DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR IMMUNOSUPPRESSIVE AGENT USING UV- SPECTROPHOTOMETRY AND RP-HPLC.

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Abstract:

This dissertation focuses on the development and validation of analytical methods for the quantification of immunosuppressive agents, specifically Tacrolimus, using UV spectrophotometry and reverse-phase high-performance liquid chromatography (RP-HPLC) in both bulk and dosage forms. A simple and rapid UV-Vis spectrophotometric method for the estimation of Tacrolimus was developed, with calibration curves constructed and the linearity range determined. Additionally, a precise, accurate, and robust HPLC method for the quantification of Tacrolimus in bulk and pharmaceutical formulations was established, optimizing chromatographic conditions including mobile phase composition, flow rate, detection wavelength, and column selection. The HPLC method was validated according to ICH guidelines, demonstrating excellent specificity, linearity, accuracy, precision, LOD, LOQ, and robustness. Comparative analysis between the UV-Vis spectrophotometric and HPLC methods highlighted the former as a viable alternative for Tacrolimus quantification. These validated methods support routine quality control and stability studies, ensuring regulatory compliance, and facilitating the development of innovative drug delivery systems. They also contribute to the understanding of Tacrolimus's physicochemical properties, ultimately enhancing its therapeutic management.

Keyword: UV Spectrophotometry, RP-HPLC, Immunosuppressive Agent, Method Development, Method Validation, Pharmaceutical Analysis, Tacrolimus. Dosage Form. Bulk Analysis, Analytical Chemistry

Introduction:

Tacrolimus is an immunosuppressive drug primarily used to prevent organ transplant rejection, particularly in kidney, liver, and heart transplantsTacrolimus chemically The initial oral dose for adults undergoing kidney, liver, or heart transplantation typically ranges from 0.1-0.2 mg/kg/day divided into two doses. Dose adjustments are made based on blood level monitoring and clinical response.

UV-Visible (UV-Vis) spectroscopy is based on the principle that molecules absorb light at specific wavelengths, causing electronic transitions from a lower energy ground state to a higher energy excited state. High-Performance Liquid Chromatography (HPLC) is a powerful analytical technique used to separate, identify, and quantify components in a mixture. It is widely used in various fields such as pharmaceuticals, environmental science, and biochemistry due to its high precision, accuracy, and versatility. HPLC operates on the principle of liquid chromatography,

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where a liquid mobile phase carries the sample through a column packed with a solid stationary phase. The different components of the sample interact differently with the stationary phase, leading to their separation as they move through the column at different rates. The separation is based on differences in the distribution coefficient of each component between the mobile and stationary phases

MATERIALS AND METHODS:

Tacrolimus was provided by reputed pharmaceutical company in Alkem laboratory, Maharashtra, INDIA. HPLC grade methanol and water were used; analytical reagent (AR) grade O-Phosphoric Acid was used. HPLC 3000 Series binary gradient system, Analytical Technologies Ltd. was used and data were processed by HPLC workstation software.

Chromatographic conditions

Based on the solubility studies and selected analytical method, the initial chromatographic conditions were established. This involved the selection of the detection wavelength, mobile phase composition, stationary phase. The selected detection wavelength was 292 nm based on the UV spectroscopic analysis. Methanol was considered for composition of the mobile phase on the basis of the requirement of the method and solubility of analytes. The acidic pH was preferred based on the chemistry of the analytes. The Cosmosil C18 (250mm x 4.6ID, Particle size: 5 micron) column was selected as the stationary phase. The injection volume was set to 20 µl.

Preparation of Standard Stock Solution:

10 mg of Tacrolimus were transferred to separate 10 mL volumetric flasks. A small amount of methanol (5 mL) was added to flask, and the contents were sonicated to dissolve the drugs. The volumes of solution were then made up to the mark with methanol to yield standard stock solution of 1000 µg/mL of Tacrolimus volumetric flasks. These standard stock solutions were appropriately diluted with methanol to obtain concentrations of 100 µg/mL for Tacrolimus.

Preparation of sample Stock Solution:

The weight of ointment equivalent to 10 mg of was transferred to a 10 mL volumetric flask. 5 mL of methanol was added, and the mixture was sonicated to dissolve the ointment. The volume was then made up to the mark with methanol, resulting in sample stock solution with concentrations of 1000 µg/mL for Tacrolimus. These sample stock solutions were appropriately diluted with methanol to obtain final concentrations of 100 µg/mL for Tacrolimus.

Analytical Method validation

Linearity

The standard stock solution was diluted with mobile phase in a 10 ml volumetric flask to have a concentration in the range of 25 µg/ml- 125 µg/ml of CTL and 32 µg/ml-160 µg/ml of AZE, respectively. The obtained working standard stock solution was injected into the system to obtain the chromatogram and results. Regression analysis was performed to establish linearity.

Specificity

The blank, standard solution and sample solution were injected to prove the specificity of the method.

Accuracy

The accuracy of the method was performed using the standard addition method, which involved spiking of standard at the three levels i.e. 50%, 100% and 150% of the test solution. The percent recovery was calculated.

Precision:

Repeatability was performed using a minimum of six determinations of selected test concentration. Results from the determination of repeatability and intermediate precision, are expressed in the form of RSD.

Detection Limit and Quantitation limit

The DL and QL was calculated by using the standard deviation of response and slope of the calibration curve.

$$LOD = 3.3\sigma/m \text{ Equation}$$

Where, σ.=Standard Deviation of Response, m= Slope of Calibration Curve

$$LOQ = 10\sigma\ m.$$

Where, σ =Standard Deviation of Response, m= Slope of Calibration Curve

Robustness:

Robustness was performed by changing the detection wavelength and pH of the mobile phase. The detection wavelength was changed between 203-207nm and pH was changed from 2.8- 3.2.

Assay:

The analysis of the dosage form was conducted after the completion of analytical method development and validation. Appropriate aliquots of 0.96 mL of standard stock solution were withdrawn and transferred to the 10 mL volumetric flask to obtain a solution of containing 45 µg/mL of the Tacrolimus. The amount of the drug recovered was calculated by using the Equation and the percent assay was calculated using the Equation.

$$\text{(Assay (mg)} = \frac{(\text{Test area} \times \text{Standard wt} \times \text{Test dilution} \times \text{Purity of standard} \times \text{Average wt})}{standard\ area \times standard\ dilution \times test\ wt \times 100}$$

Equation Formula for determination of assay (mg)

$$Assay\ (\%Label\ Claim) = \frac{Assay\ (mg)}{Label\ Claim\ of\ formulation\ (mg)} \times 100$$

Result and Discussion:

Pre-Development Studies:

The pre development studies results are mentioned in Table 1.

Table 1 Pre-development studies of Tacrolimus

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

Predevelopment Studies	Tacrolimus
Organoleptic Properties	White colored solid crystalline powder, odourless
Melting point	126-130°C
FTIR Analysis	Based on the FTIR analysis the drugs were identified to be Tacrolimus.
Solubility Analysis	Soluble in methanol, DMSO, sparingly soluble in ethanol and acetonitrile, and very slightly soluble in water.

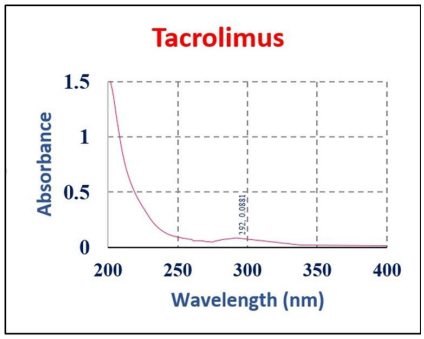
Development of UV- spectroscopic method for Tacrolimus:

Selection of solvent:

Solvent compositions were evaluated to identify the optimal solvent for UV analysis of Tacrolimus. The methanol shows produced characteristic spectra with sufficient absorption for drugs. To enhance the method's economic efficiency, the methanol solvent was selected. Consequently, Methanol was chosen as the solvent for further analysis.

Selection of detection wavelength:

The UV spectra of Tacrolimus were recorded, as shown in Figures 8. The wavelength of maximum absorption (λmax) was determined to be 292 nm for. These λmax value are critical as they represent the wavelengths at which the highest absorbance occurs, ensuring maximum sensitivity and accuracy in subsequent quantitative analyses.



Standard Calibration Curve:

The calibration curves for Tacrolimus were constructed by plotting the absorbance against the respective concentrations of the analyte. The absorbance values and corresponding concentrations are presented in the tables No. 15.

Table 15 Standard calibration curve

Sr. No.	Concentration Tacrolimus (µg/ml)	Absorbance Tacrolimus (AU) (λmax=292nm)
1	35	0.1852
2	40	0.2221
3	45	0.2532
4	50	0.2869
5	55	0.3082

The calibration curves for Tacrolimus were obtained by plotting the absorbance against the respective concentrations of each analyte, as shown in figure.9

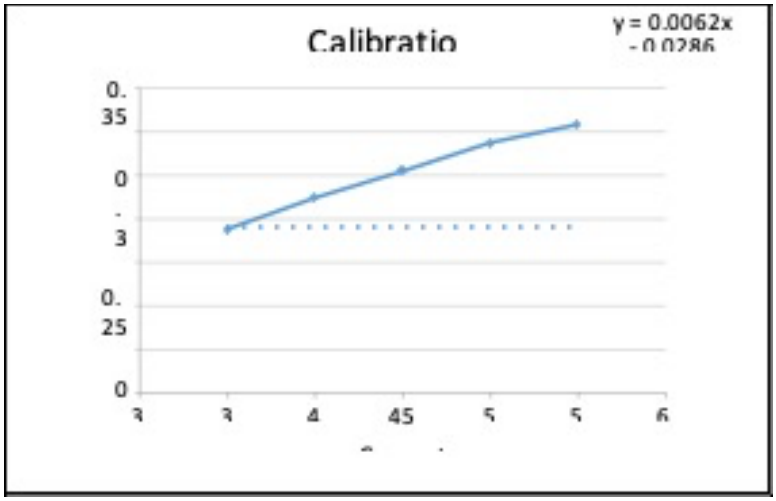


Figure 9 Calibration curve of UV spectroscopy

Table 16 Regression analysis data for calibration curve

Characteristics	Results
Sample	Tacrolimus
Solvent	Methanol
Regression Line Equation	Y= 0.0062x - 0.0286
Slope (m)	0.0062
Y intercept	0.0286
Standard Error	0.004851
Residual sum of squares	0.00007

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

Analytical Method Validation:

Range

Response Calibration Model (Linearity):

To evaluate the linearity of the response, linear regression analysis was performed on the calibration data for Tacrolimus, within the concentration ranges of 35-55 µg/mL. The regression analysis included the calculation of the regression line using the method of least squares. The results, including the correlation coefficients, regression line equations, and other relevant parameters, are summarized in Table 17.

