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Research Paper

Analytical Method Development and Validation of Apixaban by RP-HPLC

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ABSTRACT:
The aim and objective of a paper is to develop and validate a simple, precise and sensitive method for estimation of Apixaban in bulk drug and its marketed formulation using the RP-HPLC method. The separation was achieved on C18 column (4.6 id x 250mm; 5µm) using mobile phase (Water: ACN) in the ratio of 40:60(v/v) with a run time of 7 minutes and wavelength for estimation of Apixaban was taken as 280 nm. Literature survey reveals that there are very few HPLC, UV methods available, Hence RP-HPLC method for estimation of Apixaban was developed. Various parameters likeLinearity, Accuracy, Repeatability, Precision, Robustness, LOD and LOQ, % Recoverywas validated as per ICH guideline.
KEYWORDS: *RP-HPLC method, Validation, Accuracy, Precision.*

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I. INTRODUCTION

Apixaban is a pyrazolopyridine that is 7-oxo-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide substituted at position 1 by a 4-methoxyphenyl group and at position 6 by a 4-(2-oxopiperidin-1-yl)phenyl group. It has a role as an anticoagulant and an EC 3.4.21.6 (coagulation factor Xa) inhibitor. It is a pyrazolopyridine, a member of piperidones, a lactam and an aromatic ether.[1]

Apixaban is oral anti coagulant used help prevent strokes or blood clots in people who have atrial fibrillation (a condition in which the heart beats irregularly, increasing the chance of clots forming in the body and possibly causing strokes) that is not caused by heart valve disease. Apixaban is also used to prevent deep vein thrombosis (DVT; a blood clot, usually in the leg) and pulmonary embolism (PE; a blood clot in the lung) in people who are having hip replacement or knee replacement surgery. Apixaban is also used to treat DVT and PE and may be continued to prevent DVT and PE from happening again after the initial treatment is completed.[1] [2]

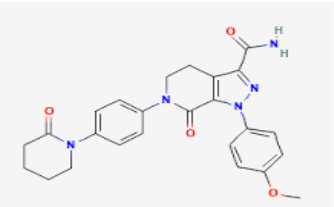


Figure 1: Apixaban Drug

CHROMATOGRAPHY: Chromatography is a separation process that is achieved by distributing the components of a mixture between two phases, a stationary phase and a mobile phase. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase.[3]

Normal Phase Chromatography: In normal phase chromatography, the stationary phase is a polar adsorbent and the mobile phase is generally a mixture of non-aqueous solvents. The silica structure is saturated with silanol groups at the end. These OH groups are active sites (very polar) in the stationary phase.This forms a weak type of bond with any molecule in the vicinity when any of the following interactions are present

1.Dipole-induced dipole 2.Dipole-dipole 3.Hydrogenbonding 4. π-Complex bonding

Reversed Phase Chromatography: Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase, i.e., the sorbent.[4]

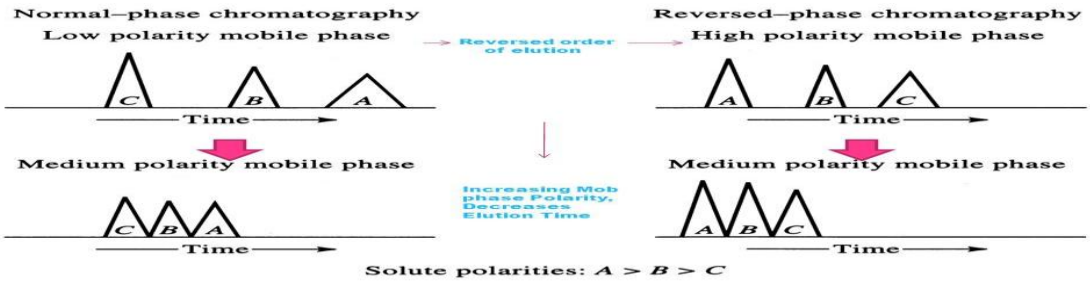
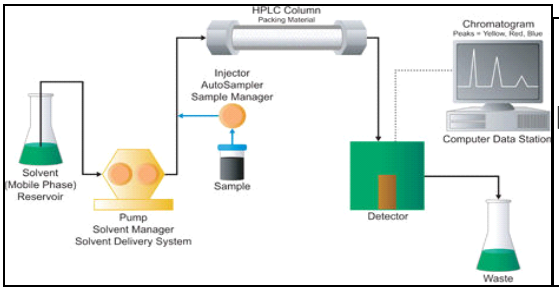


Figure 2: Relationship between polarity and elution times for NP and RP Chromatography



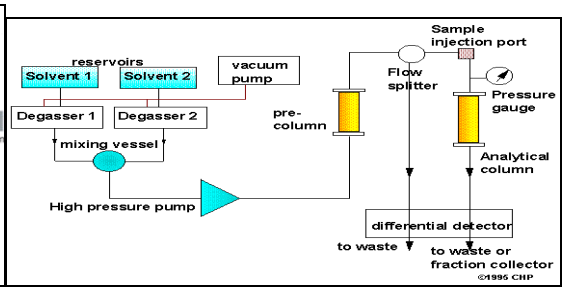


Figure 3: HPLC System Figure 4: Instrumentation HPLC System

HighPerformance Liquid Chromatography which is also known as High Pressure Liquid Chromatography. It is a popular analytical technique used for the separation, identification and quantification of each constituent ofmixture.[12] HPLC is an advanced technique of column liquid chromatography. The solvent usually flows through column with the help of gravity but in HPLC technique the solvent will be forced under high pressures upto 400 atmospheres so that sample can be separated into different constituents with the help of difference in relative affinities.[4]

ANALYTICAL METHODS DEVELOPMENT AND VALIDATION: Method validation can be defined as establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics [20].Method validation is a part and parcel of an industry. It refers to documented observation which ensures that a specific method, and the instruments involved in the method, will provide appropriate results that accurately fulfill the desired properties of the product.

