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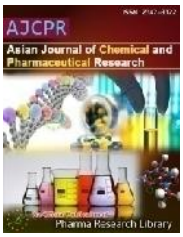

Research Article **Open Access**

Analytical Method Development and Validation by RP-HPLC Method in Bulk and Tablet Dosage form of Tacrolimus and Guggulsterone

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A B S T R A C T

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of tacrolimus and guggulsterone was done by RP-HPLC. Present work is aimed to develop a new, simple, fast, rapid, accurate, efficient and reproducible RP-HPLC method for the simultaneous analysis of Tacrolimus and guggulsterone. The Phosphate buffer was p^H 3.0 and the mobile phase was optimized with consists of Methanol: Phosphate buffer mixed in the ratio of 70:30 % v/ v. symmetry C_{18} column C18 (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 256 nm. The solutions were chromatographed at a constant flow rate of 0.8 ml/min. the linearity range of tacrolimus and guggulsterone were found to be from 10-50 µg/ml of tacrolimus and 60-300 µg/ml of guggulsterone. Linear regression coefficient was not more than 0.999. The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of tacrolimus and guggulsterone .LOD and LOQ were found to be within limit.

Keywords: Tacrolimus and guggulsteron, RP-HPLC, symmetry C18 column.

A R T I C L E I N F O

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1. Introduction

Tacrolimus is a macrolide calcineurin inhibitor. In T-cells, activation of the T-cell receptor normally increases intracellular calcium, which acts via calmodulin to activate calcineurin. Calcineurin then dephosphorylates the transcription factor nuclear factor of activated T-cells (NF-AT), which moves to the nucleus of the T-cell and increases the activity of genes coding for IL-2 and related cytokines. Tacrolimus prevents the dephosphorylation of NF-AT. [6] In detail, Tacrolimus reduces peptidyl-prolyl isomerase activity by binding to the immunophilin FKBP12 (FK506 binding protein) creating a new complex. This FKBP12-FK506 complex interacts with and inhibits calcineurin, thus inhibiting both T-lymphocyte signal transduction and IL-2 transcription.[7] Although this activity is similar to that of cyclosporin, the incidence of acute rejection is reduced by tacrolimus use over cyclosporin.[8] Although short-term immune suppression concerning patient and graft survival is found to be similar between the two drugs, tacrolimus results in a more favorable lipid profile, and this may have important long-term implications given the prognostic influence of rejection on graft survival. [9]

Molecular formulae: $C_{44}H_{69}NO_{12}$
Molecular Weight: 804.018 g/mol
Trade Name: Acroli, biomus, crolim
Category: Immunosuppressive, Immunomodulator, anti fungal

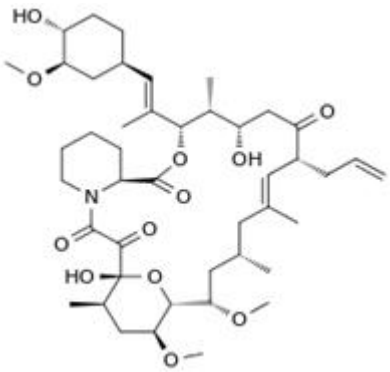

Figure 1: Tacrolimus

Guggulsterone is a plant steroid found in the resin of the guggul plant, *Commiphora mukul*. Guggulsterone can exist as either of two stereoisomers, *E*-guggulsterone & *Z*-guggulsterone. In humans, it acts as an antagonist of the farnesoid X receptor, which was once believed to result in decreased cholesterol synthesis in the liver. Several studies have been published that indicate no overall reduction in total cholesterol occurs using various dosages of guggulsterone, and levels of low-density lipoprotein ("bad cholesterol") increased in many people.[1][2] Nevertheless, guggulsterone is an ingredient in many nutritional supplements..

Molecular formulae : $C_{21}H_{28}O_2$
Molecular Weight: 312.45 $g \cdot mol^{-1}$
Trade Names: Thyroidenergy 90v caps
Category: heal arthritis, to remove body toxins

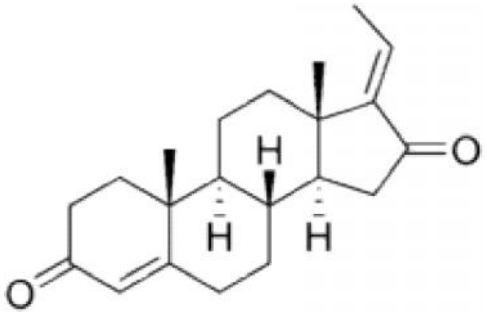

Figure 2: Guggulsterone

2. Materials and Methods

Apparatus

The instrument used for the study was Waters HPLC Auto Sampler, Separation module 2695, photo diode array detector with Empower-software version-2.

