Advanced Pharmaceutical Bulletin, **2012**, 2(2), 135-139
doi: 10.5681/apb.2012.021
http://apb.tbzmed.ac.ir/

Development and Validation of an HPLC Method for the Analysis of Sirolimus in Drug Products

Ziba Islambulchilar [1,2,3], Saeed Ghanbarzadeh [1,4], Shahram Emami [3], Hadi Valizadeh [1,5], Parvin Zakeri-Milani [1,6*]

[1] *Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.*

[2] *Faculty of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran.*

[3] *Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran.*

[4] *Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*

[5] *Research Center for Pharmaceutical Nanotechnology, Tabriz University of Medical Sciences, Tabriz, Iran.*

[6] *Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.*

ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 20 April 2012
Accepted: 10 May 2012
ePublished: 20 May 2012

Keywords:
Sirolimus
HPLC
Method development
Validation
Ultraviolet
Chromatography

ABSTRACT

***Purpose*:** The aim of this study was to develop a simple, rapid and sensitive reverse phase high performance liquid chromatography (RP-HPLC) method for quantification of sirolimus (SRL) in pharmaceutical dosage forms**.** ***Methods*:** The chromatographic system employs isocratic elution using a Knauer- C18, 5 mm, 4.6 × 150 mm. Mobile phase consisting of acetonitril and ammonium acetate buffer set at flow rate 1.5 ml/min. The analyte was detected and quantified at 278nm using ultraviolet detector. The method was validated as per ICH guidelines. ***Results*:** The standard curve was found to have a linear relationship ($r^2 > 0.99$) over the analytical range of 125–2000ng/ml. For all quality control (QC) standards in intraday and interday assay, accuracy and precision range were -0.96 to 6.30 and 0.86 to 13.74 respectively, demonstrating the precision and accuracy over the analytical range. Samples were stable during preparation and analysis procedure. ***Conclusion*:** Therefore the rapid and sensitive developed method can be used for the routine analysis of sirolimus such as dissolution and stability assays of pre- and post-marketed dosage forms.

Introduction

Sirolimus (SRL, formerly rapamycin: $C_{51}H_{79}NO_{13}$, CAS: 53123-88-9) is a lactone-lactam macrolide antibiotic with immunosuppressant and anticancer effects. It was first identified as an antifungal agent produced by the Streptomyces hygroscopicus bacterium and was subsequently demonstrated to be a potent immunosuppressive agent that was approved by the U.S. Food and Drug Administration (FDA) to be used beside cyclosporine or tacrolimus in kidney transplantation. In contrast to tacrolimus and cyclosporine, which inhibit the production of cytokines, sirolimus binds to the FK (peptidyl-prolyl cis-trans isomerase) binding protein which modulates the activity of the mammalian Target Of Rapamycin (mTOR).The mTOR inhibits Interleukin-2-mediated signal transduction, resulting in cell cycle stop in the G1-S phase and prevents cell cycle progression and proliferation, hence blocks the response of T- and B-cell activation by cytokines. These pharmacological properties allow rapamycin not only to be a promising immunosuppressant with the absence of nephrotoxicity but also to be a possible chemotherapeutic agent against many types of solid tumor. SRL is a white to off-white powder, insoluble in water, very slightly soluble in hexane and petroleum ether, soluble in methanol, diethyl ether, and N, N-dimethylformamide (DMF) and freely soluble in benzyl alcohol, chloroform, acetone, and acetonitrile (ACN).[1-9] Validation of new analytical methods for pharmaceutical products is a requirement of Current Good Manufacturing Practice (cGMP) regulations. As stated by the current International Conference on Harmonization (ICH) guideline, accuracy, precision, linearity and stability of test methods are some of the analytical parameters which require assays validation.[3,10-13] Several types of analytical methods have been used for quantification of SRL, including immunoassay, HPLC ultraviolet, and HPLC mass spectrometric detection (HPLC-MS or HPLC/MS/MS). The immunoassay is not commercially available, and there is an acute need for an accurate, rapid, and simple chromatographic assay to determine SRL in drug development procedure and also post marketing investigations.[4,7,14,15] Considering the less complication

***Corresponding author:** Parvin Zakeri-Milani (PhD), Department of Pharmaceutics, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran. 51664. Tel: +98 (411) 3392593, Fax: +98 (411) 3344798, E-mail: pzakeri@tbzmed.ac.ir

and readily availability of HPLC-UV method in research laboratories and clinic a simple and rapid chromatographic method in the present study was developed to routine analysis of sirolimus concentration.