Table 17 Regression analysis data of linearity

Characteristics	Results
Sample	Tacrolimus
Solvent	Methanol
Regression Line Equation	Y= 0.0062x - 0.0286
Slope (m)	0.0062
Y intercept	0.0286
Standard Error	0.004851
Residual sum of squares	0.00007

The correlation coefficients were found to be 0.9927 for tacrolimus indicating a strong linear relationship between analyte concentration and absorbance

The linearity assessment shows a strong correlation between analyte concentration and absorbance for Tacrolimus within the tested concentration ranges. The high correlation coefficients, along with the other regression parameters, confirm the method's ability to provide reliable and accurate quantification of these analytes.

Lower Range Limits: The lower range limits were validated by calculating the detection limit and quantitation limit for tacrolimus. The results for lower range limits validation are shown in table 18.

Table 18 Lower range limits for tacrolimus

Sample	Detection Limit (DL)	Quantitation Limit (QL)
Tacrolimus	**2.594032**	**7.824193**

Based on the lower detection limit and quantitation limit, it can be confirmed that the developed method is able to detect the analyte easily.

Accuracy:

The accuracy of the method was assessed based on the analysis of spiked samples. Accuracy was expressed as the percent recovery of the known added analyte in the sample. The results of the accuracy study are shown in Table 19. The percentage recovery was found to be in the range of 97.11-98.96 % for Tacrolimus, which is within the acceptable limits.

Table 19 Accuracy study data of tacrolimus

Accuracy Level	Known amount of analyte added Added (µg/ml)	Amount of analyte recovered (µg/ml)	% Recovery of known amount of analyte added
	Tacrolimus	Tacrolimus	Tacrolimus
50%	60	58.27	97.11
	60	58.36	97.26
	60	59.01	98.35
100%	80	79.05	98.81
	80	79.03	98.78
	80	79.1	98.87
150%	100	98.96	98.96
	100	98.69	98.69
	100	98.87	98.87

The standard deviation (SD) and relative standard deviation (RSD) of the percent recovery were calculated, with results presented in Table 20. The RSD of the percent recovery was found to be less than 2%, indicating precision in the results of the accuracy study.

Table 20 statistical validation of accuracy study data

Accuracy Level	% Mean	SD	%RSD
	Tacrolimus	Tacrolimus	Tacrolimus
50%	97.56	0.5494	0.5632
100%	98.82	0.0450	0.0456
150%	98.84	0.1122	0.1135

These results demonstrate the method's accuracy, showing that the percent recovery of the known added analyte is within acceptable limits, and precision of accuracy study is confirmed by the low RSD values.

Precision:

The precision of the current UV spectrophotometric method was assessed by performing validation tests including repeatability and intermediate precision.

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

Repeatability:

Repeatability was assessed through six determinations of homogeneous samples of Tacrolimus, performed within a short interval of time. The results for repeatability study are shown in table 21.

Table 21 Repeatability study data of tacrolimus

TIME	Amount Added (µg/ml)	Amount Recovered (µg/ml)	% Recovery
INTRADAY	**Tacrolimus**	**Tacrolimus**	**Tacrolimus**
10 AM	45	45.56	101.25
12 AM	45	45.27	100.60
1 PM	45	45.09	100.21
2 PM	45	44.91	99.82
3 PM	45	45.04	100.10
4 PM	45	44.88	99.74

The RSD values obtained were within the acceptance limit (i.e., <2) for Tacrolimus, indicating method's consistency under the same operating conditions over a short period.

Table 22 statistical data for repeatability

Name	% Mean	SD	%RSD
Tacrolimus	100.28	0.5137	0.5123

Intermediate Precision:

Intermediate precision was evaluated by performing six determinations of the same homogeneous samples over two consecutive days. The results for intermediate precision study are shown in table 23.

Table 23 repeatability data for Tacrolimus

TIME	Amount Added (µg/ml)	Amount Recovered (µg/ml)	% Recovery
INTERDAY	**Tacrolimus**	**Tacrolimus**	**Tacrolimus**
Day 1	45	45.56	101.25
	45	45.27	100.6
	45	45.09	100.21

Day 2	45	44.52	98.93
	45	44.89	99.75
	45	44.82	99.6

The results, as shown in Table 8.13, demonstrated that the RSD values were also within the acceptance limit (i.e., <2) for both drugs, ensuring the method's reliability under varying conditions, such as different days.

Table 24 Analytical data for repeatability

TIME	%Mean	SD	%RSD
Tacrolimus	100.28	0.7445	0.7424

Based on the precision study, the low relative standard deviation indicates and confirms developed method's ability to produce consistent results

Assay:

The analysis of the dosage form was conducted to verify the accuracy and precision of the developed UV spectrophotometric method in a real sample matrix. The dosage form, containing Tacrolimus was subjected to the UV spectrophotometric analysis, and the amount of analyte recovered and the percent assay was calculated, and the results were recorded, as shown in table 25.

Table 25 Analytical data for assay

Sample	Amount added (µg/ml)	Amount Recovered (µg/ml)	Percent Assay
Tacrolimus	45	45.45	101.02

The results demonstrate that the developed method accurately and precisely quantifies Tacrolimus in the dosage form

Development of HPLC method for Tacrolimus:

High performance liquid chromatographic method was developed for determination of Tacrolimus in bulk form. Optimized chromatographic condition was shown in Table 26

Table 26 chromatographic condition

Parameters	Condition
Sample Name	Tacrolimus
Mobile Phase	Methanol: phosphate buffer (pH 3) 60:40% V/V

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

Column	Cosmosil C18 (250mm x 4.6ID, Particle size : 5micron)
Detection Wavelength	292nm
Flow Rate	0.8ml/min
Injection Volume	20µL
Pressure	12-13MPa

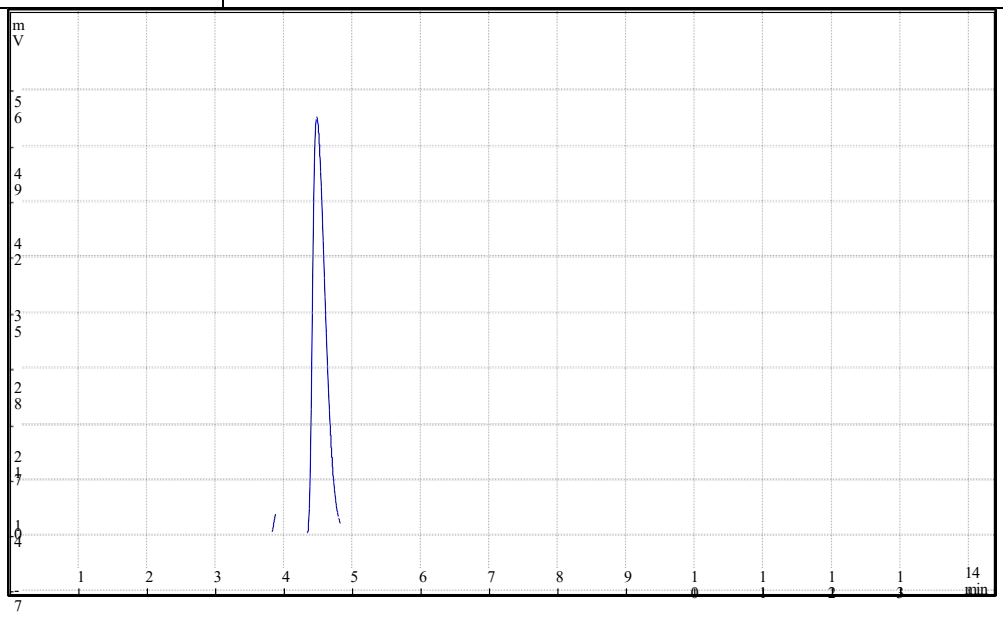


Figure 10 optimized chromatogram

Table 27 Analytical data for optimized chromatogram

Sr. No	Name	Retention Time(min)	Area(AU)	Run time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.470	696939	8.38 min	8674	1.24

ANALYTICAL METHOD VALIDATION

Specificity:

The specificity was demonstrated by analyzing the chromatograms obtained by injecting the Tacrolimus's blank, standard solution, and sample solution. From the obtained chromatograms, the method shows no interference by the presence of components such as mobile phase, and excipients in sample solution. This shows the specificity nature of the process.

Blank Chromatogram:

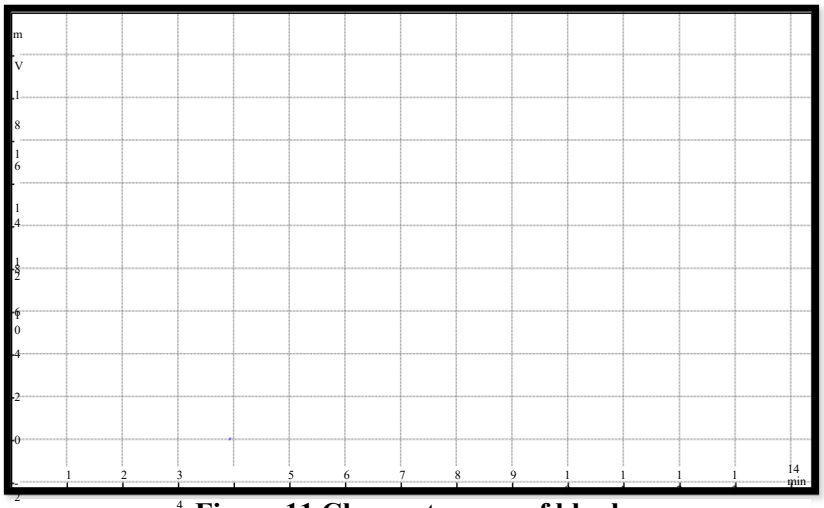


Figure 11 Chromatogram of blank

Standard chromatogram:

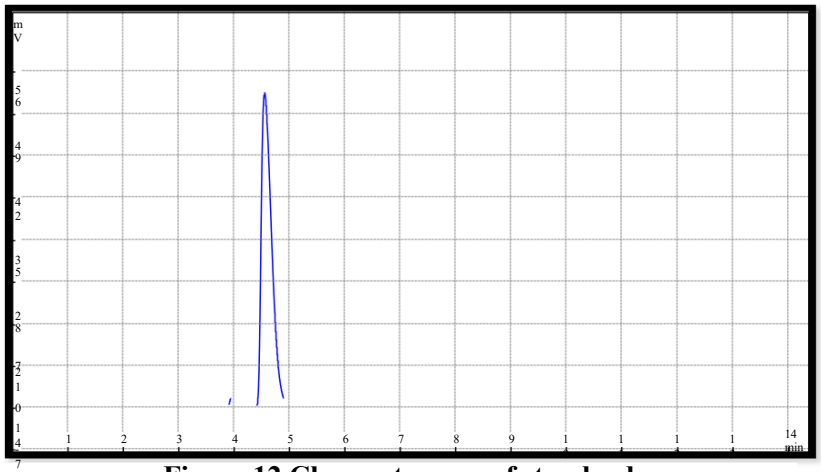


Figure 12 Chromatogram of standard

Sample Solution Chromatogram:

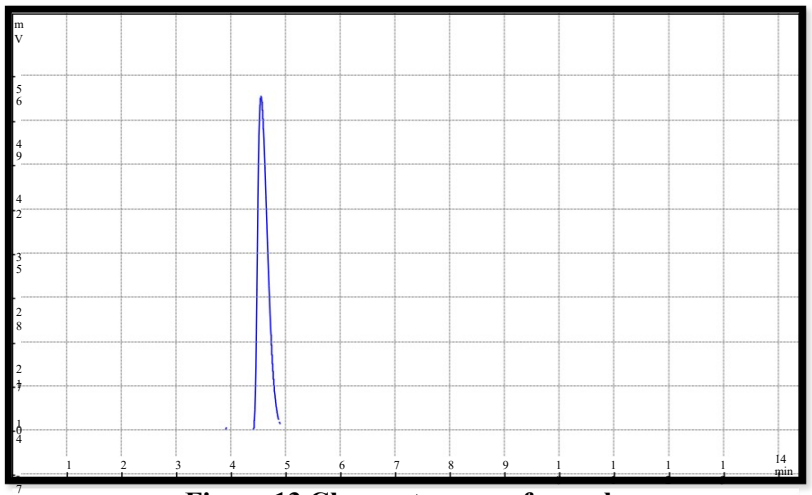


Figure 13 Chromatogram of sample

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

Range

Calibration model for response (Linearity)

To evaluate the linearity of the response, the plot of concentration versus peak area was obtained for Tacrolimus (10-50 µg/ml) as shown in fig 14. The linearity chromatograms for each concentration level are shown in Figures 14. The regression line was calculated using the method of least squares, and the derived data are presented in Table 28. The correlation coefficients for the analyte concentration and peak area were 0.9996 for Tacrolimus, indicating a strong linear relationship between the analyte concentration and the response. Data of calibration curve of Tacrolimus by HPLC method.

Table 28 linearity range of Tacrolimus

Sr. No.	Concentration (µg/ml)	Area (AU)
1	10	206672
2	20	427393
3	30	682890
4	40	926291
5	50	1172611

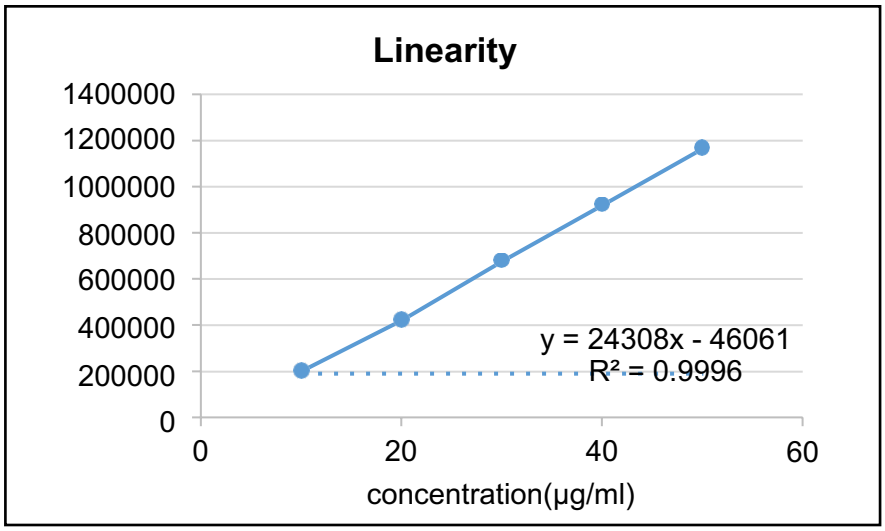


Figure 14 Linearity plot for Tacrolimus

Regression Analysis Data for Tacrolimus

Table no 29 summarizes the optical characteristics and Analytical data for linearity for Tacrolimus by RP-HPLC method.

Sr. No	Parameters	High-Performance Liquid Chromatography Method
1	λmax (nm)	292nm
2	Range (µg/mL)	10-50 µg/mL
3	Regression line equation[y]	y = 24308x - 46061
4	Slope[m]	24308
5	Y Intercept [c]	46061
6	Correlation Coefficient [R2]	0.9996
7	Residual some of squares (SS)	2.65x 10^8
8	Standard Error	9405.903

Table 29 Regression analysis data of tacrolimus

Linearity Level 1

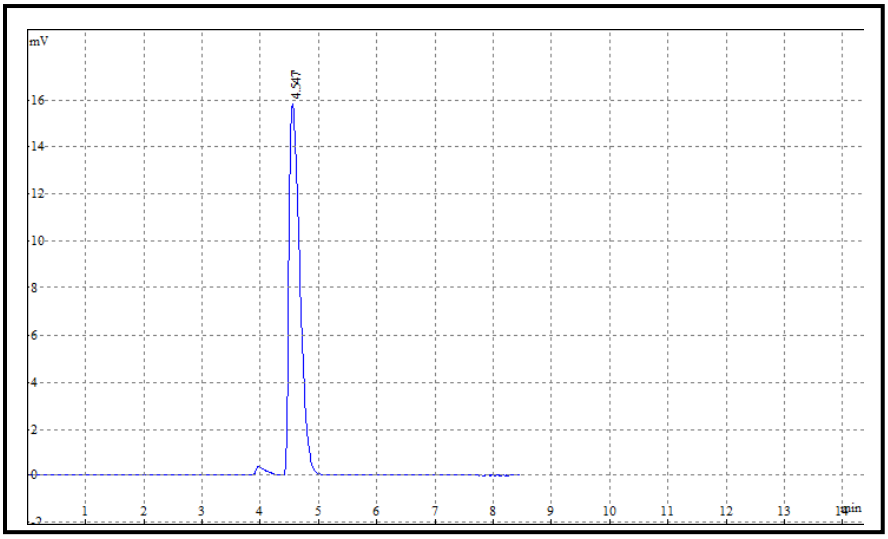


Figure 15 Chromatogram of tacrolimus at linearity level 1

In the chromatogram of analyte the retention time was observed 4.447 min, the area was obtained 206672 and theoretical plates are obtained 8834.

Table 30 Analytical data for tacrolimus chromatogram at linearity level 1

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

Sr. No	Name	Retention Time(min)	Area(AU)	Run time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.547	206672	8.47	8834	1.24

Linearity level 2

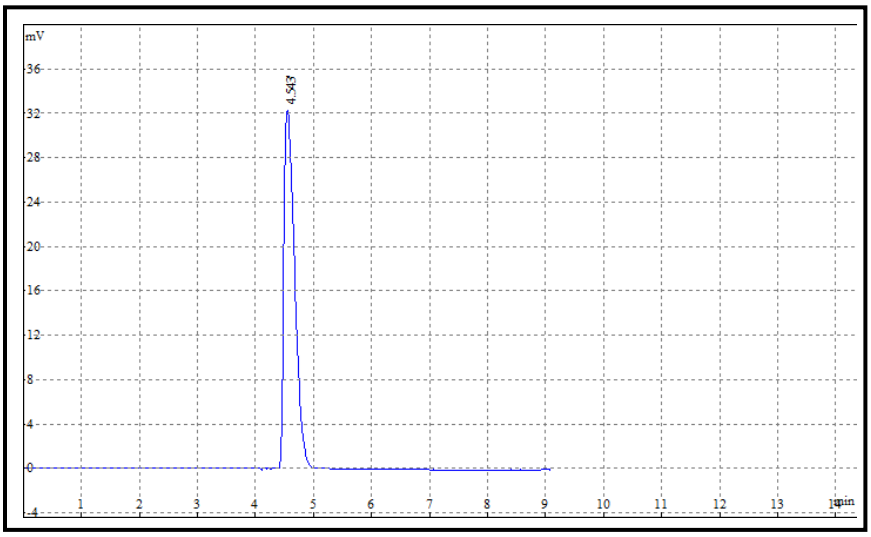


Figure 16 Chromatogram of tacrolimus at linearity leve

In the chromatogram of analyte the retention time was observed 4.543 min, the area was obtained 206672 and theoretical plates are obtained 8785.

Table 31 Analytical data for chromatogram at linearity level 2

Sr. No	Name	Retention Time(min)	Area (AU)	Run time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.543	427393	9.09	8785	1.26

Linearity level 3:

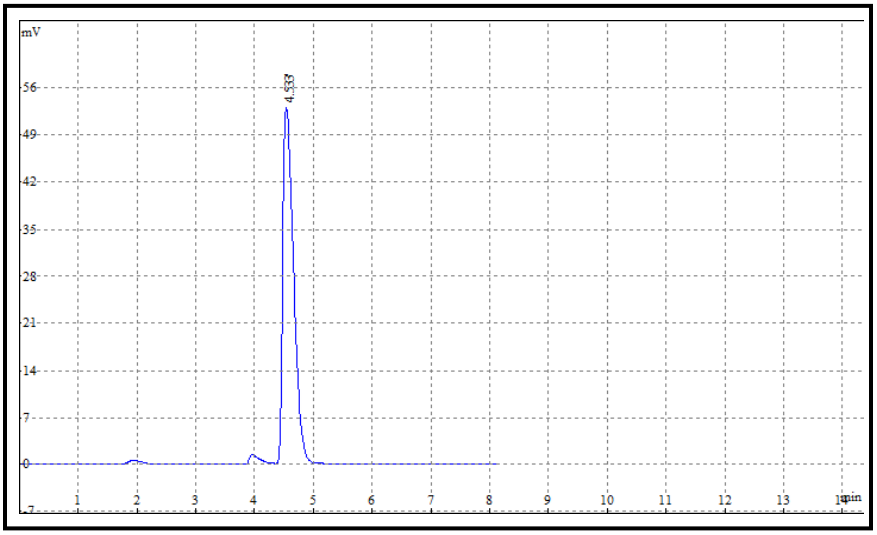


Figure 17 Chromatogram of tacrolimus at linearity level 3

Table No. 8. 1 Analytical data for tacrolimus chromatogram at linearity level 3

Sr. No	Name	Retention Time(min)	Area(AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.533	682890	8.13min	8881	1.27