Validation: It is accepted that during the course of a typical drug product development program, a defined analytical method will many modifications because composition changes, lower strength may be added or percentage of coating material may change on the formulation. Because of the changes the analytical method may be modified and if modified it should be verified so it requires different levels of validation. Two different levels/types of method validations, complete validation and partial validation or mini, validation, are defined and characterized as follows.[26]

Complete validation: Complete validation is necessary before executing clinical batch or registration batch of drug product. If any modification in the formulation or if any impurity found in the stability study the existing method to be modified and validated again[28]and the parameters are detailed below for the complete validation given table 2.[36]

Mini Validation: Mini validations is required for all the test methods like Assay, Related substance, UOD and Blend Uniformity for analyzing the routine samples prior starting the complete validation some parameters to be checked as per ICH Guidelines detailed below given table 3 and acceptance criteria discuss.

Table: 1Parameters for an Analytical Procedure[26]

Parameters	Identification	Impurities Quantitative Limit		Assay
Accuracy	-	+	-	+
Precision	-	+	-	+
Specificity	+	+	+	+
Detection limit	-	-	+	-
Quantitation limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+
Robustness	+	+	+	+

Table: 2 ICH Guidelines and Acceptance Criteria[26]

Analytical methods	Parameters
Assay, Content or blend Uniformity, Uniformity of Dosage Units, Dissolution	Precision, Accuracy, specificity only blank and placebo interference, Linearity and solution stability.
Related substance	Precision, Accuracy, specificity includes forced degradation study, LOD and LOQ, Linearity and solution stability
Residual solvents	Precision, Accuracy, specificity only blank and placebo interference, LOD and LOQ, and Linearity.

II. MATERIALS AND METHODS

Table 3. Drug sample [1] [2]

Sr. No	Name of drug/ Chemicals	Drug suppliers
1	Apixaban	Glenmark Pharma ,Nashik
2	Methanol HPLC Grade	
3	Water HPLC Grade	

1. Selection of chromatographic Mode: The reverse phase HPLC was selected for separation because it is convenient and rugged than other forms of the liquid chromatography and is more likely to result in a satisfactory final separation. [30]

2. Selection of stationary phase: On the basis of reversed phase HPLC mode and number of carbon present in molecule (analyte) stationary phase with Grace C18 (250mm× 4.6 ID) phase and particle size 5 micron was selected.

3. Selection of mobile Phase: After assessing the solubility of drug in different solvents as well on the basis of literature survey; methanol and water was selected.

4. Preparation of stock standard solution: 0.01 gm was weighed accurately and transferred to separate 10 ml volumetric flask dissolved in methanol and water in a proportion of 70:30 (in %) to give a stock solution of 1000ppm.[29]

5. Optimization of Chromatographic Parameters: Optimization in HPLC is the process of finding a set of conditions that adequately separate and enable the quantification of the analyte from the endogenous material with acceptable accuracy, precision, sensitivity cost, ease and speed.

6. Optimization of Chromatographic Parameters: Optimization in HPLC is the process of finding a set of conditions that adequately separate and enable the quantification of the analyte from the endogenous material with acceptable accuracy, precision, sensitivity, specificity, cost, ease and speed.

7. Selection analytical wavelength: Appropriate dilution of each stock solution with mobile phase, various concentration of Apixaban were prepared separately .Each solution was scanned in the spectrum mode between the range 200 to 400 nm were overlaid .The wavelength selected for the analysis was 280 nm at which Apixaban drug showed significant absorbance.[30]

Table: 4 Chromatographic Conditions[26] [43]

Chromatographic Mode	Chromatic Condition
HPLC System	HPLC Agilent Technologies System
Model no.	HPLC 1220 Infinity Series
Pump	P-3000-M Reciprocating (40 MPa)
Detector	UV-3000-M
Software	HPLC Workstation
Column	Grace C18 (250×4.6 ID,66particle size :5 micron)
Mobile Phase	Methanol: Water (70:30)
Detection Wavelength	280nm
Flow Rate	1.0 ml/min
Sample Size	20µL
Sonicator	Wenser Ultra SonicatorModel: WUC-4LCapacity: 4 Liter
Balance	Wenser High Precision BalanceModel: PGB 100Min: 0.001gm

Parameters for Method Validation

The analytical methods which need to be validated are classified as per ICH are classified as following.

1. Linearity: The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.[28]

For the establishment of linearity, a minimum of 5 concentrations is recommended.

2. Detection Limit (LOD): The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

The detection limit (DL) may be expressed as: $DL=3.3\ \sigma/S$

Where σ = the standard deviation of the response, S = the slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte[29].

