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STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION FOR ANTICOAGULANT DRUG APIXABAN IN APIXABAN DRUG SUBSTANCE AND DRUG PRODUCT

Shriram Pandurang Suryawanshi[1*] and Prof. Dr. Gayatri Barabde[2]

[1&2]Department of Chemistry, The Institute of Science Dr. Homi Bhabha State University, Mumbai, Maharashtra, India. Pin Code 400032.

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***Corresponding Author**
Shriram Pandurang Suryawanshi
Department of Chemistry, The Institute of Science Dr. Homi Bhabha State University, Mumbai, Maharashtra, India. Pin Code 400032.

ABSTRACT

The aimed of this research is to develop the method on reverse phase liquid chromatographic (RP-HPLC) for the assay determination of Apixaban in both drug substance and tablet dosage form. A Inertsil ODS C18 (5 µm particle size, 150 mm x 4.6 mm). was used for the RP-HPLC separation of Apixaban. The mobile phase consisted of a buffer solution pH 4 and methanol in a ratio of 50:50 v/v. The flow rate was set at 1.2 ml/minute, with an injection volume of 10 µL. The column oven temperature was maintained at 40°C, while the autosampler temperature was set to 25ºC. Detection of Apixaban was performed at a wavelength of 230 nm. The developed stability indicating analytical method underwent to validation in accordance with ICH guidelines. **Results:** The chromatographic condition, results showed that the peak retention time of Apixaban was 2.2 min. The linearity of Apixaban was observed in the concentration range of 7.5 µg/mL to 37.5 µg/mL, with a percent accuracy was observed in rangingfrom 100.0% to 101.2%. Stress testing was conducted under acidic, basic, and oxidation conditions. The linearity correlation coefficient and system suitability criteria met the requirements specified by ICH guidelines. **Conclusions:** The developed RP-HPLC method exhibited linearity and accuracy, with correlation coefficients and percent recovery falling within the acceptance criteria. The method proved to be simple, rapid, stability-indicating and cost-effective for thedetermination of Apixaban in both drug substance and tablet form.

KEYWORDS: Apixaban, RP-HPLC, Stability indicating, Assay, Validation, Tablets.

BACKGROUND

The chemical name of Apixaban is 1-(4-methoxyphenyl)–7–oxo–6–[4–(2– oxopi-peridinin-1-yl) phenyl]–4, 5,6, 7–tetrahydro-1H-pyrazolo[3,4–c] pyridine-3-carboxamide, is a white to pale yellow colored powder. It has a molecular formula of C25H25N5O4 and a molecular weight of 459.5 g/mol. Apixaban belongs to the class of oral anticoagulant drugs and selectively inhibits coagulation factor Xa.[1] Refer figure (1).

Apixaban is sold under the brand name Eliquis. Apixaban acts by interfering with the conversion of prothrombin to thrombin, thereby inhibiting the formation of cross-linked fibrin clots. It is specifically indicated for the prophylaxis of deep vein thrombosis and is used for thromboprophylaxis in patients undergoing hip and knee replacement surgeries to prevent blood clots.[2] The approval of Apixaban (Eliquis, Bristol-Myers Squibb/Pfizer) in Europe in May 2011 and in the United States in December 2012 was for the prevention of stroke and systemic embolism in patients with nonvalvular atrial fibrillation (AF). The World Health Organization (WHO) lists Apixaban as an essential medicine.[20] From a survey of the literature, there is a limited number of analytical methods available for its estimation, either as a standalone drug or in combination with other medications.[16,17] Existing analytical method for the quantification of Apixaban include spectrophotometry[4-8], HPLC[9-13], LC-MS[14] and Bioanalytical method[15] are available for active drug substance and drug product. However, there is a need for a method that is rapid, easy to perform, reproducible, accurate, and suitable for stability sample analysis.

This research aims to develop a reverse phase high-performance liquid chromatography (RP-HPLC) method for the estimation of Apixaban in drug substances and finished drugs product. The RP-HPLC technique offers excellent separation and quantification capabilities, making it a valuable tool for pharmaceutical analysis. The goal is to establish a method that fulfills the requirements of being rapid, easy to implement, reproducible, accurate, and capable of analyzing stability samples. The developed method will enable the determination of the amount of Apixaban in drug substances and finished dosage forms.

Overall, this research addresses the need for a comprehensive and suitable analytical method for the estimation of Apixaban, contributing to the advancement of pharmaceutical analysis and ensuring the quality of Apixaban-containing formulations.

