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Quantification of Everolimus in bulk and tablet dosage forms by a HPLC method coupled with a photodiode array detector

Suresh Kumar Raju Vidyadharani[1]*, Ravoori Sreenivasulu[2]
1. Analytical development laboratory, Mylan laboratories limited, Hyderabad, India.
2. Department of chemistry, Sri Krishnadevaraya University College, Anantapuram, India .

ABSTRACT

A rapid, sensitive, precise, and accurate HPLC method coupled with a photodiode array detector was developed, optimized, and validated for the estimation of everolimus in bulk and tablet dosage forms. The chromatographic separation was achieved using a kromosil C8 column (200 mm × 4.6 mm i.d., 5 µm particle size) at 30°C temperature. The isocratic mobile phase consisted of 0.1M dipotassium hydrogen phosphate and methanol (60:40, v/v). The mobile phase was delivered at 1.0 ml/min and the analyte was monitored at 277 nm. The method was successfully validated in accordance to International Conference on Harmonization. The retention time of everolimus was found to be 3.496 min, and the calibration curve was linear in the concentration range of 50-150 µg/ml (R^2 = 0.9992). The limit of detection and the limit of quantitation were found to be 0.109 µg/ml and 0.364 µg/ml, respectively. The proposed method is accurate (percent recoveries were in the range of 99.56-100.09%) and precise (percent relative standard deviation - 0.047 %). No chromatographic interferences from the tablet excipients and components of mobile phase were found. The proposed method was found to be suitable for the quantitative determination of everolimus in tablet dosage forms.

Keywords: Everolimus, Afinitor, HPLC, validation, analysis

*Corresponding Author Email: pssuresh_78@rediffmail.com
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INTRODUCTION

Everolimus, chemically described as dihydroxy-12-[(2*R*)-1-[(1*S*,3*R*,4*R*)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]propan-2-yl]-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-azatricyclo[30.3.1.0 hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentone (Figure 1), is a 40-*O*-(2-hydroxyethyl) derivative of sirolimus with immunosuppressant and anti-angiogenic properties [1]. Everolimus is used to prevent graft rejection of heart and kidney transplants by blocking cell proliferation signals and also in the treatment of renal cell cancer and other tumours [2-4].

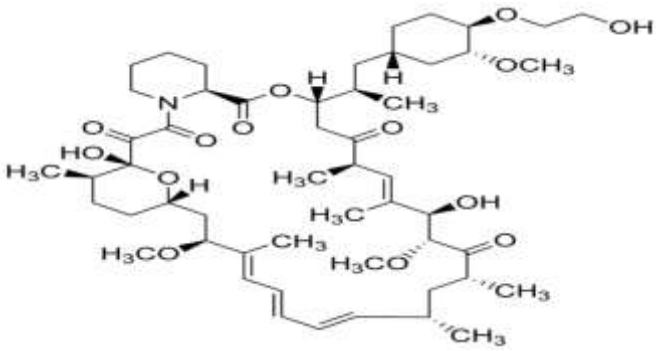

Figure 1: Structure of everolimus

Everolimus exhibits its activity by acting as an inhibitor for protein kinase, cytochrome P450 3A4, P-glycoprotein and cytochrome P450 2D6. Everolimus binds to FK Binding Protein-12 with high affinity to generate an immunosuppressive complex. This complex in turn binds and inhibits the activation of the mammalian Target of Rapamycin (mTOR), which is a key regulatory kinase. The inhibition of mTOR results in lessening cell proliferation, angiogenesis and glucose uptake [5].

Various methods have been reported for the determination of everolimus. They include UV spectrophotometry [6], fluorescence polarization immunoassay [7,8], HPLC-MS/MS [9-13], surface-assisted laser desorption/ionization mass spectrometry [14] and HPLC [15-18]. UV spectrophotometric method is used for the determination of everolimus in surfactant-containing dissolution media [6]. Fluorescence polarization immunoassay methods are involved in the measurement of everolimus blood concentrations in clinical transplantation [7,8]. HPLC-MS/MS methods are used for the quantification of everolimus in human blood and are suited to the therapeutic drug monitoring of everolimus [9-13]. Surface-assisted laser desorption/ionization mass spectrometry method was successfully applied for the determination of immunosuppressive drugs in human urine and serum samples [14].

