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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF APIXABAN BY RP-HPLC METHOD

[1]Mrs.Kanchan Chauhan, [1]Mr.Ansari Saif Iqbal[*]

[1]Department of Pharmaceutical Quality Assurance.

[1]Allana College of Pharmacy Pune, India

Abstract:

A new sensitive and rapid HPLC method was developed for the Apixaban bulk and pharmaceutical dosage forms, it was validated according to ICH guidelines. RP-HPLC analysis was performed on the shimadzu LC-2010C HT with a LabSolutions software on a Inertsil ODS-3 C18 (150 mm × 4.6 mm 5 µm) column, with a mixture of Phosphate Buffer and Acetonitrile in the ratio of 55:45 (v/v) as the mobile phase, at the constant flow rate of 1.0 ml/min with column temperature of 30°C. The λ_{max} was found to be 280 nm and the retention time of Apixaban was found to be 3.9 min. The calibration plot gave linear relationship over the concentration range of 20–140 µg/ml. The accuracy of the proposed method was determined by recovery studies and was found to be 100.0%. The precision study was found to be within the acceptable limits and %RSD of the of precision was <2%. The results of robustness and solutions stability studies were within the acceptable limits as well. The proposed method showed excellent system suitability, specificity, linearity, accuracy, precision, solution stability, and robustness results within the acceptance criteria.

Keyword: Apixaban, RP-HPLC, Validation.

INTRODUCTION

For the treatment and prevention of thromboembolic illness, warfarin was the only oral anticoagulant available in the United States from the 1940s. It is used to reduce thromboembolic events in people with atrial fibrillation, [1,2]. Apixaban structure is shown in **Fig.1** and the IUPAC name of apixaban is 1-(4-methoxyphenyl)–7–oxo-6-[4-(2–oxopiperidin-1-yl) phenyl]-4, 5, 6, 7tetrahydro-1H–pyrazolo[3,4-c] pyridine– 3-carboxamide. Apixaban as a typical of BCS Class III drug. The molecular formula is $C_{25}H_{25}N_5O_4$.Very few methods has been reported for development of apixaban. The development of analytical techniques that are validated in terms of system suitability, specificity, linearity and range, accuracy, precision, robustness, according to the ICH guidelines Q2(R1)[3].

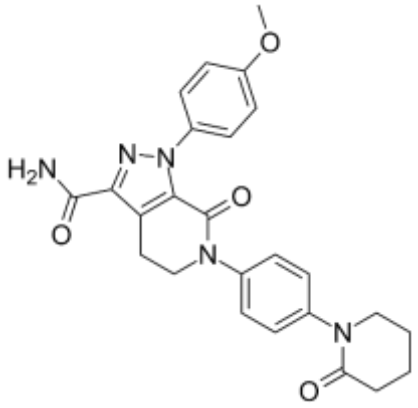

Figure 1: Structure of Apixaban

MATERIALS & METHODS

Chemicals & Reagents

A pharmaceutical grade sample of Apixaban API was obtained as a gift from Enaltec pharmaceutical laboratory. Apixaban tablets containing 5 mg Apixaban were purchased from the local market. Potassium dihydrogen phosphate and Acetonitrile HPLC grade were purchased from merck company, sodium hydroxide pellets of Emparta grade, 0.45µ membrane disc filter of Millipore purchased from MDI.

Instruments

Shimadzu HPLC system was used for liquid chromatography method development and validation (model LC-2010C HT), and a column of Inertsil ODS-3, C18 (150 mm × 4.6 mm, 5 µm) and the detector consisted of UV/VIS operated at 280 nm. LabSolution Software was used for data processing and evaluation. pH meter (thermo scientific, Labindia) model orion star A211. Analytical Balance is of Mettler Toledo model (XS205DU).

EXPERIMENTAL WORKS

Solvent selection

The separation will be achieved because the analytes have distinct chemical affinities for each of the phases. The analytes having a lower affinity for the stationary phase will elute sooner, while the analytes with a higher affinity will elute later. The mobile phase can be modified to alter the relative analyte affinity, which in turn affects the separation's selectivity (chemical separating power) and retention time.