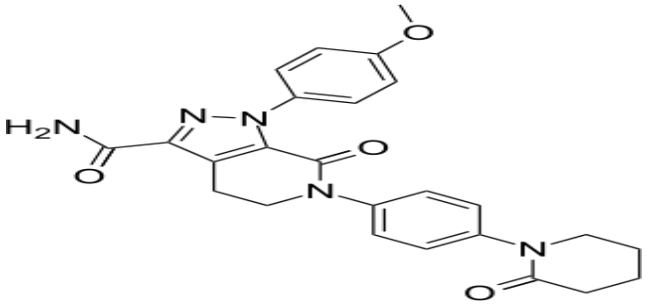

Figure 1: Structure of Apixaban.

METHODS

Method Development

The aimed of this study was to develop a reliable and efficient method for the analysis of Apixaban on a C18 column. different combinations of mobile phases or buffer systems were tried to achieve optimal peak shapes and a suitable run time. It was found that a mobile phase composed of buffer pH 4.0 and acetonitrile (50:50 v/v) provided adequate peak shape with a reduced elution time. Thus, this optimized mobile phase was selected for further analysis. The flow rate was set at 1.2 mL/min to ensure run time was minimized. For optimal separation, an Inertsil ODS C18 column (150 mm x 4.6 mm, particle size 5 µm) was chosen as the stationary phase. different detection wavelengths were evaluated and 230 nm was selected to have the highest absorption for Apixaban. The retention time of Apixaban was found to be 2.2 minutes.

Instrumentation

The HPLC analysis was performed using an Ultraviolet (UV) detector and the Waters Alliance 2695 separation model chromatographic data acquisition system. The data was collected and processed using Empower software.

The optimized chromatographic conditions and system suitability result are summarized in Table 1. This table provides essential information regarding the method used for the analysis of apixaban. These parameters were carefully determined to ensure accurate and reliable analysis of apixaban in both active substance and finished dosage forms.

Table 1: Chromatographic conditions and system suitability data.

Chromatographic Conditions:	
Column:	Inertsil ODS C18 (5 µm particle size, 150 mm x 4.6 mm)
Flow rate (mL/min.):	1.2

Injection volume (µL)	10
Wavelength (nm):	230
Column oven temperature (°C):	40
Autosampler temperature (°C):	25
Run time (min.):	5
Retention time (min.):	2.2
System suitability parameters For Apixaban	
Tailing factor (Limit: ≤ 2.0):	1.37
Theoretical plates (Limit: ≥ 2000):	4572
% Relative standard deviation (% RSD):	0.40

Reagents and chemicals

Apixaban tablets were procured from a commercial source, Medley Pharmaceutical Limited provided the standard. Chemcluse lifescience Pvt Ltd provided HPLC-grade Acetonitrile, AR grade Sodium perchlorate and All the other chemicals were AR grade.

Preparation of Buffer

1.4 g of sodium perchlorate to 1 liter of milli-Q water, mixed thoroughly and adjusted the pH to 4 with perchloric acid.

Preparation of mobile phase

The mobile phase was prepared by mixing the buffer solution and acetonitrile in a ratio of 50:50 (v/v). The mobile phase was degassed before use.

Diluent solution

The diluent solution was prepared by mixing water and acetonitrile in a ratio of 50:50 (v/v).

Blank Solution

The diluent solution was used as a blank solution.

Preparation of Stock standard solution

25 mg of Apixaban working standard was accurately weighed and transferred into a 100 mL volumetric flask. 70 mL of diluent solution was added to this flask and the mixture was sonicated until the standard was completely dissolved. The flask was then diluted to the 100 ml mark with diluent and mixed thoroughly. (Conc.: 250 ppm).

Preparation of Standard solution (Final)

A 5 mL volume of the standard stock solution was transferred into a 50 mL volumetric flask. The flask was then filled up to the mark with the diluent solution, and thoroughly mixed

(Conc.:25ppm).

Preparation of sample solution

Twenty tablets were weighed and the average weight determined. The tablets were then triturated to form a powder. An amount of powder equivalent to 25 mg of Apixaban is transferred into a 100 ml volumetric flask. 70 mL of diluent solution was added to this flask and the mixture was sonicated for 30 min with intermittent shaking. The solution was then diluted to the 100 mL mark with diluent and mixed thoroughly. Then the solution was further diluted, 5 ml of the resulting sample solution was transferred to a 50 ml volumetric flask and the flask was filled to the mark with the diluent solution. The contents were thoroughly mixed. The sample solution was filtered through a 0.45 µm nylon syringe filter prior to analysis. (Concentration: 25 ppm)

METHOD VALIDATION

As specified in ICH guidelines[21], the purpose of method validation is to demonstrate that the method suitability for its intended purpose. The method was validated according to ICH standards, which are used to determine the performance characteristics of a method (expressed in terms of analytical parameters) that meet the requirements of the method's intended application. They were evaluated using the most efficient chromatographic conditions and instruments.

