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Development of RP-HPLC method and validation for the quantitative estimation of everolimus in bulk form and marketed tablet dosage form

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Abstract

A selective, accurate and precise RP-HPLC method was developed and validated for the estimation of Everolimus in bulk and marketed pharmaceutical dosage forms. The Everolimus was resolved on a Symmetry ODS C_{18} (4.6mm × 250mm, 5µm) using Methanol: Phosphate Buffer used in the ratio of 35:65% v/v as the mobile phase. The detection wavelength was 235 nm. The retention time obtained for Everolimus were 3.006 min. The linearity ranges were 6-14 with Regression coefficients of 0.9996. The % R.S.D. of precision studies was found to be 0.175. The accuracy of the proposed method was determined by recovery studies and the mean recovery was 100.72%. The limit of detection and the limit of quantitation were found to be 1.2µg/ml and 3.6µg/ml, respectively. Different analytical performance parameters such as precision, accuracy, limit of detection, limit of quantification and robustness were determined according to International Conference on Harmonization (ICH) guidelines. Thus the novel proposed method for Everolimus was found to be feasible for the estimation of Everolimus in bulk as well as the pharmaceutical dosage form.

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Introduction

Everolimus is a derivative of Rapamycin (Sirolimus), and works similarly to Rapamycin as an mTOR (mammalian target of Rapamycin) inhibitor. It is currently used as an immunosuppressant to prevent rejection of organ transplants. In a similar fashion to other mTOR inhibitors Everolimus' effect is solely on the mTORC1 protein and not on the mTORC2 protein. Oral Everolimus is absorbed rapidly, and reaches peak concentration after 1.3-1.8 hours. Steady state is reached within 7 days, and steady-state peak and trough concentrations, and area under the concentration-time curve (AUC), are proportional to dosage. Everolimus [1] is an mTOR inhibitor that binds with high affinity to the FK506 binding protein-12 (FKBP-12), thereby forming a drug complex that inhibits the activation of mTOR. This inhibition reduces the activity of effectors downstream, which leads to a blockage in the progression of cells from G1 into S phase, and subsequently inducing cell growth arrest and apoptosis. Everolimus [2] also inhibits the expression of hypoxia-inducible factor, leading to a decrease in the expression of vascular endothelial growth factor. The result of Everolimus [3] inhibition of mTOR is a reduction in cell proliferation, angiogenesis, and glucose uptake. The IUPAC Name of Everolimus is (1R, 9S, 12S, 15R, 16E, 18R, 19R, 21R, 23S, 24E, 26E, 28E, 30S, 32S, 35R)-1, 18-dihydroxy-12-[(2R)-1-[(1S, 3R, 4R)-4-(2-hydroxy ethoxy)-3-methoxy cyclo hexyl] propan-2-yl]-19, 30-dimethoxy-15, 17, 21, 23, 29, 35-hexa methyl-11, 36-dioxa-4-azatri cyclo [30.3.1.04, 9] hexatriaconta-16, 24, 26, 28-tetraene-2, 3, 10, 14, 20-pentone. The Chemical Structure of Everolimus as shown in fig-1.

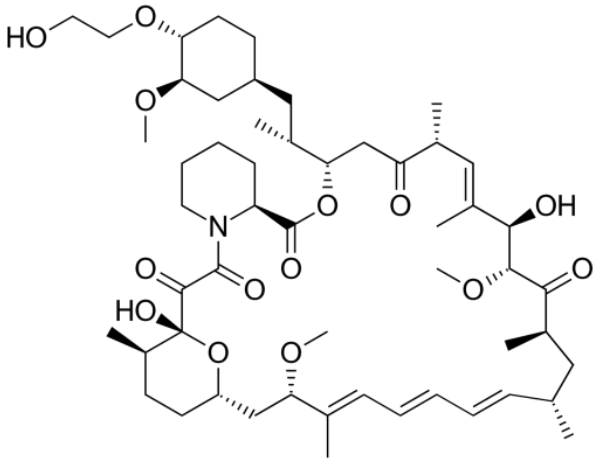

Fig 1: Chemical Structure of Everolimus

Materials and Methods

Instruments Used

Table 1: Instruments used

S. No	Instruments And Glass wares	Model
1	HPLC	HPLC with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2	pH meter	Lab India
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

Chemicals Used

Table 2: Chemicals used

S. No	Chemical	Brand names
1	Everolimus (Pure)	Local Market
2	Water and Methanol for HPLC	LICHROSOLV (MERCK)
3	Acetonitrile for HPLC	Merck

HPLC Method Development

Preparation of standard solution

Accurately weigh and transfer 10 mg of Everolimus working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.1ml of the above Everolimus stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines [30].