3. Quantitation Limit (LOQ): The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products. Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

The quantitation limit (QL) may be expressed as: $QL=10/S$

Where σ = the standard deviation of the response, S = the slope of the calibration curve. The slope S may be estimated from the calibration curve of the analyte.

4. Accuracy: The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness. Accuracy should be established across the specified range of the analytical procedure.

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.[43]

5. Precision: The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels:a) Repeatability, b) Intermediate precision c) Reproducibility.

Recommended Data: The standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision investigated.[41]

6. Robustness: The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters[37].

7. Ruggedness: Ruggedness is measure of reproducibility test results under the variation in conditions normally expected from laboratory to laboratory and from analyst to analyst. The Ruggedness of an analytical method is

degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as; different laboratories, analysts, instruments, reagents, temperature, time etc.[35]

8.Range: The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The following minimum specified ranges should be considered:

For the assay of a drug substance or a finished (drug) product: normally from 80 to 120 percent of the test concentration;

For content uniformity: covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified;

For Dissolution Testing: +/-20 % over the specified range[34]

III. RESULT AND DISCUSSION

The proposed method was validated as per ICH guidelines. The solution of the drugs was prepared as per the earlier adopted procedure given in the experiment.

Selection of detector and detection wavelength: From the UV spectra 280nm was selected for the estimation of the drug. **(Figure: 5)**

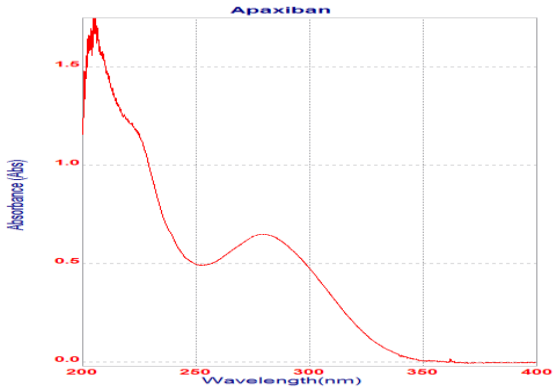


Figure 5: UV Spectra of Apixaban

Application of method for laboratory mixture In order to see the feasibility of the method in the marketed formulation, it was first tried in physical laboratory mixture.

Accurately weighed quantity of 10 mg Apixaban were transferred to 100 ml volumetric flask containing methanol and water (70:30). Volume was adjusted up to mark. It was further diluted to get concentration 10µg/ml. Constant volume 20 µL was injected into column and peak area was recorded. The concentration of the drugs determined from their respective linearity curves. The procedure was repeated for six times; results are shown chromatogram in **Figure: 6**

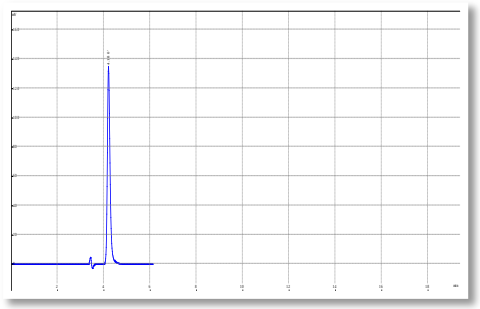


Figure 6: Chromatogram of Apixaban standard stock solution

1. Linearity Studies From the stock standard solution, aliquots portion (0.1-0.5 ml) were transferred into series of 10 ml volumetric flasks and diluted up to the mark with mobile phase to obtain final concentration in the range of 10 to 50 µg/ml for Apixaban .A constant volume of 20 µL of each sample injected with the help of Hamilton Syringe.All measurements were repeated five times for each concentration and calibration curve was constructed by plotting the peak area verses the drug concentration.

Limit: The 'R' square value should be near to 1
Table: 5 Calibration curve

Concentration	Area
10	205079
20	429387
30	6996677
40	958443
50	1233101

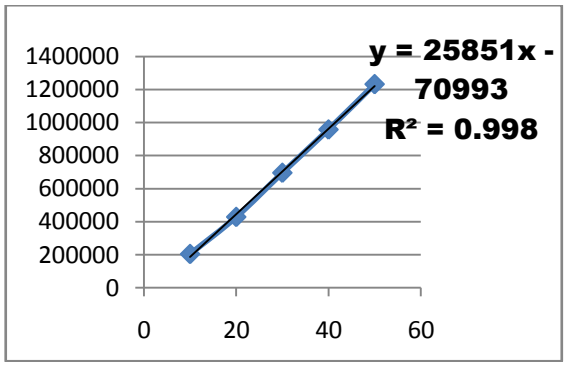
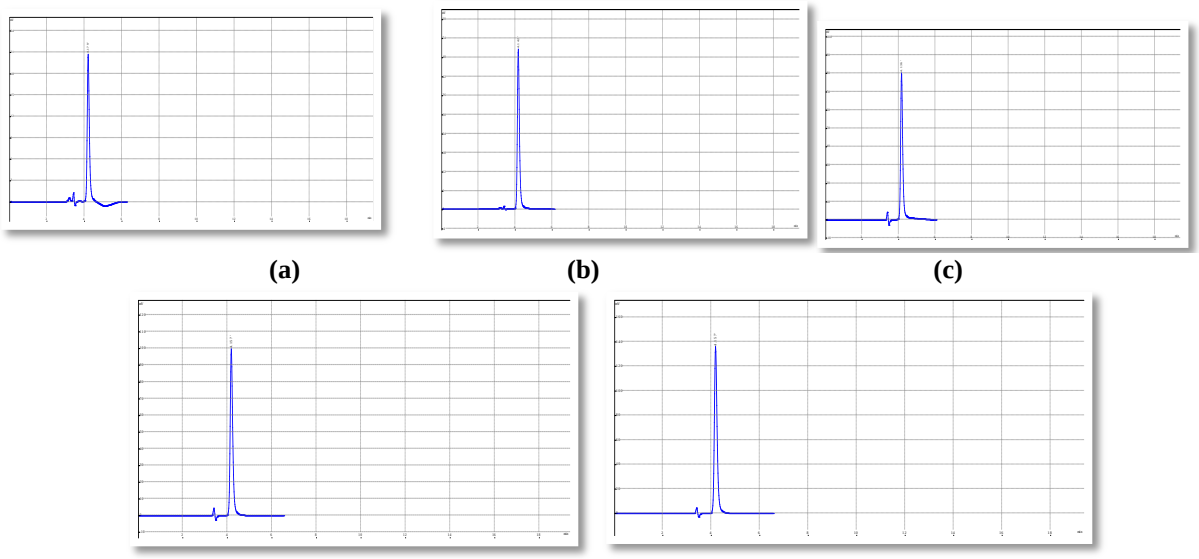


