation

The RP-HPLC assay validation was done as per ICH Q2A and Q2B guidelines. The linearity of our method was tested by constructing a curve from 0.1-10 µg/ml. The linearity was established by linear regression of the peak area ratio of SRL/I.S. versus the concentration. The limit of detection (LOD) and the lower limit of quantification (LLOQ) were measured according to the FDA's guidance for bioanalytical method validation. The limit of detection was defined as the lowest concentration of SRL resulting in a peak height greater or equal to three times from background noise (S/N ≥ 3). The LOQ was investigated in extracted samples from five different days. The intra-assay precision was verified at four concentrations (1, 3, 5, 10 µg/ml) for five analyses. The inter-day precision was determined at the same five concentrations in five replicates. The inaccuracy was calculated as the percentage error from the true value.

The overall recovery for SRL and I.S. was calculated by comparing the peak-area ratios of spiked samples with those obtained by direct injections of the same amount of both compounds. The suitability parameters that are evaluated for HPLC method includes peak area reproducibility and retention time.

RESULTS

Chromatography

To facilitate pharmacokinetics study of SRL, a sensitive and reproducible HPLC method has been developed and validated for quantitative determination of SRL *in-vitro* samples. After the pretreatment with a rapid single liquid-liquid extraction SRL was separated by reverse HPLC with PDA detection at 278 nm. The representative chromatograms of SRL spiked in unextracted standard concentration, blank, *in-vitro* release studies of SES and TDM samples were shown in Fig. 2-5. The retention time of KTZ and SRL was 4.30 min and 5.23 min respectively with good base line separation.

Linearity, limit of detection (LOD) and limit of quantitation (LOQ)

Peak areas ratio of sirolimus and ketoconazole were measured. A representative calibration graph of peak area

ratio versus concentration in the range of 0.1-10 μ g/ml resulted in regression equation of the calibration curve was calculated as $y = 57.56x + 3671$ (correlation coefficient, $R^2 = 0.997$), where y is the peak area ratio of SRL/KTZ and x is the concentration of SRL. These results demonstrated a good linearity between the peak area ratios versus concentrations. The limit of detection (LOD) and lower limit of quantitation (LLOQ) was found to be 3ng/ml and 10ng/ml respectively.

Precision and accuracy

The precision of the assay method was validated by the determination of the intra- and inter-day coefficient of variation (%RSD) and percentage deviation. The intra-day and inter-day precision has been done over the concentration range of 0.1-10 μ g/ml. The average %RSD of intra-day and inter-day precision was within the acceptable limits only. The accuracy of the method was verified by comparing the concentrations measured for SRL spiked from extracted sample with actual added concentrations. The intra- and inter-day data expressed as percentage deviation and the data were shown in Table 1.

Recovery

The recovery of SRL after liquid-liquid extraction procedures was evaluated at four concentrations of 1, 3, 5, 10 μ g/ml. Absolute recovery was calculated by comparing the peak areas for direct injection of pure SRL in acetonitrile with those obtained by dichloromethane extracted samples containing same amount of sirolimus. Table 2 shows the recovery efficiency of sirolimus from *in-vitro* samples and the average extraction efficiency of were found to be 98.3%.

System suitability

The %CV for area response for the drug was 1.65%, which is within the acceptance value $\pm 2\%$. The %CV for retention time for the drug was 0.3% respectively, which is within the acceptance range of $\pm 2\%$.

Table 1: Intraday and interday accuracy and precision of HPLC assay for Sirolimus

Theoretical concentration (μ g/ml)	Observed concentration (μ g/ml, mean \pm S.D, n=5)	%RSD
Intra-day (n=5)		
1	1.02 \pm 0.01	0.980
3	2.98 \pm 0.02	0.671
5	4.93 \pm 0.04	0.811
10	9.98 \pm 0.09	0.901
Inter-day (n=5)		
1	1.11 \pm 0.01	0.900
3	3.01 \pm 0.04	1.328
5	4.99 \pm 0.10	2.004
10	10.02 \pm 0.09	0.898

Table 2: Recovery of Sirolimus from *in-vitro* samples

QC samples (μ g/ml)	Concentration of QC samples after extraction	Recovery (%)
1	0.98	98
3	2.95	98.3
5	4.90	98
10	9.89	98.9

DISCUSSION

The present method for the determination of SRL with KTZ as internal standard from *in-vitro* samples is rapid, sensitive and reproducible. The excellent separation was demonstrated in the chromatograms and no interfering peaks were observed. The calibration curve was linear linearity between the peak area ratios versus concentrations and the method was suitable for the analysis of *in-vitro* samples over the

range of 0.1-10 μ g/ml. The lower limit of quantification (LLOQ) was found to be 10ng/ml.

The accuracy of the method was in compliance with the proposed limits and the precision of the method was satisfactory. The system suitability of the method shows that the performance of the chromatographic system is not significantly influenced by variations of the operational parameters inside an accepted domain. This method shows the system suitability parameters are within the limits only. A rapid single-step liquid-liquid extraction with dichloromethane shows good recovery. This method offers short run time (less than 6min) provides a cost effective analysis. The method described here is appropriate for a clinical study that does not require analysis of SRL metabolites.