Mobile Phase Optimization

Initially the mobile phase[4] tried was Methanol and Methanol: Water with varying proportions. Finally, the mobile phase was optimized to Methanol: Phosphate Buffer in proportion 35:65% v/v.

Optimization of Column

The method was performed with various C18 columns like, X- bridge column, Xterra, and C18 column. Symmetry ODS C18 (4.6 x 250mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow [5].

Preparation of Buffer and Mobile Phase

Preparation of Potassium dihydrogen Phosphate (KH2PO4) buffer (pH-3.6)

Dissolve 6.8043 of potassium dihydrogen phosphate in 1000 ml HPLC water and adjust the pH 3.6 with diluted orthophosphoric acid. Filter and sonicate the solution by vacuum filtration [6] and ultra-sonication.

Preparation of Mobile Phase

Accurately measured 350 ml (35%) of Methanol, 650 ml of Phosphate buffer (65%) were mixed and degassed in digital ultra sonicater for 15 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation

The Mobile phase was used as the diluent.

Method Validation Parameters

System Suitability

Accurately weigh and transfer 10 mg of Everolimus working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Everolimus stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits [7].

Specificity

Preparation of Standard Solution

Accurately weigh and transfer 10 mg of Everolimus working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Everolimus stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Preparation of Sample Solution

Weight 10 mg equivalent weight of Everolimus sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.1ml of Everolimus above stock solution into a 10ml volumetric flask and dilute up to the mark with diluent.

Procedure

Inject the three replicate injections of standard and sample solutions and calculate the assay [8] by using formula:

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

Linearity

Accurately weigh and transfer 10 mg of Everolimus working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents [9] and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Preparation of Level – I (6ppm of Everolimus)

Take 0.6ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – II (8ppm of Everolimus)

Take 0.8ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – III (10ppm of Everolimus)

Take 0.1ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Preparation of Level – IV (12ppm of Everolimus)

Take 0.12ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator [10].

Preparation of Level-V (14ppm of Everolimus)

Take 0.14ml of stock solution in to 10ml of volumetric flask and make up the volume up to mark with diluents and sonicate the solution for bubble entrapment using ultrasonicator.

Procedure

Inject each level into the chromatographic system [11] and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient [12].

Precision

Repeatability

Preparation of Everolimus Product Solution for Precision

Accurately weigh and transfer 10 mg of Everolimus working standard into a 10ml of clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Everolimus stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Intermediate Precision

To evaluate the intermediate precision [13] (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure

Analyst 1

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Analyst 2

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy

For preparation of 50% Standard stock solution

Accurately weigh and transfer 10 mg of Everolimus working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.05ml of the above Everolimus stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 100% Standard stock solution

Accurately weigh and transfer 10 mg of Everolimus working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Everolimus stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

For preparation of 150% Standard stock solution

Accurately weigh and transfer 10 mg of Everolimus working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15ml of the above Everolimus stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions [14]. Recorded the chromatograms and measured the peak responses. Calculate the Amount found

and Amount added for Everolimus and calculate the individual recovery and mean recovery values [15].

Robustness

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For preparation of Standard solution

Accurately weigh and transfer 10 mg of Everolimus working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Everolimus stock solution into a 10ml volumetric flask and dilute up to the mark with diluents.

Effect of Variation of flow conditions

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Results and Discussion

Method Development

Optimized Chromatographic Conditions

Mobile phase ratio	:	Methanol: Phosphate Buffer (35:65) V/V
Column	:	Symmetry ODS C18 (4.6×250mm, 5µm)
Column temperature	:	Ambient
Wavelength	:	235nm
Flow rate	:	1ml/min
Injection volume	:	10µl
Run time	:	8min