Among the four HPLC methods reported for the quantification of everolimus, two HPLC methods are applied for the human blood samples [15,16]. Only two HPLC methods are described in the

literature for the estimation of everolimus in pharmaceutical dosage forms [17,18]. However, the previously reported HPLC methods for everolimus estimation in pharmaceutical dosage forms suffered from one or more disadvantage such as narrow range of linearity [18], poor sensitivity [18], less precise [17,18] and less accurate [17]. Therefore, it is necessary to develop and validate a sensitive, precise and accurate HPLC method for the quantification of everolimus in bulk and tablet dosage forms.

MATERIALS AND METHOD

Apparatus

In the present investigation, Waters HPLC system with a binary HPLC pumps model 2695, photodiode-array (PDA) detector model 2998 and a vacuum degasser. The HPLC system was controlled by Waters Empower2 software.

Reference drug, Chemicals and solvents:

Reference standard of everolimus was obtained from Lara Drugs Private Limited, Telangana, India. The reference standard was used as received. Afinitor tablets (manufactured by Novartis Pharma stein AG, Switzerland) labeled to 10 mg of everolimus was used in the current investigation. Analytical reagent grade dipotassium hydrogen phosphate was from Sd. Fine Chemicals Ltd., Mumbai, India. HPLC grade methanol was purchased from Merck India Ltd., Mumbai, India. Milli-Q-water was used all through the experiment.

Chromatographic conditions

The chromatographic column used was the Kromosil C8 column (200 mm x 4.6 mm i.d., 5 µm particle). The optimum mobile phase was prepared by mixing the 0.1M dipotassium hydrogen phosphate and methanol in the ratio of 60:40 (*v/v*). The mobile phase was filtered using a 0.45 µm millipore membrane filter and was degassed by sonication for 20 minutes prior to use. A wavelength of 277 nm was chosen for the determination of the everolimus. The flow rate used was 1.0 ml/min. The injection volume was 20 µl and the temperature of the column was 30 ^{o}C. The total run time was only 6 minutes.

Preparation of standard solutions

The stock standard solution (1 mg/ml) of everolimus was prepared by dissolving accurately weighed 100 mg of everolimus reference standard in 80 ml of mobile phase, then sonicated for 10 minutes and then diluted up to 100 ml with the mobile phase. The stock standard solution was diluted appropriately with mobile phase to get working standard solutions of everolimus in the concentrations 50 µg/ml, 75 µg/ml, 100 µg/ml, 125 µg/ml and 150 µg/ml.

Preparation of tablet sample solution

Twenty tablets were weighed accurately and ground into a fine powder. The tablet sample solution was prepared by transferring an accurately weighed amount of the powdered tablet equivalent to 100 mg of everolimus to a 100 ml volumetric flask containing 80 ml of mobile phase, sonicated for 10 minutes, and then diluted up to 100 ml with the same solvent. The prepared solution was further diluted with mobile phase to get a concentration of 100 µg/ml everolimus for analysis. The solution was filtered using a 0.45 µm membrane filter before injecting into the system.

Preparation of placebo blank:

A placebo blank containing starch (40 mg), acacia (30 mg), sodium citrate (40 mg), hydroxyl cellulose (40 mg), magnesium stearate (50 mg) and talc (40 mg) was prepared by mixing all the components into a homogeneous mixture. A 100 mg of the placebo blank was accurately weighed and its solution was prepared as described under section 'Preparation of tablet sample solution'.

Calibration curve

The calibration curve was established by analyzing the different concentrations of everolimus standard solution ranging from 50-150 µg/ml. The calibration curve was constructed by linear regression analysis of the peak area against the respective concentration of everolimus. The estimation of unknown concentration of everolimus was done using this calibration curve.