Specificity

Studies the interference of the diluent (Blank), placebo, degradation components, or the presence of impurities with the Apixaban peak using chromatographic spectral peak purity measurements. The chromatograms of the blank, test, and placebo solutions were compared for specificity.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to produce test results that are directly proportional to the concentration of analyte in sample. The Apixaban linearity was determined by analyzing serial dilutions of a standard stock solution. According to Table (2), five concentrations of Apixaban in range 7.5 to 37.5 µg/mL were prepared and analyzed. The limit for the correlation coefficient & %Y-axis intercept should be met as per ICH.

Table 2: Linearity Concentration Levels of Apixaban.

Linearity Level (%)	Standard stock solution (250ppm) volume (mL)	Diluted to (ml)	Final concentration in ppm
Level (25%) - 1	1.5	50	7.5
Level (50%) - 2	2.5	50	12.5
Level (80%) -3	4.0	50	20.0
Level (100%) -4	5.0	50	25.0
Level (120%) -5	6.0	50	30.0
Level (150%) -6	7.5	50	37.5

Accuracy

The analytical method's accuracy or recovery was tested at known analyte concentrations. It is demonstrated by spiking known quantity of actives in the placebo. Accuracy evaluated for three different spiked Apixaban solutions. Solutions were prepared in triplicate at each recovery level. Data obtained from triplicate determinations was collected at 50%, 100%, 150% levels with respective sample concentration. calculated the individual level recovery and mean recovery. Refer tables 3 for accuracy solution preparation.

Table 3: Accuracy Concentration Levels of Apixaban.

Accuracy Level (%)	Placebo Wt. (mg)	Apixaban Added (mg)	Dilutedto (mL)	Volume taken (mL)	Diluted to (mL)	Final Conc. (µg/mL)
Level I (50%)	1030	12.5	100	5	50	12.5
Level III (100%)	1030	25	100	5	50	25
Level III (150%)	1030	37.5	100	5	50	37.5

Precision

The precision or repeatability of an analytical procedure expresses the degree of agreement between a set of measurements obtained from numerous samplings of the same homogeneous sample under prescribed conditions. Six separate tests of Apixaban tablets at a concentration of 25 µg/ml were performed to ensure repeatability of the technique. The mean area and percentage relative standard deviation (RSD) were calculated. The RSD percentage should be below 2%.

Robustness

To order to evaluate the robustness of the analytical method, deliberate variations of method parameters were presented. The following parameters were tested for their influence on the

analysis of Apixaban like mobile phase composition, buffer solution pH, mobile phase flow rate, and detection wavelength. These variations were designed to simulate potential minor changes that may occur during routine laboratory operations. System suitability parameters and chromatographic performance were assessed under these modifications conditions for ensuring the reliability and robustness of the method.

RESULTS

The goal of the method validation was to demonstration that the method can be used for its declared purpose, as per ICH guidelines. The proposed stability indication method for the assay determination of apixaban in drug substance and drug product was developed and validated for specificity, accuracy (repeatability), linearity, accuracy and robustness and Force degradation in accordance with ICH guidelines. The results for the individual parameters are shown below.

Specificity

Chromatograms of the blank, reference, and test solutions are comparing. No peak interference is observed at the retention time of Apixaban. The retention time of the main peak in the chromatograms of the reference solution and the tested solution is the same. This proved the method's specificity. Refer to figures (2) to (5).

