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DEVELOPMENT AND VALIDATION BY ANALYTICAL METHOD RP-HPLC FOR APIXABAN IN TABLET DOSAGE FORM

Manjusha U. Kakde[1]*, Vijayalaxmi A. Chavan[2] and Snehal S. Manekar[3]

[1]Student, Shri Bhagwan College of Pharmacy, Aurangabad- 431003, Maharashtra.
[2]Assistant Professor, Shri Bhagwan College of Pharmacy, Aurangabad-431003, Maharashtra.
[3]Assistant Professor, Dr. Rajendra Gode Institute of Pharmacy, Amravati.

***Corresponding Author: Manjusha U. Kakde**
Student, Shri Bhagwan College of Pharmacy, Aurangabad- 431003, Maharashtra.

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ABSTRACT
For the Apixaban, a new, simple, sensitive, and inexpensive RP-HPLC analytical method has been developed in the current investigation. Due to the short analytical run time, the developed and validated RP-HPLC technique was shown to be more cost-effective. The mobile phase of Methanol: Water with 0.15% orthophosphoric acid (OPA) produced the best results. The peaks were suitably clear and symmetric. When evaluated spectrophotometrically, apixaban was shown to exhibit significant absorbance at 277 nm; hence, this wavelength was chosen as the detecting wavelength. The retention time was 4.9 minutes. By injecting 100g/ml of the standard concentration of apixaban, the System suitability was evaluated. The acceptable level of RT, peak area, tailing factor, and theoretical plate were verified. The developed method was validated using Q2 R1 of the ICH guidelines, and parameters like linearity, precision, accuracy, robustness, the limit of detection (LOD), and the limit of quantitation (LOQ) were validated. LOD and LOQ were determined to be respectively 1.3852 ug/ml and 4.202 ug/ml. As a result, quality control laboratories can use the established RP-HPLC method for the regular examination of apixaban in bulk and pharmaceutical dosage forms.

KEYWORDS: Reverse phase-high-performance liquid chromatography, Validation, Apixaban, Thrombosis.

INTRODUCTION
Blood flow can be obstructed by thrombosis in both veins and arteries.[1] Depending on where the thrombosis occurs, it could lead to complications.[2] The most severe issues are heart attack, stroke, and severe breathing issues[3] Deep vein thrombosis (DVT) is primarily brought on by damage to a vein following surgery, inflammation, infection, or injury.[4,5] The US Food and Drug Administration (FDA) approved the use of apixaban, a new oral anticoagulant (NOAC), in patients with non-valvular atrial fibrillation in 2012 to lower their risk of stroke and blood clots.[6] It was later authorised in 2014 to treat pulmonary embolism (PE) and deep vein thrombosis (DVT). Apixaban is now used to treat thrombosis after it was authorised in 2014 for usage in patients to lower their risk of blood clots (DVT and PE) after hip and knee replacement surgery.[7,8] The process of developing analytical methods and then validating those methods is crucial to the process of discovering new drugs.[9] Despite having good potency, the medicine cannot be sold because there is no certified analytical method. It also offers a way to assess the biological safety of the medications by addressing the SIAMs (stability indicating assay methods).[10,11]

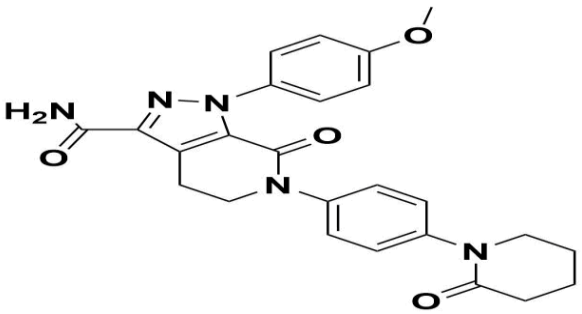


Figure 01: Structure of apixaban.