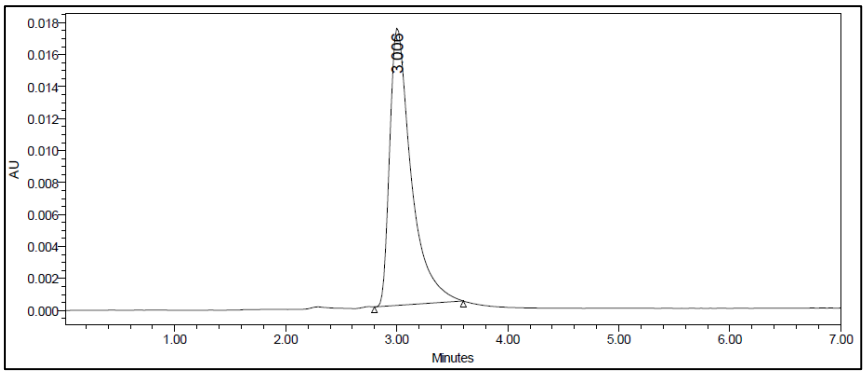

Fig 2: Optimized Chromatographic Condition

Validation of Analytical Method

The most widely applied validation characteristics are system suitability, accuracy, precision, specificity, linearity, range, robustness, and the limit of detection, limit of quantification, limit of detection and limit of quantification.

System Suitability

The chromato-graphic systems used for analysis must pass system suitability [16] before going to start the experiment. At first HPLC system is stabilized for forty minutes. Inject blank preparation (single injection) and standard preparation (five replicates) and record the chromatograms to evaluate the system suitability parameters such as tailing factor (NMT 1.5), theoretical plate count (NLT 2000) and retention time. The %RSD for the peak area of five replicate injections of Everolimus standard NMT 2.0. The parameters, such as tailing factor, % RSD, and theoretical plates, were studied.

Table 3: Results of system suitability for Everolimus

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Everolimus	3.008	1652847	185647	6589	1.24
2	Everolimus	3.005	1653658	186254	6587	1.26
3	Everolimus	3.001	1654521	185475	6584	1.28
4	Everolimus	3.000	1653564	186594	6582	1.29
5	Everolimus	3.001	1658745	185684	6895	1.24
Mean			1654667			
Std. Dev.			2355.764			
% RSD			0.142371			

Specificity

The ICH documents define specificity [17] as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Analytical method was tested for specificity to measure accurately quantitates Everolimus in drug product.

Linearity

Linearity [18] is the ability of the method to elicit test results that are directly or by a well-defined mathematical transformation to analyte concentration within a given range. The range [19] is the interval between the upper and lower levels of analyte. The linearity determined for Everolimus concentration range of 6-14 µg/ml. As shown in Table 4 and Fig. 3. The linearity of the method was evaluated by linear

regression analysis [20].

Chromatographic Data for Linearity Study

Table 4: Data for Linearity of Everolimus

Concentration µg/ml	Average Peak Area
6	1078475
8	1461129
10	1808358
12	2211573
14	2593778

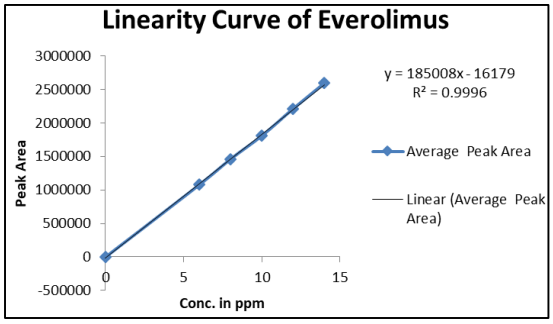

Fig 3: Linearity Curve of Everolimus

Linearity Plot

The plot of Concentration (x) versus the Average Peak Area (y) data of Everolimus is a straight line.

Y = mx + c

Slope (m) = 185008

Intercept (c) = 16179

Correlation Coefficient (r) = 0.999

Validation Criteria: The response linearity is verified if the Correlation Coefficient [21] is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 0.16179. These values meet the validation criteria.

Precision

The precision [22] of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Repeatability: Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

Table 5: Results of repeatability for Everolimus

S. No	Peak name	Retention time	Area(µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Everolimus	3.008	1658954	186958	1.26	6785
2	Everolimus	3.000	1658745	187548	1.27	6854
3	Everolimus	3.013	1659865	189854	1.26	6852
4	Everolimus	3.006	1653254	186985	1.25	6784
5	Everolimus	3.001	1654781	189542	1.24	6895
Mean			1657120			
Std.dev			2913.592			
%RSD			0.175823			

Intermediate Precision

The Intermediate Precision [23] consists of two methods:-

Intra Day: In Intra Day process, the 50%, 100% and 150% concentration are injected at different intervals of time in same day.