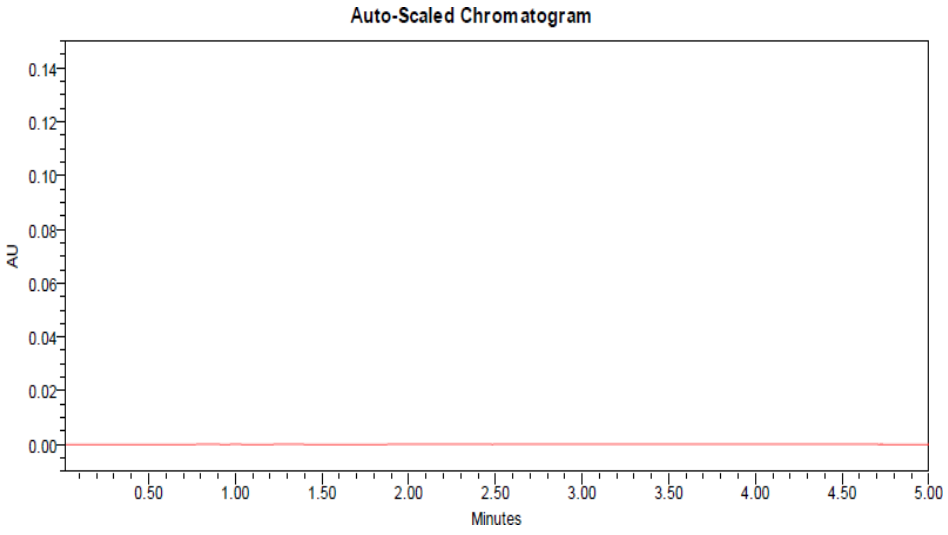

Figure 2: Representative Chromatogram of Blank.

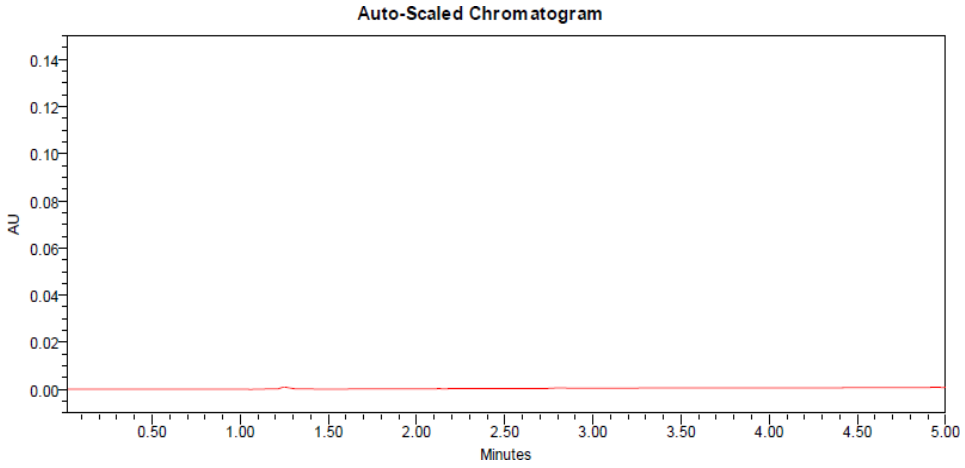

Figure 3: Representative Chromatogram of Placebo Sample.