Reagents and Materials

The solvents used were sodium perchlorate, perchloric acid, Methanol, acetontrile, Water, Ortho phosphoric acid, potassium dihydrogen orthophosphate, 0.45µm Nylon filter, 0.45µm PVDF filter

Analytical Method Development

A. Selection of wavelength

A solution of 10 µg/ml of Tacrolimus and Guggulsterone were prepared in milli Q water. The resulting solutions were scanned individually on HPLC PDA detector from 190 to 400 nm and also in UV-Visible spectrophotometer. The optimal response for three of them was obtained at 256 nm. Hence the complete method was processed at the wavelength of 256nm.

B. Selection of chromatographic condition

Proper selection of the method depends up on the nature of the sample (ionic/ ionisable/neutral molecule), its molecular weight and solubility. The drugs selected in the present study, were polar in nature. Thus reverse phase HPLC was selected for the initial separation because of its simplicity, suitability, ruggedness and its wider usage.

C. Initial separation condition

The mobile phase selected to elute the drug from the stationary phase was milliQ water and HPLC methanol, because of its favorable UV transmittance, low viscosity and low back pressure.

Preparation of standard solution: 10 mg of Tacrolimus and 10mg of Guggulsterone were accurately weighed and transferred into a 10 ml clean dry volumetric flask, about 7 ml of diluent was added, sonicated to dissolve it completely and the volume was made up to the mark with the same solvent to give a concentration of 1000 µg/ml. (Stock solution) Further 0.3 and 1.8 ml were pipetted out fromthe above stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to give a concentration of 30 µg/ml and 180µg/ml respectively.

Preparation of sample solution: 10 Tablets of contents were weighed and triturated in glass mortar. The quantity of powder equivalent to 10 mg of active ingredient present in Tacrolimus and Guggulsterone was transferred into a 10 ml clean dry volumetric flask, 7 ml of diluent was added to it and was shaken by mechanical stirrer and sonicated for

about 30 minutes by shaking at intervals of five minutes each and was diluted up to the mark with diluent to give a concentration of 1000 µg/ml and allowed to stand until the residue settles before taking an aliquot for further dilution (stock solution). 0.3 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark to give the respective concentrations as par with standard solution. The solution was filtered through 0.45 µm filter before injecting into HPLC system.

Preparation of Placebo:

The amount of powdered inactive ingredient supposed to be present in 10 tablets were accurately weighed and transferred in to 10 ml volumetric flask, 7 ml of diluent was added and shaken by mechanical stirrer and sonicated for about 30 minutes by shaking at intervals of five minutes and was diluted up to the mark with diluent and allowed to stand until the residue settles before taking an aliquot for dilution. 0.1 ml of upper clear solution was transferred to a 10 ml volumetric flask and diluted with diluent up to the mark and the solution was filtered through 0.45 µm filter before injecting into HPLC system.

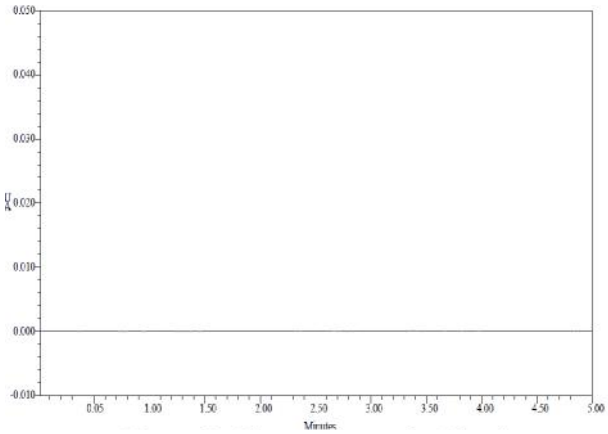

Figure 3: Chromatogram for Placebo

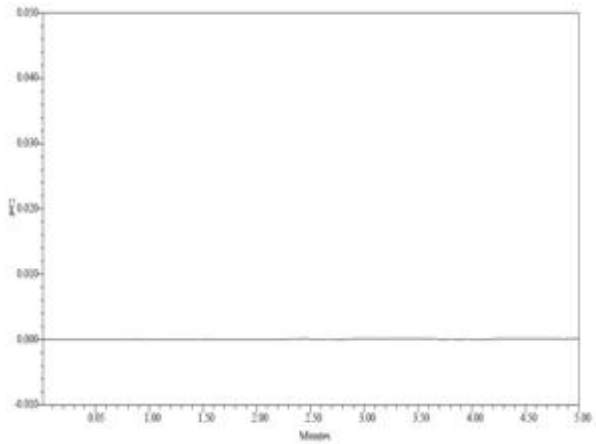

Figure 4: Chromatogram of Blank

Trial-1

Method development for the drugs was initiated based on the individual chemical characteristics' and their methods given in individual journals.

