IJPSR (2021), Volume 12, Issue 1 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 29 December 2019; received in revised form, 05 April 2020; accepted, 10 October 2020; published 01 January 2021

DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING RP-HPLC METHOD OF APIXABAN IN COMMERCIAL DOSAGE FORM

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Keywords:

Apixaban, Validation, Stability, HPLC, Eliquis

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ABSTRACT: The aim of this study is to develop a sensitive, specific, rapid, and precise reverse-phase high-performance liquid chromatography (RP-HPLC) method and validate it for the quantification of process-related and degradation impurities of apixaban; an anticoagulant drug. The chromatographic separation was achieved on a Sigma-Aldrich's Ascentis Express® C18 (4.6 mm × 100 mm, 2.7 µ) HPLC column with a runtime of 40 min. Mobile phase-A and mobile phase-B were phosphate buffer and acetonitrile, respectively. The column oven temperature was set at 35 °C, and the photodiode array detector was set at 225 nm. The newly developed method was utilized to detect nine process-related impurities (Imp-1 to Imp-9) in a test sample of Apixaban. Forced degradation study was carried out under acidic, alkaline, oxidative, photolytic, and thermal conditions to demonstrate the stability-indicating nature of the developed RP-HPLC method. The developed method was validated as per ICH guidelines and found to be specific, precise, sensitive, and robust. In conclusion, the RP-HPLC method was successfully developed and validated then effectively applied to analyze both; Apixaban drug substance and product.

INTRODUCTION: Apixaban is an anticoagulant drug chemically known as l-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-l-yl) phenyl]- 4, 5, 6, 7-tetrahydro- lH-pyrazolo [3, 4-c] pyridine- 3- carbo-xamide **Fig. 1**. [1]

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.12(1).241-51

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.12(1).241-51

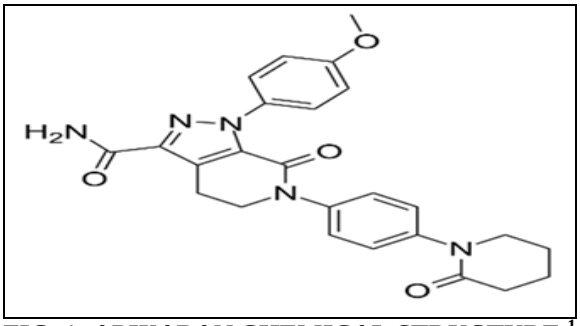

FIG. 1: APIXABAN CHEMICAL STRUCTURE [1]

"Eliquis", the trade name of apixaban, is prescribed to treat atrial fibrillation (a heart rhythm disorder)

and to lower the risk of stroke caused by blood clots. It is a selective, reversible, direct inhibitor of factor X_a indicated to reduce the risk of stroke and systemic embolism in patients with non-valvular atrial fibrillation [2, 3]. Furthermore, it is indicated to prevent deep vein thrombosis (DVT), which may lead to pulmonary embolism, especially after hip or knee replacement surgery [4-6]. It was designed by Aderis Pharmaceuticals and jointly developed by Pfizer and Bristol-Myers Squibb. "Eliquis" was officially permitted in the USA and Europe on December 2012 and January 2010, respectively [7].

During the development of any drug substance or product in the pharmaceutical industry, the development of an accurate and efficient analytical method to determine the quality of the product is a crucial step [8]. The current research focused on the development of an efficient analytical method using a core-shell chromatography column for the quantitative analysis. Silica particles are mostly used in ultra-pressure liquid chromatography (UPLC) columns. They are porous with a particle size of sub-2 µ, thus yields good resolution, speed, sensitivity, and at the same time, they offer high backpressure [9-11]. The cost of UPLC investment and its columns for routine analysis is higher than that of conventional HPLC and its columns. Hence, core-shell columns have been preferred to overcome such limitations [12, 13]. Several analytical methods reported in the literature described the investigation of the pharmacokinetics of apixaban. Most of them were in the content of Apixaban and/or its metabolites investigated in human plasma by liquid chromatography-mass spectrometry method [14-16]. Nevertheless, researchers did not succeed in providing the details of process and degradation related impurities due to the employed stress conditions. One of the articles reported on stability, indicating the HPLC method for apixaban bulk drug sample, found the method to be non-selective at our end [17]. Hence, there is a need for the development of a selective, fast, and stability-indicating HPLC method on the core shell column.

To the best of our knowledge, no method on the core-shell column has been reported for the determination of apixaban and its potential process-related impurities in drug substance and drug product for regular analysis and stability studies in quality control laboratories.