Linearity Level 4:

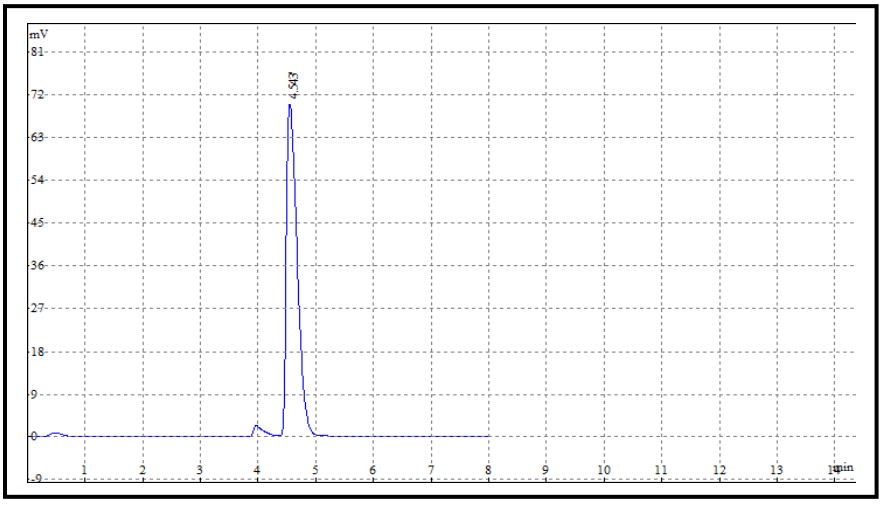


Figure 18 Chromatogram of tacrolimus at linearity level 4

In the chromatogram of analyte the retention time was observed 4.543 min, the area was obtained 926291 and

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

Table 32 Analytical data for tacrolimus chromatogram at linearity level 4

Sr. No	Name	Retention Time(min)	Area (AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.543	926291	8.00min	8812	1.25

Linearity level No.5

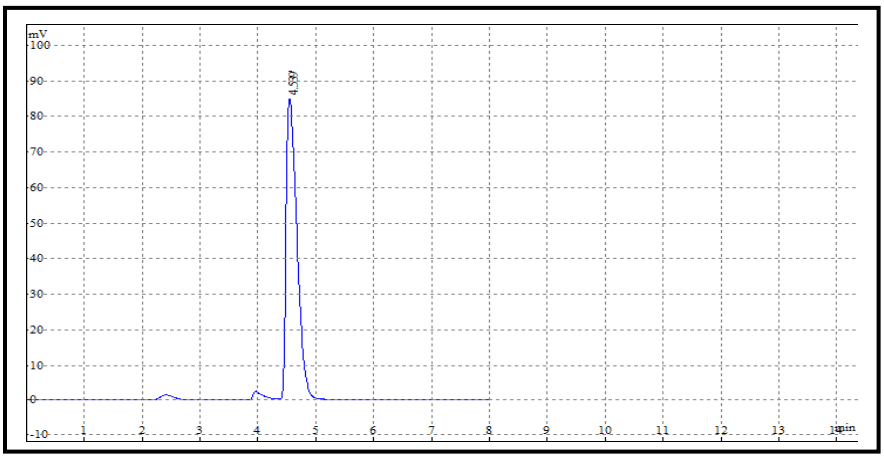


Figure 19 Chromatogram of tacrolimus at linearity level

In the chromatogram of analyte the retention time was observed 4.539 min, the area was obtained 1172611and theoretical plates are obtained 8794.

Table 33 Chromatogram of tacrolimus at linearity level 5

Sr. No	Name	Retention Time(min)	Area(AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.539	1172611	8.00min	8794	1.26

Lower Range Limit: (Detection limit and Quantitation limit)

The detection limit and quantitation limit was calculated based on the standard deviation of the response and slope of the regression line of tacrolimus.

Table 34 Lower range limits for tacrolimus

Drugs Name	LOD (µg/mL)	LOQ (µg/mL)
Tacrolimus	0.055932203	0.16949153

Accuracy Level

Accuracy:

AccuracyLevel	Standard Added	Conc. (µg/ml)	Total conc.	Area obtained	Std area	Drug recovered	%Recovery
50%	10	20	30	680476	682890	29.893951	99.6465024
	10	20	30	685074		30.095945	100.319817
	10	20	30	685155		30.099504	100.331679
100%	20	20	40	923255	926291	39.868896	99.6722412
	20	20	40	929988		40.159647	100.399119
	20	20	40	929988		40.159647	100.399119
150%	30	20	50	1170580	1172611	49.913398	99.8267968
	30	20	50	1176132		50.150135	100.30027
	30	20	50	1178211		50.238783	100.477567

Table 35 Accuracy study data for tacrolimus

Accuracy was verified by the spiking a known amount of analyte of interest in the matrix of the solution at three levels (50%, 100%, and 150%) of the test concentration, and the % recovery found was within acceptable limit. Results of accuracy are shown in Table no. the percent recovery was found in the range of 99.64 to 100.47% for Tacrolimus

Statistical validation of Tacrolimus by HPLC method

Table 36 statistical validation data for accuracy study of tacrolimus

Accuracy Level	% Mean	SD	%RSD
50%	99.90179	0.39232	0.392706
100%	99.84459	0.419369	0.420022
150%	99.79961	0.335535	0.336209

Chromatogram of Sample:

For 50% Recovery

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

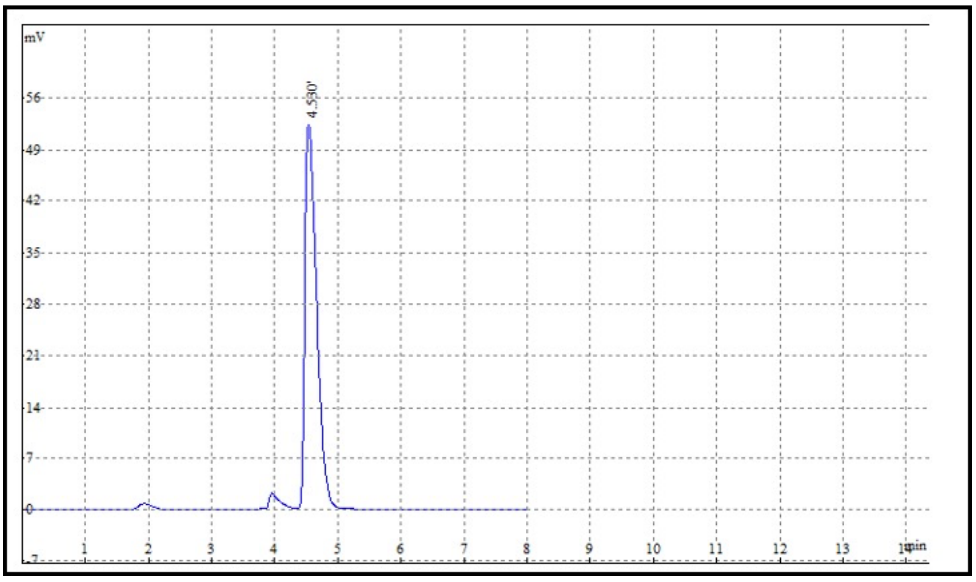


Figure 20 Chromatogram of tacrolimus at 50% accuracy level

the chromatogram of analyte the retention time was observed 4.530 min, the area was obtained 680476 and theoretical plates are obtained 8763.

Table 37 Analytical data for accuracy study at 50% level

Sr. No	Name	Conc. Found	Retention Time(min)	Area (AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	29.895	4.530	680476	8min	8763	1.28

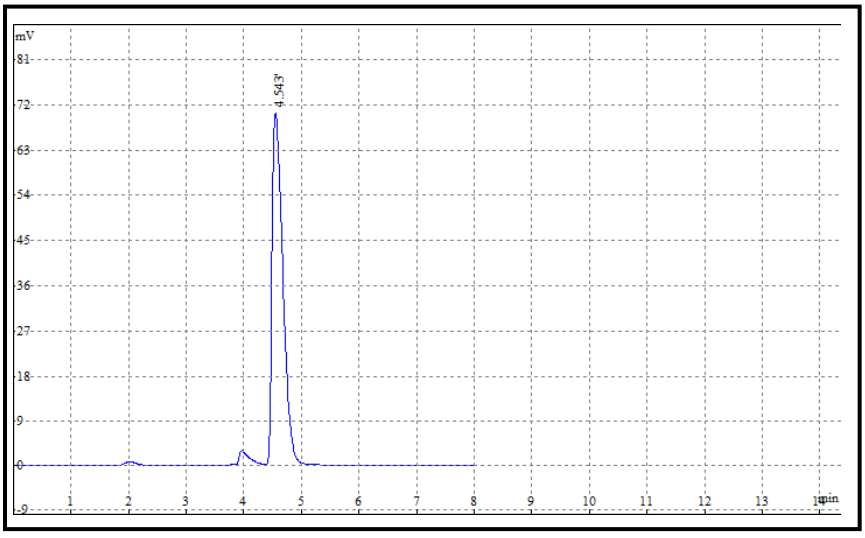


Figure 21 Chromatogram for tacrolimus at 100% accuracy level
In the chromatogram of analyte the retention time was observed 4.543 min, the area was obtained 923255 and theoretical plates are obtained 8796.

Table 38 Analytical data for accuracy study at 100% level

Sr. No	Name	Conc. Found	Retention Time(min0	Area(AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	39.86	4.543	923255	8min	8796	1.28

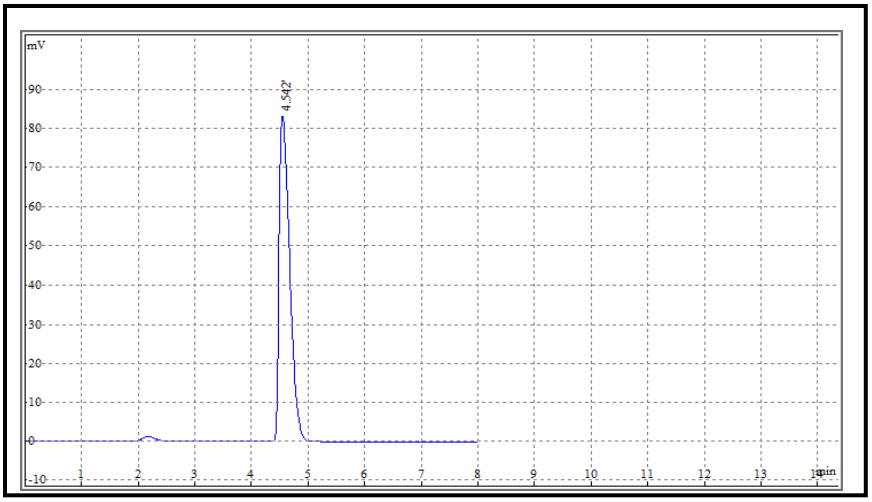


Figure 22 Chromatogram for tacrolimus at 150% accuracy level

In the chromatogram of analyte the retention time was observed 4.542 min, the area was obtained 1170580 and theoretical plates are obtained 8822.