Mobile phase: Methanol: Phosphate buffer P^H3 (70:30)

Diluent: Methanol

Chromatographic conditions:

Flow rate : 1ml/min

Column : Symmetry C_{18} (4.6 x 150mm, 5µm)

Detector wavelength : 256 nm

Column oven : Ambient

Injection volume : 10µl

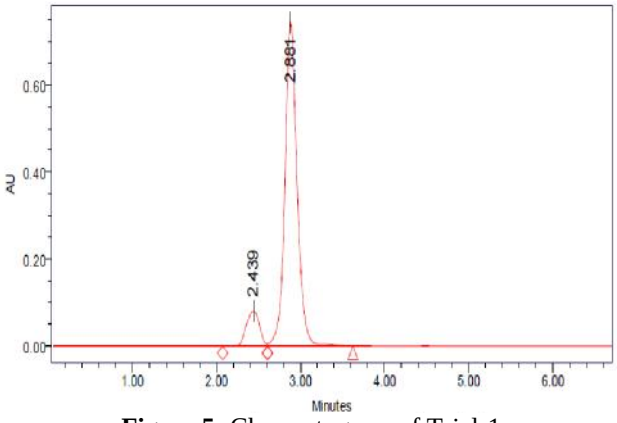

Figure 5: Chromatogram of Trial-1

Observation:

There was no proper peak separation. The Chromatogram for trial 1 was shown in Fig: 5.

Trail-2

Inorder to improve resolution and remove fronting of the peak and avoid unwanted peaks interfering, mobile phase was changed and again the same experiment was performed.

Mobile phase: Methanol: Sodium acetate P^H 4 (60:40)

Diluent: methanol

Chromatographic conditions:

Flow rate : 0.8ml per min

Column :Symmetry C_{18} (4.6 x 150mm, 5µm)

Detector wavelength : 256 nm

Column oven : Ambient

Injection volume : 10µl

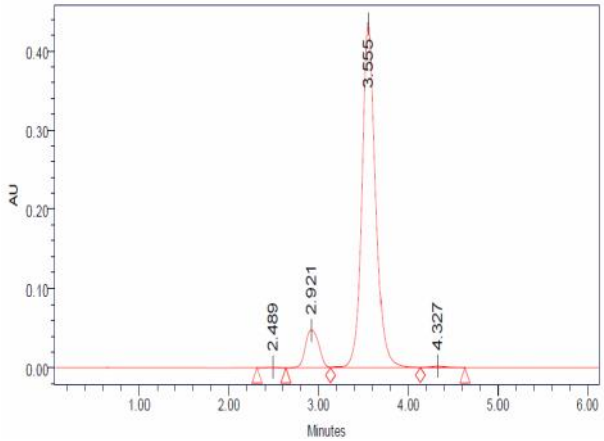

Figure 6: Chromatogram of Trial-2

Observation:

Peaks are separated but tailing has been observed. The Chromatogram for trial 2 was shown in Fig: 6.

Trial-3

In order to avoid poor response and tailing mobile phase was changed i.e organic phase was changed

Mobile phase: Methanol: Ammonium acetate P^H 3(70:30)

Diluent: methanol.

Chromatographic conditions:

Flow rate : **2** ml per min

Column : Symmetry C_{18} (4.6 x 150mm, 5µm)

Detector wavelength : 260 nm

Column oven : Ambient

Injection volume : 20µl.

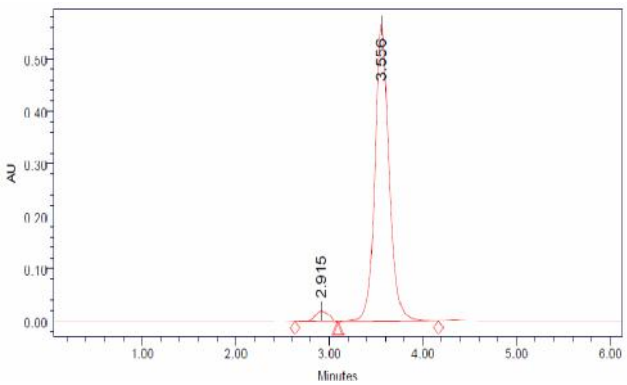

Figure 7: Chromatogram of Trial-3

Observation:

Peaks were eluted but with less resolution peaks were seen. . The chromatogram for trial 3 was shown in Fig: 7.