Inter Day: In Inter Day process, the 50%, 100% and 150% concentration are injected at same intervals of time in different days.

Table 6: Results of intra-assay & inter-assay

Conc. of Everolimus (API) (µg/ml)	Observed Conc. of Everolimus (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
50	49.38	0.56	49.45	0.56
100	100.17	0.71	99.70	0.77
150	150.89	0.89	149.91	0.85

Observations: The intra & inter day variation of the method was carried out for standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Everolimus revealed that the proposed method is precise.

Accuracy

Accuracy at different concentrations (50%, 100%, and 150%) was prepared and the % recovery was calculated. The accuracy [24] of a method is defined as the closeness of a measured value to the true value. The recovery studies were carried out at 50%, 100% and 150% of the target level in the standard in triplicate each in the injection.

Table 7: The Accuracy Results for Everolimus

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	109068.3	5	5.021	100.420%	100.72%
100%	202187	10	10.054	100.540%	
150%	297032.3	15	15.181	101.206%	

Limit of Detection for Everolimus

The detection limit [25] of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

$$LOD = 3.3 \times \sigma / s$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result

= 1.2µg/ml

Quantitation Limit

The quantitation limit [26] of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

$$LOQ = 10 \times \sigma / S$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve [27]

Result

= 3.6µg/ml

Robustness

The robustness [28] was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Everolimus. The method is robust only in less flow condition. The standard of Everolimus was injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Table 8: Result of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.68
Flow (0.9 ml/min)	0.39
Temperature (27^0C)	0.54
Temperature (23^0C)	0.63
Wavelength of Detection (280 nm)	0.91
Wavelength of detection (270 nm)	0.93

Acceptance Criteria: The tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.

7. Estimation of Everolimus in Pharmaceutical Dosage Form

Twenty Tablets were taken and the I.P. method was followed to determine the average weight. Above weighed tablets were finally powdered and triturated well. A quantity of powder equivalent to 25 mg of drugs were transferred to 25 ml volumetric flask, make and solution was sonicated for 15 minutes, there after volume was made up to 25 ml with same solvent. Then 10 ml of the above solution was diluted to 100 ml with mobile phase. The solution was filtered through a membrane filter (0.45 µm) and sonicated to degas. The solution prepared was injected in five replicates into the HPLC system and the observations were recorded.

The data are shown in Table-9.

ASSAY

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg}$$

Where:

AT = Peak Area of drug obtained with test preparation

AS = Peak Area of drug obtained with standard preparation

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

DT = Dilution of sample solution

P = Percentage purity [29] of working standard

Table 9: Recovery Data for estimation Everolimus in Rolimus 10 Tablet

Brand name of Everolimus	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Rolimus 10 Tablet (Cipla)	10mg	9.867 (± 0.468)	99.825 (± 0.418)

Result & Discussion

The amount of drug in Rolimus 10 Tablet was found to be 9.867 (± 0.468) mg/tab for Everolimus & %Purity was 99.825 %.

Summary and Conclusion

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Everolimus, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry ODS C18 (4.6×250mm, 5µm) column was preferred because using this column peak shape, resolution and absorbance were good. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Everolimus it is evident that most of the HPLC work can be accomplished in the wavelength range of 235 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 10µl were found to be the best analysis. The result shows the developed method is yet another suitable method for assay which can help in the analysis of Everolimus in different formulations. A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Everolimus API. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. The result shows the developed method is yet another suitable method for assay, purity which can help in the analysis of Everolimus in different formulations.

References

1. https://go.drugbank.com/drugs/DB01590
2. https://en.wikipedia.org/wiki/Everolimus
3. https://pubchem.ncbi.nlm.nih.gov/compound/Everolimus