Figure 7: Calibration Curve of Apixaban

Table 6: Linearity studies for different concentration

	10ppm	20ppm	30ppm	40ppm	50ppm
Wavelength	280nm	280nm	280nm	280nm	280nm
Mobile phase	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)
Sample volume	20µl	20µl	20µl	20µl	20µl
Flow rate	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min
Pressure	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa
Time	6.25min	6.04min	6.10min	6.53min	6.55min



(a) **(b)** **(c)**

(d) **(e)**

Figure 8: Chromatogram of linearity [a) 10ppm b)20ppm c)30ppm d) 40ppm e) 50ppm]

Table 7: Chromatogram of Linearity

Sr. no	Time (min)	Area	Resolution Time	Plate No.	Asymmetry
1	4.179	205079	0.00	7444	1.32
2	4.142	429387	0.00	7589	1.32
3	4.136	696677	0.00	7056	1.34
4	4.157	958443	0.00	7778	1.31
5	4.157	1233101	0.00	7088	1.31

Conclusion for Linearity Study-: As per ICH guidelines, the proposed method was validated and linearity studies were performed with measurements repeated five times for each concentration and calibration curve was constructed by plotting peak area Vs Drug Concentration. After Linearity Studies observations shows that, as the concentration for the drug Apixaban increases the peak area over the graph also increases.
R^2 found to be 0.998 which is near to 1.

2. Accuracy Accuracy studies were performed to validate the accuracy of developed method for preanalysed

drug solution of Apixaban. The definite concentration of 10%, 30%, 50% was added and then accuracy was analyzed. Limit: %SD and %RSD value should be less than 2%

Table 8: Accuracy Study for different concentration

	10ppm 01	**10ppm 02**	**10ppm 03**	**30ppm 01**	**30ppm 02**	**30ppm 03**	**50ppm 01**	**50ppm 02**	**50ppm 03**
Wave Length	280nm	280nm	280nm	280nm	280nm	280nm	280nm	280nm	280nm
Mobile phase	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)
Sample volume	20µl	20µl	20µl	20µl	20µl	20µl	20µl	20µl	20µl
Flow rate	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min
Pressure	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa
Time	6.25min	6.05min	6.06min	6.04min	6.24min	6.05min	6.55min	6.10min	6.17min

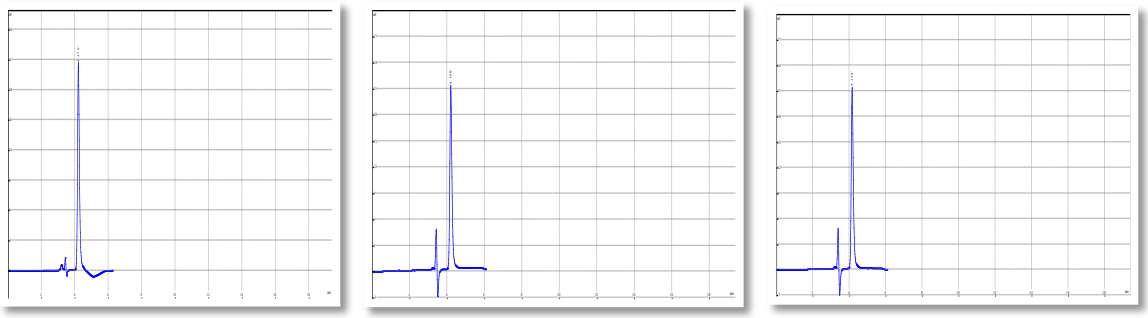


Figure 9: Chromatogram of Accuracy (10ppm 01, 10 ppm 02, 10 ppm 03)