Analysis of everolimus in tablet dosage forms:

20 µl of tablet sample solution prepared in the section 'Preparation of tablet sample solution' was injected into the HPLC column in triplicate. The peak areas were determined. The concentration of everolimus in tablet was determined using the calibration curve.

RESULTS AND DISCUSSION

Method development:

Different mobile phases have been employed in order to optimize the desired HPLC method. The best conditions selected were based on minimizing peak tailing, improving peak symmetry, column efficiency and total analysis time. Different combination ratios of potassium dihdrogen phosphate, dipotassium hydrogen phosphate, water and methanol were tested. The best chromatographic separation was achieved on kromosil C8 column (200 mm × 4.6 mm i.d., particle size 5 µm) using a mobile phase composed of 0.1M dipotassium hydrogen phosophate: methanol (60:40, *v/v*) pumped with a flow rate of 1 ml/min. The column temperature was kept constant at 30 °C. The ultraviolet absorption spectra of the everolimus demonstrated an optimum response at a

wavelength 277 nm, and it was therefore chosen as detection wavelength during the entire study. A typical HPLC chromatogram of everolimus under the optimized conditions is shown in Figure 2.

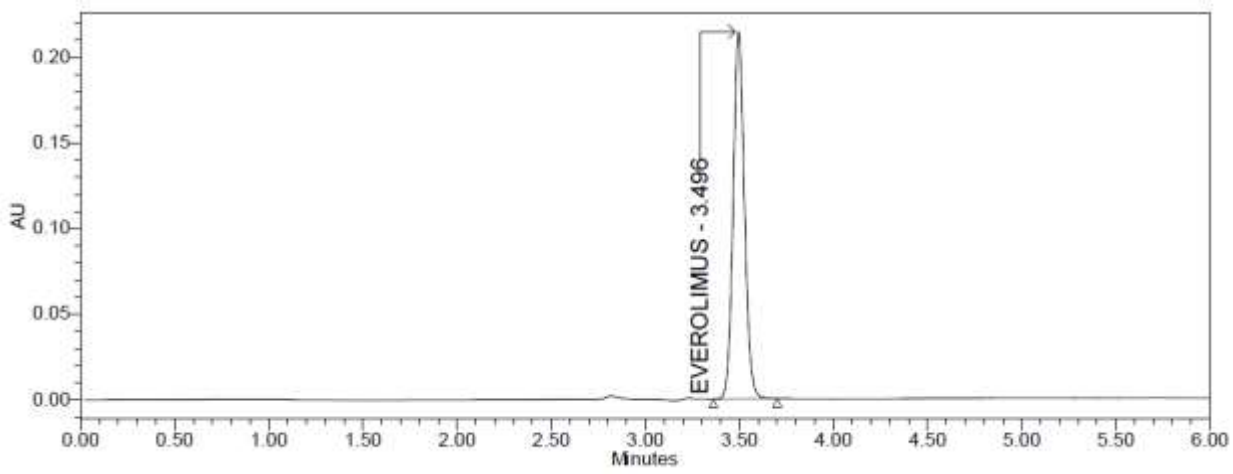

Figure 2: Typical HPLC chromatogram of everolimus (100 µg/ml)

Method Validation

The optimized HPLC method was validated according to the ICH guidelines [19]. Parameters such as system suitability, selectivity, linearity, limit of detection, limit of quantitation, precision, accuracy and robustness were validated.

System suitability:

The system suitability was evaluated by making five replicate injections of the everolimus standard solution (100 µg/ml) and analyzing each active ingredient for its peak area, peak USP tailing factor and USP plate count. The proposed accepted criteria are ≤ 2% for RSD%, ≤ 2 for USP tailing factor and ≥ 2000 for the USP plate count. The system suitability results showed a % RSD value of 0.0136% and 0.0121% for everolimus retention time and peak area, respectively. The peak tailing factor everolimus was 1.10. The USP plate count for the peak of everolimus was 16887. These values indicate that the proposed PLC method met the accepted requirements.