The main objective of the current research was to develop a precise, fast, sensitive, and stability-indicating RP-HPLC method for the determination of process and degradation related impurities of apixaban. And to validate the method according to "USP Validation of Compendia Procedures" and ICH guidelines [18-23].

MATERIALS AND METHODS:

Chemicals and Reagents: Apixaban reference standard was kindly given as a gift sample from United Pharmaceuticals. Methanol and acetonitrile (HPLC grade) Milli-Q water. Hydrochloric acid HCL (AR) 37%, sodium hydroxide NaOH (AR-grade), ortho-phosphoric acid (HPLC - grade) 85%, ammonium HOAC $-NH_3$ acetate (GR-Q), potassium dihydrogen phosphate -$KHPO_4$(AR-grade), hydrogen peroxide H_2O_2 (ACS-Q) 50%, formic acid HCOOH (AR-Q), triethylamine TEA (HPLC- grade), sodium lauryl sulphate Na-L-SO_4, polysorbate Na-L-PO_4- 80 (GR-Q) were procured from Merck-R, Fischer Scientific-R and Sigma -J.

Instruments: The HPLC system used was an Agilent 1260 infinity equipped with a quaternary solvent pump, a thermostated autosampler, a degasser, UV/VWD detector, and a column oven. A double beam UV-Visible spectrophotometer (Shimadzu 1800), USP Type II-Paddle apparatus (Labindia DS2000 with Ismatec high precision multichannel pump as autosampler), photostability chamber (Atlas Sun test XLS+), HPLC water purifier (Millipore–Merck Millipore), orbital shaker (Thermo scientific) and a centrifuge (REMI research centrifuge) were used for this analysis.

Preparation of Solutions:

UV Studies:

Preparation of Standard Stock Solution (10 ppm): Apixaban reference standard (1 mg) was accurately weighed and transferred into a 100 ml volumetric flask. 30 ml solvent (acetonitrile: water 90:10 v/v) was added, and the mixture was sonicated. The solution was diluted up to the mark with the diluents to give the standard stock solution.

The λ_{max} was determined using a UV-VIS spectrophotometer. A working standard range from 2 to 7 ppm was prepared from the stock solution of 10 ppm and used for linearity studies.

Preparation of Standard Stock Solution (90 ppm): Apixaban reference standard (25 mg) was weighed and transferred into a 25 ml volumetric flask to obtain a solution of 1000 ppm in duplicate. 18 ml of methanol was added and sonicated. This solution was diluted to volume with methanol and mixed well. 9.0 ml of standard stock solution was taken and diluted to 100 ml with diluent to give a standard stock solution of 90 ppm.

Preparation of Sample Solution: Five apixaban tablets were weighed and transferred to 250 ml volumetric flask. Then, 100 ml of water was added and sonicated for 1 h to disperse the contents. Further, 50 ml of water was added, and the flask was kept in an orbital shaker at 50 rpm for 2 h at room temperature (RT). The flasks were made up to the mark with water. The solution was then filtered using a 0.45 µ Whatman Teflon filter. The filter was saturated by discarding the first 10 ml of filtrate. 5.0 ml of the above filtrate was further diluted to 100 ml with diluents and mixed well to get the desired concentration.

Compatibility Studies of Filters: Filter compatibility tests were conducted to get a particle-free solution, which also should reproduce a 100% response as per the respective content. The filtrate from each filter was compared with the centrifuged sample. The filter complying with all the parameters and showing the same concentration as that of the centrifuged sample was selected for further studies. Five intact tablets were weighed and transferred to 250 ml volumetric flask. 25 ml of miliQ water was added and sonicated for 10 min. 150 ml of acetonitrile was added and sonicated for 20 min with intermittent swirling. This was further diluted with acetonitrile mixed and filtered using a 0.45µ Whatman Teflon filter. 10 ml of filtrate was discarded, and a further 5 ml was diluted to 100 ml with diluent. A 20 ml aliquot was subjected to centrifuge at 5000 rpm for 20 min, and another 20 ml per filter was passed through the filters. The four solutions were chromatographed and were compared for peak shape and peak area. The filter that matched the centrifuged sample data was selected for further studies.

Selection of Diluent (HPLC Compatible Solvent) and Solution Stability Studies: The stability of the solution under study was established by keeping the solution at room temperature for 24 h. A standard solution was prepared using acetonitrile: water (90: 10 v/v, 50: 50 v/v, and 10:90 v/v), methanol: water (90: 10 v/v, 50: 50 v/v and 10: 90 v/v). The samples were injected at different time intervals of 5 min, 1 d, and 1 w.