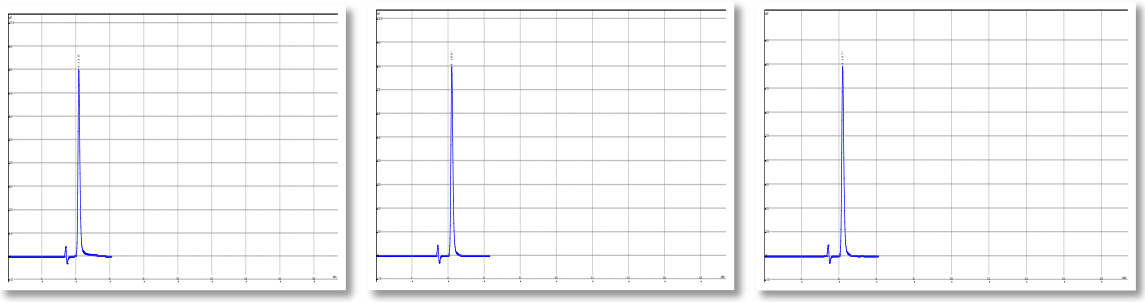


Figure 10: Chromatogram of Accuracy (30ppm 01, 30 ppm 02, 30 ppm 03)

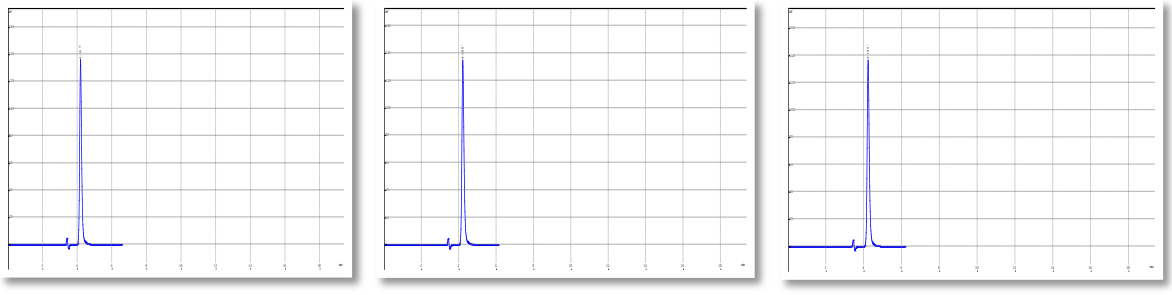


Figure 11: Chromatogram of Accuracy (50ppm 01, 50 ppm 02, 50 ppm 03)

Table 9: Chromatogram readings for accuracy of different concentration

Solution	**Time**	**Area**	**Resolution Time**	**Plate No.**	**Asymmetry**
10ppm	4.179	205079	0.00	7444	1.32
	4.168	204846	0.00	7783	1.30
	4.129	204753	0.00	7323	1.31

30ppm	4.136	696677	0.00	7056	1.34
	4.166	694616	0.00	7061	1.31
	4.157	698788	0.00	7652	1.30
50ppm	4.157	1233101	0.00	7088	1.31
	4.180	1229235	0.00	7268	1.31
	4.195	1230628	0.00	7673	1.31

Table 10: Mean Accuracy of different concentration

Sr. No	Conc.	Area	Mean	SD	%SD	%RSD
	10	204846	204815	53.69357503	0.02621565	0.026215646
	10	204846				
	10	204753				
	30	696677	696693.6667	2086.049935	0.2994214	0.299421401
	30	694616				
	30	698788				
	50	1233101	1230988	1957.980848	0.15905767	0.159057671
	50	1229235				
	50	1230628				

Conclusion for Accuracy

After the proposed method for validation of Apixaban. Accuracy was assessed using minimum of 9 determinations over a minimum 3 concentration levels of 10% , 30% ,50% with 3 replicates of each shown that values for %SD and %RSD are less than 2% which is within limits and express the closeness of accepted reference value and values found .

3. Precision

Precision of the method was verified by repeatability and intermediate precision studies.

Repeatability was measured by multiple injections of a homogenous sample of 30ppm.

Intra-day precision was studied by analyzing 30ppm of Apixaban for three times on the same day.

Inter-day precision was checked analyzing the same concentration for two different data

INTERDAY

Table 11: Precision Study for different concentration (Interday)

	Day 1			Day 2		
	30ppm 01	**30ppm 02**	**30ppm 03**	**30ppm 01**	**30ppm 02**	**30ppm 03**
Wavelength	280nm	280nm	280nm	280nm	280nm	280nm
Mobile phase	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)
Sample volume	20µl	20µl	20µl	20µl	20µl	20µl
Flow rate	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min
Pressure	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa
Time	6.04min	6.24min	6.05min	6.20min	6.03min	6.13min

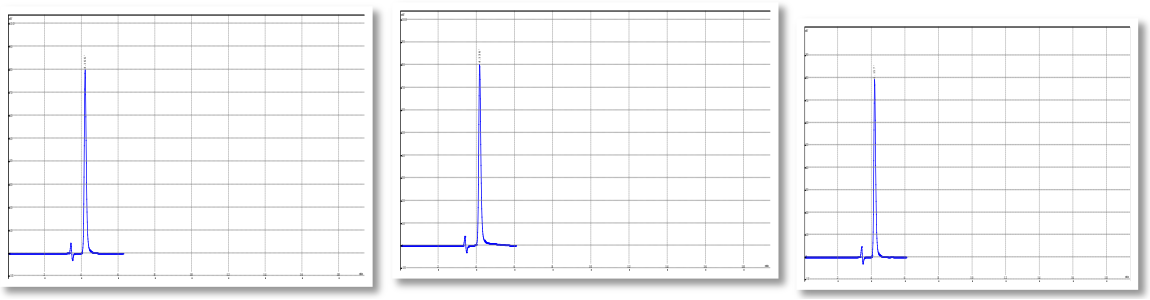


Figure 12 : Chromatogram of precision study Day 01 (Interday)