Selectivity:

The chromatograms of the placebo blank, mobile phase blank, everolimus standard (100 µg/ml) and tablet sample (100 µg/ml) solutions were recorded at the same wavelength of 277 nm in order to check the selectivity of the proposed method. The retention time of everolimus tablet sample solution peak match the peak of the standard solution. No peaks were found at these retention times in the placebo blank and mobile phase blank chromatograms (Figures 3-6). Therefore, the proposed method is selective for the quantification of the everolimus in tablet dosage forms.

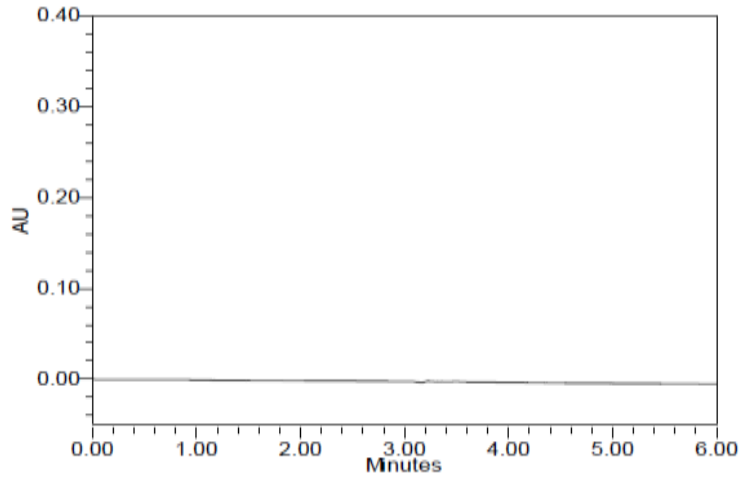

Figure 3: Chromatogram of mobile phase blank

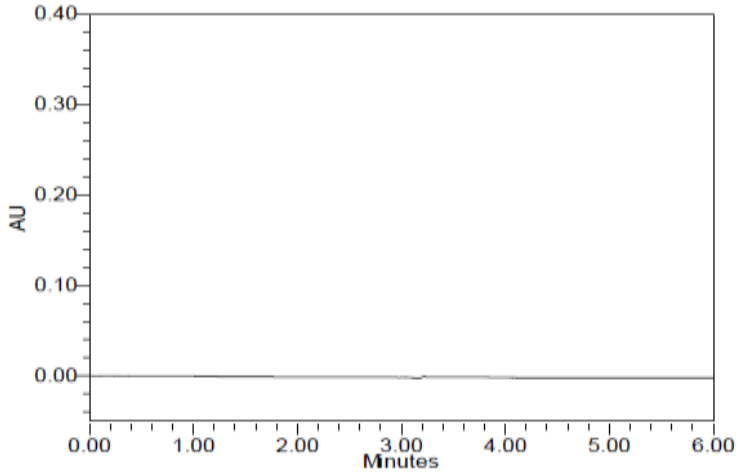

Figure 4: Chromatogram of placebo blank solution

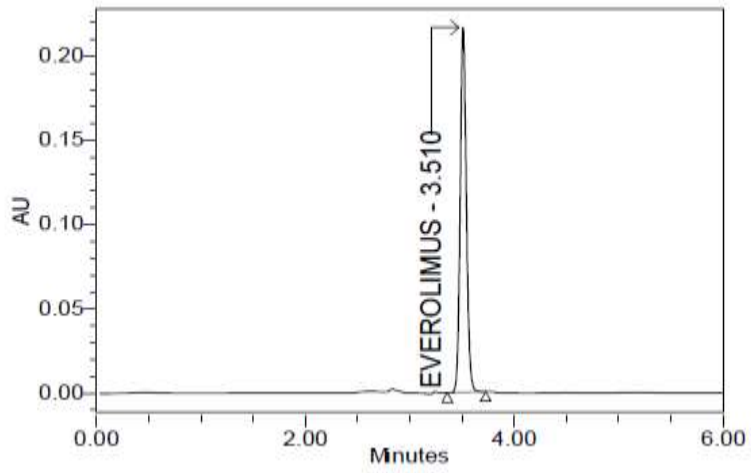

Figure 5: Chromatogram of everolimus standard solution

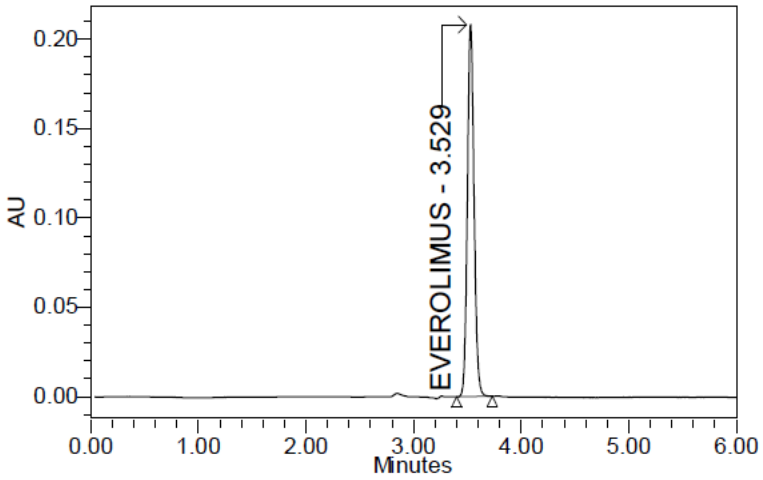

Figure 6: Chromatogram of everolimus tablet sample solution

Linearity:

The linearity in the range of 50-150 µg/ml was investigated. The regression line demonstrated linearity in the tested range. The regression lines were linear with R^2 of 0.9992 for everolimus (Figure 7).

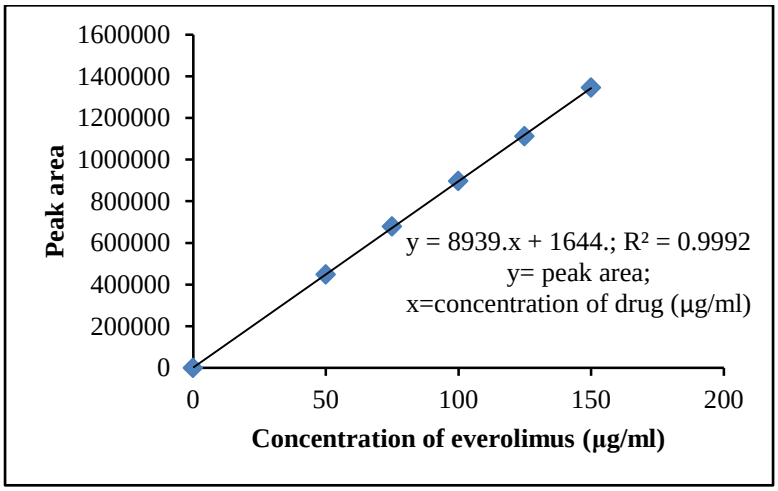

Figure 7: Linearity range for everolimus

Sensitivity:

The sensitivity of the proposed method was evaluated by establishing the limit of detection (LOD) and the limit of quantitation (LOQ) for everolimus at a signal-to-noise ratio of three and ten, respectively. The LOD and LOQ values of 0.109 µg/ml and 0.364 µg/ml for everolimus were obtained, respectively. The values indicated the adequate sensitivity of the proposed method.

Precision and accuracy:

The precision and accuracy of the proposed method was assessed by the determination of working standard solution of everolimus at a concentration of 100 µg/ml for six successive times. The precision were expressed as % relative standard deviation and accuracy as % recovery. The results

are shown in Table 1. The low values of % RSD and good % recovery values confirm the satisfactory precision and accuracy of the proposed method.

