e-ISSN:2321-0812
p-ISSN:2347-2340

Research and Reviews: Journal of Pharmaceutical Analysis

Analytical Methodologies for the Determination of Sirolimus: A Review.

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Review Article

Received: 11/01/2014
Revised: 05/02/2014
Accepted: 07/02/2014

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Keywords: Sirolimus (SRL), cyclosporine (CsA)

ABSTRACT

Sirolimus is a macrolide lactone obtained from fermentations of a solid mold, *Streptomyces hygroscopicus*. Sirolimus is a potent immunosuppressive agent used to prevent rejection in organ transplantation especially useful in kidney transplants. Sirolimus was approved in 1999, by the US Food and Drugs Administration (FDA) for renal transplants and also known as Rapamycin® (RAPA). A wide variety of analytical methods have been reported for the determination of SRL in biological fluids. Methods for the measurement of Sirolimus in biological fluid have included various chromatographic methods like RP-HPLC-UV, LC-ESI-MS/MS and RP-HPLC-PDA and also included various enzymatic methods like RRA and MEIA by IMx analyzer. The applications of these methods for the determination of Sirolimus in biological sample (human or dog blood) have also been discussed in this article.

INTRODUCTION

Sirolimus (SRL) is a 31-membered triene macrolide lactone with a hemiketal masked a, b- dicarboxamide and produced by fermentation of *Streptomyces hygroscopicus*. It is a novel anti-rejection drug with potent immunosuppressive activity both in vitro and in vivo and also has antiproliferative activity; it is especially useful in kidney transplants.[1] It prevents activation of T cells and B cells by inhibiting their response to interleukin-2 (IL-2) and other T-cell growth factor receptors like Cyclosporine A (CsA) and Tacrolimus (TAC). SRL requires formation of a complex with an immunophilin (cystolic proteins bind with Immunosuppressive drugs), i.e. FK Binding Protein-12 (FKBP-12). However, the SRL-FKBP-12 complex does not affect calcineurin activity and inhibits a protein kinase, designated *mammalian target of rapamycin* (mTOR), which is a key enzyme in cell cycle progression. Inhibition of mTOR blocks cell-cycle progression at the $G_1 \rightarrow S$ phase transition [2,3].

SRL is effective alone or administered in combination with other immunosuppressive agents, such as CsA. The biologic activity of SRL potentiates the immunosuppressive effects of CsA [4]. SRL was approved in mid-September, 1999, by the US Food and Drugs Administration (FDA) for renal transplants for use in combination with CsA and steroids and also known as Rapamycin® (RAPA). The available marketed formulations of LEF are enlisted in Table no. 1 [5].

Table 1: Marketed formulations of LEF

Marketed name	Dosage form	Strength	Company
RAPAMUNE®	Tablet	0.5, 1 and 2 mg	Pfizer
RAPAMUNE®	solution	1 mg/ml	Pfizer
RAPACAN®	Tablet	1 and 2 mg	Biocon

Development and validation of analytical methods are of basic importance to optimize the analysis of drugs in the pharmaceutical industry and to guarantee quality of the commercialized product [6]. A wide variety of analytical methods have been reported for the determination of SRL in biological fluids. It includes Reverse Phase - High Performance Liquid Chromatography - UV Spectroscopy (RP-HPLC-UV), Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS), Liquid Chromatography - Electron Spray Ionization - Tandem Mass Spectrometry (LC-ESI-MS/MS) Reverse Phase - High Performance Liquid Chromatography - Photo Diode Array Detector (RP-HPLC-PDA), Radio Receptor Assay (RRA) and Micro particle Enzyme Immuno Assay (MEIA) by Tandem Mass Analyzer (MS/MS) and IMx analyzer. Among HPLC methods different internal standards, reversed phase columns with different particle size, different internal diameters and different mobile phase compositions have been used for the

quantification purpose. The aim of this review is to summarize these validated techniques for the determination of SRL in biological matrix.