Selection of Flow Rate and load Volume: Flow rates (0.8 ml/min. and 1.3 ml/min.) and loading volumes (1, 4, 5, 6, 10, 20, 50 µl) were analyzed to get a precise and reproducible peak of apixaban with minimum tailing or fronting.

Selection and Optimization of Mobile Phase: Various mobile-phase compositions like acetonitrile: water (50: 50v/v, 90: 10 v/v, 60: 40 v/v, 40: 60 v/v and 10: 90 v/v), acetonitrile: methanol: water (80: 10: 10 v/v, 50: 25: 25 v/v, 30: 40: 30 v/v, 40:30: 30 v/v and 10: 45: 45 v/v), acetonitrile: TEA buffer in water (50:50 v/v ratio with 0.25 ml and 0.5 ml concentration of TEA per 500 ml), acetonitrile: TEA and FA buffer in water (50: 50 v/v ratio with0.25 ml: 0.25 ml and 0.5 ml: 0.5 ml concentration of TEA and FA per 500 ml.), acetonitrile: buffer [pH range 3 to 7.5] (50: 50 v/v, 80: 20 v/v and 20: 80 v/v) were evaluated in an effort to get symmetrical, sharp and reproducible peaks of apixaban.

Selection and Optimization of Stationary Phase: Using the temporary developed chromatographic method a variety of columns with different attributes like polarity, particle size (3.5 µ and5 µ), carbon loading (C8 and C18) and dimensions were screened in an effort to arrive at the best column for the given analysis. Some of the columns studied were; Symmetry C18 50 × 4.6 mm 3.5 µ, Akzonobel - Kromasilc C18 150 × 4.6 mm 5 µ, Waters Xterrac C18 150 × 4.6 mm 3.5 µ, BDS Hypersilthermoc C8 50 × 4.6 mm 5 µ, FortiscC8 50 × 4.6 mm 5µ, and Agilent zorbaxc RX C18 150 × 4.6 mm, *etc*.

Selection of Dissolution Media: Apixaban standard solution was prepared, and the final dilution was done using water, 0.1 N HCl, pH 4.5 buffer, pH 6.8 buffer, SLS media and tween media, respectively. Five tablets were weighed and transferred to a 250 ml volumetric flask. 100 ml of water was added and sonicated for 1h to disperse the contents. Further, 50 ml of water were added,

and the flask was kept in an orbital shaker at 50 rpm for 2 h at room temperature. The solutions were made up to the mark with water. The solutions were mixed well and filtered using a 0.45µ Whatman Teflon filter. The filter was saturated by discarding 10 ml of filtrate, and 5.0 ml of the above solution was diluted to 100 ml with diluent and mixed well. Six tablets were weighed and transferred to each jar of Dissolution Rate Apparatus USP II was chosen, and the temp. was maintained at 37 °C, stirring rate 75 rpm, and the volume of dissolution media was 900 ml. At specified intervals of time (5, 10, 20, 30, 45, and 60 min), 10 ml of sample aliquots were withdrawn and replenished with the same volume of fresh media. The samples were filtered with a 0.45µ Whatman Teflon filter. Around 8 ml of the sample was discarded to saturate the filter. Similarly, the procedure was repeated using 0.1 N HCl, pH 4.5 buffer, pH 6.8 buffer, 0.1% SLS media, 0.2% SLS media and 0.1% Tween 80 media, 0.2% Tween 80 media. The release of the apixaban into the respective media was calculated using the formula;

$$\text{Release} = At/As \times Ws/25 \times 25/5 \times P/100 \times 100/LC$$

Where, AT = Peak area response of apixaban in the chromatogram obtained from test solution, AS = Average peak area response of apixaban in the chromatograms obtained from replicate injections of standard solution, WS = Weight of apixaban standard taken in mg in the standard stock solution, P = % Purity of apixaban standard, LC =Label claim of apixaban in mg per tablet.

Assay of Apixaban Tablets: The samples were analyzed using the developed chromatographic method, and the % content of the drug in the tablets was determined.

TABLE 1: GRADIENT SEQUENCE FOR RELATED SUBSTANCES STUDIES

Time	% acetonitrile	% formic (FA) : Triethylamon (TEA) 1:1 v/v in warwe
0	4	55
10	20	80
20	50	50
30	80	20
40	45	55

0.1 mg of known impurity (Acetal impurity, RS-13) was accurately weighed and transferred to a 10 ml volumetric flask. 5 ml of diluent was added, sonicated, and diluted to volume with diluent to get 10 ppm of impurity solution. The chromatograms of apixaban reference standard, drug sample and known impurity solutions were compared using the gradient run below in **Table 1**.