EXPERIMENTAL WORK
MATERIALS AND REAGENTS
Apixaban (Active Pharmaceutical Ingredient) was provided as a gift sample by Wockhardt Pvt Ltd. The apixaban tablets (label claim 5mg of Apixaban) were purchased from a local market. Methanol and water of HPLC grade (produced by the Research lab) were utilized. Other compounds were all of the HPLC grades. Since all reagents are HPLC grade, no additional characterization was done.

Determination of wavelength

100 ml of solvent was used to dissolve 100 mg of pure apixaban, which is equivalent to 1000 ug/ml. this solution was again diluted to 100ug/ml. Using solvent as a blank, the final solution was scanned using a UV-Visible spectrometer between a range of 400 and 200 nm.

HPLC Method Development

Preparation of stock solution

To produce a concentration of 1000 ug/ml, accurately weigh 100 mg of apixaban after it has been dissolved in 100 ml of methanol. To achieve 100ugm/ml, the solution was further diluted. The resulting stock solution was sonicated three times over a period of 10 minutes each time, then filtered using a 0.45 membrane filter.

Preparation of mobile phase

As the mobile phase, HPLC grade methanol and water in a 90:10 ratio were chosen and sufficiently sonicated. The same was used for the Development and validation of the HPLC method.

System suitability testing

To make a final solution, the stock solution was pipette out up to 10ml and dilute up to 100 ml to obtain the resultant solution of 100µgm/ml. Results for retention time, peak area, tailing factor, and theoretical plate were recorded after the injection of this solution. The results of the studies and the acceptability level of all variables were verified, and all the parameters were checked.

Method validation[12]

Linearity

By using a calibration curve of five reference drug concentrations, linearity was achieved. Pipetting 0.5, 1.0, 1.5, 2.0, and 2.5 ml of the stock solution resulted in 5, 10, 15, 20, and 25 ug/ml of the final solution, respectively. The resultant mixture was sonicated three times over a period of 10 minutes before being filtered using a 0.45 membrane filter. A calibration curve was constructed between peak area and concentration. Results for the Equation of the line were recorded, and the correlation coefficient and intercept were calculated.

Precision

By analyzing three standard solutions (LQC, MQC, and NQC) at concentrations of 10, 15, and 20 per ml, the repeatability of the procedure was assessed. The suggested method's repeatability was tested by doing drug assays at various intervals both on the same day (intra-day) and on three different days (Inter-day precision). The data was recorded to calculate the mean, SD, %RSD, etc.

Accuracy

The definition of the accuracy of an analytical procedure is "The degree to which the test result obtained by that method resembles the true value." This Accuracy was assessed over the range at three concentration levels by ICH guideline Q2R1. To perform Accuracy, the known quantity of apixaban standard was added to the apixaban sample. Utilizing improved chromatographic conditions, the amount of drug recovered was calculated by adding 80, 100, and 120% of the working level concentration and mixing. Triplicate samples were used to calculate the amount recovered (ug/ml).

Robustness

For the parameters like Flow rate, wavelength, and mobile phase, the chosen solution was used for a robustness assessment. The table's parameters were injected, and the areas of each parameter were measured. More than 5% RSD should not be present in the variation. The percent assay should also fall between 98 and 102%. To determine the impact, one factor was modified at a time.

Table 01: Robustness variation table.

Condition	Normal	Variation1	Variation 2
Mobile phase	90:10	91:9 91:9	89:11
Flow rate	0.7ml/min 0.7ml/min	0.6 ml/min	0.8 ml/min
Wavelength	277	276	278

LOD and LOQ

The limit of detection is the lowest concentration of an analyte that can be detected in a sample but not necessarily quantitated, under the given experimental conditions. The limit of quantitation is the quality of quantitative assays for low amounts of chemicals in sample matrices, such as contaminants in bulk substances and degradation products in finished medications. It is the lowest concentration of analyte in a sample that can be accurately and precisely identified under the given experimental conditions.