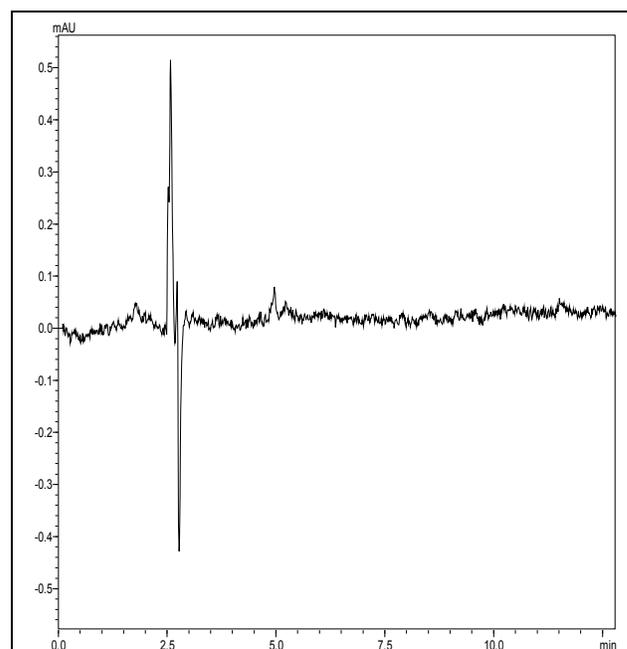


Fig. 2: Blank chromatogram

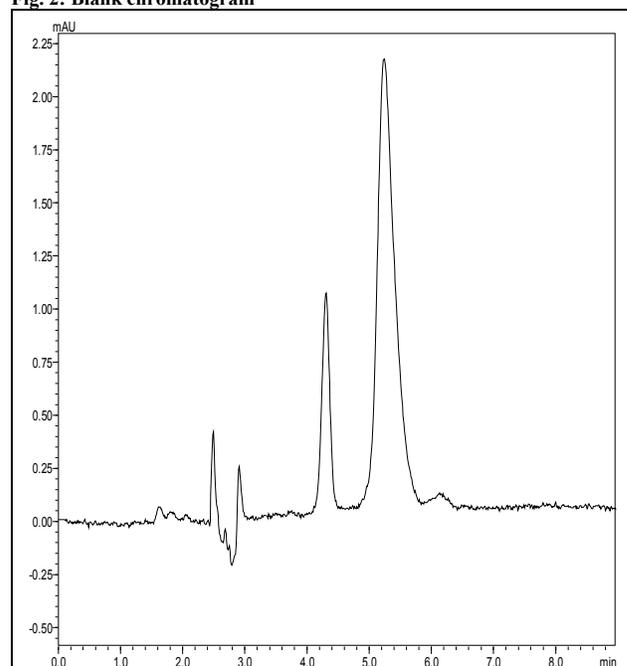


Fig. 3: Standard chromatogram of Sirolimus (700ng/ml)