Related Substances Studies: The isocratic mode of the RP-HPLC method was modified to a gradient mode as given below with a run time of 40 min.

Forced Degradation Studies: With slight modifications, the developed chromatographic method was used to get accurate results. The optimized chromatographic method was kept same and run time was 12.0 min. Forced degradation studies of apixaban were conducted using solution stressors like distilled water, 0.1 N HCl, 0.1 N NaOH, hydrogen peroxide 50%. Photostability studies were conducted at a flux of 4.6 × 1014 photons for 24 h, and thermostability studies were conducted at a temperature of 80 °C for 24 h. The results were compared with the untreated standards. Five tablets were weighed accurately and transferred to 250 ml volumetric flask. 25 ml miliQ water was added and sonicated for 10 min. 150 ml of acetonitrile was added and sonicated for 20 min with intermittent swirling. 25 ml of the respective stressor was added and heated at 80 °C for 24 h on a water bath previously maintained at 80 °C. The sample was cooled to RT and neutralized. It was filtered using a 0.45 µ Whatman Teflon filter. 10 ml of filtrate was used for filter saturation, and the remaining 7 ml was used. 5 ml of filtrate was diluted to100 ml with diluent. The results were compared with the untreated sample. For the solid degradation analysis, the tablets were treated with the respective stressor, and the results were compared with the untreated sample.

Validation Studies:

Accuracy: The accuracy of the method was determined by calculating % recovery. A known amount of apixaban was added to a placebo and the amounts were estimated by measuring the peak area. These studies were carried out in triplicate over the specified concentration range and the amount of apixaban was estimated by measuring the peak area ratios. The percentage recovery and standard deviation of percentage recovery was calculated.

Precision: The precision of the method was determined in terms of Intra-day and inter-day precision. For intra-day precision studies, a standard solution of 90 ppm was injected at various time intervals and percent related standard deviation (%RSD) was estimated. The inter-day precision was studied by injecting the same concentration of the standard solution on consecutive days, and the % RSD of the signal was calculated. The repeatability, intermediate precision, and reproducibility of the developed method was determined

Specificity: Specificity is the ability to assess the analyte unequivocally in the presence of components *etc*. The blank (diluent), placebo, standard (90 ppm), sample (90 ppm) were prepared and injected to prove that the method developed was specific to apixaban

Linearity and Range: The linearity of the method was determined at six concentration levels ranging from 1-7 ppm of apixaban. A regression line was plotted of peak area v/s concentration. The correlation coefficient and equation of the regression line were calculated. The interval of lowest assessed concentration to the highest is the linearity range of the procedure.

Limit of Detection (LOD) and Limit of Quantification (LOQ): 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. 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. Where, = the standard deviation of the response, Slope = slope of the calibration curve.

Robustness: Robustness of the developed method was studied by changing the flow rate and column temperature. The effect of flow rate was studied by keeping all chromatographic conditions the same except the flow rate, *i.e.,* 1.2 ml/min and in the next run 1.4 ml/min, respectively. Similarly, the effect of temperature was studied by keeping all chromatographic conditions the same except the temperature, *i.e.,* 35 °C and in the next run with 25 °C respectively.

System Suitability: The system suitability parameters like retention time, the number of USP theoretical plates, USP tailing, peak area, and peak height were evaluated.

RESULTS AND DISCUSSION:

UV Studies: The UV spectrum of apixaban in acetonitrile: water (90: 10 v/v) indicated the λ_{max} to be 256 nm **Fig. 2**. The response was linear, and the regression equation was found to be y = 0.1331x + 0.0858, as seen in UV-scan, **Fig. 2**.

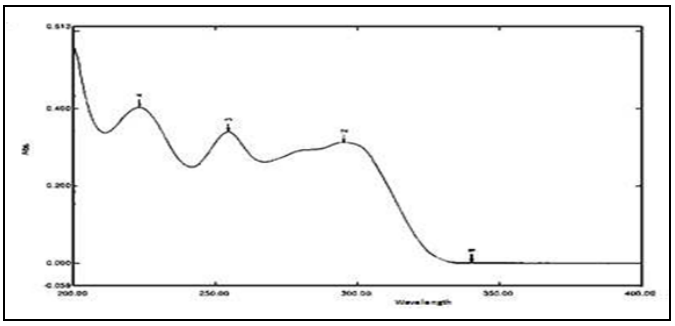

FIG. 2: UV-SPECTRUM SCAN OF APIXABAN IN ACETONITRILE TO WATER (90:10 v/v) IN CALIBRATION CURVE

Filter Compatibility Studies and Stability: A temporary chromatographic method was used for the selection of the best filter. Whatman Nylon w/GMF membrane filter pore size of0.45µ was selected after comparing the peak area and concentration **Table 2** of centrifuged drug solution to other drug samples.