Selection of wavelength

100 mg of Apixaban was accurately weighed and dissolved in 100 ml of mobile phase to yield a concentration of 1000 µg/ml, which was then diluted to yield a concentration of 10 µg/ml. Using mobile phase as a blank, the UV spectrum of a solution with a concentration of 10 µg/ml was recorded. It has a high absorbance at 280 nm, so it was chosen as the maximum absorbance for Apixaban.

Preparation of mobile phase

Phosphate buffer of pH 3.8 and Acetonitrile in the ratio of 55:45 were prepared mix well and degassed.

Preparation of standard stock solution

Weigh about 50 mg of Apixaban Working Standard and transfer into 100 ml volumetric flask, add 70 ml of diluent, sonicate to dissolve, and dilute up to the mark with diluent. Mix well, to make the concentration of Apixaban 500 µg/ml

Preparation of standard solution

Further transfer accurately 5.0 ml of above Standard Stock Solution in to a 25 ml volumetric flask, make up to the mark with diluent and mix well, to make the concentration of Apixaban 100 µg/ml

Preparation of sample solution

Determine the average weight of 20 tablets. Transfer 10 intact tablets into a 100 ml volumetric flask. Add about 70 ml of diluent and sonicate for 30 minutes with intermittent shaking. Cool to room temperature and make volume up to the mark with diluent, mix well. Sonicate this solution for 10 minutes. Filter this solution through 0.45µ Nylon Syringe filter. Further transfer accurately 5.0 ml of this supernatant solution in to a 25 ml volumetric f1ask, make up to the mark with diluent and mix well, to make the concentration of Apixaban 100 µg/ml

METHOD VALIDATION

System Suitability

The system appropriateness characteristics were evaluated by creating standard Apixaban solutions. Six injections of 100 µg/ml solutions were performed, and different characteristics such as retention time, theoretical plates, tailing factor, and peak area were calculated.

Specificity

Specificity, standard and sample solution were prepared of 100 g/ml and injected into the HPLC apparatus, where the peak area and retention time was measured.

Linearity and Range

Different standard solutions were made by diluting the standard stock solution with the mobile phase in different concentrations of apixaban range from 20, 40, 60, 80, 100, 120 and 140 µg/ml. This was done to assess the linearity and range of the method. The calibration curve's linearity was assessed using linear regression analysis utilizing the least squares method.

LOD & LOQ

The formula LOD = 3.3(Standard deviation/Slope) used to derive the detection limit was based on the standard deviation of the peak area. Additionally, the Quantification limit was established using a formula LOQ = 10(Standard deviation/Slope) based on the peak area's standard deviation.

Accuracy

Recovery experiments at three concentrations levels 50%, 100%, and 150% were used to gauge the assay method's accuracy. Three samples from each concentration were injected. For each of the replicate samples, the %RSD and the percentage recovery of added Apixaban were calculated.

Precision

The method's precision was determined by its repeatability as well as its intraday and interday precisions. The intraday and interday precision of the sample solution were established by analyzing them in six independent sample preparations of 5 mg strength and injecting them into the HPLC apparatus. Evaluate precision by performing the precision for test Assay as using same lot on different day, by different analyst, on different HPLC system and with different column. Evaluate the reproducibility by comparing the results obtained from interday with those obtained from intraday.

Solution Stability

Analyzing the sample preparations for up to 52 hours at 10°C in the refrigerator and 30°C in ambient room temperature determined the stability of analytical solutions. Three injections of each solution were examined, and the average peak and %RSD were calculated.

Robustness

The analytical method robustness was evaluated by examining the effect of minor changes in HPLC conditions on the proposed methods testing revealed that a minor change in method conditions such as mobile phase composition, temperature and wavelength were varied by ±2% and the %RSD was calculated.