Table 39 Analytical data for accuracy study at 150%

Sr. No	Name	Conc. Found	Retention Time	Area	Run time	Theoretical Plates	Tailing Factor
1	Tacrolimus	49.91	4.542	1170580	8 min	8822	1.27

% Assay:

Table 40 Results of analysis of dosage form (% Assay)

Sr. No.	Conc. added	Area of Standard	Area of Sample	% Assay
1	30 (µg/ml)	682048	682890	98.887%

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

Chromatogram of Standard:

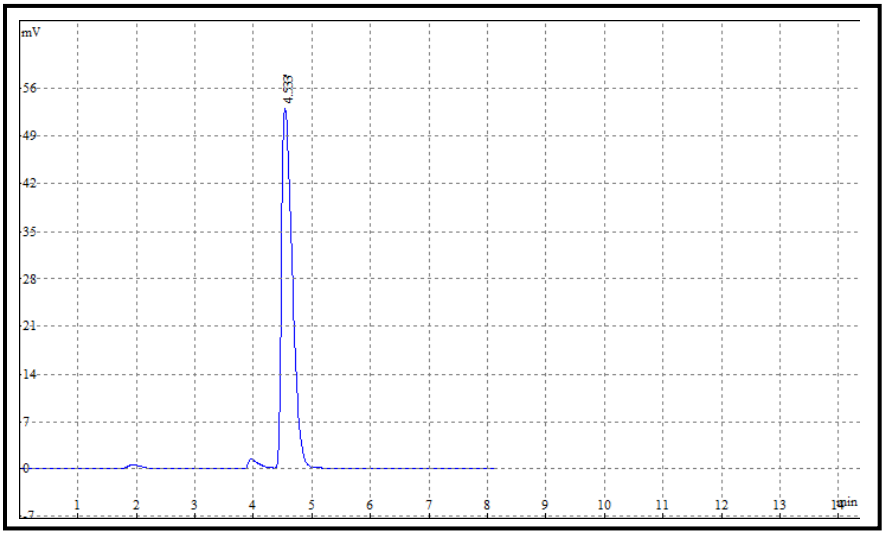


Figure 23 Chromatogram of standard

In the chromatogram of analyte the retention time was observed 4.533 min, the area was obtained 682890 and theoretical plates are obtained 8881.

Sr. No	Name	Conc. Found	Retention Time	Area	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	29.96	4.533	682890	12.330	8881	1.27

Table 41 Analytical data for chromatogram of standard

Chromatogram of sample:

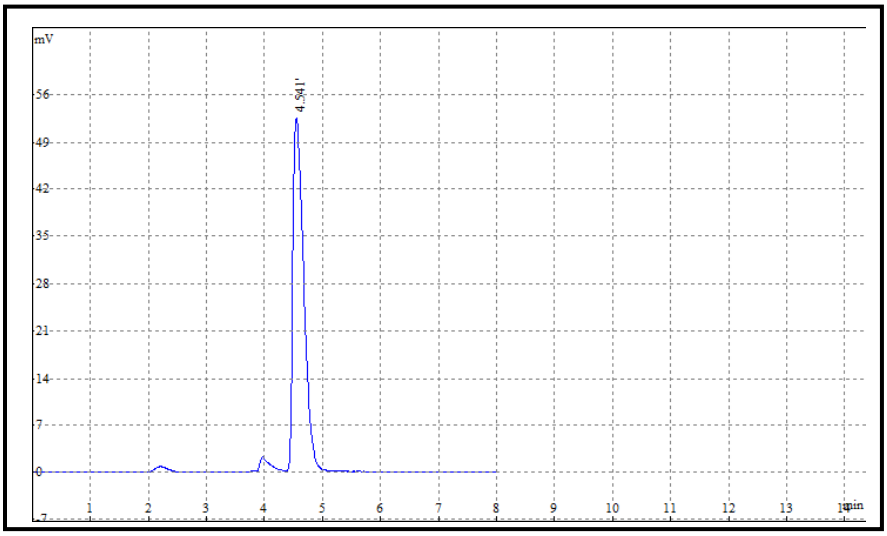


Figure 24 Chromatogram of sample

In the chromatogram of analyte the retention time was observed 4.541 min, the area was obtained 682048and theoretical plates are obtained 8778.

Table 42 Analytical data for chromatogram of sample

Sr. No	Name	Retention Time(min)	Area(AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.541	682048	12.269	8778	1.27

Precision:

Repeatability

Repeatability of developed method was performed by using six determination of homogenous samples of tacrolimus over a short interval of time under the same operating condition by the same analyst. The results for repeatability are shown in table 43.

Time	Conc.	Area	Conc. Found	%Recovery	mean	SD	%RSD
10:00 AM	30	682890	30	100	100.2756422	0.06624834	0.066066234
11:00 AM	30	685074	30.095945	100.3198172			
1:00 PM	30	685155	30.099504	100.3316786			
2:00 PM	30	686394	30.153934	100.5131134			
4:00 PM	30	683000	30.004832	100.016108			
5:00 PM	30	686121	30.141941	100.4731362			

Table 43 Repeatability study data for tacrolimus

Morning: Sample 1 (10 AM)

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

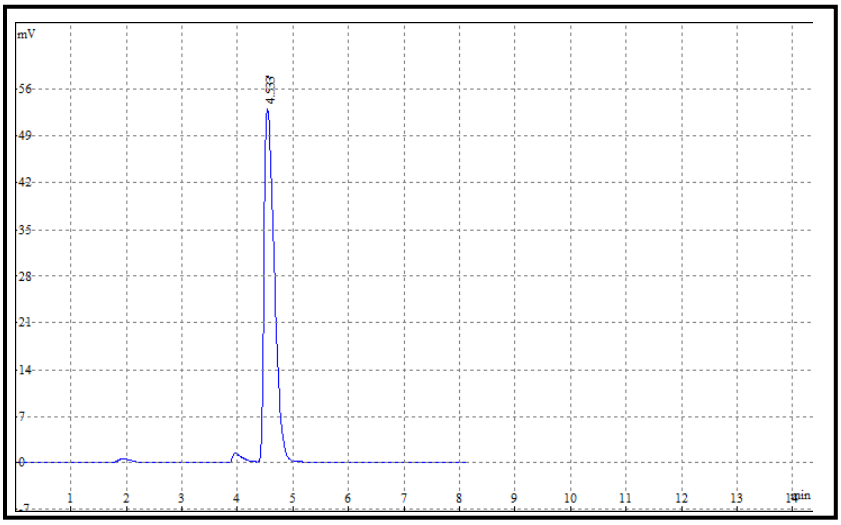


Figure 25 Chromatogram of tacrolimus at 10 AM

In the chromatogram of analyte the retention time was observed 4.533 min, the area was obtained 682890 and theoretical plates are obtained 8881.

Table 44 Analytical data for repeatability study at 10 AM

Sr. No	Name	Retention Time(min)	Area(AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.533	682890	8.13 min	8881	1.27

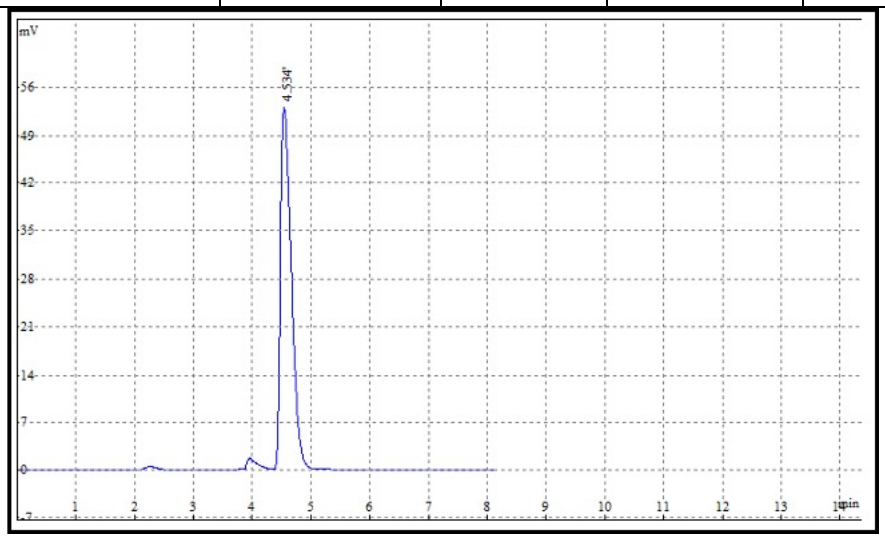


Figure 26 Chromatogram of tacrolimus at 11 AM

In the chromatogram of analyte the retention time was observed 4.534 min, the area was obtained 685074 and theoretical plates are obtained 8839.

Table 45 Analytical data for repeatability study of tacrolimus at 11 AM

Sr. No	Name	Retention Time(min)	Area (AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.534	685074	8.12 min	8839	1.28

Afternoon: Sample 1 (1 PM)

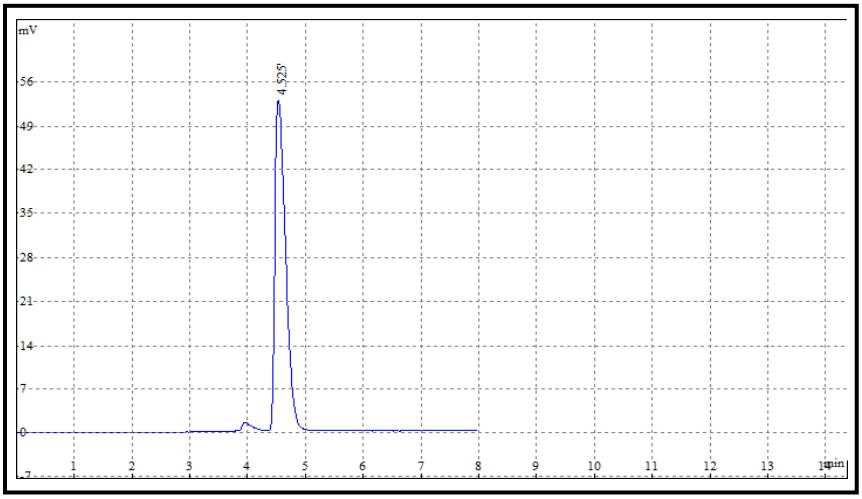


Figure 27 Chromatogram of tacrolimus at 1 PM

In the chromatogram of analyte the retention time was observed 4.525 min, the area was obtained 685155 and theoretical plates are obtained 8914.