TABLE 2: FILTER COMPATIBILITY STUDIES

Sample no.	Sample analyzed form	Concentration (ppm)	Peak Area
1	Centrifuge	89.631134583	0.45µ
2	Whatman	89.621134437	Nylon w/GMF

Based on these observations, acetonitrile: water; 90: 10 v/v was selected as the diluent for further studies. The stability of the drug sample was determined by keeping the sample in contact with the diluent for 5 min, 24 h and 1 w, which indicated no change in the concentration **Fig. 3-5** and **Table 3**.

TABLE 3: EFFECT OF DILUENT ON CHROMATOGRAM AFTER A SPECIFIC TIME INTERVALS

Sample no.	Analysis time (after)	Retention time (min)	Peak Area
1	5 min	9.010	1134437
2	24 min	9.010	1134437
2	one min	8.010	1134432

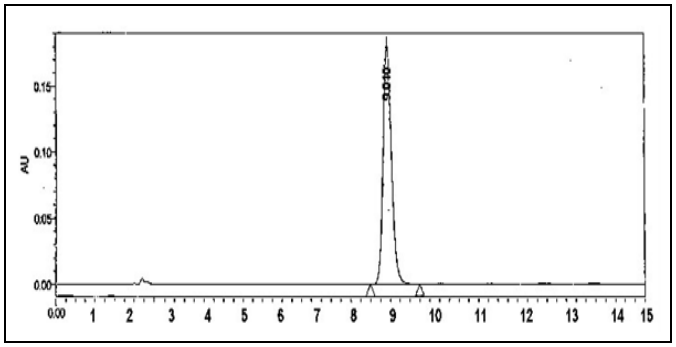

FIG. 3: CHROMATOGRAM OF APIXABAN IN STANDARD SOLUTION IN CONTACT WITH DILUENT AFTER 5 min

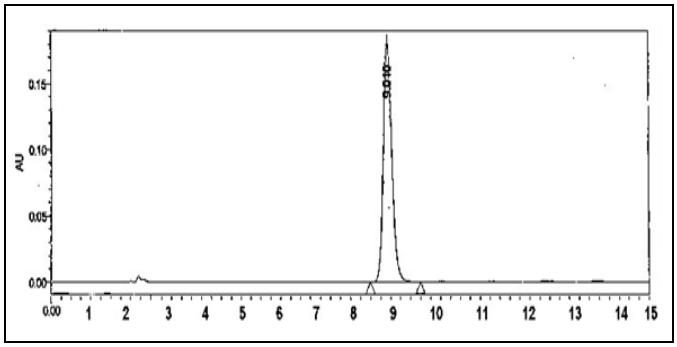

FIG. 4: CHROMATOGRAM OF APIXABAN IN STANDARD SOLUTION IN CONTACT WITH DILUENT AFTER ON DAY (24 h)

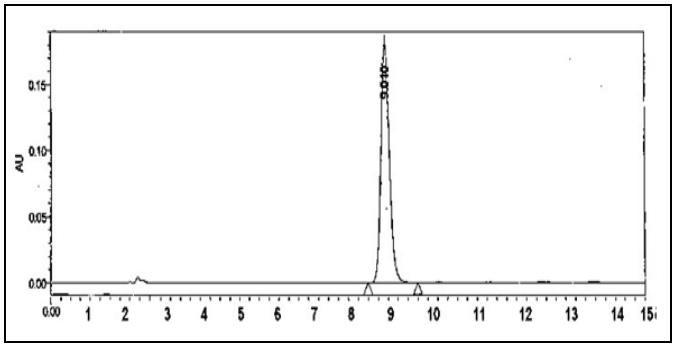

FIG. 5: CHROMATOGRAM OF APIXABAN IN STANDARD SOLUTION IN CONTACT WITH DILUENT AFTER SEVEN DAYS (ONE WEEK)

Selection of Mobile Phase and Stationary Phase: According to the observations of peak shape and retention time as **Fig. 6**, a flow rate of 1.3 ml and loading volume 10 µl was selected. Based on the data in **Table 5**, acetonitrile: formic acid: tri-ethylamine in water 50: 50 v/v (0.5 ml: 0.5 ml) was selected as the mobile phase for the analysis as shown in **Fig. 7**.