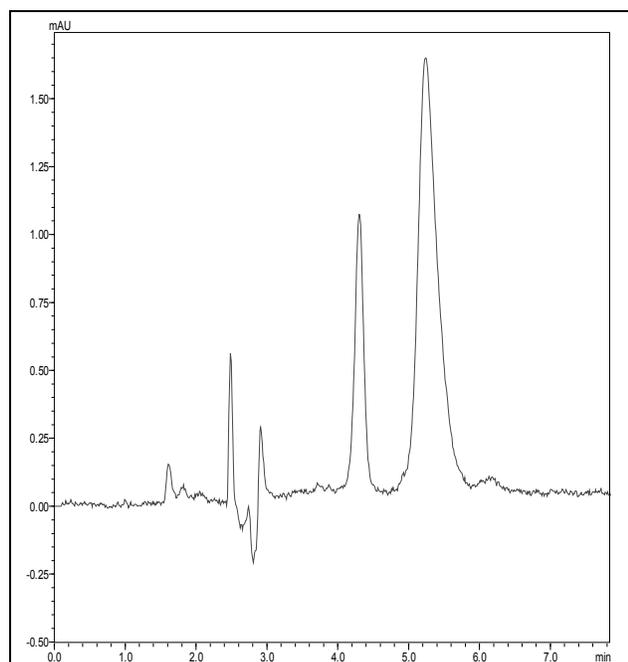


Fig. 4: Chromatogram of Sirolimus from invitro release samples of SES

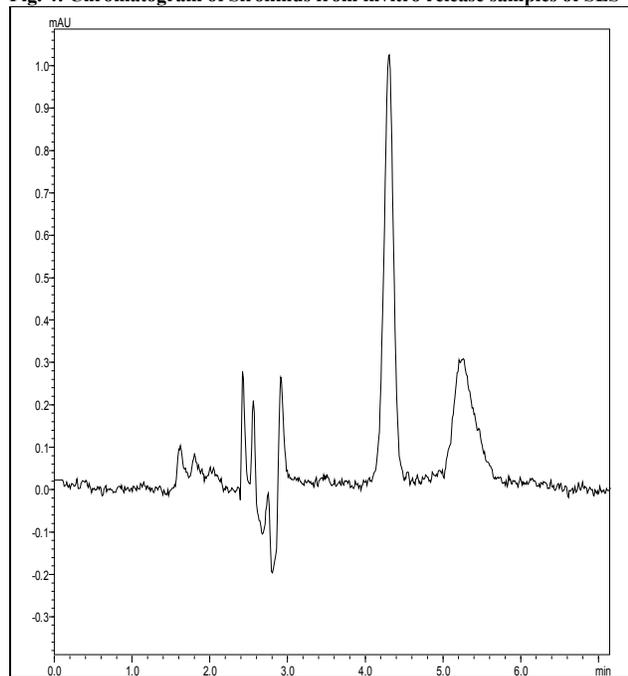


Fig. 5: Chromatogram of Sirolimus from plasma samples

In conclusion, the method can be successfully applied for estimation of SRL from *in-vitro* elution studies of sirolimus eluting stents, simultaneous estimation of KTZ and SRL in therapeutic drug monitoring and other pharmacokinetic studies.

REFERENCES

1. Groth CG, Backman L, Morales JM, Calne R, Kreis H, Lang L, Touraine JL, Claesson K, Campistol JM, Durand D, Wranner L, Brattstrom C, Charpentier B. Sirolimus (rapamycin) - based therapy in human renal transplantation: similar efficacy and different toxicity compared with cyclosporine: Sirolimus European Renal Transplant Study Group. *Transplantation* 1999; 67: 1036-1042.
2. Marx SO, Jayaraman T, Go LO, Marks AR. Rapamycin- FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. *Circ. Res.* 1995; 76: 412-417.
3. Poon M, Marx SO, Gallo R, Badimon JJ, Taubman MB, Marks AR. Rapamycin inhibits vascular smooth muscle cell migration. *J. Clin. Invest.* 1996; 98: 2277-2283.
4. Bhargava B, Karthikeyan G, Abizaid AS, Mehran R. New approaches to preventing restenosis. *Clin. Rev. B. M. J.* 2003; 327: 274-279.
5. Gregory CR, Huang X, Pratt RE, Dzau VJ, Shorthouse B, Billingham ME, Morris RE. Treatment with rapamycin and mycophenolic acid reduces arterial intimal thickening produced by mechanical injury and allows endothelial replacement. *Transplantation* 1995; 59: 655-661.
6. Gallo R, Padurean A, Jayaraman T, Marx S, Roque M, Adelman S, Chesebro J, Fallon J, Fuster V, Marks A, Badimon JJ. Inhibition of thickening after balloon angioplasty in porcine coronary arteries by targeting regulators of the cell cycle. *Circulation* 1999; 99: 2164-2170.
7. Fryer J, Yatskoff RW, Pascoe EA, Thliveris J. The relationship of blood concentration of rapamycin and cyclosporine to suppression of allograft rejection in a rabbit heterotopic heart transplant model. *Transplantation* 1993; 55: 340-345.
8. Yakimets WJ, Lakey JR, Yatskoff RW, Katyal D, Finegood DT, Rajotte RV, Kneteman NM. Prolongation of canine pancreatic islet allograft survival with combined rapamycin and cyclosporine therapy at low doses. Rapamycin efficacy is blood level related. *Transplantation* 1993; 56: 1293-1298.
9. Kahan BD, Napoli K, Kelly PA, Podbielski J, Hussein I, Urbauer DL, Katz SH, Van Buren CT. Therapeutic drug monitoring of sirolimus: correlations with efficacy and toxicity. *Clin. Transplant.* 2000; 14: 97-109.
10. Thomas PP, Manivannan J, John GT, Jacob CK. Sirolimus and ketoconazole co-prescription in renal transplant recipients. *Transplantation* 2004; 77(3): 474-475.
11. Vincent WJ, Jeffrey SA, Susan W, Paulina TT, Amy OC, Anne C, Xiang-Qing YU, Daniel O, Zeibun R. Sirolimus oral absorption in rats is increased by ketoconazole but is not affected by D-alpha-tocopheryl poly (ethylene glycol 1000) succinate. *The Journal of Pharmacology and Experimental Therapeutics* 2002; 303(1): 308-313.
12. Findlay JA, Radics L. On the chemistry and high field nuclear magnetic resonance spectroscopy of rapamycin. *Can J Chem.* 1980; 58: 579-90.
13. Sehgal SN, Baker H. Rapamycin (AY-22, 989), a new antifungal antibiotic II Fermentation, isolation and characterization. *J Antibiot (Tokyo).* 1975; 28: 727-730.
14. Morris RE. Rapamycins: antifungal, antitumor, anti proliferative, and immunosuppressive macrolides. *Transplant Rev.* 1992; 6: 39-87.
15. Stella VI, Kennedy PE. Prodrugs of rapamycin. US Patent no 4, 650, 803. 1987.