RESULTS & DISCUSSION

HPLC method optimization and System suitability

Various mobile phases were tested in a variety of ratios for method optimization, including acetonitrile: water (60:40) v/v, acetonitrile: methanol: water (50:25:25v/v/v), and acetonitrile: phosphate buffer pH 3.2 (60:40v/v), among others. Due to tailing, fronting, and a lack of crispness at the peak, all of these mobile phases were unacceptable. Following numerous tests, the mobile phase of phosphate buffer pH 3.8: acetonitrile (55:45v/v) was chosen because it produced sharp peaks without tailing or fronting. The chromatogram of standard solution of apixaban was shown in **Fig 2**. Optimized chromatographic conditions and system suitability parameter were shown in **Table 1**.

Table 1: Optimized chromatographic condition and system suitability parameter

Column	Inertsil ODS-3, C18
Wavelength	280
Column temperature	30°C
Injection volume	10 µl
Run time	12 min.
Flow rate	1 ml/min
Pump mode	Isocratic
Mobile phase	Phosphate buffer 3.8: Acetonitrile (55:45)
Retention time	3.90 minutes
Tailing Factor	1.305
Theoretical plate	3821
Peak area	2281143

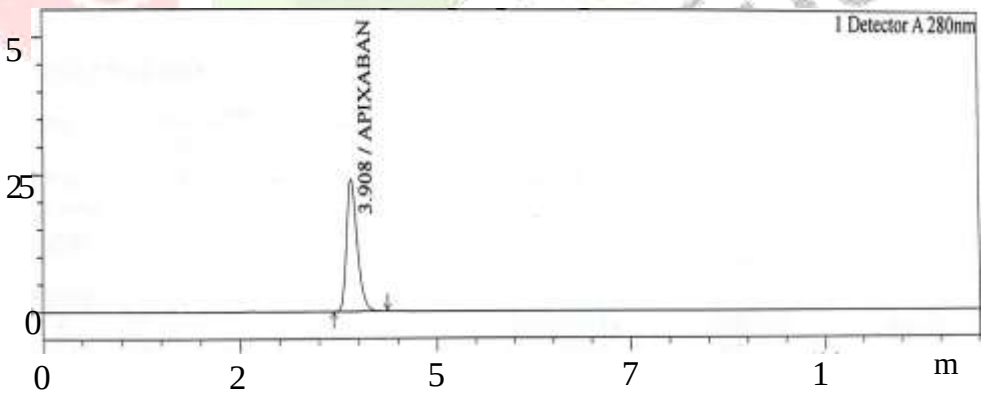

Figure 2: chromatogram of Apixaban standard

Specificity

From **Table 2** demonstrates that the retention times of the standard and sample solutions are the same, indicating that there is no interference, proving that the method is extremely selective.

Table 2: Specificity data by HPLC method

Sr.no	Solution	Peak Area	Retention Time
1	Standard solution	2013153	3.908
2	Sample solution	2007282	3.812

Linearity

To draw the calibration graph, the mean peak area from the HPLC was plotted against corresponding concentrations according to the findings of the linearity investigation **(Fig. 3)**, apixaban has a linear relationship over the concentration range of 20, 40, 60, 80, 100, 120, 140 µg/ml. The goodness-of-fit (R^2) value was determined to be 0.9993, demonstrating a linear relationship between the analyte concentration and area under the peak. A linear equation, Y=21903x + 46030, was derived from the regression analysis. Results of linearity is shown in **Table 3** and overlay chromatogram of linearity was shown in **Fig. 4**.

Table 3: Linearity data by HPLC

Sr.no	Concentration (µg/ml)	Mean peak Area
1	20	587656
2	40	1112259
3	60	1834339
4	80	2316198
5	100	2740186
6	120	3364550
7	140	4453458