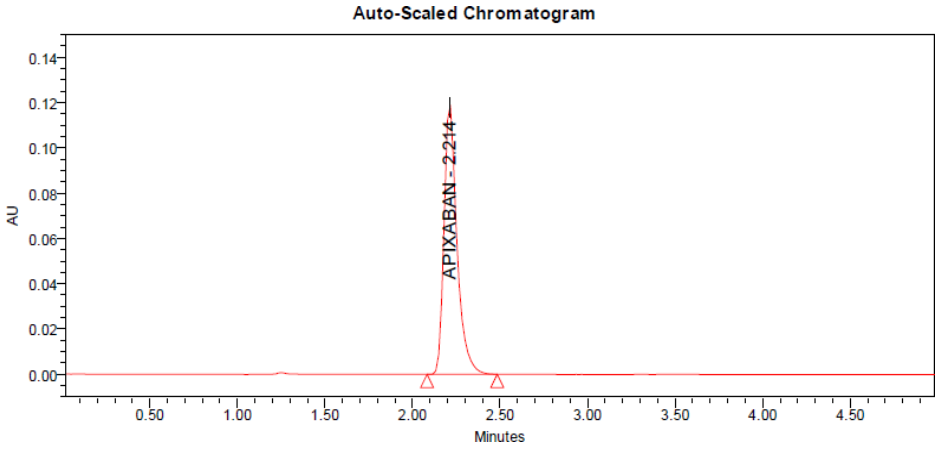

Figure 4: Representative chromatogram of Apixaban standard

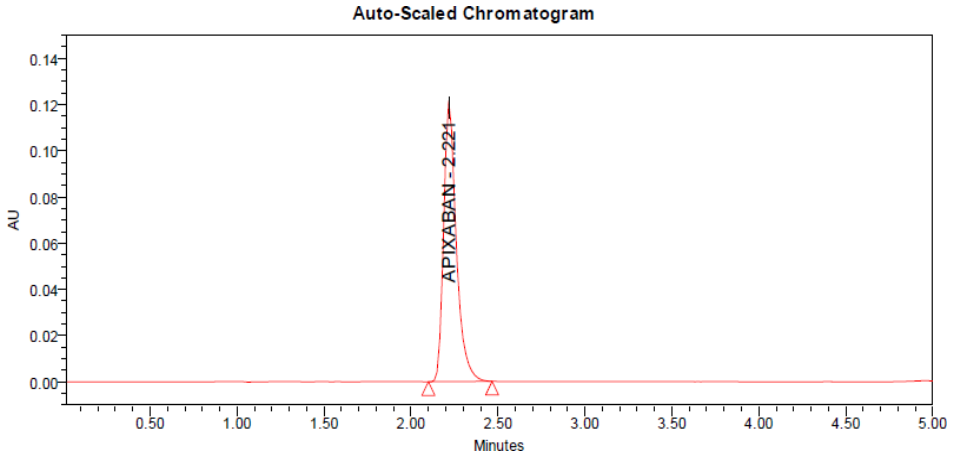

Figure 5: Representative chromatogram of Apixaban sample.

Linearity

Five concentrations of Apixaban, including 7.5, 12.5, 20.0, 25.0, 30.0 and 37.5 µg/ml, were prepared, and a linearity graph of concentration versus peak area was plotted as shown in

figure (6). The graph of residuals against concentration was also plotted, as seen in figure 7. A linearity correlation between peak areas and analyte concentration was determined in the range of 25% to 150% (7.5-37.5 µg/ml). The outcomes are shown in Table (4).

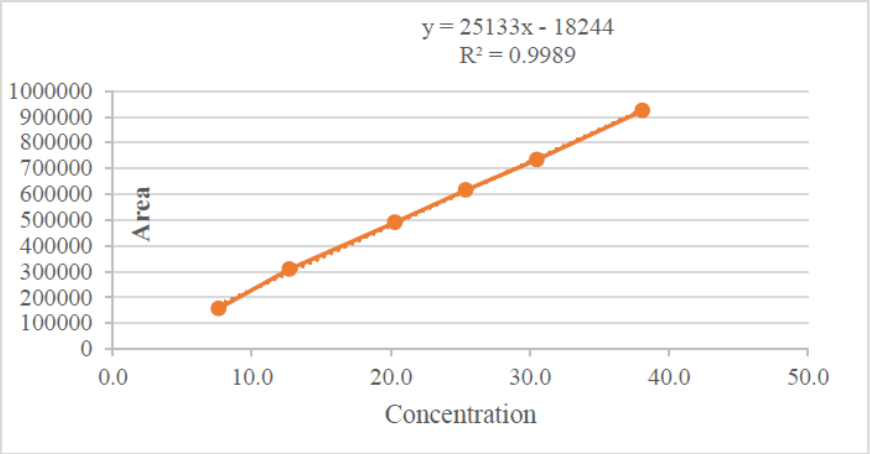

Figure 6: Linearity plot for Apixaban.

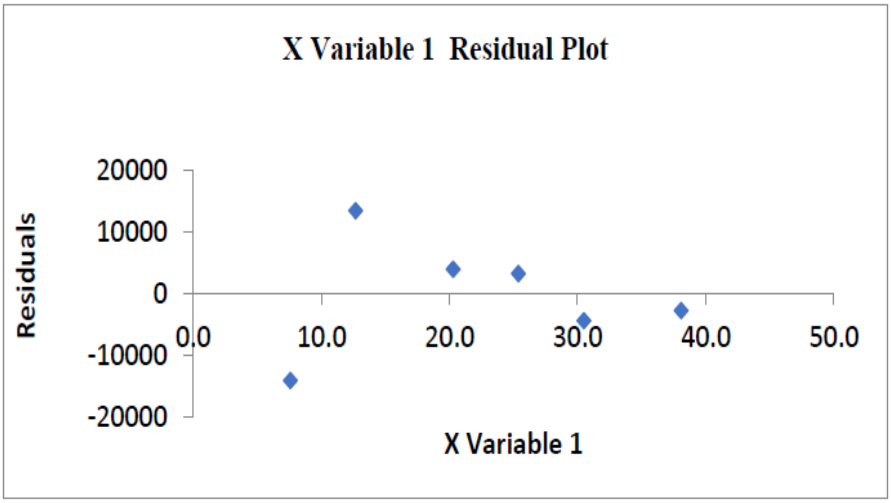

Figure 7: Plot of Area Residuals against concentration of Apixaban.

Table 4: Linearity of Apixaban.