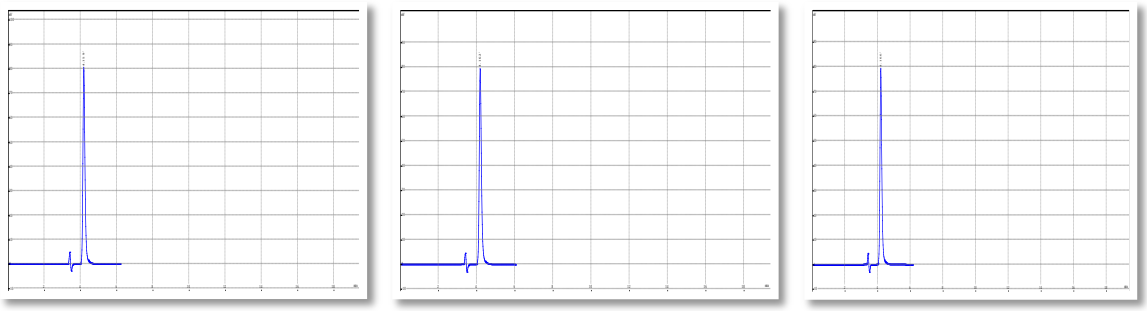


Figure 13 : Chromatogram of precision study Day 02 (Interday)

Table 12: Precision Study for different concentration (Intraday)

	Morning			**Evening**		
	30ppm 01	**30ppm 02**	**30ppm 03**	**30ppm 01**	**30ppm 02**	**30ppm 03**
Wavelength	280nm	280nm	280nm	280nm	280nm	280nm
Mobile phase	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)
Sample volume	20µl	20µl	20µl	20µl	20µl	20µl
Flow rate	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min
Pressure	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa
Time	6.04min	6.24min	6.05min	6.10min	6.34min	7.12min

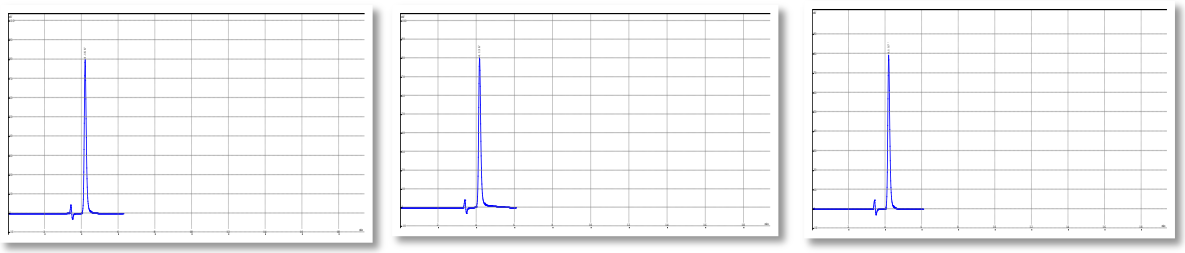


Figure 14 : Chromatogram of precision study Morning (Intraday)

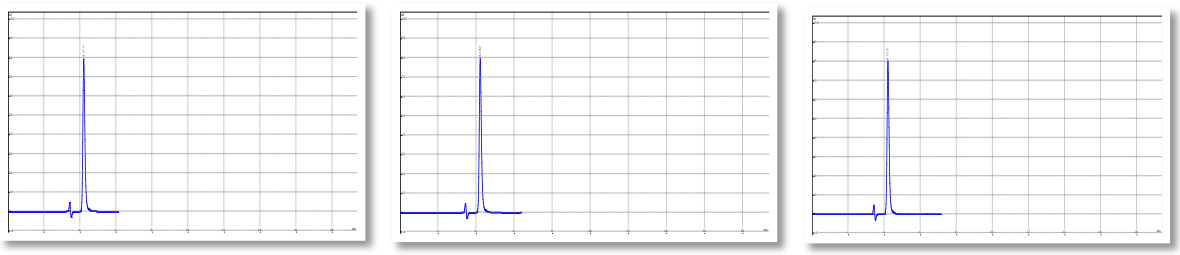


Figure 15 : Chromatogram of precision study Evening (Intraday)

Table 13 : Chromatogram readings for Precision (Interday and Intraday)

A) INTERDAY

Interday		**Time**	**Area**	**Resolution Time**	**Plate No.**	**Asymmetry**
	Day 01	4.136	696677	0.00	7056	1.34
		4.166	694616	0.00	7061	1.31
		4.157	698788	0.00	7652	1.30
	Day 02	4.159	697587	0.00	7659	1.30
		4.1623	693477	0.00	7134	1.30
		4.166	695160	0.00	7355	1.30
Intraday	Morning	4.136	696677	0.00	7056	1.34
		4.166	694616	0.00	7061	1.31
		4.157	698788	0.00	7652	1.30
	Evening	4.177	692793	0.00	7715	1.30
		4.183	694334	0.00	7979	1.31
		4.173	693799	0.00	7647	1.30

Day 1

30ppm	30ppm	30ppm
696677	694616	698788

Mean	%RSD
695160	0.29%

Day 2

30ppm	30ppm	30ppm
697587	693477	695160

B) INTRADAY

Morning

30ppm	30ppm	30ppm
696677	694616	698788

Mean	%RSD
695767.8	0.31%

Evening

30ppm	30ppm	30ppm
692793	694334	693799

4. LOD AND LOQ

LOD = 3.3×S.D/Slope

3.3 ×53.6935/26790.46 = 0.0066

LOQ = 10×S.D/Slope

10×53.6935/26790.46 = 0020042.