Materials and Methods

Chemicals

Sirolimus was obtained from Poli (Lazio, Italy). HPLC grade solvents, such as methanol and acetonitril (ACN) were purchased from Merck (Darmstadt, Germany). Double-distilled water was used during the entire HPLC procedure. Analytical grade ammonium acetate, sodium lauryl sulphate (SLS) and sodium hydroxide were obtained from Merck (Darmstadt, Germany).

HPLC conditions

The RP-HPLC (Beckman, Florida, USA) with a variable wavelength ultraviolet spectrophotometric detector (166 gold) set at 278 nm. System Gold software was used for data acquisition and system Gold nouveau software was used for data reporting and analysis.

For the preliminary analysis different columns in combination with different solvent systems were tried out. Analytical column used for analysis was Knauer ODS 5 mm, 4.6 × 150 mm. Column temperature was set at 55°C. The mobile phase was a mixture of 70% ACN and 30% ammonium acetate buffer (The buffer was prepared by dissolving 0.8 g ammonium acetate in 1000 ml water, adjusted to pH 5.8 with NaOH 1N). Injection volume was 150 µl which injected into the column using a Hamilton (Bonaduz, Switzerland) injector syringe and the isocratic flow rate was set at 1.5 ml/min. SRL was detected by UV absorption at 278 nm.

Preparation of standard solutions

The primary stock solution of sirolimus was prepared by dissolving 10 mg sirolimus in methanol in a 50 ml volumetric flask to yield a concentration of 200 µg/ml. One ml of obtained solution was diluted in 100 ml volumetric flask with sodium lauryl sulphate (0.4% in water) solution. Finally, working standard solutions were prepared by serially dilution with SLS solution to obtain sirolimus concentrations of 125, 250, 500, 1000, 1500, and 2000 ng/ml.

Method Validation

The optimized RP-HPLC method was validated according to ICH guidelines with respect to linearity range, sensitivity, accuracy, precision, and stability.

Linearity

The linearity of an analytical method indicates its ability to obtain the response directly proportional to the concentration of the analyte in the sample within a definite range. The standard curves in the range of 125-2000 ng/ml were prepared on 3 consecutive days. Each set of standards was injected into the column from lowest to highest concentration.

Limits of Detection (LOD) and Quantitation (LOQ)

LOD and LOQ settle on the sensitivity of the method. LOD is the lowest concentration of the analyte detected by the method and LOQ is the minimum measurable concentration. There are three different methods indicated in ICH guidelines for determination of LOD and LOQ. The first method relies on visual evaluation method, second, signal to noise ratio method (the signal-to-noise ratio of 3:1 and 10:1 were taken as LOD and LOQ respectively) and third is the slope method (were calculated by the equations LOD = yb + 3.3sb, and LOQ = yb + 10sb where yb is the signal of the blank and sb is its SD), which were calculated using Gold nouveau software.[7,10,12,13,16-19] In this study we used the slope method for determination of LOD and LOQ.

Accuracy and Precision

Three quality control samples with concentrations at three levels (low, medium and high) within calibration range were used in triplicates to determine the accuracy and precision of the method. The mean measured concentrations for all prepared samples were considered for intraday and interday accuracy and precision evaluation. Accuracy was assessed via calculating the percentage deviation of the calculated concentration and the theoretical concentration while the precision was determined by calculating the coefficient of variation percentage (CV %) for intra- and inter-day runs. The acceptable value for accuracy is less than ±15% deviation from the nominal values and, CV% ≤ ±15% for precision.[1,3,7,13,16,18,20]

Stability

The stability of the drug in analytical solution was checked by replicate analysis (N = 3) of the three control samples (in low, medium and high concentrations) for 6 h (considering the approximate time required for preparation of samples and analysis by HPLC method) after storage at ambient temperature. In all sample stability experiments, the results were compared with the sirolimus initial concentrations. The acceptable relative error was considered to be ±15 %.[13,14,21-23]

Results and Discussion

Linearity

Linearity of the method was confirmed by preparing sirolimus standard curves for the analytical range of 125–2000 ng/ml. Statistical analysis using least square regression indicated excellent linearity for SRL in the mentioned range. A good correlation between SRL peak heights and drug concentration was observed with $r^2 \geq 0.99$ for all standard curves (Table 1). Concentration curves for SRL had a mean slope, intercept and r^2 of 0.099, -9.82 and 0.996 respectively.