Linearity Parameter	Values	Limits
Correlation coefficient R	0.9995	≥ 0.999
%Y –intercept	-3.39	≤ ± 5 %
Regression line Slope	24984.59	To be report

The method was considered linear in the range of 7.5 to 37.5 µg/mL for apixaban because the correlation coefficient and % Y-intercept were within the acceptable range.

Accuracy

By assessing the accuracy of Apixaban, the recoveries of the assay method were confirmed. The mean and individual recovery values for Apixaban were calculated. The methods were found to be accurate as the percentage of individual recovery and average recovery are within the acceptable criteria (100.0 to 101.2%) as shown in Table 5. It demonstrated that the method is unaffected by excipients present in the formulation.

Table 5: Accuracy for Apixaban Tablets.

(%) Accuracy	% Recovery	% Means recovery	% Minimum recovery	% Maximum recovery
50%	100.4	100.4	100.0	100.8
	100.0			
	100.8			
100%	101.0	101.0	100.9	101.0
	100.9			
	101.0			
150%	101.2	100.9	100.7	101.2
	100.8			
	100.7			

Precision

The method exactness as defined by precision and method was precise as since the percentage relative standard deviation from six individual sample preparation at same condition was well within the acceptance limit ±2 %. Refer table (6).

Table 6: Apixaban Tablets Method Precision.

Sample preparation	Apixaban in Tablets (%)
Sample Preparation-1	100.6
Sample Preparation -2	100.8
Sample Preparation -3	101.1
Sample Preparation -4	101.4
Sample Preparation -5	101.0
Sample Preparation -6	101.1
Mean	101.0
STD Dev	0.28
% RSD	0.28

Robustness studies

Method proved to be robust as all evalauted robustness parameters met the system suitability criteria. The robustness of the developed RP-HPLC method was demonstrated by the fact that

the deliberate modification of parameter had no significant effect on its performance. The outcomes are shown in Table (7).

Table 7: Apixaban Tablets Robustness Result.

Robustness Parameter	% RSD System Suitability	% Apixaban in tablets
Optimized condition		
Normal Condition	0.28	101.0
Change in pH		
pH 4.2	0.42	99.5
pH 3.8	0.74	99.0
Change in Flow rate		
1.0 mL/Minutes	0.20	99.3
1.4 mL/minutes	0.39	100.5
Change in Wavelength		
228 nm	0.40	100.4
232 nm	0.47	100.4

Filter selection study

Filter analysis was conducted on a sample solution of Apixaban Tablets. The samples were prepared with a centrifuge and nylon filter. The subsequent results were obtained. The outcomes were displayed in Table (8).

Table 8: Filter selection study.

Parameter	% Assay	% Variation
Centrifuge	100.7	-
Test Filtered through Nylon 0.45 µm	100.8	0.10
Test Filtered through PVDF 0.45 µm	101.0	0.20

Solution stability study

Sample and standard preparations were tested against a freshly prepared standard preparation for 48 hours. The observed results are within the acceptable range (±2%). Refer table (9).

Table 9: Apixaban Solution stability study.

Solution Stability	% Assay Apixaban Standard	% Assay Apixaban Test Sample
Initial	99.4	100.7
48 Hrs	99.8	101.5
% Variation	0.40	0.8

Sample Stress Studies

In accordance with the International Conference on Harmonization (ICH) guidelines on stability testing, stress degradation studies were conducted on Apixaban Tablets using the developed method to evaluate the inherent stability characteristics of the active substance.

Acidic Degradation

For Acidic degradation study, a amount of Apixaban tablets grinded fine powder equivalent to 25 mg was transferred into a 100 mL volumetric flask. added the 30 mL of diluent. The mixture was sonicated for 30 minutes with occasional shaking. Then, 2 mL of 5N hydrochloric acid solution was added to the volumetric flask and thoroughly mixed. The flask was placed in a water bath at 80°C for 30 minutes. After cooling the sample solution, 2 mL of 5N sodium hydroxide solution was added to neutralize the acidic environment. The solution was then diluted with diluent to the mark, mixed properly, and further diluted by transferring 5 mL of the sample solution into a 50 mL volumetric flask. The flask was filled with diluent to the mark and thoroughly mixed. The sample solution was filtered through a 0.45 µm nylon syringe filter prior to analysis (Concentration: 25ppm). refer figure (8).