5. Robustness

The Robustness of a method was studied making deliberate changes in few parameters. *viz;* change in flow rate, pH and mobile phase composition .The effects on the results were studied by injecting 20µg/ml .one factor was changed at one time to estimate the effects.

Table 14: Robustness Studies

A) Change in flow rate

Conc.	Area	Mean	SD	%SD
20	429387	429730	1084.55	0.25238013
20	430945			
20	428859			

B) Change in wavelength

Conc.	Area	Mean	SD	%SD
20	429387	428590	2078.08	0.48486407
20	426231			
20	430151			

Table 15: Robustness studies for Flow rate and wavelength

	Flow Rate			Wavelength		
	20ppm 01	**20ppm 02**	**20ppm 03**	**20ppm 01**	**20ppm 02**	**20ppm 03**
Wavelength	280nm	280nm	280nm	280nm	280nm	280nm
Mobile phase	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)
Sample volume	20µl	20µl	20µl	20µl	20µl	20µl
Flow rate	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min
Pressure	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa
Time	6.10min	6.04min	6.62min	6.10min	6.09min	6.08min

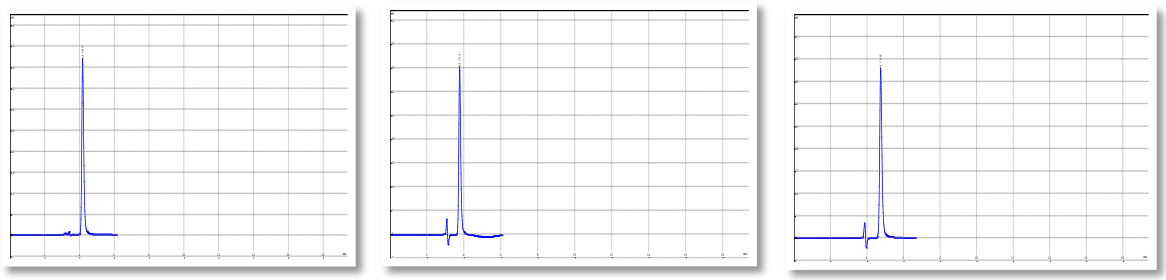


Figure 16 : Chromatogram of Robustness study (Flowrate)

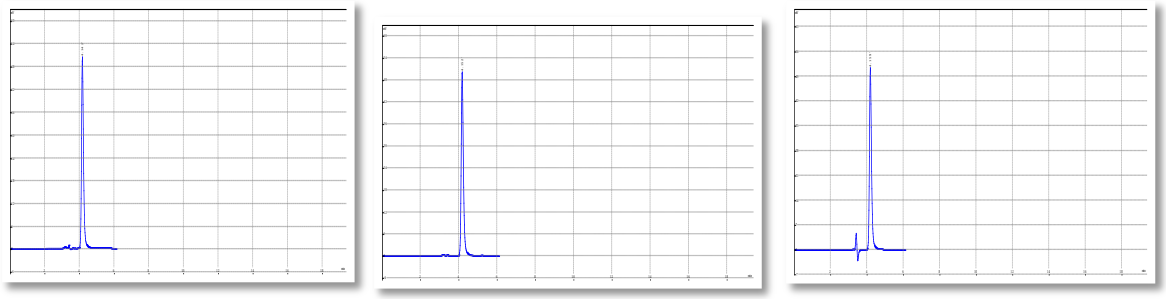


Figure 17 : Chromatogram of Robustness study (Wavelength)

Table 16 : Chromatogram readings for Robustness studies (Flow rate and wavelength)

	Time	Area	Resolution Time	Plate No.	Asymmetry
Flow rate	4.142	429387	0.00	7589	1.32
	3.733	430945	0.00	7413	1.31
	4.708	428859	0.00	8050	1.31
Wavelength	4.142	429387	0.00	7589	1.32
	4.152	426231	0.00	7937	1.32
	4.159	430150	0.00	7819	1.31

Conclusion for Robustness Study

The robustness of the method was studied by making deliberate variations in chromatographic conditions and the effects on the results were examined as % RSD, (less than 2). The low values of % RSD indicate robustness of the method.