Various Analytical Methods Developed For Sirolimus

Biological Samples

For biological material human blood or dog blood was used. Sample-preparation is usually performed by solid-phase extraction (SPE) or liquid–liquid extraction (LLE). Methods for the measurement of SRL in biological fluid have included various chromatographic methods like RP-HPLC-UV, LC-MS/MS, LC-ESI-MS/MS andRP-HPLC-PDA and also included various enzymatic methods like RRA and MEIA by MS/MS analyzer and IMx analyzer.

Chromatographic Methods

Taylor *et al.* 1998 reported a HPLC- ESI- MS/MS detection method for the analysis of SRL in blood. Samples were prepared by pre-treatment with acetonitrile: 15 m*M* zinc sulphate (70:30, v/v) using 32-o-desmethoxysirolimus as an internal standard (IS) and C_{18} solid-phase extraction. The chromatographic separation was carried out on a Novapak C_{18} column (150 × 32.1 mm I.D., 4 µm particle size), at 50°C temperature using mobile phase consisting of methanol: 50 m*M* ammonium acetate buffer (pH 5.1) (80:20% v/v) at a flow rate of 0.2 ml/min. Detection was done on triple quadruple instrument using selected reaction monitoring of the mass transitions 931.8→864.6 *m/z* and 901.8→834.4 *m/z* for SRL and IS, respectively. The method validation parameters are tabulated in Table no. 2 [7].

Table 2: Validation Parameters reported by Paul J. Taylor *et al.* 1998

Parameters	Result
Linearity Range	0.2 - 100.0 mg/L
Recovery	SRL - 80.5% IS - 81.3%
Accuracy	94.8% - 104.4%
Inter-day Precision (% RSD)	1.4% to 5.0%,

Kirchner *et al.* 1999 developed and validated a semi-automated HPLC-ESI-MS assay for the simultaneous quantification of SRL and CsA in blood. The semi-automated sample preparation consisted of a manual deproteinization step with a mixture of methanol and zinc sulphate and automatic column switching on-line HPLC extraction on guard column (30 × 4 mm, 10 µm) filled with C_{18}Nucleosil® 100 by using water (pH 7.0) as a mobile phase at a flow rate 0.35 ml/min. For chromatography, analytical Hypersil® ODS column (250 × 2 mm, 5 µm) as a stationary phase at 35^{0}C temperature andmethanol: water (90:10, v/v) as a mobile phase at a flow rate 0.2 ml/min wereused. Detection was done by mass analyzer by measuring sodium adduct ions $[M+Na]^+$ of SRL (*m/z* 936.6) and CsA (*m/z 1224.9*) with a dwell time of 0.5 s. The method result parameters are tabulated in Table no. 3. SRL and CsA were stable in blood for at least 4 months at -20^{0}C with 98% mean [8].

Table 3: Validation parameters described by Kirchner *et al.* 1999

Parameters	Result	
	SRL	CsA
Retention Time	9.7 ± 0.03 min	11.5 ± 0.07 min
linearity Range	0.4 - 100 µg/L	2 - 1500 µg/L
Regression Coefficient	0.998	0.987
Recovery	98%	96%
Intra - day Precision (%CV)	2.9 - 4.6 %	2.5 - 5.6 %
Inter - day Precision (%CV)	7.8 - 9.5%	8.5 - 10.2 %

Halt DW *et al.* 2000 described a RP- HPLC-UV method for estimation of SRL levels in human whole blood sample by using desmethoxyrapamycin as an IS. The chromatographic separation was carried out on ultra-sphere C_{18} bonded silica column (25 cm × 4.6 mm, 5 µm), heated at 50^{0}C with mobile phase consisting of acetonitrile: deionized water (65:35, v/v) at a flow rate of 1.5 ml/min by using UV detector at 278 nm. The result parameters are enlisted in Table no. 4. Samples were stable for 3 freeze-thaw cycles when stored at -20^{0}C and for ≥2 days when stored at ambient temperature [9].

e-ISSN:2321-0812
p-ISSN:2347-2340

Table 4: Validation parameters described by Halt DW *et al.* 2000

Parameters	Result
Linearity Range	6.5 - 356.4 ng/ml
Recovery	SRL: 81.5 ± 4.3% IS: 62.7 ± 3.6%
Within assay Repeatability (%CV)	≤ 5%
Between assay Reproducibility (%CV)	≤ 6.6%