Table 46 Analytical data for repeatability study at 1 PM

Sr. No	Name	Retention Time(min)	Area (AU)	Run time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.525	685155	7.98 min	8914	1.24

Afternoon: Sample 2

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

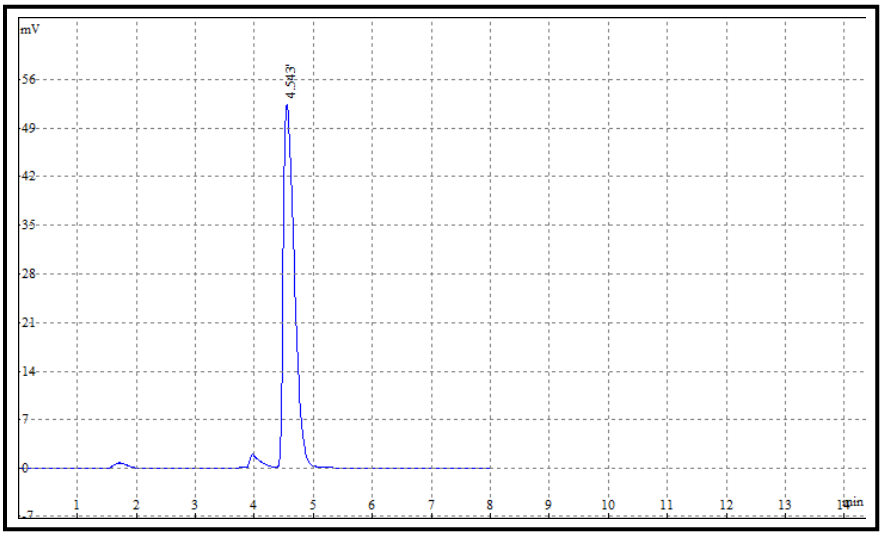


Figure 28 Chromatogram of tacrolimus at 2 PM

In the chromatogram of analyte the retention time was observed 4.543 min, the area was obtained 686394 and theoretical plates are obtained 8806.

Table 47 Repeatability study data for tacrolimus at 2 PM

Sr. No	Name	Retention Time	Area	Run time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.543	686394	8 min	8806	1.26

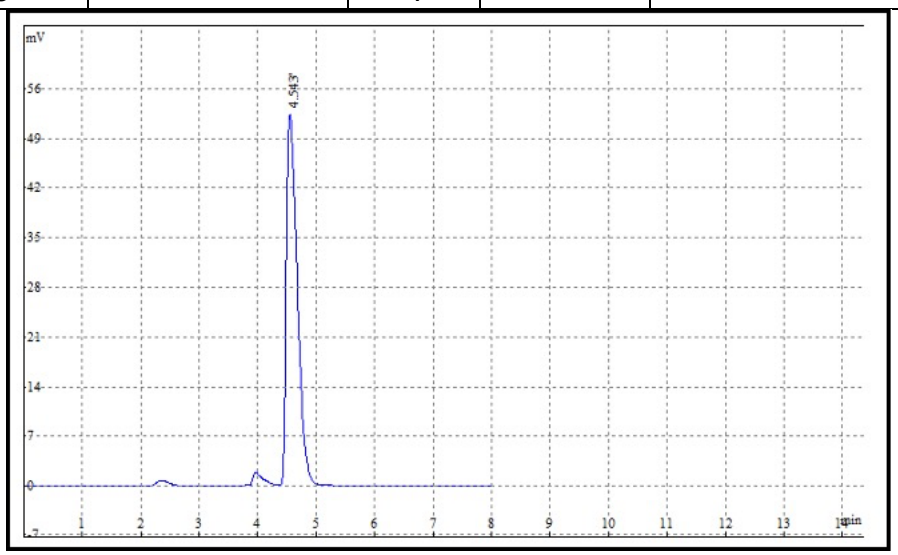


Figure 29 Chromatogram of tacrolimus at 4 PM

In the chromatogram of analyte the retention time was observed 4.543 min, the area was obtained 683000 and theoretical plates are obtained 8837.

Table 48 Analytical data for repeatability study for tacrolimus at 4 PM

Sr. No	Name	Retention Time(min)	Area(AU)	Run time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.543	683000	8 min	8837	1.26

Evening: Sample 2

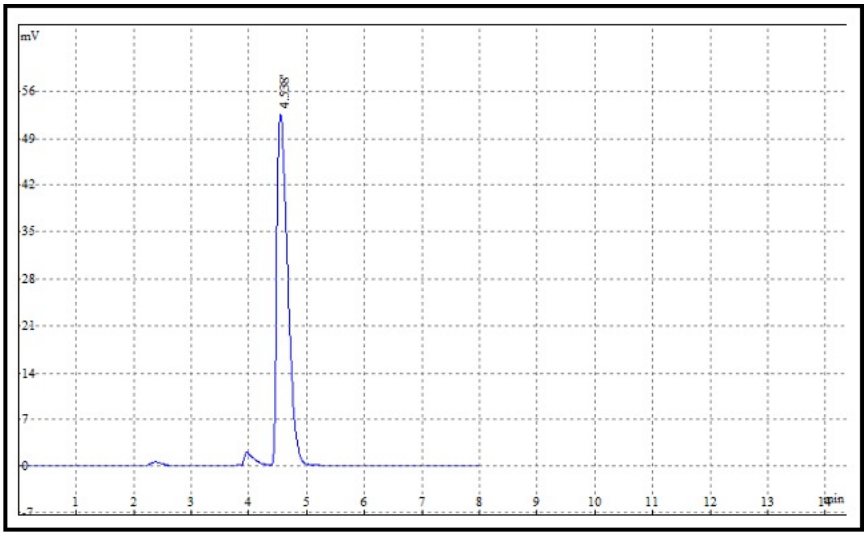


Figure 30 Chromatogram of tacrolimus at 5 PM

In the chromatogram of analyte the retention time was observed 4.538 min, the area was obtained 686121 and theoretical plates are obtained 8814.

Table 49 Analytical data for repeatability study at 5 PM

Sr. No	Name	Retention Time(min)	Area(AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.538	686121	8 min	8814	1.26

Intermediate precision:

The intermediate precision was performed using six determinations of homogenous sample of tacrolimus in two successive days. The percent recovery was calculated and the result are shown in table 50. The intermediate precision is expressed as %RSD of % recovery, shown in the table 50. The chromatogram for intermediate precision are shown in figure 31

Table 50 Intermediate precision study data for tacrolimus

Time	Conc. (µg/ml)	Area(AU)	Conc. Found	%Recovery	Mean	SD	%RSD
10:00 AM	30	682890	30	100			

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

11:00 AM	30	685074	30.095945	100.3198172	100.0631629	0.267398279	0.267229489
1:00 PM	30	685155	30.099504	100.3316786			
2:00 PM	30	682484	29.982164	99.94054679			
4:00 PM	30	683988	30.048236	100.1607872			
5:00 PM	30	680337	29.887844	99.6261477			

Day 1: sample 1

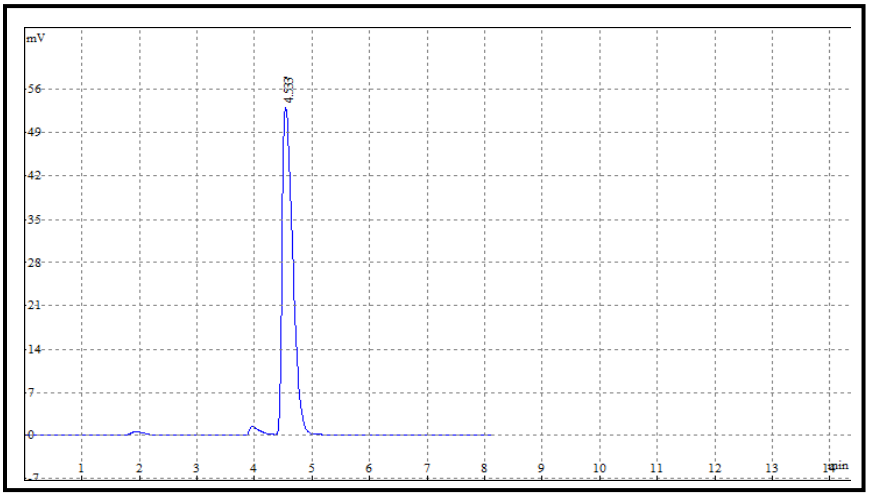


Figure 31 Chromatogram of tacrolimus day 1- sample 1

In the chromatogram of analyte the retention time was observed 4.533 min, the area was obtained 682890 and theoretical plates are obtained 8881

Table 51 Intermediate precision study data for tacrolimus Day 1 - Sample 1

Sr. No	Name	Retention Time(min)	Area (AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.533	682890	8.13 min	8881	1.27

Day 1: Sample 2

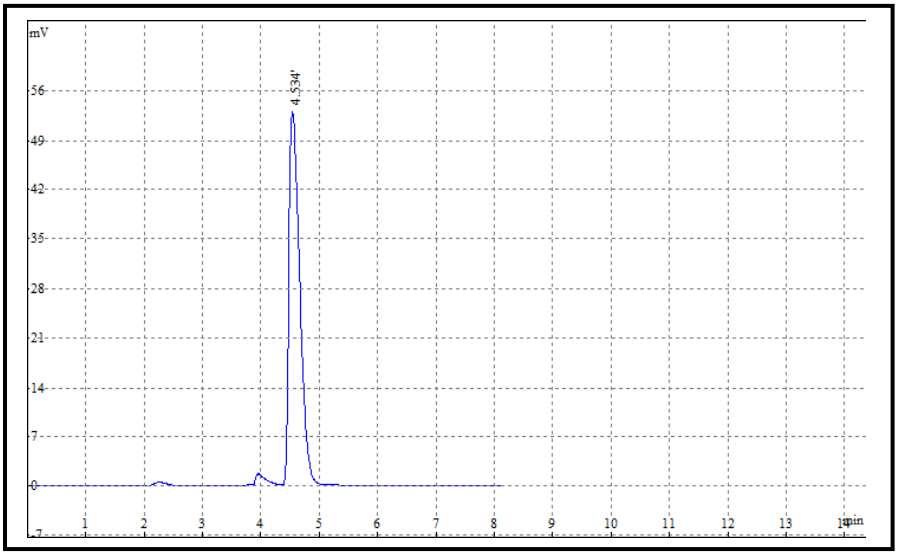


Figure 32 Chromatogram of tacrolimus day 1- sample 2

In the chromatogram of analyte the retention time was observed 4.534 min, the area was obtained 685074 and theoretical plates are obtained 8839.