4. R Snyder, J Kirkland, L Glajch. Practical HPLC Method Development, john Wiley and sons International publication, II Edn., 2011.
5. S Ashutoshkar. Pharmaceutical Drug Analysis 2nd Edn, New Age International Private Limited Publishers, 2005, 452-474.
6. H Beckett, JB Stenlake. Practical Pharmaceutical Chemistry, 4th End. C.B.S. Publishers and Distributors New Delhi, 1-9, 157-167.
7. HH Williard, LL Merit, FA Dean, FA Settle. Instrumental Methods Of Analysis, 6th Edn, C.B.S. Publishers and Distributors, New Delhi. 430-440, 495-504,529-545.
8. BK Sharma. Instrumental Methods of Chemical Analysis. GOEL Publishing House, Meerut, 286-300.
9. Instant notes on analytical chemistry by D. Kealey and P.J. Haines, © BIOS Scientific Publishers Limited, UK, 2002, 6-7.
10. Gurdeep R Chatwal, Sham K Anand. Instrumental methods of Chemical Analysis, 5th edition, Himalaya Publishing House (Mumbai), 2005, P-2.566.
11. ME Swartz. Journal of liquid chromatography. 2005; 28(7/8):1253-1263.
12. Journal of Chromatography. B, Analytical Technologies in the Biomedical and life Sciences. 2008 March 1. Published on. 2008; 863(2):258-2650
13. International Conference on Harmonization Harmonized Tripartite Guideline. Validation of Analytical Procedures. Text and Methodology. Q2 (R1), 2005.
14. International Conference on Harmonization (ICH). Validation of Analytical Methods: Definitions and Terminology. ICH Q2A, 1994.
15. JM Green. A practical guide to analytical method validation, anal. Chem. News & features, 1996, 305a-309a.
16. PA Winslow, rf Meyer. Defining a master plan for the validation of analytical methods, J Validation technology, 1997, 361-367.
17. Aoac peer-verified methods program, manual on policies and procedures, Arlington, Va., USA, 1998.
18. R Patil. J of Chromatographia. 2008; 67:575.
19. Baht, Leena. J of Liq. Chrom. 2007; 30:309.
20. HH Williard, LL Merit, FA Dean, FA Settle. Instrumental methods of analysis, 7th edition, C.B.S. Publishers, New Delhi, 2002.
21. GN Menon, LB White. Department of Analytical Research, Abbott Laboratories, (pub med-index for MEDLINE).
22. Food, Drug Administration (FDA), Analytical Procedures and Methods Validation: Chemistry, Manufacturing and Controls Documentation;" Federal Register (Notices). 2000; 65(169):52776-52777.
23. Vibha G, *et al*. Development and validation of HPLC method - a review. International Research Journal of Pharmaceutical and Applied Sciences. 2012; 2(4):22-23.
24. Bliesner DM. In: Validating Chromatographic Methods. John Wiley & sons Inc., 2006, 88-92.
25. Validation of Analytical Procedures: Methodology. ICH-Guidelines Q2B, Geneva. 1996, 11. (CPMP/ICH/281/95).
26. Development and validation of HPLC method - A Review, Vibha Gupta *et al*, International Research Journal of Pharmaceutical and Applied Sciences. 2012; 2(4):17-25.
27. A Review: HPLC Method Development and Validation, Santosh Kumar Bhardwaj **et al*. International Journal of Analytical and Bioanalytical Chemistry, 2015.
28. Method Development. A Guide to Basics Quantitative & Qualitative HPLC, LC, GC chromacademy.
29. Lalit V Sonawane, Bioanalytical Method Validation and Its Pharmaceutical Application-A Review Pharmaceutica Analytical Acta Center for Drug Evaluation and Research (CDER) Reviewer Guidance. 2014; 5:3.
30. ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology.
31. 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 Journal of Pharm Sciences 2015; 77(5):599-604, doi: 10.4103/0250-474x.169044.
32. Somana Siva Prasad, GV Krishna Mohan, A Naga Babu. Development of Simple and Robust RP-HPLC Method for Determination of Everolimus and its Impurities in Oral Solid Dosage Form, Asian Journal of Chemistry. 2019; 31(5):1002-1008.
33. Rushikesh Mulay, Rishikesh Bachhav. Stability Indicating RP-HPLC Method Development and Validation of Everolimus in Bulk and Pharmaceutical Dosage Form, Published in International Journal of Trend in Scientific Research and Development (ijtsrd), ISSN: 2456-6470, Volume-5 | Issue-6, October 2021, pp.199-207, URL: https://www.ijtsrd.com/papers/ijtsrd46341.pdf.
34. Devavath Renuka, Nalla Kumar P, Bhagavan Raju M. Method Development and Validation of Everolimus by Using RP-HPLC, World Journal of Pharmaceutical Research, 8(13), 949-957.