Trail 4 (Optimized Method)

Preparation of mobile phase:

Take 24 gm of Ammonium acetate into 1000ml volumetric flask dissolved in HPLC grated water and adjust Phupto 3 with ortho phosphoric acid. From the above prepared buffer take 350 ml (35%) and 650ml Methanol (65%) HPLC were mixed and degassed in ultrasonic water bath for 5 minutes and was filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation: Mobile phase was used as Diluent.

Chromatographic conditions:

Flow rate : 1.2 ml per min

Column : Symmetry C_{18} (4.6 x 150mm, 5µm)

Detector wavelength: 256nm

Column oven : Ambient

Injection volume : 10µl

Run time : 8 min

Test Procedure:

20 µl of the Standard, Sample and Blank preparations in duplicate were injected separately into HPLC system and the peak responses for Tacrolimus and Guggulsterone were measured. The quantities from the peak area in mg of Tacrolimus and Guggulsterone were calculated per tablet taken. The developed RP-HPLC method for the simultaneous estimation of Tacrolimus and Guggulsterone were carried out On symmetry C_{18} (4.6 x 150mm), 5µm column in gradient mode using mobile phase composition of Methanol: Ammonium acetate buffer Ph 3 [65: 35, v / v] with flow rate of 1.2 ml / min at 256nm.

Observation: Resolution between two analytes were good. No peak asymmetry was observed. No other impurity interference was seen. All the results were found to be within the acceptance criteria. Hence the method was considered to be optimized.

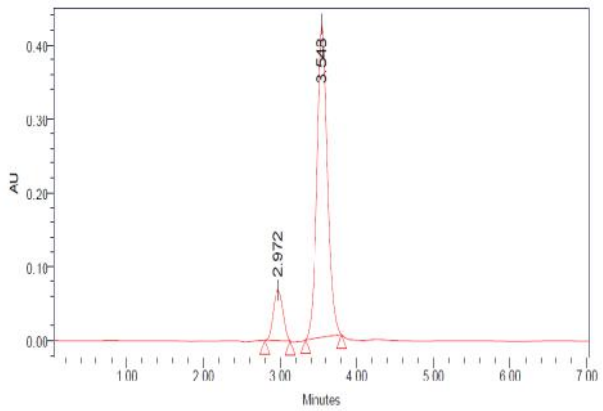

Figure 8: Standard Chromatogram for Optimised Method

Calculation: The amount of drug present was calculated by using the following formula:

For Tacrolimus:

$$\text{Assay\%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:

AT = average area counts of sample preparation.

As= average area counts of standard preparation.

WS = Weight of working standard taken in mg.

P = Percentage purity of working standard

LC = label claim of Tacrolimus mg/ml.

For Guggulsterone:

$$\text{Assay\%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:

AT = average area counts of sample preparation.

As= average area counts of standard preparation.

WS = Weight of working standard taken in mg.

P = Percentage purity of working standard

LC = label claim of Guggulsterone mg/ml.

Observation: In this trial no peak was observed, still more trials was required for good peaks

3. Results and discussion

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. According to ICH guidelines, typical analytical performance characteristics that should be considered in the validation of the type of methods are: System suitability, Linearity, Precision, Accuracy, Specificity, Robustness, Ruggedness, Limit of detection, Limit of quantification.

1. System Suitability

1. Tacrolimus

Table 1: Chromatogram values for System suitability

Injection	R_t	Peak Area	USP Plate count	USP Tailing
1	0.430	1250763	2487	1.62
2	0.432	1247867	2489	1.58
3	0.434	1255849	2496	1.64
Mean	0.432	1251360		
SD	0.002	3850.679		
% RSD	0.4629	0.30722		

Tailing factor Obtained from the standard injection is 1.7
Theoretical Plates Obtained from the standard injection is 2496

2. Guggulsterone

Table 2: Chromatogram values for System suitability

Injection	R_t	Peak Area	USP Plate count	USP Tailing	USP Resolution
1	0.660	940627	2281	1.51	3.04
2	0.671	931161	2244	1.47	3.09
3	0.667	940306	2261	1.47	3.05
Mean	0.666	937364.7			
SD	0.005568	5374.93			
% RSD	0.8360	0.573409			

Tailing factor Obtained from the standard injection is 1.51
Theoretical Plates Obtained from the standard injection is 2281

Acceptance criteria

- The % RSD for the retention times of principal peak from 3 replicate injections of each Standard solution should be not more than 2.0 %
- The number of theoretical plates (N) for the Tacrolinus and Guggulsterone peaks should be not less than 2000.
- The Tailing factor (T) for the Tacrolinus and Guggulsterone peaks should be not more than 2.0.