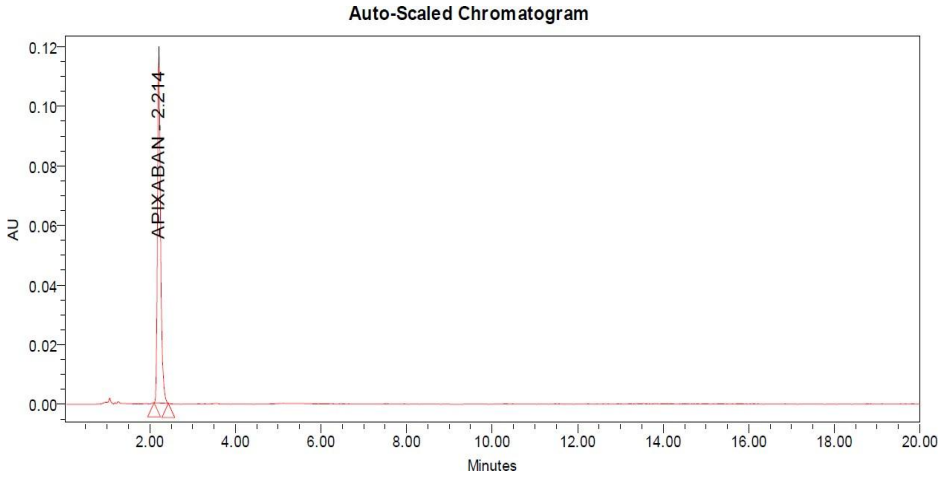

Figure 8: Representetive chromatogram of Apixaban Acidic degradation Sample.

Base Degradation

For base degradation study, a amount of Apixaban tablets grinded fine powder equivalent to 25 mg was transferred into a 100 mL volumetric flask. Added the 30 mL of diluent. The mixture was sonicated for 30 minutes with occasional shaking. Then, 3 mL of 5N sodium hydroxide solution was added to the volumetric flask and thoroughly mixed. The flask was placed in a water bath at 80°C for 30 minutes. After cooling the sample, 3 mL of 5N hydrochloric acid solution was added to neutralize the alkaline environment. The solution

was then diluted with diluent to the mark, mixed properly, and further diluted by transferring 5 mL of the sample solution into a 50 mL volumetric flask. The flask was filled with diluent to the mark and thoroughly mixed. The sample solution was filtered through a 0.45 µm nylon syringe filter prior to analysis (Concentration: 25ppm). refer figure (9)

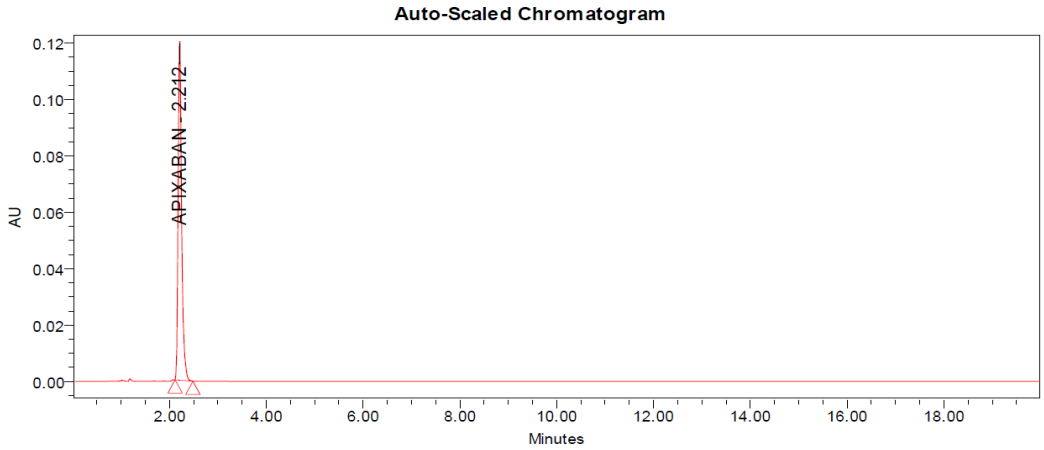

Figure 9: Representetive chromatogram of Apixaban Base degradation Sample.