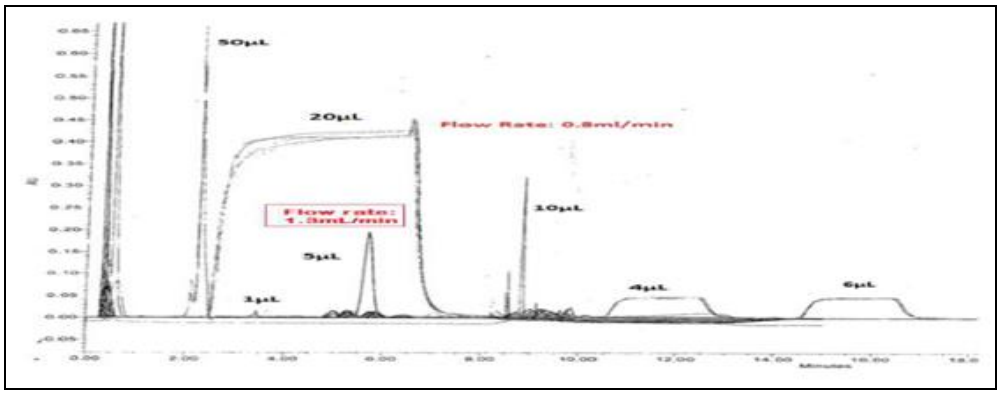

FIG. 6: FLOW RATE EFFECT AND VOLUME LOADING ON SHAPE OF PEAK FOR APIXABAN

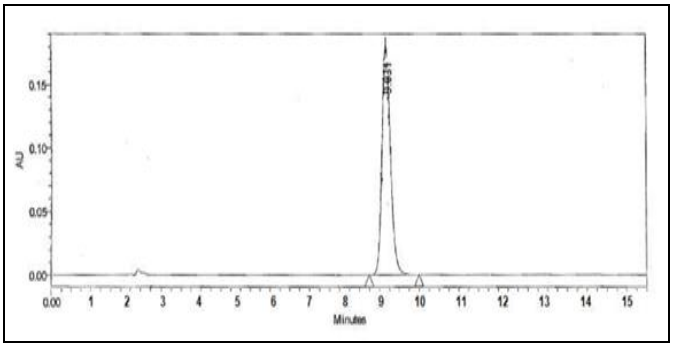

FIG. 7: APIXABAN CHROMATOGRAM WITH ACETONIT-RILE ACN: FORMIC ACID: TRI-ETHYLAMINE (BUFFER) IN H_2O_2 50: 50 v/v (0.5 ml: 0.5 ml) -MOBILE PHASE

A variety of columns with different attributes like polarity, particle size, carbon loading, and dimensions were screened in an effort to arrive at the best column for developing the analytical method of apixaban in API and tablet formulation. **Table 4** Akzonobel Kromasilc C18 150 × 4.6 mm 5µ was selected as the column for the analysis of Apixaban as **Fig. 8**.

Table 5 shows the results of the effect of the stationary phase on the chromatogram.

TABLE 4: EFFECT OF MOBILE PHASE COMPOSITION ON CHROMATOGRAM

Sample no.	Mobile phase composition	Observation	
		Retention time	Peak shape
1	Acetonitrile: water in 50:50 v/v	8.913	Tailing
2	Acetonitrile: water 90 : 10 v/v	6.341	Sharp
3	Acetonitrile: water 60:40 v/v	6.341	Tailing
4	Acetonitrile: water in 40:60 v/v	12.252	Sharp
5	Acetonitrile: water in 10:90 v/v	11.472	Shoulder
6	Acetonitrile: methanol : water 80:10 :10 v/v	9.001	Sharp
7	Acetonitrile: methanol : water 50:25:25 v/v	7.882	Sharp
8	Acetonitrile: methanol : water 30:40:30 v/v	9.624	Sharp
9	Acetonitrile: methanol : water 40:30:30 v/v	12.113	Sharp
10	Acetonitrile: methanol : water 10:45:45 v/v	8.624	Sharp
11	Acetonitrile: triethylamine in water 9.991 (TEA buffer) 50:50 v/v	0.25 ml	Tailing
12	Acetonitrile: triethylamine in water 9.100 (TEA buffer) 50:50 v/v	0.5	Tailing
13	Acetonitrile: triethylamine in water 9.931 (TEA buffer) 50:50 v/v	0.25ml : 0.25 ml	Sharp
14	Acetonitrile: triethylamine in water 9.434 (TEA buffer) 50:50 v/v	0.5ml : 0.5 ml	Sharp
15	Acetonitrile: buffer pH 3.0 in 50:50:v/v	7.021	Merged
16	Acetonitrile: buffer pH 4.5 IN 50:50 v/v	9.982	Merged
17	Acetonitrile: buffer pH 6.8 in 50:50 v/v	3.718	Tailing
18	Acetonitrile: buffer pH 7.5 in 50:50 v/v	-	-