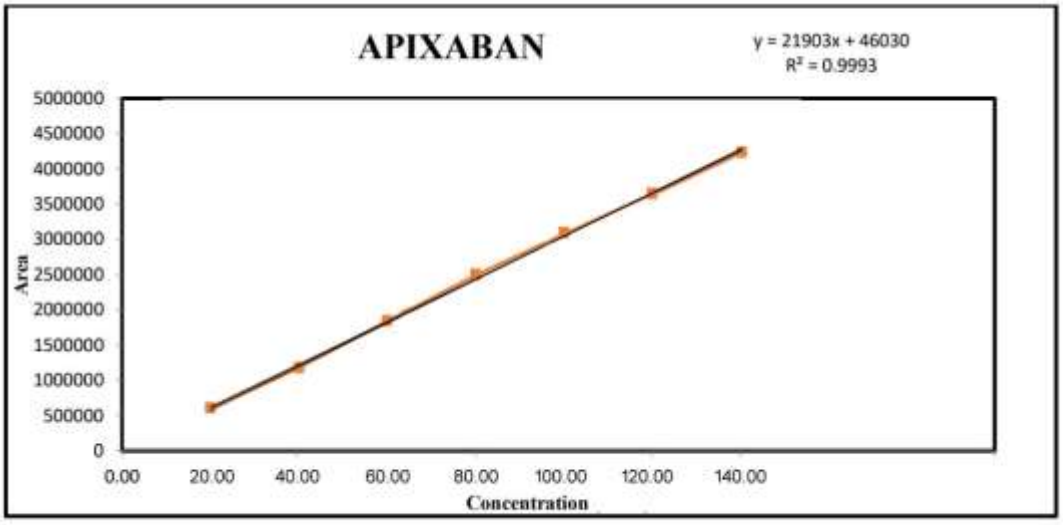

Figure 3: Linearity graph of apixaban

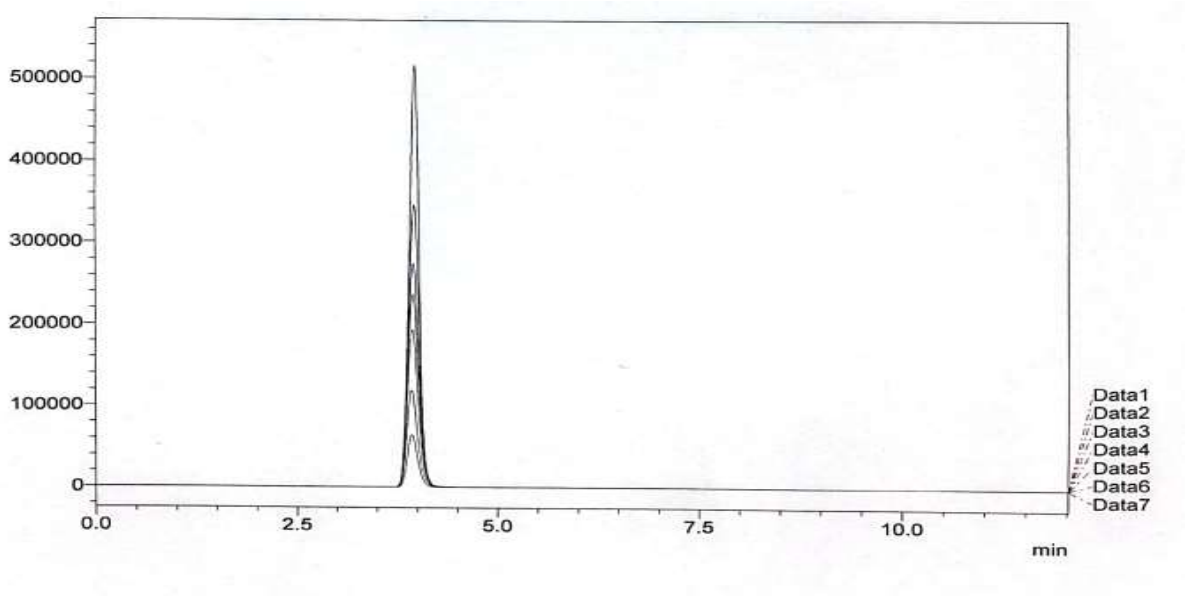

Figure 4: overlay chromatogram of linearity

LOD and LOQ

LOD and LOQ were calculated from the equation and were found to be 1.3049 µg/ml and 3.9543 µg/ml respectively and shown in **Table 4**.