Table 1: Precision and accuracy of the proposed method

Sample No.	Concentration of drug (µg/ml)	Peak area	Recovery (%)
1	100	893687	98.94
2	100	893989	98.97
3	100	892969	98.86
4	100	893289	98.89
5	100	893405	98.90
6	100	894061	98.98
Mean	100	893566.67	98.92
% RSD	-	0.047	0.046

Recovery study:

The accuracy of the proposed HPLC method was further checked by the standard addition method. The preanalyzed sample solution was spiked with known concentration of everolimus at three different concentration levels (50%, 100% and 150%).The average % recovery of everolimus was calculated and the results are shown in Table 2. From the results, it was observed that the proposed method was accurate for the determination of everolimus in tablet dosage forms. Common excipients in tablets did not interfere with the assay.

Table 2: Recovery data of the proposed method

Spiked level (%)	Concentration of everolimus(*µ*g/ml)		Recovery (%)	Mean (%)
	Added	Found		
50	49.50	49.39	99.78	99.56
	49.50	49.20	99.39	
	49.50	49.26	99.51	
100	99.00	98.88	99.87	99.90
	99.00	98.94	99.94	
	99.00	98.90	99.90	
150	148.50	148.49	99.99	100.09
	148.50	148.41	99.94	
	148.50	148.99	100.33	

Robustness

The method robustness is the ability of the method to remain unaffected by small, but deliberate changes in method parameters. Effects of change in factors such as mobile phase flow rate (±0.1 ml/min) and temperature (±2 ^{o}C) on analysis parameters such as response area, tailing factor, USP plate count were observed in robustness studies. The results obtained were shown in Table 3. The system suitability parameters are well within the acceptable limits, indicating that the proposed HPLC method remained robust under the optimized conditions.

Table 3: Robustness data of the proposed method

Parameter	Concentration of drug (*µ*g/ml)	USP Tailing	USP plate count	Peak area
Flow rate 1.0 + 1 ml/min	100	1.11	17291	1127478
Flow rate 1.0 - 1 ml/min	100	1.08	14464	1144118
Temperature 30 + 2 ^{o}C	100	1.09	17025	1124741
Temperature 30 - 2 ^{o}C	100	1.08	14750	1141947

CONCLUSION:

The validated HPLC method developed for the determination of everolimus in bulk and tablet dosage forms was evaluated over linearity, sensitivity, system suitability, selectivity, precision, accuracy and robustness. All the validation results were within the allowed specifications. The developed method is proven to be rapid, precise and accurate for the determination of the everolimus in tablet dosage forms in the presence of excipients. The absence of interfering peaks in the chromatogram suggests that the tablet excipients and components of mobile phase do not interfere with the estimation of everolimus by proposed method. As a result, the proposed HPLC method could be useful for the quantitative quality control and routine analysis of everolimus in tablet dosage forms.

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REFERENCES:

1. Mohamed A. Hussein, Ali El-Hindawi, Gaafar Ragab. Everolimus in Erdheim–Chester disease. Egypt J Intern Med 2013;25 (3), 159-63.
2. Stephan W. Hirt, Christoph Bara, Markus J. Barten, Tobias Deuse, Andreas O. Doesch, Ingo Kaczmarek, Uwe Schulz, Jörg Stypmann, Assad Haneya, Hans B. Lehmkuhl. Everolimus in heart transplantation: An update. Journal of Transplantation, 2013, vol. 2013, Article ID 683964, 12 pages, 2013. doi:10.1155/2013/683964
3. Julio Pascual. The use of everolimus in renal-transplant patients. Int J Nephrol Renovasc Dis. 2009; 2: 9–21.
4. Lothar Bergmann, Ulrich Kube, Christian Doehn, Thomas Steiner, Peter J Goebell, Manfred Kindler, Edwin Herrmann, Jan Janssen, Steffen Weikert, Michael T Scheffler, Joerg Schmitz, Michael Albrecht, Michael Staehler. Everolimus in metastatic renal cell

carcinoma after failure of initial anti–VEGF therapy: final results of a noninterventional study. BMC Cancer, 2015, 15:303 **DOI:** 10.1186/s12885-015-1309-7