LOD and LOQ were determined using the following formulas

$LOD = 3.3 \times (SD)/S$

$LOQ = 10\times (SD)/S$

Where,

SD = Standard deviation

S = Slope

% Recovery

Preparation of stock from the dosage form

The average weight of twenty tablets each containing the label's stated 5 mg of apixaban was calculated before

they were powdered. Powder equivalent to 100mg of Apixaban was transferred to 100 ml of Mobile phase. Filtered the solution through a 0.45 membrane filter and sonicated it three times over a period of 10 minutes before being filtered using a 0.45 membrane filter. To obtain the resulting solution of 10 ug/ml, 1 ml solution from the stock solution was pipetted out and diluted up to 100 ml using a mobile phase.

Preparation of test solution for % recovery by spike method

To conduct the recovery experiment, a standard sample of 10ugm/ml was spiked into test solutions made from the finished product at levels of 80, 100, and 120%. A standard addition method was used to conduct the recovery study, adding known quantities of the apixaban standard solution (10 microgram/ml) to each level of the test solution as previously mentioned. The resulting solution was injected in three separate injections, and the area obtained for each drug was tracked. To determine the actual area corresponding to the test sample, the mean area of the standard drug sample was subtracted from the area obtained at each level.

RESULT AND DISCUSSION

HPLC Method Development

Selection of analytical column

HPLC system with phenomena (4.6*250mm) analytical column and UV 730 detector was chosen for method development. In the mobile phase, the Apixaban standard solution was prepared. As the mobile phase for the chromatogram development, various HPLC grade solvents with various polarities were tested.

Selection of mobile phase

Methanol and water (0.1% OPA) (90:10v/v) were found to be the best mobile phase combination for apixaban. This mobile phase was chosen because it offers the best resolution, the best retention time, and the appropriate tailing factor.

Selection of analytical wavelength

It is standard procedure in HPLC to choose the drug's isosbestic point from the UV spectrum when choosing the detection wavelength. Some trials will be successful. The detection wavelength that was ultimately chosen from all of the trials was 277nm, where the drug's peak height was acceptable.

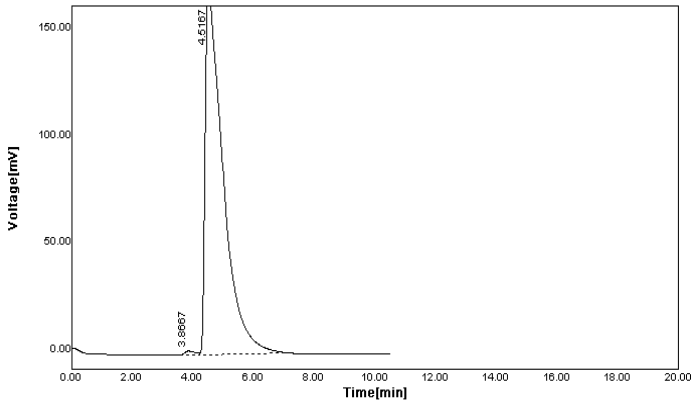
Through trial and error, the following chromatographic conditions were established and maintained throughout the experiment.

Table 02: Chromatographic conditions

Sr. No.	Parameters	Specification
1	Column	C18(4.68250mm)
2	Particle size packing	5um
3	Mobile Phase	Methanol: water (0.15%OPA)
4	Detection Wavelength	277.0 nm
5	Flow rate	0.7ml/min
6	Temperature	Ambient
7	Sample size	20ul

Method Development Trial:

Trial 1: 1 APIXABAN 20 MCG (80 MEOH+10 (0.1 % OPA)-278-0.7 ML (250X 4.6) YCM

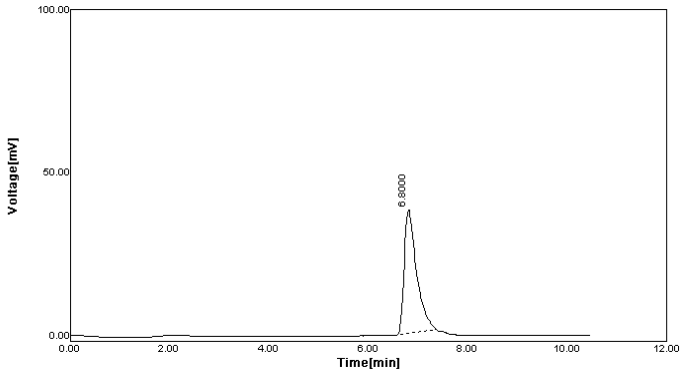


No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	3.8667	36.0402	0.51	649.5	1.3146	0.0000

2	4.5167	6975.5645	99.49	297.3	3.7266	0.7856
Sum		7011.6045				

Figure 02: Trial 1: 1 Apixaban 20 MCG.

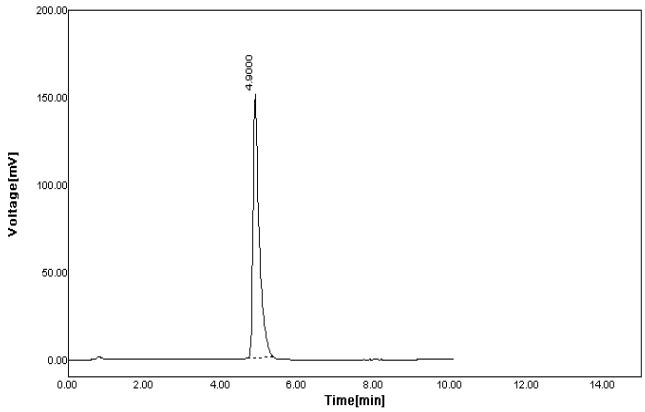
Trial 2: 1 APIXABAN 20 MCG (50 MEOH+10 (0.1 % OPA)-278-0.7 ML (250X 4.6) YCM



No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	6.8000	661.1403	100.00	4173.6	1.8400	0.0000
Sum		661.1403				

Figure 03: Trial 2: 1 APIXABAN 20 MCG.

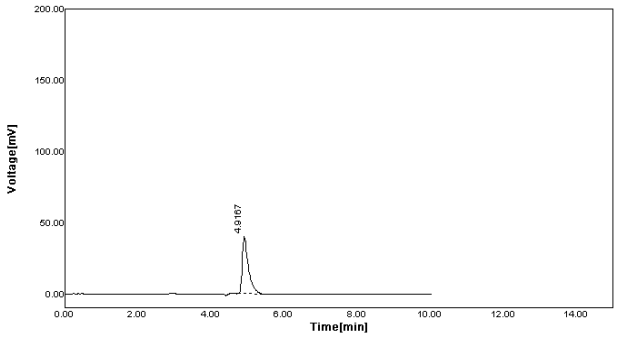
Trial 3: 1 APIXABAN 20 MCG (90 MEOH+10 (0.1 % OPA)-278-0.7 ML (250X 4.6) YCM



No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	4.9000	1669.6498	100.00	5515.6	1.3520	0.0000
Sum		1669.6498				

Figure04: Trial 3 : 1 APIXABAN 20 MCG

Trial 4: APIXABAN 05 MCG (90 MEOH+10 (0.1 % OPA)-278-0.7 ML (250X 4.6) YCM



No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	4.9167	450.8249	100.00	6503.2	1.3658	0.0000
Sum		450.8249				

Figure 05: Trial 4: APIXABAN 05 MCG

System suitability testing

Once experiment results were obtained, all parameters were checked for acceptability level as well as other parameters were also verified for acceptability level.