Maleki *et al.* 2000 established a RP-HPLC-UV method using desmethoxysirolimus as an IS for therapeutic drug monitoring of SRL in human whole blood. In this method, samples were prepared by extraction with l-chlorobutane and the chromatographic separation was performed on a C_{18} column (4.6 mm x 15 cm, 3 pm) using mobile phase consisting of methanol: acetonitrile: water (68:2:30, v/v/v) with flow rate of 1.0 ml/min at temperature 60^{0}C and detected by UV detector at 278 nm. The validation parameters are tabulated in Table no. 5 [10].

Table 5: Validation parameters reported by Maleki S *et al.* 2000

Parameters	Result
Linearity Range	2.5-150.0 ng/mL
Recovery	88.0% to 106.3%
Intra-day Precision (% RSD)	12.0% to 14.4%
Inter-day Precision (% RSD)	2.6% to 13.0%

Cattaneo *et al.* 2002 presented RP-HPLC-UV method for the analysis of SRL in whole blood using 32-o-desmethoxyrapamycin as an IS. Here, the samples were purified by zinc sulfate and then extracted with acetone followed by solid-phase extraction. The chromatographic separation was performed on a column packed with Ultra sphere C_8 (7534.6 mm, 3 mm) and heated at 50^{0}C by using a mixture of distilled water: methanol: acetonitrile (34:30:36, v/v/v) at a flow rate of 1 ml/min and detected by UV detector at 278 nm. The method result parameters are tabulated in Table no. 6 [11].

Table 6: Validation parameters reported by Cattaneo *et al.* 2002

Parameters	Result
Retention Time	SRL : 13.1 min IS : 14.5 min
Linearity Range	2.5 - 60 ng/ml
Regression Coefficient	0.999
Limit of Detection (LOD)	1 ng/ml
Recovery	SRL: 61.1 ± 3.1% IS: 60.1 ± 2.1%
Intra - day Precision (%CV)	1.5 – 8.0%
Inter - day Precision (%CV)	1.8 – 6.6%

Campanero MA *et al.* 2004 established RP-HPLC-PDA method for TDM of SRL in blood samples from renal, cardiac and hepatic transplants. Here, SRL and Desmethoxyrapamycin (IS) samples were purified by a combination of a precipitating blood matrix with zinc sulphate and a single step liquid-liquid extraction with acetone and 1-chlorobutane. The Separation was carried out at 50^{0}C on a C18 column (150 × 2.1mm, 5µm), in isocratic mode using mobile phase consisting of distilled water: methanol: acetonitrile (26:50:24, v/v/v), at a flow rate of 0.25 ml/min and detected by UV diode array detector at 278 nm. The method validation parameters are tabulated in Table no. 7 [12].

Table 7: Validation Parameters reported by Campanero MA *et al.* 2004

Parameters	Result
Retention Time	SRL: 13.1 min IS: 16.0 min
Linearity Range	2.5 - 100 ng/ml
Regression Coefficient	0.996
Limit of Detection (LOD)	0.68 ng/ml
Limit of Quantification (LOQ)	1.75 ng/ml
Recovery	SRL: 65.7 ± 1.18 IS: 66.2 ± 2.37%,
Intra - day Precision (%CV)	4.56 - 7.89%
Inter - day Precision (%CV)	4.04 - 8.28%

Lee *et al.* 2010 proposed LC-MS/MS method for determination of SRL in dog blood by using Tacrolimus (TAC) as an IS. Here, the concentration of SRL was quantified in blood samples for up to 36 h after the dog had

received a 3 mg/kg dose of SRL. The chromatography was achieved on a Prodigy Phenyl-3 column (2.0mm × 50mm, 5µm), in isocratic mode using mobile phase composed of acetonitrile: methanol: ammonium bicarbonate (10 m*M*) (68:17:15, v/v/v) at a flow rate of 0.25 ml/min and detection was carried out by triple quadrupole mass spectrometer for optimizing multiple reaction monitoring (MRM) conditions of the analytes in positive ion mode. Here, precursor→product ion transition for SRL (*m/z*931.7→864.5) and IS (*m/z* 821.6→768.5) were monitored. The method result parameters are tabulated in Table no. 8 [13].