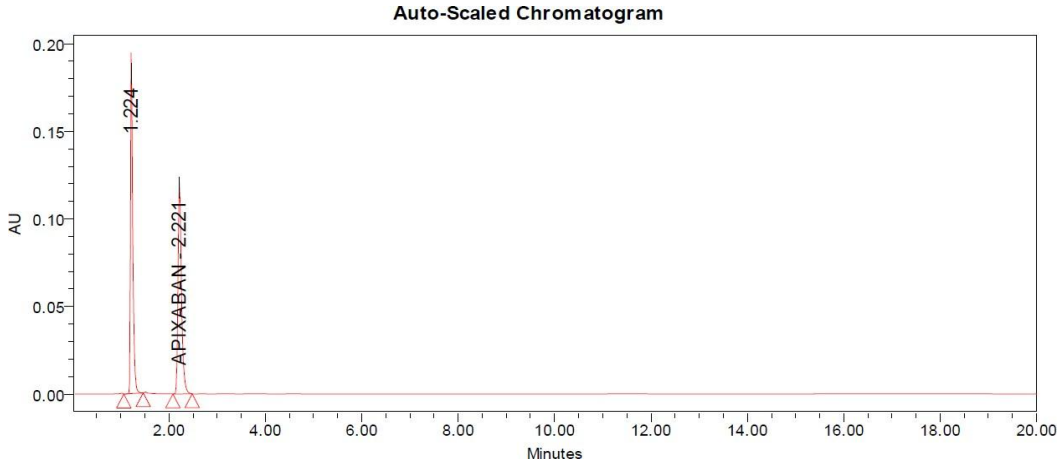

Figure 10: Representetive chromatogram of Apixaban Oxidation degradation Sample.

Oxidative Degradation

For oxidation degradation study, equivalent to 25 mg was transferred into a 100 mL volumetric flask added the 30 mL of diluent. The mixture was sonicated for 30 minutes with occasional shaking. Then, 2 mL of 30% hydrogen peroxide solution was added to the volumetric flask and thoroughly mixed. The flask was kept at room temperature for 24 hours. The solution was then diluted with diluent to the mark, mixed properly, and further diluted by transferring 5 mL of the sample solution into a 50 mL volumetric flask. The flask was filled with diluent to the mark and thoroughly mixed. The sample solution was filtered through a 0.45 µm nylon syringe filter prior to analysis (Concentration: 25ppm). refer figure (10).

Table 10: Force degradation for Apixaban Tablets.

Stress Condition	%Degradation
Acidic Degradation	2.5
Alkaline Degradation	2.9
Oxidative Degradation	0.6

DISCUSSION

The development and validation of the RP-HPLC method for the analysis of Apixaban was successfully completed in accordance with ICH guidelines. The method exhibits desirable properties such as specificity, linearity, precision, accuracy, robustness and solution stability. Specificity testing confirmed that there were no interfering peaks that could affect the retention time of Apixaban, indicating the method's specificity. The linearity of the method was established over the concentration range of 7.5 - 37.5 µg/mL for Apixaban, demonstrating its ability to produce assay results that are directly proportional to analyte concentration.

The precision of the method were evaluated through reproducibility. The method shown good precision with small percentage relative standard deviation (RSD), indicating its repeatability. The accuracy of the method were evaluated through recovery rate determination at different analyte concentration levels (50%, 100%, and 150%). The accuracy of the method was demonstrated by the satisfactory recovery rates at the different spiked levels, indicating its ability to accurately quantify Apixaban in the presence of other matrix components.

The method also showed robustness as it remained unaffected by minor variations in optimized method parameters such as mobile phase composition, flow rate, pH, and wavelength. This indicates its reliability under normal conditions and provides flexibility in method execution.

Furthermore, the solution stability of the reference and test solutions was evaluated and found to be acceptable for up to 48 hours, indicating the method's suitability for analysis over a reasonable time period.

CONCLUSIONS

In summary, the developed RP-HPLC method for the analysis of Apixaban demonstrates several key attributes that make it suitable for routine analysis in the pharmaceutical industry. It is a simple, selective, linear, precise, accurate, robust, and stability-indicating method,

aligning with the ICH guidelines.

The method introduces a sample procedure with short run time to economic used for efficient analysis of a large number of samples in the quality control department. This contributes to cost and time savings in the analytical process. The method's reliability and robustness make it suitable for the routine analysis of Apixaban in both drug substance (bulk) and drug product (pharmaceutical dosage form).

Overall, the developed Apixaban assay method provides a valuable tool for pharmaceutical companies, facilitating the efficient and reliable analysis of Apixaban in various pharmaceutical formulations.

ABBREVIATIONS

RP-HPLC: Reverse phase high performance liquid chromatography, RSD: Relative standard deviation, NMT: not more than, NLT: Not less than, UV: Ultraviolet, HPLC: High performance liquid chromatography, UPLC: Ultra-performance liquid chromatography, LCMS: Liquid chromatography-mass spectrometry, HPTLC: High-performance thin layer chromatography ICH: International Council for Harmonisation, PL: Placebo.

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