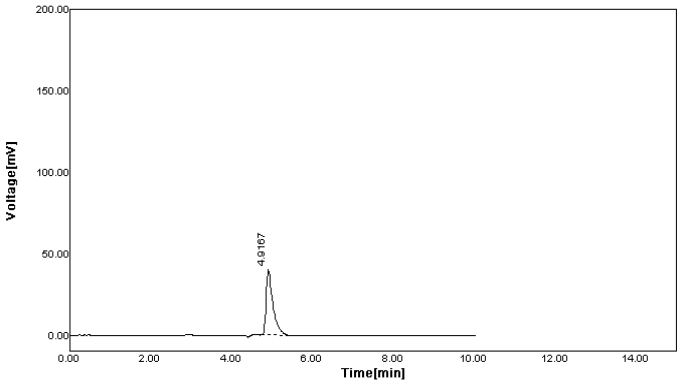


Figure 06: System suitability testing.

Table 03: System suitability testing.

Sr. no.	Parameter	Mean Area	Limit	Interference
1	Area	45.08	% RSD (<2%)	Passed
2	Retention time	4.9	<10-5	Passed
3	Theoretical plate	6503	>2000	Passed
4	Tailing factor	1.3	<2	Passed

Method Validation by HPLC

Linearity

The drug's peak areas, which were within the concentration range of 5- 25ugm/ml, were plotted to create the calibration curve. The peak area ratio and standard analyte concentrations were found to be well correlated because the correlation (r^2) was determined to be 0.999, which is to the ICH guideline.

Table 04: Observation For Apixaban

Sr. No.	CONCENTRATION (µg/ml)	AREA
1	5	434.85
2	10	849.42
3	15	1304.4
4	20	1738.85
5	25	2237.27

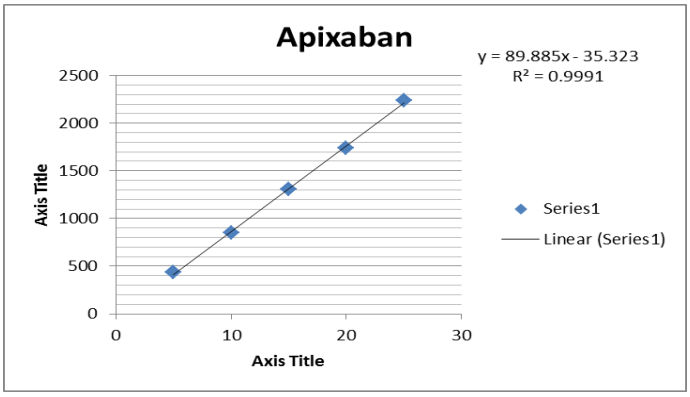
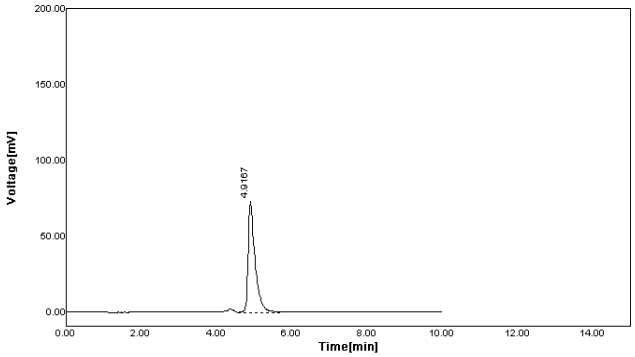


Figure 07: Linearity for apixaban.

Precision

Several parameters, including area, retention time, tailing factor, and theoretical plates, were observed throughout the precision investigation. As per ICH guideline Q2R1, all of these metrics were within acceptable ranges. As a result, the approach was accurate for the drug's provided range. Apixaban was accurate within the prescribed drug range.