Table 8: Validation parameters reported by Lee *et al.* 2010

Parameters	Result
Linearity Range	0.2 - 20 ng/ml
Correlation Coefficient	0.9987
Intra - day Precision	-0.2 - 4.9%
Inter - day Precision	4.8 - 10.1%

Mano *et al.* 2011 established LC/ESI–MS/MS method for quantitation of SRL in human whole blood. For sample preparation, first pre-treatment was done with a zinc sulphate protein precipitation, an extraction using octadecylsilyl-silica gel for eliminating water-soluble and hydrophilic compounds, and Hybrid SPE Cartridge treatment to eliminate phospholipids. The separation was performed using Ascomycin as an IS on a Capcell Pak MG II (150mm×2.0mm i.d., 5µm) at 50^{0}C column temperature using 10m*M* ammonium acetate: methanol (20:80, v/v) as a mobile phase at a flow rate of 0.2 mL/min. Detection was done by selected reaction monitoring (SRM) at the transitions of m/z 931.6–864.5 and m/z 809.5–756.5 were used for monitoring SRL and Ascomycin, respectively, and dwell time was 500 ms. The method validated parameters are tabulated in Table no. 9. Sample was stable for at least 46 days under -20^{0}C storage for at least three freeze-thaw cycles [14,15] .

Table 9: Validation Parameters described by Mano *et al.* 2011

Parameters	Result
Linearity Range	0.5 - 50 ng/ml
Regression Coefficient	1.000
Limit of Detection (LOD)	500 fg/ml
Limit of Quantification (LOQ)	0.1 ng/ml
Intra - day Precision	1.41 - 2.85%
Inter - day Precision	3.86 - 6.92%

Anil Kumar *et al.* 2012 performed analytical method RP-HPLC with PDA detection for quantification of SRLin blood samples using ketoconazole (KTZ) as IS. Extraction was performed using dichloromethane under nitrogen atmosphere andseparation was accomplished by waters Xterra MS C_{18} analytical column (250 × 4.6 mm, 5µm), in isocratic mode using mobile phase consisting of methanol: water: glacial acetic acid (90:10:0.1%, v/v) at a flow rate of 1 ml/min and detected by PDA detector at 278 nm. The method validation parameters are tabulated in Table no. 10 [16].

Table 10: Validation Parameters reported by Anil Kumar *et al.* 2012

Parameters	Result
Retention Time	SRL: 5.23 min KTZ: 4.30 min
Linearity Range	0.1 – 10 µg/ml
Regression Coefficient	0.997
Limit of Detection (LOD)	3 ng/ml
Lower Limit of Quantification (LLOQ)	10 ng/ml
Recovery	98.3%
Intra - day Precision (%CV)	o.671 – 0.980%
Inter - day Precision(%CV)	0.598 – 1.324%

Enzymatic Methods

Goodyear N. *et al.* 1996 described a RRA for estimation of SRL in whole blood. for this, SRL was resuspended from the dried tubes with [3H]-dihydro FK506 at 160,000 disintegrations per min (dpm) followed by direct methanol extraction, using 15-25 µg of protein containing the 14 or 52 kDa protein for final diluent. After this, samples applied to Sephadex LH-20 columns, bed volume 1.8 mL previously equilibrated with LH-20 buffer (20 mmol/L Tris (pH 7.2), 5 mmol/L β-mercaptoethanol, and 0.5 g/L sodium azide), and eluted with 150 µL followed by 1.25 mL LH-20 buffer to separate free [3H]-dihydro FK506 from bound and the bound portion was measured using a scintillation counter with a counting efficiency of 59%. Nonspecific binding was estimated using a 1 mg/L concentration of unlabeled SRL. The validation parameters are tabulated in Table no. 11. In interference study, no interference observed in RRA for SRL with CsA, Methotrexate, Dexamethasone and Prednisolone [17].