Table 4: LOD and LOQ of Apixaban

Sr no	Parameter	Result
1	LOD	1.3049 µg/ml
2	LOQ	3.9543 µg/ml

Accuracy

The accuracy of an analytical procedure represents the proximity of results achieved by the method to the true value, the accuracy results showed that the percentage recovery at all three levels was in the range of 99.9%–100.5% and the %RDS values were in the range of 0.51%, the percentage recovery and %RSD was found to be within the acceptable range of 98.0% to 102.0%, demonstrating the method's suitability for routine drug analysis. System suitability data of accuracy was found to be within acceptable limit and Accuracy of apixaban is shown in **Table 5**.

Table 5: Accuracy data by HPLC method

Sr.no	Level	Amount of API added (mg)	Conc. Sample Added µg/ml	Peak Area Response	Conc. Recovered µg/ml	%Recovery
1	50	25	50	565977	50.2	100.4
2	100	50	50	2222467	98.8	99.9
3	150	100	50	4521278	148.3	100.5
MEAN						**100.0**
SD						**0.5102**
Overall % RSD						**0.51**

Precision

The interday, intraday, precision findings demonstrated good reproducibility, and the %RSD values were within limits, demonstrating that the approach was highly exact. **Table 6** and **Table 7** shows the results.

Table 6: Interday precision

Sr.no	Conc.(µg/ml)	Area	SD	%RSD
1	20	2243450	17087.235	0.753156
2	40	2267615	19494.933	0.859280
3	60	2295185	36582.169	1.612437

Table 7: Intraday precision

Sr.no	Conc.(µg/ml)	Area	SD	%RSD
1	20	2235300	14505.589	0.644277
2	40	2255814	5252.389	0.233288
3	60	2263242	19757.978	0.877566

Solution stability

Showing good stability of the sample solutions for 52 hours at both conditions, where it was also determined that the results was found to be within acceptable limits. Result of stability of sample solution is shown in **Table 8**.

Table 8: Stability of sample solution data by HPLC method

		At 10° C			At Room temperature		
Sr. no	Time (Hours)	Area	Mean Area	Cumulative RSD	Area	Mean Area	Cumulative RSD
1	Initial	3029960	NA	NA	2995918	NA	NA
2	5 Hr.	3038480	3034220	0.20	2996633	2996276	0.02
3	11 Hr.	3054374	3040938	0.41	3008720	3000424	0.24
4	17 Hr.	3059865	3045670	0.45	3015845	3004279	0.32
5	24 Hr.	3075534	3051643	0.59	3005816	3004586	0.28
6	30 Hr.	3079199	3056235	0.64	2984268	3001200	0.37
7	36 Hr.	3110961	3069736	0.89	3037019	3008050	0.56
8	43 Hr.	3123195	3083855	1.06	3075315	3021164	0.96
9	48 Hr.	3131179	3096656	1.18	3096485	3035791	1.27
10	52 Hr.	3146482	3111092	1.31	3129001	3054651	1.61

Robustness:

The method is robust and found within the acceptable limit the results are summarized in **Table 9** in all modifications, good separation of Apixaban was achieved, and the absolute difference of %Assay value in each modified condition was found to be within acceptable limits.

Table 9: Robustness data by HPLC method

Sr.no	Changes in parameters	Values	% Estimation	Mean	SD	% RSD
1	Change in Column Oven temperature (± 2°C)	32°C	98.6	98.8	1.060	1.072
		28°C	99.0			
2	Change in Wavelength (± 2 nm)	282 nm	100.3	100.3	0.318	0.317
		278 nm	100.3			
3	Change in pH of buffer for mobile phase (± 0.2 unit)	4.0	99.6	99.85	0.742	0.743
		3.6	100.1			

CONCLUSION

The method has been shown to be specific for the determination of % Assay of Apixaban in Apixaban Film Coated Tablet 5 mg. The method has been shown to be Linear, precise and accurate across the suitable analytical range. Standard solution has been seen to be stable for 52 hours when studied at Room temperature and at 10°C.The method has been shown to be robust towards deliberate minor changes in the method parameters. The method can be used in quality control laboratory for release of production batches and stability study.

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