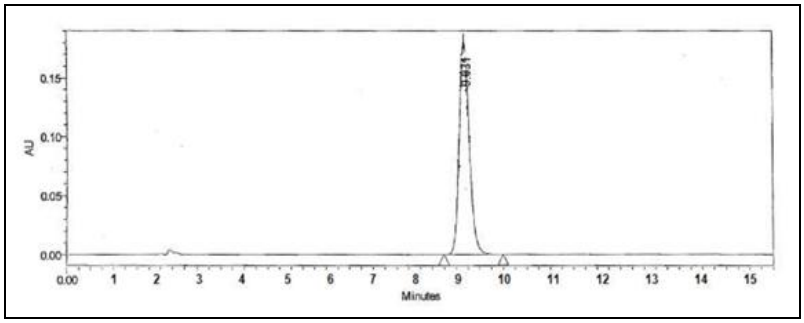

FIG. 8: APIXABAN CHROMATOGRAM WHEN AKZONOBEL - KROMASIL© C18 150 × 4.6 mm, 5µ APPLIED

TABLE 5: EFFECT OF STATIONARY PHASE ON CHROMATOGRAM

Sample no.	Stationary phase	Observation	
		Retention time (min)	Peak shape
1	Symmetry © C18 50 × 4.6 mm 3.5 µ	9.850	Sharp
2	Waters Xterra© C18 150 × 4.6 mm 3.5µ	8.989	Broad peak
3	Fortis© C8 50 × 4.6 mm 5 µ	8.989	Symmetric
4	inertsil ODS© C18 250 × 4.6mm 5 µ	9.434	Sharp
5	AkzonobelKromasil© C18 150 × 4.6 mm 5 µ	9.001	Sharp
6	BDS hypersil thermo© C8 50 × 4.6 m 5 µ	13.001	Fronting
7	YMC© c 18 150 × 4.6 mm 5 µ	14.101	Broad peak
8	supelcosilSupelco©C18 300 × 3mm 3 µ	9.130	Sharp peak
9	Hypersil thermo© C18 300 × 3mm 5 µ	8.022	Sharp peak
10	Agilent ZORBAX©SB C18 150 × 4.6 mm 5 µ	9.031	Sharp
11	Agilent zorbax©RX C18 150 × 4. Mm 5 µ	7.812	Sharp
12	Synergy hydro phenomenex© C18 50 × mm 5 µ	7.992	Broad peak

Selection of Dissolution Media: Different dissolution media were tried. After a series of trials using different solvents, **Table 6** 0.2% tween 80 was selected as the solvent which shown in **Fig. 9**.

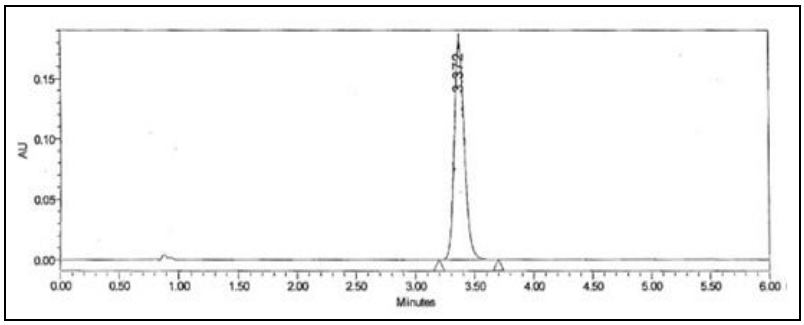

FIG. 9: APIXABAN CHROMATOGRAM IN 0.2% TWEEN 80 IN WATER AS SOLVENT RATIO

TABLE 6: EFFECT OF VARIOUS MEDIA ON THE STABILITY OF APIXABAN

Sample no.	Solvent	Peak area		% content
		Standard	Sample	
1	Distilled water	-	-	No peak
2	0.1 N HCl	1921	1850	101.23
3	pH 4.5 buffer	10421	10393	99.73
4	pH 6.8 buffer	120105	121021	100.283
5	pH 7.5 buffer	-	-	No peak
6	0.1 % SLS	1042301	1042498	99.63
7	0.2% SLS	1042598	1042502	10.00
8	0.1% Tween 80	1075400	1075412	99.61
9	0.2% Tween 80	1134682	1132817	99.48

Dissolution Studies and Assay of Apixaban Tablets: The results of dissolution studies indicated that the developed RP-HPLC method was suitable for this purpose in **Table 7**.