From the system suitability studies it was observed that all the parameters were within limit. Hence it was concluded that the Instrument, Reagents and Column were suitable to perform the Assay. The results were expressed in **Table:** 1& 2.

2. Linearity

Table 3: Linearity values of Tacrolimus

S.No	Linearity Level	Concentration	Area
1	I	10 ppm	839286
2	II	20 ppm	1067774
3	III	30 ppm	1246474
4	IV	40 ppm	1439991
5	V	50 ppm	1639065
Correlation Coefficient			0.99932

Table 4: Linearity values of guggulsteron

S.No	Linearity Level	Concentration	Area
1	I	10 ppm	626221
2	II	20 ppm	778750
3	III	30 ppm	931447
4	IV	40 ppm	1070162
5	V	50 ppm	1196060
Correlation Coefficient			0.99916

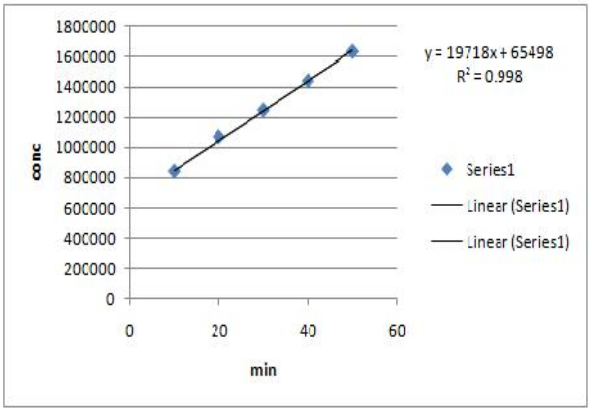

Figure 9: Calibration curve of Tacrolimus

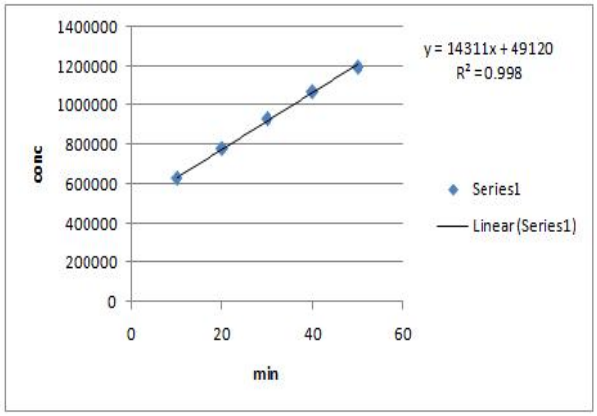

Figure 10: Calibration curve of Guggulsterone

Acceptance criteria

1. Correlation Coefficient should be not less than 0.9990.
2. % RSD of peak areas for Solution 1, 2, 3, 4 and 5 should be not more than 2.0 %.

3. Precision

Table 5: Precision of Tacrolimus

Injection No	Peak Area	R_t
1	1247256	0.431
2	1248579	0.432
3	1243273	0.432
4	1243262	0.433
5	1249574	0.437
Avg	1246389	0.433
SD	2965.62	0.002345
% RSD	0.23793	0.5366

Table 6: Precision of Guggulsterone

Injection No	Peak Area	R_t
1	935035	0.659
2	929353	0.659
3	930459	0.659
4	932389	0.659
5	922057	0.665
Avg	929858.6	0.6602
SD	4865.16	0.002683
% RSD	0.5232	0.4061

Acceptance Criteria: The % RSD for the area and R_t of five standard injections results should not be morethan 2%.

4. Ruggedness

Intermediate precision (Analyst to Analyst variability):

Table 7: Ruggedness values of Tacrolimus

Injection No	Peak Area	R_t
1	1231404	0.429
2	1233196	0.430
3	1231008	0.431
4	1238575	0.434
5	1232407	0.435
Mean	1233318	0.431
SD	3061.06	0.00258
%RSD	0.2481	0.5994

Table 8: Ruggedness values of Guggulsterone

Injection No	Peak Area	R_t
1	912412	0.656
2	913062	0.658
3	909642	0.658
4	916881	0.660
5	914005	0.662
Mean	913200.4	0.6588
SD	2621.886	0.00228
% RSD	0.287	0.3461

The % RSD for the areas and Rt'sof five standard injections results should not be more than 2%.