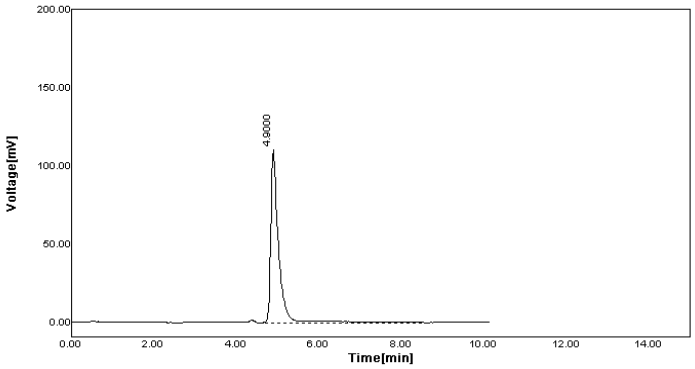
A) Precision: 10MCG Sample



No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	4.9167	865.55	100.00	5548.8	1.3124	0.0000
Sum		865.55				

Figure 08: Precision: 10MCG Sample

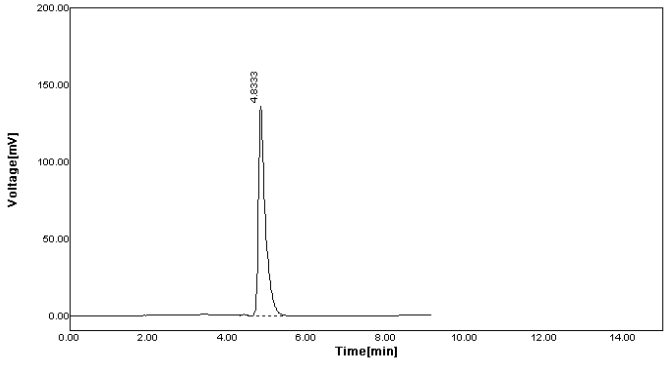
B) Precision: 15 MCG sample



No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	4.9000	1320.0658	100.00	2562.4	1.3 879	0.0000
Sum		1320.0658				

Figure 09: Precision: 15 MCG sample.

C) Precision: 20MCG sample



No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	4.8333	1737.3334	100.00	5093.1	1.3931	0.0000
Sum		1737.3334				

Figure 10: A) Precision: 20MCG sample

Table 05: Intraday

Sr. No.	Conc.	Area	II	Mean	Amount Found	% Amount found	SD	RSD
1	10	865.55	874.49	870.02	10.07	100.70	6.32	0.73
2	15	1320.06	1325.14	1322.60	15.10	100.67	3.59	0.27
3	20	1753.99	1737.33	1745.66	19.91	99.55	11.78	0.67

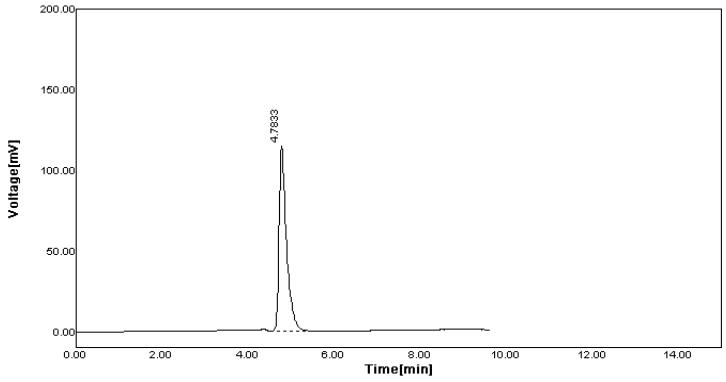
Table 06: Interday

Sr No.	Conc.	Area	II	Mean	Amount found	%Amount Found	SD	RSD
1	10	864.33	872.87	868.60	10.05	100.50	6.04	0.70
2	15	1322.99	1327.65	1325.32	15.13	100.87	3.30	0.25
3	20	1749.28	1759.87	1754.58	19.91	99.55	7.49	0.43

Accuracy

Recovery studies were carried out to verify the developed method's accuracy.