e-ISSN:2321-0812
p-ISSN:2347-2340

Table 11: Validation parameters by Goodyear N. *et al.*, 1996

Parameters	Result
Sensitivity	1.0 pg/L
Linearity Range	52 kDa Immunophilin: 2.5- 40 µg/L 14 kDa Immunophilin: 5 - 50 µg/L
Recovery	93 - 103%
Between run Precision (%CV)	5.9 - 12.9%

Jones K *et al.* 2000 studied a MEIA for prepared sample measurement in whole blood and HPLC-MS/MS method used for SRL estimation. In this method, samples were prepared by protein precipitation extraction technique. Specificity was determined by the addition of 2 sirolimus metabolites (Hydroxysirolimus and 4l-o-demethylsirolimus) to sirolimus free human whole blood. Method validation parameters are tabulated in Table no. 12. In stability study, no loss of SRL during a total of 3 freeze-thaw cycles and samples were stabled for 10 days at 4^{0}C and at ambient temperature (22^{0}C protected from light) [18].

Table 12: Validation Parameters by Jones K *et al.* 2000

Parameters	Result	
Linearity Range	3-30 ng/ml	
Limit of Detection (LOD)	1 ng/ml	
Recovery	Fresh: 95.6% - 109.5% Frozen: 93.9% - 98.0%	
Specificity		
1. Hydroxysirolimus	%CV	1 - 1.9%
2. 4-o-demethylsirolimus		2 - 4.6%
	Cross Reactivity	1 - 44%
		2 - 86%
Repeatability	<6%	
Reproducibility	<11%	
Assay	Spiked samples - 8% patient samples - 21%	

Wilson D *et al.* 2006 described microparticle enzyme immunoassay (MEIA) for sirolimus in whole blood by using IMx analyser. In this method, MEIA was a one-step competitive assay utilizing a murine monoclonal antibody covalently coupled to carboxylated micro particles. Here, samples were pretreated with a methanolic precipitating reagent and the IMx probe/electrode assembly combines pre-treated sample, micro particles, and conjugate to the incubation well of the disposable MEIA reaction cell. After this, it is reacted with alkaline phosphate and formed substrate, 4-Methylumbelliferyl phosphate, is converted to a fluorescent product which is measured by the IMx optical assembly. For Specificity, SRL was metabolized into primarily hydroxylated and/or demethylated forms by the CYP3A4 enzyme system. Method result parameters are tabulated in Table no. 13 [19].

Table 13: Validation Parameters by Wilson D *et al.* 2006

Parameters	Result
Antibody Specificity	6 - 63% cross reactivity
Limit of Detection (LOD)	0.68 µg/L
Lower Limit of Quantification (LLOQ)	1.5 µg/L
Recovery	105%
Precision (%CV)	5.7 - 12.6%

CONCLUSION

This review presents the specific, sensitive and accurate analytical techniques for the estimation of SRL in blood are based on the separation by HPLC with different detectors and some enzymatic methods. Spectrophotometric, spectrofluorimetric and Chromatographic methods other than HPLC are not reported for SRL quantification in both pharmaceutical preparations and biological fluids. However in future some newer aspects can be thought for the development and validation of stability indicating method as well as degradation kinetic study and impurity study.

REFERENCES

1. Gummert JF, Morris RE, Ikonen T. Anti-Inflammatory and Immunosupressant drugs. In: Rang HP, Dale MM. General principles of Pharmacology. Sixth edition: Churchill Livingstone publisher; 2007. p. 243.