5. Accuracy

Table 9: Chromatogram Values for Accuracy of Tacrolimus

Sample No.	Spike Level	Amount (µg/ml) added	Amount (µg/ml) found	% Recovery	Mean % Recovery
1	50 %	5	4.9	98%	100%
		5	5.1	102%	
		5	5	100%	
2	100 %	10	9.88	98.8%	99.13%
		10	9.91	99.1%	
		10	9.95	99.5%	
3	150 %	15	14.89	99.2%	99.69%
		15	14.86	99.0%	
		15	14.82	99.79%	

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%.

Table 10: Chromatogram Values for Accuracy of Guggulsterone

Sample No.	Spike Level	Amount (µg/ml) added	Amount (µg/ml) found	% Recovery	Mean % Recovery
1	50 %	5	4.9	98%	100%
		5	5.1	102%	
		5	5	100%	
2	100 %	10	9.88	98.8%	99.31%
		10	9.91	99.1%	
		10	9.95	99.5%	
3	150 %	15	14.89	99.2%	99.89%
		15	14.86	99.0%	
		15	14.99	99.79%	

Acceptance Criteria: The % Recovery for each level should be between 98.0 to 102.0%

6. Specificity

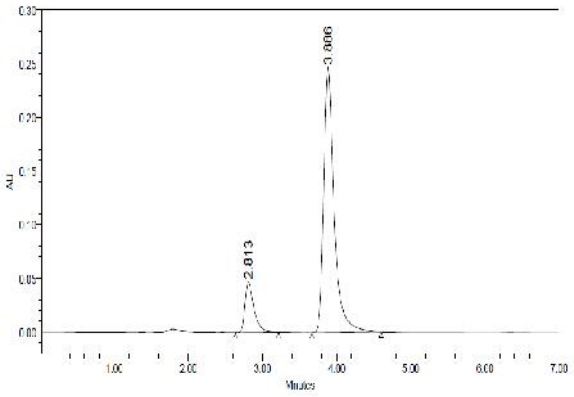

Figure 11: Specificity Chromatogram for Tacrolimus and Guggulsterone

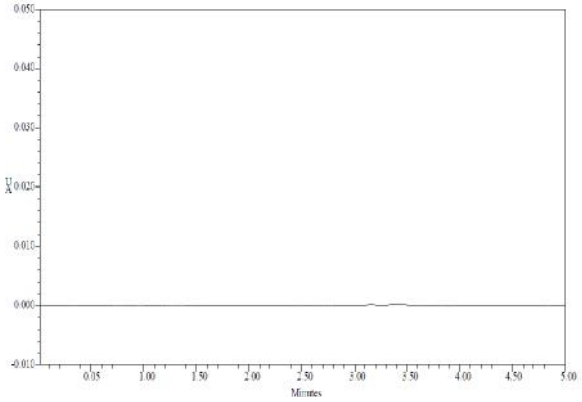

Figure 12: Chromatogram for Blank Interference

Acceptance criteria

Chromatogram of blank should not show any peak at the retention time of analyte peak. There is no interference due to blank at the retention time of analyte. Hence the method is specific.

7. Robustness

a) Effect of variation in flow rate:

Table 11: Robustness results for tacrolimus

S.No	Drug	Flow Rate ml/min		
		0.2ml/min R_t	0.3 ml/min	0.4m l/min
1	tacrolimus Robustness Results	0.516	0.429	0.362
2		0.514	0.430	0.368
3		0.513	0.431	0.368
4		0.512	0.434	0.370
5		0.510	0.435	0.372
mean		0.513	0.431	0.368
SD		0.002236	0.00258	0.0037
% RSD		0.4358	0.5994	1.0157
USP Plate count		2511	2490	2484

Table 12: Robustness results for guggulsteron

S.No	Drug	Flow Rate ml/min		
		0.2 ml/min R_t	0.3ml/min R_t	0.4 m l/min R_t
1	guggulsterone Robustness Results	0.796	0.556	0.560
2		0.795	0.558	0.561
3		0.791	0.558	0.561
4		0.788	0.560	0.564
5		0.786	0.562	0.566
mean		0.7912	0.6588	0.5624
SD		0.004324	0.00228	0.00251
% RSD		0.5465	0.3461	0.4462
USP Plate count		2279	2268	2183
USP Tailing		1.47	1.48	1.48

b) Effect of variation in mobile phase composition:

Table 13: Robustness results for tacrolimus

S.No	Drug	Mobile phase		
		More organic	organic	Less organic
1	tacrolimus Robustness Results	0.424	0.431	0.432
2		0.428	0.432	0.433
3		0.428	0.432	0.434
4		0.430	0.433	0.434
5		0.432	0.437	0.436
mean		0.4284	0.433	0.4338
SD		0.0295	0.00234	0.00148
% RSD		0.6924	0.5366	0.3419
USP Plate count		2510	2490	2492
USP Tailing		1.54	1.62	1.63