Representative chromatogram of serial concentrations of SRL in the range 125 nm to 2000 nm is shown in Figure 1. The retention time was approximately 8 min.

LOD and LOQ

The LOD and LOQ were calculated by the equations LOD = yb + 3.3sb and LOQ = yb + 10sb where yb is the signal of the blank and sb is its SD. Based on these equations, the calculated LOD and LOQ values for SRL were 22.03 and 56.2 ng/ml, respectively.

Table 1. Linearity data of SRL calibration curves on 3 consecutive days

Standard curve	Slope	Intercept	r^2
Day 1	0.078	14.34	0.996
Day 2	0.067	-79.51	0.994
Day 3	0.091	35.70	0.996
Mean	0.099	-9.82	0.996
RSD	0.15	-6.24	0.001

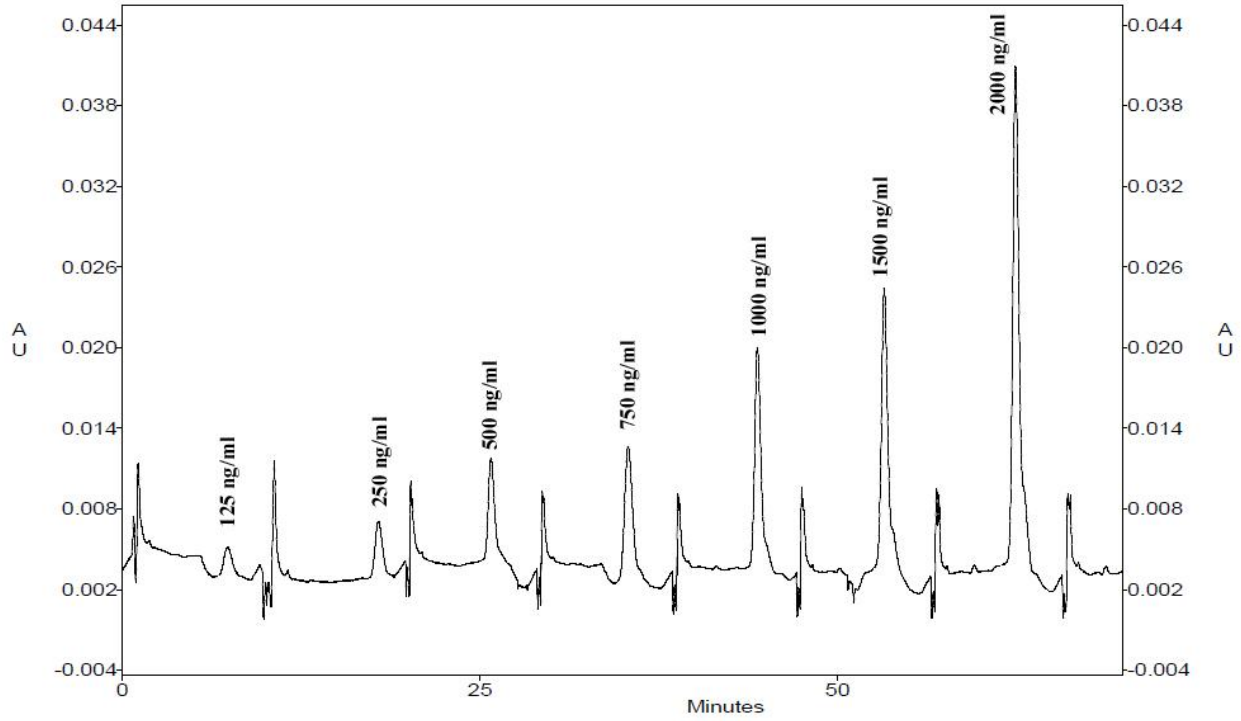

Figure 1. Chromatogram of serial concentrations of sirolimus

Accuracy and Precision

In this study intra- and inter-day precision was expressed as percent of coefficient of variation (CV %). Accuracy was expressed as the mean percentage of analyte recovered in the assay. The Intra-day precision, accuracy and relative errors range were 0.86-9.18%, 100.59-102.44% and 0.59-2.44%, respectively. Corresponding results for inter-day evaluations were 12.29-13.74%, 99.04-106.30% and 0.96-6.3%, respectively. Precision (CV %) and relative error percent acquired at each concentration should not exceed ±15%. The results of the precision and accuracy for intra- and inter-day are shown in Table 2. The results illustrate a good repeatability of response of HPLC system to different concentrations.