Accuracy: 80%



No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	4.7833	1580.0941	100.00	4548.7	1.2034	0.0000
Sum		1580.0941				

Figure 11: Accuracy: 80%

Accuracy: 100%

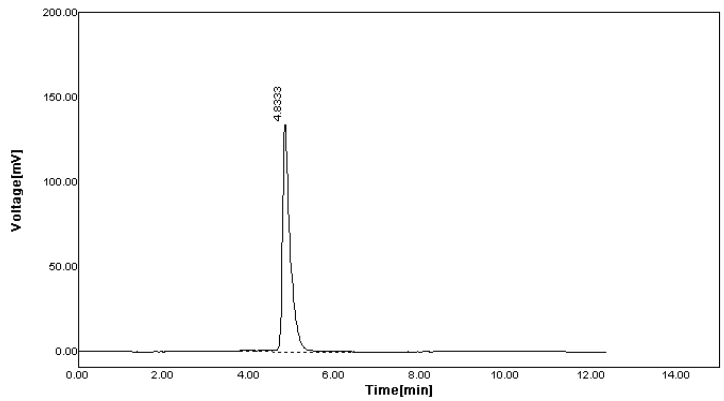
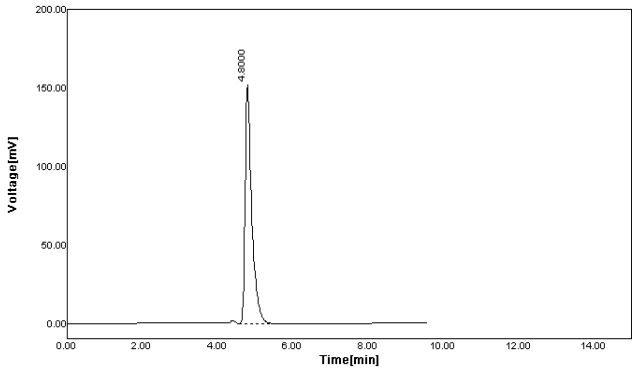


Figure 12: Accuracy: 100%

Accuracy: 120%



No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	4.8000	1950.3482	100.00	4505.3	1.2952	0.0000
Sum		1950.3482				

Figure 13: Accuracy: 120%

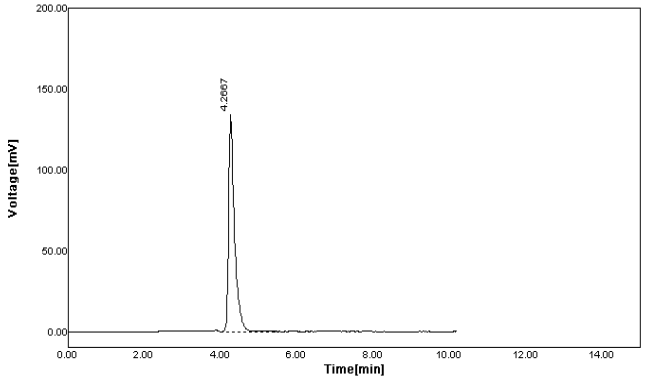
Table 07: Accuracy for apixaban.

Sr. No.	Concentration (µg/ml)	Mean area	Amount Recover(µg/ml)	% Assay	Limit (98-103)
1	10	87.00	10.7	100.7	passed
2	15	13.22	15.10	100.67	Passed
3	20	17.45	19.91	99.55	passed

Robustness

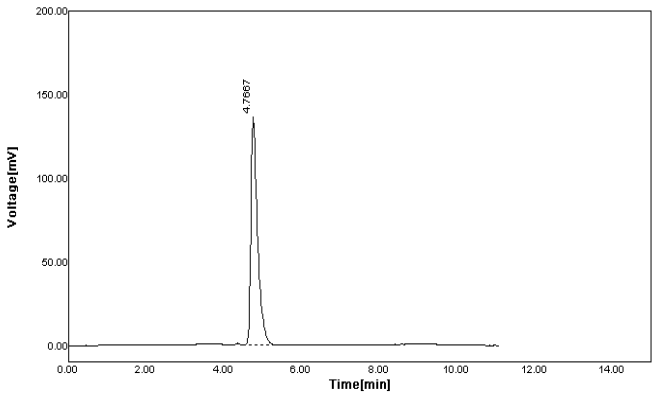
We looked at variables including flow rate, wavelength, and mobile phase. For variations, RSD, and % assay were determined, as indicated in the figure and tables below,