5. Koldehoff M, Beelen DW, Elmaagacli AH. Inhibition of mTOR with everolimus and silencing by vascular endothelial cell growth factor-specific siRNA induces synergistic antitumor activity in multiple myeloma cells. Cancer Gene Ther. 2014 Jul;21(7):275-82. doi: 10.1038/cgt.2014.27. Epub 2014 Jun 6.
6. Kamberi M, Tran TN. UV-visible spectroscopy as an alternative to liquid chromatography for determination of everolimus in surfactant-containing dissolution media: a useful approach based on solid-phase extraction. J Pharm Biomed Anal. 2012 Nov;70:94-100. doi: 10.1016/j.jpba.2012.05.038. Epub 2012 Jun 4.
7. Sara Baldelli, Alberto Crippa, Roberta Gabrieli, Roberto Fiocchi, Norberto Perico, Simona Merlini, Cosimo Ottomano, Dario Cattaneo. Comparison of the Innofluor® certican assay with HPLC-UV for the determination of everolimus concentrations in heart transplantation. Clinical Biochemistry, 39 (12), 2006, 1152–1159.
8. Khoschsorur Gholam Ali, Fruehwirth F, Zelzer S, Stettin M, Comparison of fluorescent polarization immunoassay (FPIA) versus HPLC to measure everolimus blood concentrations in clinical transplantation, Clinica Chimica Acta 2007; 380(1-2):217-21.
9. Korecka M, Solari SG, Shaw LM. Sensitive, high throughput HPLC-MS/MS method with on-line sample clean-up for everolimus measurement. Ther Drug Monit. 2006 Aug;28(4):484-90.
10. Korecka MA, Patel R, Shaw LM. Evaluation of performance of new, isotopically labeled internal standard (13c2d4]RAD001) for everolimus using a novel high-performance liquid chromatography tandem mass spectrometry method. Ther Drug Monit. 2011 Aug;33(4):460-3. doi: 10.1097/IAE.0b013e3182219af8.
11. Kumar A and VidyaSagar J, LC and LC/MS approach for quantification of everolimus and its degradants and application in stress analysis. J Pharm Res 2014; 8(2): 152-161.
12. Salm P, Taylor PJ, Lynch SV, Pillans PI, Quantification and stability of everolimus (SDZ RAD) in human blood by high-performance liquid chromatography-electrospray tandem mass Spectrometry. J Chromatogr B, AnalytTechnol Biomed Life Sci. 2002; 772(2):283-90.
13. Paul J. Taylor , Michael E. Franklin, Kendon S. Graham, Peter I. Pillans. A HPLC–mass spectrometric method suitable for the therapeutic drug monitoring of everolimus. Journal of Chromatography B, 848 (2), 2007, 208–214.

14. Pin-Shiuan Chen, Yu-Han Cheng, Sheng-Yu Lin, Sarah Y. Chang. Determination of immunosuppressive drugs in human urine and serum by surface-assisted laser desorption/ionization mass spectrometry with dispersive liquid-liquid microextraction. Analytical and Bioanalytical Chemistry, January 2016, Volume 408, Issue 2, pp 629–637
15. Boudennaia TY, Napoli KL. Validation of a practical liquid chomatography with ultraviolet detection method for quantification of whole-blood everolimus in a clinical TDM laboratory. Ther Drug Monit. 2005 Apr;27(2):171-7.
16. Spandana Kari, Srinivas P, Anil Kumar A, Vidya Sagar J. Rapid and sensitive HPLC method for quantification of everolimus and its application in release kinetics of everolimus eluting coronary stents. Current Research in Biological and Pharmaceutical Sciences, 2 (5) Sept.-Oct. 2013, 01-05.
17. D. Sharmila, A. Lakshmana rao, L. Kalyani. Development and validation of stability-indicating high performance liquid chromatographic method for the estimation of everolimus in tablets. Indian J Pharm Sci 2015;77(5):599-604.
18. Sureshbabu Kapavarapu, Rambabu Chintala. Stability indicating RP-HPLC method for the estimation of everolimus in pharmaceutical formulations. Am. J. PharmTech Res. 2015; 5(2), 323-344.
19. International Conference of Harmonization. Validation of Analytical Procedures: Text and Methodology. Geneva, Switzerland; 2005, ICH Q2 (R1).