6. % Recovery

Table 17: % Recovery for different concentration

	30ppm	40ppm	50ppm	20+10ppm	20+20ppm	20+30ppm
Wavelength	280nm	280nm	280nm	280nm	280nm	280nm
Mobile phase	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)	Methanol: Water pH: 3 (70:30)
Sample volume	20µl	20µl	20µl	20µl	20µl	20µl
Flow rate	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min	1.0ml/min
Pressure	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa	9-10MPa
Time	6.04min	6.53min	6.55min	6.17min	6.18min	6.14min

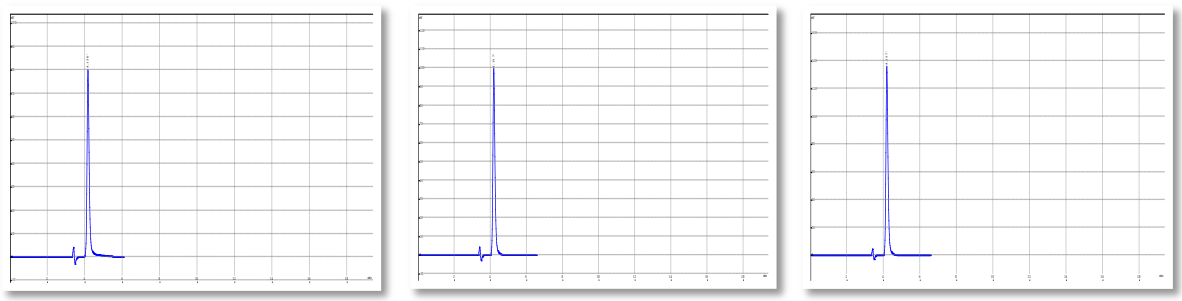


Figure 18 : Chromatogram of Standard solution (30ppm, 40ppm, 50ppm)

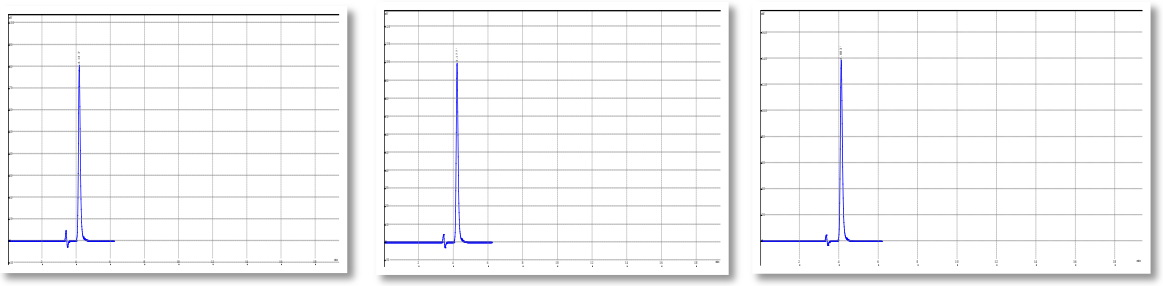


Figure 18 : Chromatogram of solution (20+10ppm, 20+200ppm, 20+30ppm)

Table 19: Chromatogram reading for Standard solution and % recovery

	Time	Area	Resolution Time	Plate No.	Asymmetry
Standard	4.136	696677	0.00	7056	1.34
	4.157	958443	0.00	7778	1.31
	4.157	1233101	0.00	7088	1.31
20+10	4.143	692506	0.00	7633	1.31
20+20	4.177	959431	0.00	7092	1.31
20+30	4.083	1230598	0.00	7045	1.31

Table 20: % Recovery

Sr. No.	% Composition	Area of Standard	Area of sample	% Recovery
1	50% Recovery	696677	692506	99.4013
2	100% Recovery	958443	959431	100.1030
3	150% Recovery	1233101	1230598	99.7970

IV. CONCLUSION

Analytical method development and Validation of Apixaban drug by RP-HPLC was perfomed. The Chromatographic separation was achieved on a HPLC Binary Gradient System Grace C18 (250×4.6 ID, particle size: 5 micron) column. Methanol: Water (70:30) was used as the mobile phase .The detection wavelength was 280 nm .and flow rate was 1.0ml/min.

The developed method was validated according to the ICH guidelines. The Linearity, accuracy, precision, LOD, LOQ, robustness and %recovery were within the limits as specified by the ICH guidelines.

After Linearity Studies observations shows that, as the concentration for the drug Apixaban increases the peak area over the graph also increases.R^2 found to be 0.998 which is near to 1. Accuracy was assessed using minimum of 9 determinations over a minimum 3 concentration levels of 10% , 30% ,50% with 3 replicates of each shown that values for %SD and %RSD are less than 2% which is within limits and express the closeness of accepted reference value and values found. Precision of the method was studied as repeatability of sample application, intra-day and inter-day precision. The results were examined as %RSD values of concentration of drugs determined. The low value of %RSD (less than 2) indicates high precision of the method. The method proved to be adequately sensitivity as indicated by low values of LOD and LOQ. The robustness of the method was studied by making deliberate variations in chromatographic conditions and the effects on the results were examined as % RSD, (less than 2). The low values of % RSD indicate robustness of the method. The results did not show any statistical difference between operators suggesting that method developed was rugged.Moreover same solvent was used throughout the experiment and it was found thatno interference from any excipient was observed in the method. Hence the proposed method was successfully applied to routineanalysis of apxiban in bulk and combined formulation. So the proposed RP-HPLC method was applied for identification of elute.

Hence method was found to be simple, accurate, precise, economic & reproducible

Table: 21Summary of Developed Method

Parameters	Apixaban
Linearity range (in ppm)	10-50ppm
Correlation Coefficient	0.998
Limit of Detection (µg)	0.0066
Limit of Quantification (µg)	0.020042
Accuracy (%RSD)	0.15-0.02
Precision (%RSD) Intra-day Inter-day	0.31 0.29

Robustness	Robust
% Recovery	99.40-100.10

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