Stability

The stability of working standard samples was determined at ambient temperature by analyzing the solutions over a period of 6 h (Table 3). The aim was to ensure that the preparation of samples and analysis time did not contribute to the degradation and indicate that the samples will remain stable during the course of the analysis .The solutions were considered stable if the variability in the assay results was less than 15 % of initial.[14,21-23] The stability of sirolimus SRL has been investigated previously and was shown that SRL even in whole blood was stable for 30 days at 4°C.[4,24] Therefore the results that SRL was stable during sample preparation and the analysis times are confirmed.

Conclusion

The analytical method described in this paper has good accuracy, precision, linearity and is suitable for SRL assay. As the method was successfully validated based on ICH guidelines, it can be readily used in quality control laboratories for the routine pharmaceutical analysis. Also this simple and rapid method can simplify performance in developing new formulations. Further investigations are necessary to adopt this method to protein binding analysis and clinical monitoring of plasma level in therapeutic drug monitoring (TDM) studies and dose adjustment.

Table 2. Intra-day and inter-day accuracy and precision obtained from calibration curves with three levels of QC samples.

Added concentration (ng/ml)	Intra-day				Inter-day			
	Mean measured (ng/ml)	Precision (CV %)	Accuracy (%)	Relative Error %	Mean calculated (ng/ml)	Precision (CV %)	Accuracy (%)	Relative Error %
1000	1021.85 ±93.37	9.188	102.18	2.18	990.41 ±151.47	12.294	99.041	0.96
1500	1508.88 ±75.32	5.020	100.59	0.59	1534.62 ±256.92	13.742	102.308	2.30
2000	2048.92 ±17.61	0.864	102.44	2.44	2126.15 ±333.58	12.689	106.307	6.30

Table 3. SRL Stability after 6h in ambient temperature

Initial concentration (ng/ml)	Measured concentration (mean ± SD, ng/ml)	Deviation %
250	263.3±7.05	5.32
1000	925.29±10.5	-7.47
2000	1855.56±12.93	-7.25

Acknowledgments

The authors would like to thank the authorities of Faculty of Pharmacy, Tabriz University of Medical Sciences, for providing analytical facilities. This paper is based on a PhD thesis (number 42 and 54) submitted in Faculty of Pharmacy, Tabriz University of Medical Sciences.

Conflict of Interest

There is no conflict of interest in this study.

References

1. Asensi Diez R, Quesada Sanz MP, Valverde Molina E, de Ramon Garrido E, Gomez Maldonado J, Munoz Castillo I. Comparison of high-resolution liquid chromatography versus microparticle enzyme immunoassay for the measurement of sirolimus levels in renal transplantation. Farm Hosp 2006;30(3):142-8.
2. Chen KH, Tsai MK, Lai IR, Lin Wu FL, Hu RH, Lee PH. Favorable results of concomitant tacrolimus and sirolimus therapy in Taiwanese renal transplant recipients at 12 months. J Formos Med Assoc 2008;107(7):533-9.
3. Di Marco GS, de Andrade MC, Felipe CR, Alfieri F, Gooding A, Silva HT Jr, et al. Determination of sirolimus blood concentration using high-performance liquid chromatography with ultraviolet detection. Ther Drug Monit 2003;25(5):558-64.
4. Jones K, Saadat-Lajevard S, Lee T, Horwatt R, Hicks D, Johnston A, et al. An immunoassay for the measurement of sirolimus. Clin Ther 2000;22(Suppl B):B49-61.
5. Molnar-Kimber KL. Mechanism of action of rapamycin (Sirolimus, Rapamune). Transplant Proc 1996;28(2):964-9.
6. Nikitin AV. [Sirolimus: mechanism of action, use in combined chemotherapy of cancer]. Antibiot Khimioter 2007;52(1-2):39-41.
7. Salm P, Taylor PJ, Pillans PI. The quantification of sirolimus by high-performance liquid chromatography-tandem mass spectrometry and microparticle enzyme immunoassay in renal transplant recipients. Clin Ther 2000;22 Suppl B:B71-85.
8. Sehgal SN. Rapamune (Sirolimus, rapamycin): an overview and mechanism of action. Ther Drug Monit 1995;17(6):660-5.
9. Sehgal SN. Rapamune (RAPA, rapamycin, sirolimus): mechanism of action immunosuppressive effect results from blockade of signal transduction and inhibition of cell cycle progression. Clin Biochem 1998;31(5):335-40.
10. Bohnert S, Vair C, Mikler J. Development and validation of a sensitive HPLC method for the quantification of HI-6 in guinea pig plasma and evaluated in domestic swine. J Chromatogr B Analyt Technol Biomed Life Sci 2010;878(17-18):1407-13.
11. Ruzilawati AB, Wahab MS, Imran A, Ismail Z, Gan SH. Method development and validation of repaglinide in human plasma by HPLC and its application in pharmacokinetic studies. J Pharm Biomed Anal 2007;43(5):1831-5.
12. Shou M, Galinada WA, Wei YC, Tang Q, Markovich RJ, Rustum AM. Development and validation of a stability-indicating HPLC method for simultaneous determination of salicylic acid, betamethasone dipropionate and their related compounds in Diprosalic Lotion. J Pharm Biomed Anal 2009;50(3):356-61.
13. Zakeri-Milani P, Barzegar-Jalali M, Tajerzadeh H, Azarmi Y, Valizadeh H. Simultaneous determination of naproxen, ketoprofen and phenol red in samples from rat intestinal permeability studies: HPLC method development and validation. J Pharm Biomed Anal 2005;39(3-4):624-30.