Table 14: Robustness results for guggulsteron

S.No	Drug	Mobile phase		
		Less organic	Normal	More organic
1	guggulosterone Robustness Results	0.785	0.659	0.576
2		0.787	0.659	0.579
3		0.789	0.659	0.579
4		0.789	0.659	0.582
5		0.790	0.665	0.585
mean		0.788	0.6602	0.5804
SD		0.002	0.00268	0.00228
% RSD		0.02538	0.4054	0.6515
USP Plate count		2432	2262	2223
USP Tailing		1.35	1.48	1.5

Acceptance criteria for variation in flow:

1. The tailing factor for Tacrolinus and Guggulsterone should be not more than 2.0 for Variation in flow.
2. The % RSD of Asymmetry and retention time for Tacrolinus and Guggulsterone should be not more than 2.0 % for variation in flow.

Acceptance criteria for variation in mobile phase:

1. Tailing Factor of Tacrolinus and Guggulsterone drugs should not be more than 2.0 for Variation in composition of mobile phase.
2. The % RSD of tailing factor and retention times of Tacrolinus and Guggulsterone drugs should be not more than 2.0 for Variation in composition of mobile phase.

Limit of Detection (LOD)

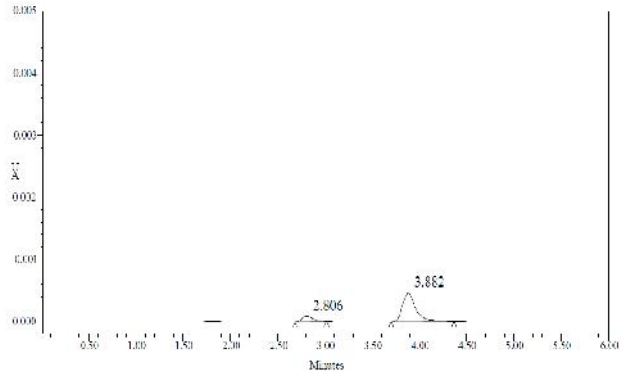

Figure 13: Chromatogram for Tacrolimus and Guggulsterone

Calculation of S/N Ratio for Tacrolimus:
Average Baseline Noise obtained from Blank: 48 µV
Signal Obtained from LOD solution (0.15% of target assay concentration): 146µV
S/N = 146/48 = 3.041
Acceptance Criteria:
S/N Ratio value shall be not more than 3 for LOD solution.
Calculation of S/N Ratio for Guggulsterone:
Average Baseline Noise obtained from Blank: 48µV
Signal Obtained from LOD solution (0.22% of target assay concentration): 148 µV
S/N = 148/48 = 3.08
Acceptance Criteria:
S/N Ratio value shall be not more than 3 for LOD solution.

Limit of Quantification (LOQ)

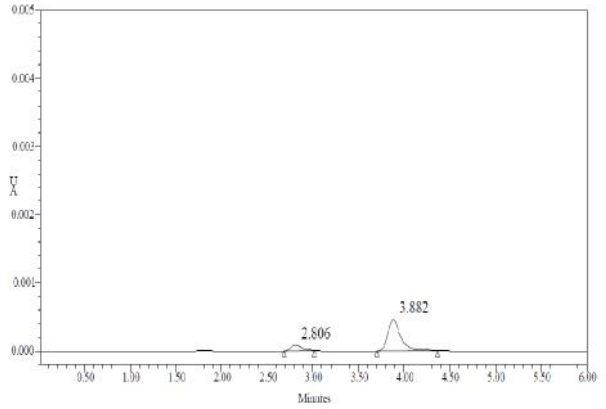

Figure 14: Chromatogram for Tacrolimus & Guggulsterone

Calculation of S/N Ratio for Tacrolimus:
Average Baseline Noise obtained from Blank: 48 µV
Signal Obtained from LOQ solution (0.05% of target assay concentration): S/N= 470/48= 9.7
Acceptance Criteria:
S/N Ratio value shall be not more than 10 for LOQ solution.
Calculation of S/N Ratio for Guggulsterone:
Average Baseline Noise obtained from Blank: 48 µV
Signal Obtained from LOQ solution (0.06% of target assay concentration): 498µV
S/N = 498/48 = 10.37
Acceptance Criteria: S/N Ratio value shall be not more than 10 for LOQ solution.