Table 52 Intermediate precision study data for tacrolimus Day 1 - Sample 2

Sr. No	Name	Retention Time(min)	Area(AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.534	685074	8.12 min	8839	1.28

Day 1: Sample 3

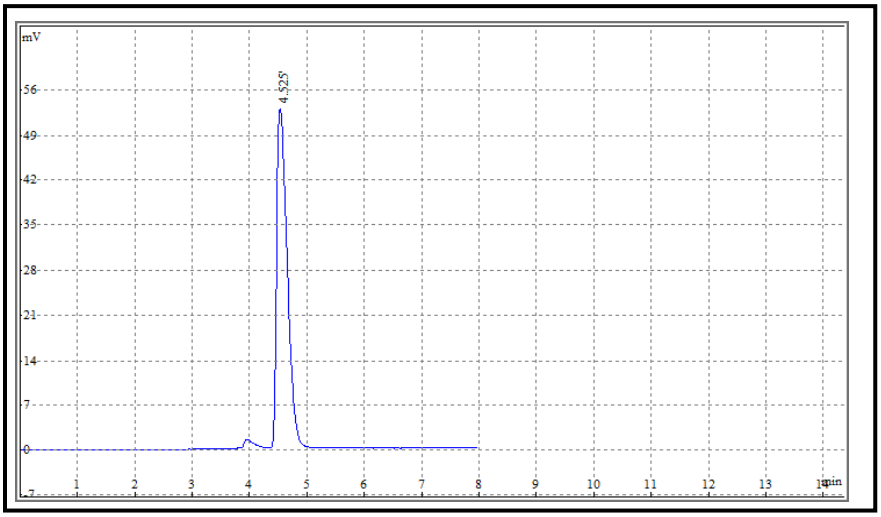


Figure 33 Chromatogram of tacrolimus day 1- sample 3

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

In the chromatogram of analyte the retention time was observed 4.525 min, the area was obtained 685155 and theoretical plates are obtained 8914.

Table 53 Intermediate precision study data for tacrolimus Day 1 - Sample 3

Sr. No	Name	Retention Time(min)	Area(AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.525	685155	7.98 min	8914	1.24

Day 2: Sample 1

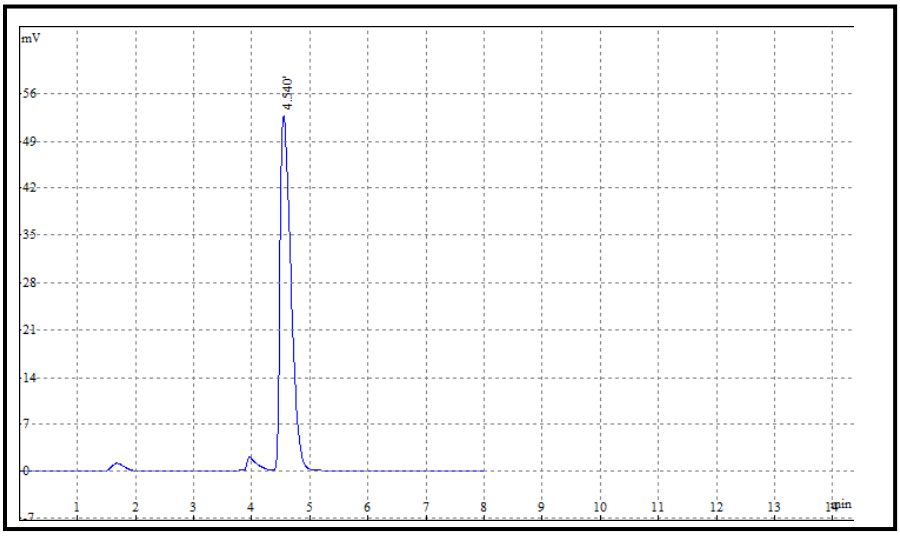


Figure 34 Chromatogram of tacrolimus day 2- sample 1

In the chromatogram of analyte the retention time was observed 4.540 min, the area was obtained 682484 and theoretical plates are obtained 8794

Table 54 Intermediate precision study data for tacrolimus Day 2 - Sample 1

Sr. No	Name	Retention Time(min)	Area(AU)	Run Time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.540	682484	8 min	8794	1.26

Day 2: Sample 2

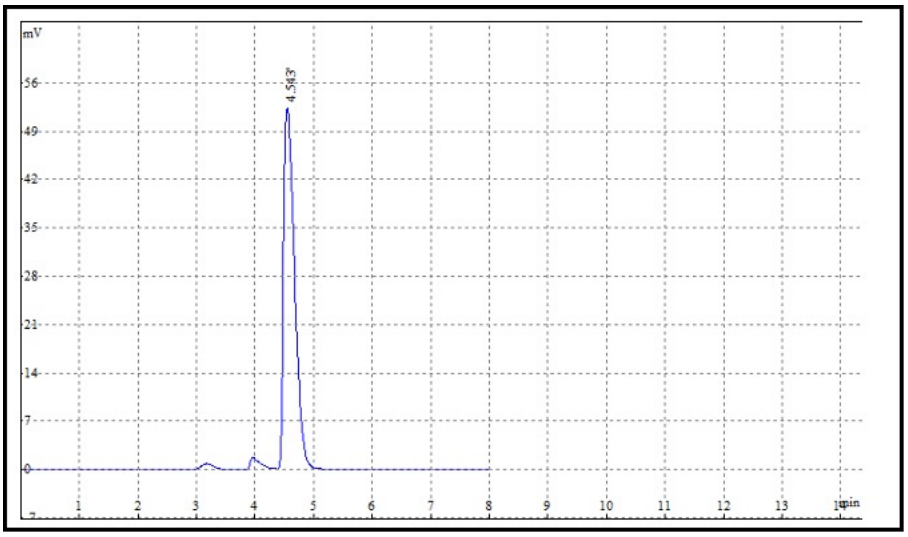


Figure 35 Chromatogram of tacrolimus day 2- sample 2

In the chromatogram of analyte the retention time was observed 4.543 min, the area was obtained 683988 and theoretical plates are obtained 8845.

Table 55 Intermediate precision study data for tacrolimus Day 2 - Sample 2

Sr. No	Name	Retention Time(min)	Area(AU)	Run time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.543	683988	8 min	8845	1.24

Day 2: Sample 3

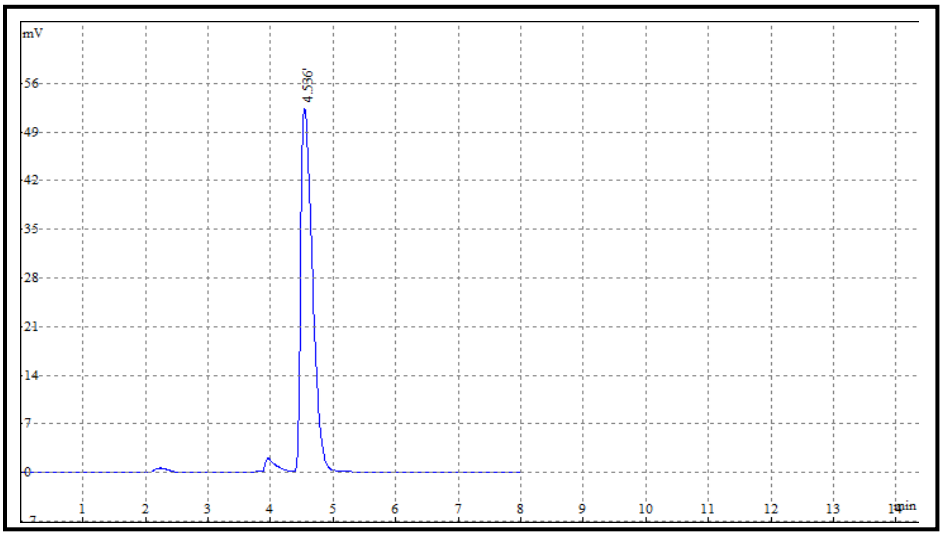


Figure 36 Chromatogram of tacrolimus day 2- sample 3

In the chromatogram of analyte the retention time was observed 4.536 min, the area was obtained 680337 and theoretical plates are obtained 8776.

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

Table 56 Intermediate precision study data for tacrolimus Day 2 - Sample 3

Sr. No	Name	Retention Time(min)	Area(AU)	Run time	Theoretical Plates	Tailing Factor
1	Tacrolimus	4.536	680337	8 min	8776	1.25

Robustness:

Robustness was studied by different deliberate variations in the chromatographic conditions. Results are shown in Table no.57

Change in the detection of wavelength

The pH of the mobile phase was small changes between 2.8-3.2. the response were recorded (table--) and found to be within an acceptable limit. The relative standard deviation of obtained responses was found to be less than 2(table 57)

Data for Robustness study of Tacrolimus by HPLC method (Change in Wavelength)

Table 57 Robustness study data - change in wavelength

Sr. No	Condition	Conc. (µg/ml)	Area(AU)	Conc. Found	SD	%RSD
1	292	20	427393	20	0.04642796	0.231676
2	290	20	428068	20.03		
3	294	20	429776	20.11		

2 Change in pH

The pH of mobile phase was small changes between 2.8-3.2. response was recorded (Table 58) and found to be within an acceptable limit. The relative standard deviation of obtained responses was found to be less than 2

Data for Robustness study of Tacrolimus by HPLC method (Change in pH)