A) Robustness for flow rate: 0.8ml



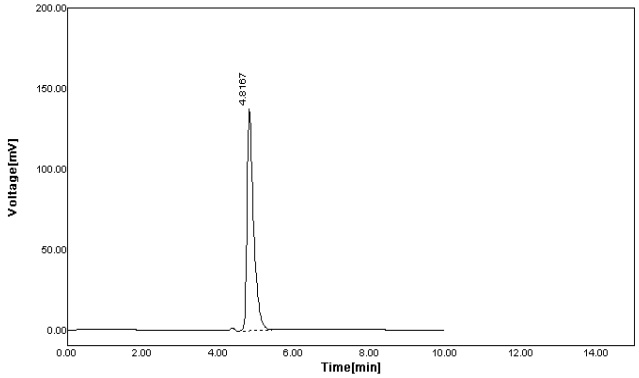
No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	4.2667	1443.0547	100.00	5621.5	1.2 994	0.0000
Sum		1443.0547				

B) Robustness for wavelength: 278



No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	4.7667	1624.6788	100.00	5287.1	1.3856	0.0000
Sum		1624.6788				

C) Robustness for Mobile phase: Methanol 91+9 water



No.	RT[min]	Area[mV*s]	Area%	TP	TF	Resolution
1	4.8167	1649.9572	100.00	4574.1	1.3514	0.0000
Sum		1649.9572				

Robustness For Flow Rate

Table 08: Robustness For Flow Rate

Sr. No.	Flow rate	Mean area	Mean of RT	%Assay	Limit (97-103%)
1	0.8	1443.33	4.2	100	passed
2	0.6	2005.58	5.4	99.20	passed

Robustness for mobile phase

Table 09: Robustness For Mobile Phase

Sr. No.	Mobile phase Ratio	Mean area	Mean of RT	%Assay	Limit (97-103%)
1	91:9(+1)	1656.2	4.7	101.1	passed
2	89:11(-)	1665.00	4.8	99.75	passed

Robustness for wavelength

Table 10: Robustness for wavelength.

Sr. No.	wavelength	Mean area	Mean of RT
1	278(+1)	1625.38	4.7
2	276(-1)	1727.59	4.7

LOD and LOQ

The LOD was found to be 1.3852ug/ml and 4.202 ug/ml was found to be the LOQ.

% Recovery

The amount of apixaban recovered in percent was calculated from the measured area. The outcomes were consistent with the specific drug's compendial standards. The recovery outcome was displayed in a table.

Table11: % Recovery.

Sr. No.	Recovery level	Concentration (µg/ml)	Amount added	Amount found	% Recovery	Limit (97-103%)
1	80%	10	8	18.03	100.38	Passed
2	100%	10	10	20.10	101.2	Passed
3	120%	10	12	22.06	101.58	Passed

CONCLUSION

According to the current investigations, a special HPLC method has been developed and validated for apixaban determination. Methanol and water were chosen as the mobile phase on a C18 stationary phase to influence the analysis of the component peaks. The most effective mixture was found to be Methanol: water (0.15%OPA) in a ratio of 90:10 v/v because the chromatographic peaks were more well-defined, resolved, and nearly tailing-free. The theoretical plate count and tailing factor are within acceptable bounds. Peak tailing, theoretical plates, and the % assay did not significantly change as a result of the method's planned modifications. This demonstrated how exact, precise, and reliable the current method was. The lowest LOD and LOQ values achieved using the suggested method show that it was sensitive. As a result, quality control laboratories can use the established RP-HPLC method for the regular examination of apixaban in bulk and pharmaceutical dosage forms.

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