4. Conclusion

High performance liquid chromatography is at present one of the most sophisticated tool of the analysis. The estimation of tacrolimus and guggulsterone was done by RP-HPLC. The Phosphate buffer was p^H 3.0 and the mobile phase was optimized with consists of Methanol: Phosphate buffer mixed in the ratio of 70:30% v/v. Inertsil C_{18} column C18 (4.6 x 150mm, 5µm) or equivalent chemically bonded to porous silica particles was used as stationary phase. The detection was carried out using UV detector at 260 nm. The solutions were chromatographed at a constant flow rate of 0.8 ml/min. the linearity range of tacrolimus and guggulsterone were found to be from 100-500 µg/ml of tacrolimus & 1-5µg/ml of guggulsterone. Linear regression coefficient was not more than 0.999. The values of % RSD are less than 2% indicating accuracy and precision of the method. The percentage recovery varies from 98-102% of tacrolimus and guggulsterone. LOD and LOQ were found to be within limit. The results obtained on the validation parameters met ICH and USP requirements .it inferred the method found to be simple, accurate, precise and linear. The method was found to be having suitable application in routine laboratory analysis with high degree of accuracy and precision.

5. References

[1] Willard HH, Merrit LL, Dean JA, Settle FA. Instrumental methods of analysis, CBS Publishers and Distributors, New Delhi, 6th edition, 1986:1-15.
[2] Douglas A. Skoog, F. James Holler, Timothy A. Nieman. Principles of instrumental analysis, Saunders Golden Sun burst Series, Philadelphia, 2nd edition, 1980:725-760.
[3] United States of Pharmacopeia, USP30-NF25, The official compendia of standards, official May 1, 2007.
[4] Validation of Analytical Procedures; Methodology. ICH Harmonized Tripartite Guidelines, Geneva, Switzerland, (1996), PP 1- 8.
[5] Kaliszan R, Wiczling P, Markuszewski MJ, pH gradient high performance liquid chromatography theory & applications Journal of Chromatography, 1060 (1-2) (2004) 165-75.

[6] Draft ICH Guidelines on Validation of Analytical Procedures Definitions and terminology. Federal Register, vol 60. IFPMA, Switzerland, (1995), PP 1126.
[7] Vijay warty, Sheila Zuckerman and Thomas starzl. Tacrolimus analysis: a comparison of different methods and matrices. The drug monit.1995, 17(2): 159-167.
[8] Meenakshi S. Akhade, Poonam A. Agrawal, and K. S. Laddha, Indian Development and Validation of RP-HPLC Method for Simultaneous Estimation of Picroside I, Plumbagin, and Z-guggulsterone in Tablet Formulation Journal of Pharmaceutical Sciences,;Jul/Aug2013, Vol. 75 Issue 4, p476
[9] Qingping Shi, Jianchun Li, Feng Ding. Development and Validation of method for the determination of related substances of Tacrolimus in Tacrolimus Capsules and Degradation Studies International Journal of Chem Tech Research. Vol.4, No.4, pp 1543-1552, Oct-Dec 2012.
[10] .Amardeep, Mukunda S. Bande, Shilpi Agarwal. Validated Spectrophotometric Method for Determination of Tacrolimus In Marketed Formulation IOSR Journal of Pharmacy Vol. 2, Issue 2, Mar.-Apr, 2012, pp. 317-321.
[11] Patel, Pranav; Panchal, Shital; Mehta, Tejal; Solanki, Sagar; Patel, Chintan RP-HPLC method for determination of tacrolimus in bulk and pharmaceutical formulation International Journal of Pharmacy & Pharmaceutical Sciences;Oct2011, Vol. 3 Issue 4, p220.
[12] Pragnesh N Dave, Lakha V Chopda. Review on biological activity and determination of E&Z Guggulsterones concentration by HPLC & HPTLC Methods International Journal of Chemical Studies, Volume 1 Issue 3,pp 167-174.
[13] Kamal YT, Mohammed Musthaba S, Singh M, Parveen R, Ahmad S, Baboota S, Ali I, Siddiqui KM, Arif Zaidi SM Development and validation of HPLC method for simultaneous estimation of piperine and guggulsterones in compound Unani formulation (tablets) and a nanoreservoir system. Ncbi. 2012, 26(10):1183-90.
[14] Dr. Kealey and P.J Haines. Analytical Chemistry, 1stedition, Bios Publisher, 2002, 1-7.
[15] Braith Wait A and Smith FJ, Chromatographic Methods, 5 thedition, Kluwer Academic Publisher, 1996, pp 1-2.
[16] Andrea Weston and Phyllisr. Brown, HPLC Principle and Practice, 1st edition, Academic press, 1997, pp: 24-37.
[17] Yuri Kazakevich and Rosario Lobrutto, HPLC for Pharmaceutical Scientists, Wiley Interscience A John Wiley & Sons, Inc., Publication, 1st Edition, 2007, pp:15-23.