14. Islambulchilar Z, Valizadeh H, Zakeri-Milani P. Rapid HPLC determination of pioglitazone in human plasma by protein precipitation and its application to pharmacokinetic studies. J AOAC Int 2010;93(3):876-81.
15. Wilson D, Johnston F, Holt D, Moreton M, Engelmayer J, Gaulier JM, et al. Multi-center evaluation of analytical performance of the microparticle enzyme immunoassay for sirolimus. Clin Biochem 2006;39(4):378-86.
16. Kuehl PJ, Angersbach BS, Stratton SP, Myrdal PB. Development of an HPLC method for the analysis of Apomine in a topical cream formulation. J Pharm Biomed Anal 2006;40(4):975-80.
17. Taylor PJ, Salm P, Lynch SV, Pillans PI. Simultaneous quantification of tacrolimus and sirolimus, in human blood, by high-performance liquid chromatography-tandem mass spectrometry. Ther Drug Monit 2000;22(5):608-12.
18. Zochowska D, Bartlomiejczyk I, Kaminska A, Senatorski G, Paczek L. High-performance liquid chromatography versus immunoassay for the measurement of sirolimus: comparison of two methods. Transplant Proc 2006;38(1):78-80.
19. Zakeri-Milani P, Valizadeh H, Islambulchilar Z. Comparative bioavailability study of two cefixime formulations administered orally in healthy male volunteers. Arzneimittelforschung 2008;58(2):97-100.
20. Zakeri-Milani P, Nemati M, Ghanbarzadeh S, Hamishehkar H, Valizadeh H. Fasted state bioavailability of two delayed release formulations of divalproex sodium in healthy Iranian volunteers. Arzneimittelforschung 2011;61(8):439-43.
21. Souri E, Jalalizadeh H, Saremi S. Development and validation of a simple and rapid HPLC method for determination of pioglitazone in human plasma and its application to a pharmacokinetic study. J Chromatogr Sci 2008;46(9):809-12.
22. Holt DW, Lee T, Johnston A. Measurement of sirolimus in whole blood using high-performance liquid chromatography with ultraviolet detection. Clin Ther 2000;22(Suppl B):B38-48.
23. Bhatt J, Jangid A, Venkatesh G, Subbaiah G, Singh S. Liquid chromatography-tandem mass spectrometry (LC-MS-MS) method for simultaneous determination of venlafaxine and its active metabolite O-desmethyl venlafaxine in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci 2005;829(1-2):75-81.
24. Gomes NA, Laud A, Pudage A, Joshi SS, Vaidya VV, Tandel JA. Validated LC-MS/MS method for determination of Alverine and one of its hydroxy metabolites in human plasma along with its application to a bioequivalence study. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877(3):197-206.