Table 58 Robustness study data for tacrolimus - change in pH

Sr. No	Condition	Conc. (µg/ml)	Area(AU)	conc. Found	SD	%RSD
1	3	20	427393	20		

2	3.2	20	428464	20.05	0.03681787	0.183722
3	2.8	20	429358	20.09		

Change in detection of wavelength:
wavelength 294nm

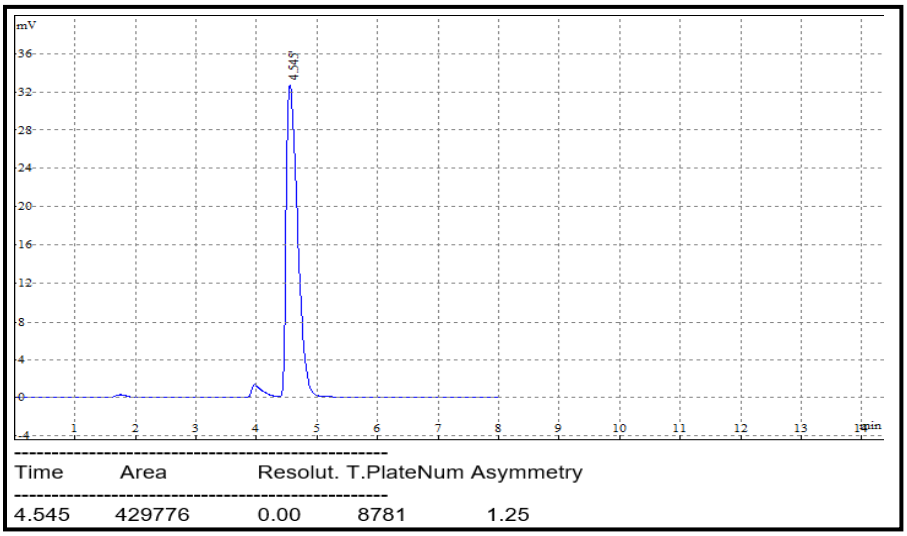


Figure 37 Chromatogram of tacrolimus at 294nm Table 59 Robustness study data at 294nm

Sr. No.	Name	Retention Time(min)	Area(AU)	Run Time	Tailing Factor	Plate Count
1	Tacrolimus	4.545	429776	8 min	1.25	8781

Wavelength 290nm

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

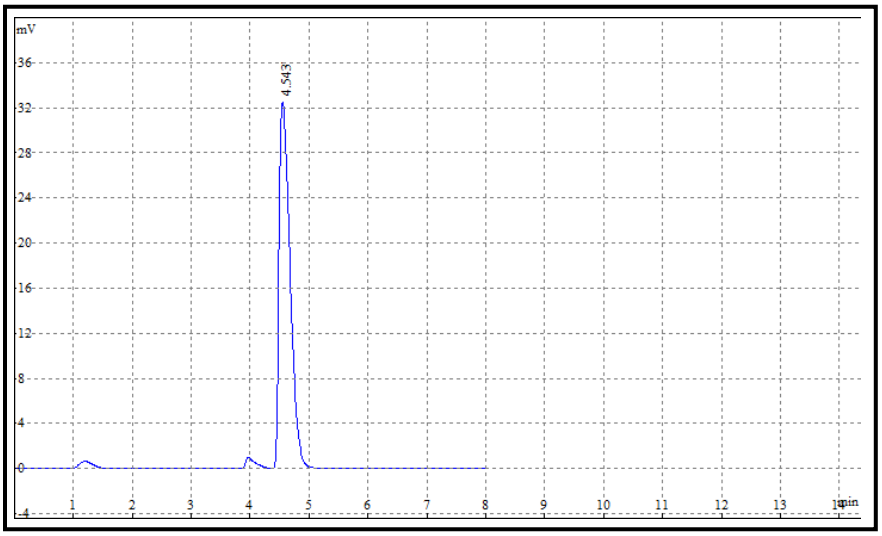


Figure 38 Chromatogram of tacrolimus at 290nm

In the chromatogram of analyte the retention time was observed 4.543 min, the area was obtained 428068 and theoretical plates are obtained 8838.

Table 60 Robustness study chromatogram for 290nm

Sr. No.	Name	Retention Time(min)	Area(AU)	Run Time	Tailing Factor	Plate Count
1	Tacrolimus	4.543	428068	8 min	1.28	8838

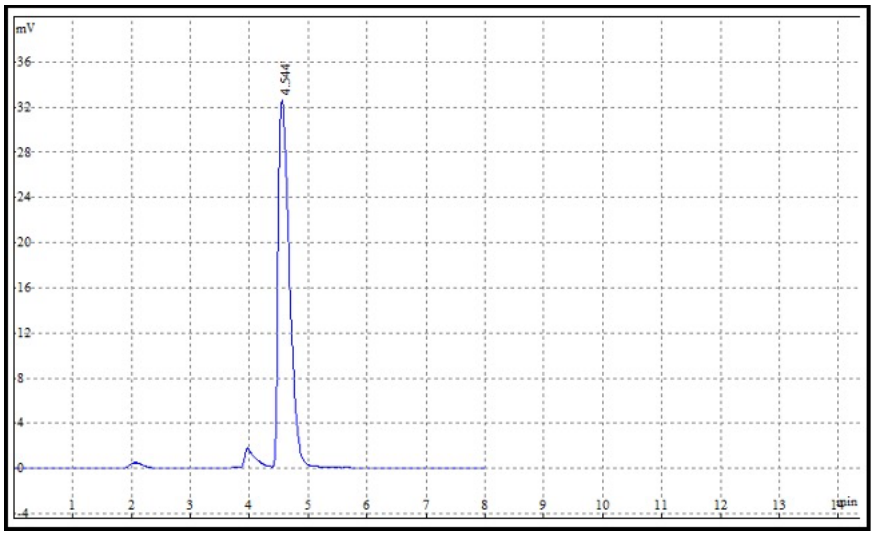
Change in pH

The pH of mobile phase was small changes between 2.8-3.2. response was recorded (table 61) and found to be within in acceptable limit. The relative standard deviation of obtained responses was found to be less than 2(table 61)

Table 61 Robustness study data -change in pH

Sr. No	Condition	Conc. (µg/ml)	Area(AU)	conc. Found	SD	%RSD
1	3	20	427393	20	0.03681787	0.183722
2	3.2	20	428464	20.05		
3	2.8	20	429358	20.09		

Figure 39 Chromatogram of tacrolimus at pH 2.8



In the chromatogram of analyte the retention time was observed 4.544 min, the area was obtained 429358and theoretical plates are obtained 8767.

Table 62 Robustness study chromatogram of tacrolimus at pH 2.8

Sr. No.	Name	Retention Time(min)	Area(AU)	Run Time	Tailing Factor	Plate Count
1	Tacrolimus	4.544	429358	8 min	1.26	8767

pH 3.2

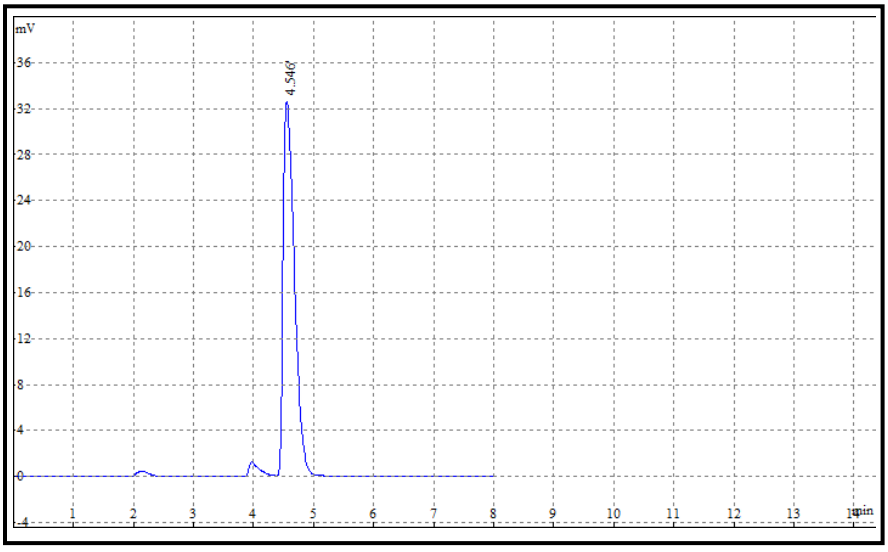


Figure 40 Chromatogram of tacrolimus at pH 3.0

In the chromatogram of analyte the retention time was observed 4.546 min, the area was obtained 428464 and theoretical plates are obtained 8805.

Acta Biomed 2024; Vol. 95, N. 2: ISSN: 0392-4203 | eISSN: 2531-6745

Table 63 Robustness study chromatogram at pH 3.2

Sr. No.	Name	Retention Time(min)	Area(AU)	Run time	Tailing Factor	Plate Count
1	Tacrolimus	4.546	428464	8 min	1.26	8805

Ruggedness:

To perform the ruggedness of the analytical of the five different concentration was taken by different analysts. The ruggedness is express in terms of percent RSD in table 64 and the chromatograms for ruggedness are shown in figure 40

Different analyst studied Ruggedness. Results obtained are shown in table 64.

Table 64 Ruggedness study data for tacrolimus

Conc.	Tacrolimus	Area(AU)	Amount found (µg/ml)	SD	%RSD
10	**Analyst I**	208327	10.08	0.04	0.398406375
	Analyst II	206672	10		
20	**Analyst I**	427263	19.99	0.005	0.025006252
	Analyst II	427393	20		
30	**Analyst I**	681770	29.95	0.025	0.083402836
	Analyst II	682890	30		
40	**Analyst I**	929933	40.15	0.075	0.187149095
	Analyst II	926291	40		
50	**Analyst I**	1173862	50.05	0.025	0.049975012
	Analyst II	1172611	50		

Conclusion

The successful development and validation of analytical methods for Tacrolimus are pivotal for its quality control, therapeutic monitoring, and formulation innovation. This thesis has systematically addressed the need for robust analytical techniques by developing both HPLC and UV-Vis spectrophotometric methods, ensuring they meet stringent regulatory standards. A precise and accurate HPLC method for Tacrolimus quantification was developed. Optimal chromatographic conditions were established, including the mobile phase composition, flow rate,

detection wavelength, and column selection. The HPLC method was validated according to ICH guidelines, demonstrating excellent specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), and robustness. This ensures the method's reliability for routine quality control and stability studies. A rapid and simple UV-Vis spectrophotometric method for Tacrolimus estimation was developed. Calibration curves were constructed, and the method's linearity range was determined. The UV-Vis method was validated for accuracy, precision, linearity, LOD, LOQ, and robustness. The method's performance was comparable to the HPLC method, providing an alternative analytical technique. The outcomes of this thesis provide a significant contribution to the analytical methodologies for Tacrolimus. The developed HPLC and UV- Vis spectrophotometric methods offer reliable and efficient tools for the drug's quantification, ensuring compliance with regulatory standards and supporting therapeutic monitoring. These methods facilitate the quality control of Tacrolimus formulations, aiding in the development of innovative drug delivery systems. Furthermore, the pre-formulation studies enrich the understanding of Tacrolimus's physicochemical properties, contributing to its effective formulation and stability. The validated methods establish a strong foundation for future research and clinical applications, enhancing the therapeutic management of patients receiving Tacrolimus. This thesis underscores the importance of robust analytical techniques in ensuring drug quality, efficacy, and safety, ultimately contributing to the advancement of pharmaceutical science

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