2. Harvey RA, Champe PC. Immunosupressants. In: Lippincott's Illustrated reviews in Pharmacology. Fourth Edition, New Delhi: published by Wolters Kluwer Pvt. Ltd.; 2009. p. 492-493.
3. Krensky AM, Vincenti F, Bennelt WM. Immunosupressants, Tolerogens and Immunostimulants. In: Brunton LL. Goodman and Gilman's, The Pharmacological basis of Therapeutics. Eleventh edition: Medical publishing division; 2006. p. 1413-1414.
4. Lake DF, Briggs AD, Agporigue ET. Immunopharmacology. In: Katzung BG. Basic and clinical pharmacology. Eleventh edition, New Delhi: Tata Mcgraw Hill Education publisher; 2009. p. 972-973.
5. Indian Drug Review (IDR), drug triple i compendium. UBM medicaindia private limited, Bangalore. Volume XIX: Issue no. 5, 2013. p. 268
6. Venn RF. Principles and practice of bioanalysis, second edition, London: Taylor & Francis; 2000. p. 299-300
7. Taylor PJ, Johnson AG. Quantitative analysis of sirolimus (Rapamycin) in blood by high performance liquid chromatography-electrospray tandem mass spectrometry. J Chromatogr B. 1998;718:251 - 257.
8. Kirchner GI, Vidal C, Jacobsen W, Franzke A, Hallensleben K , Christians U, Sewing K. Simultaneous on-line extraction and analysis of sirolimus (rapamycin) and ciclosporin in blood by liquid chromatography - electrospray mass spectrometry. J Chromatogr B. 1999;721:285 - 294.
9. Halt DW, Lee T, Johnston A. Measurement of Sirolimus in Whole Blood Using High-Performance Liquid Chromatography with Ultraviolet Detection. Clin Ther. 2000;22:B38-B48.
10. Maleki S, Graves S, Becker S, Horwatt R, Hicks D, Stroshane RM, Kincaid H. Therapeutic Monitoring of Sirolimus in Human Whole-Blood Samples by High-Performance Liquid Chromatography. Clin Ther. 2000; 22, SUPPL. B:25 - 37.
11. Cattaneo D, Perico N, Gaspari F. Assessment of sirolimus concentrations in whole blood by high performance liquid chromatography with ultraviolet detection. J Chromatogr B. 2002;774:187 - 194.
12. Campanero MA, Cardenas E, Sadaba B, Garcia-Quetglas E, Munoz-Juarez MJ, Gil-Aldea I, Pazo D, Azanza JR, Honorato J. Therapeutic drug monitoring for sirolimus in whole blood of organ transplants by high-performance liquid chromatography with ultraviolet detection. J Chromatogr A. 2004;1031:265 - 273.
13. Lee J-H, Cha K-H, Cho W, Park J, Park HJ, Cho Y, Hwang S-J. Quantitative determination of sirolimus in dog blood using liquid chromatography–tandem mass spectrometry, and its applications to pharmacokinetic studies. J Pharm Biomed Anal. 2010;53:1042 - 1047.
14. Mano N, Sato M, Nozawa M, Matsumoto Y, Mori M, Yamaguchi H, Goto J, Shimada M. An accurate quantitative LC/ESI–MS/MS method for sirolimus in human whole blood. J Chromatogr B. 2011;879:987 - 992.
15. Mano N, Nozawa M, Sato M, Mori M, Yamaguchi H, Kanda K, Nogami M, Goto J, Shimada M. Identification and elimination of ion suppression in the quantitative analysis of sirolimus in human blood by LC/ESI-MS/MS. J Chromatogr B. 2011;879:968 - 974.
16. A Anil Kumar, P Srinivas, K Spandana, N. Rama, J VidyaSagar. Rapid and Sensitive HPLC Method for the Determination of Sirolimus with Ketoconazole as Internal Standard and Its Further Applications. Int J Pharm Sci Drug Res. 2012;4(1):70 - 73.
17. Goodyear N, Napoli KL, Murthy JN, Kahan BD, Soldin SJ. Radioreceptor Assay for Sirolimus. Clin Biochem. 1996;29(5):457 – 460.
18. Jones K, Saadat-Lajevardi S, Lee T, Horwatt R, Hicks D, Johnston A, Halt DW. An Immunoassay for the Measurement of Sirolimus. Clln Ther. 2000;22:B49 - B61.
19. Wilson D, et al. Multi-center evaluation of analytical performance of the microparticle enzyme immunoassay for sirolimus. Clin Biochem. 2006;39:378 - 386.