TABLE 7: RELEASE STUDIES OF APIXABAN IN 0.2% TWEEN 80

Sample no. Time (min)	Peak area		% release
	Standard	Sample	
15	11346804	81342	42.26
210	11346805	01212	44.02
315	11346805	41822	47.58
430	11346807	55817	66.38
545	11346801	084912	95.27
660	11346801	132817	99.48

The dissolution results indicated a release of 99.4% in 60 min at 75 rpm and the chromatogram in **Fig. 10** indicates this. The assay of apixaban tablets was conducted using the developed and validated RP-HPLC method, and the drug content was found to be 99.9%. **Fig. 9** and **Table 7** show the results.

Related Substances (RS) and Forced Degradation Studies (FD): The modified analytical method with a run time of 12.0 min was used for the determination of impurities generated in the apixaban API sample after conducting forced degradation using various stressors. The results obtained in **Table 9** indicated that the developed method was able to resolve all the degradation peaks from the Apixaban, which indicted in **Fig. 12-17**.

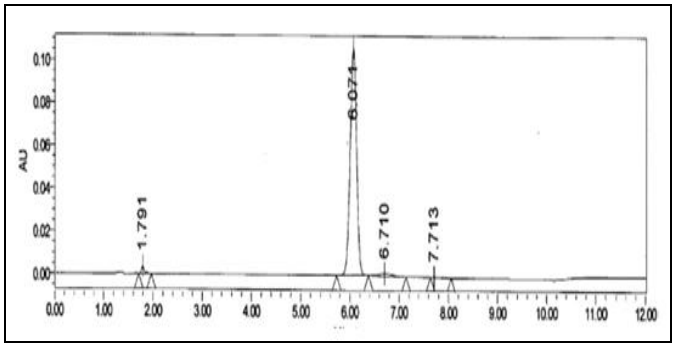

FIG. 11: APIXABAN CHROMATOGRAM IN CONDITION OF (THERMAL STABILITY)

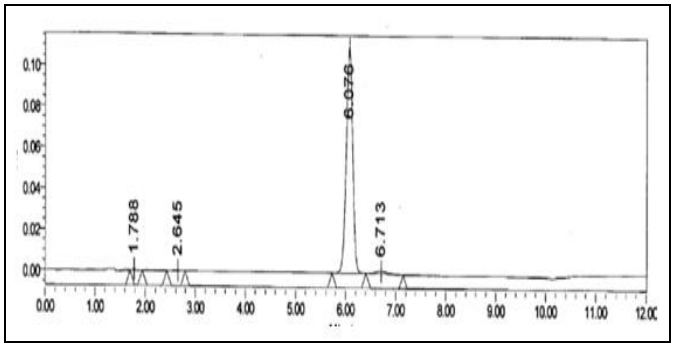

FIG. 12: APIXABAN CHROMATOGRAM IN CONDITION OF (PHOTO- STABILITY)

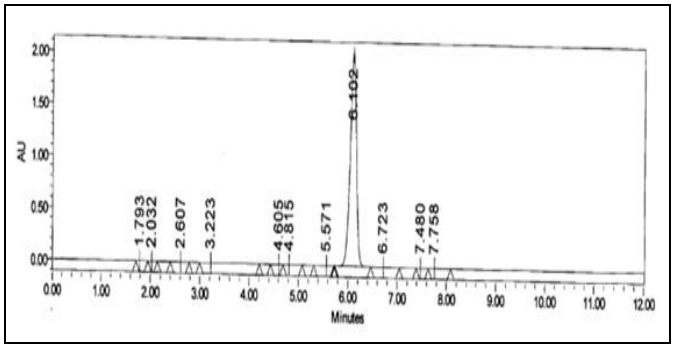

FIG. 13: APIXABAN CHROMATOGRAM IN CONDITION OF (HYDROLYTIC STABILITY)

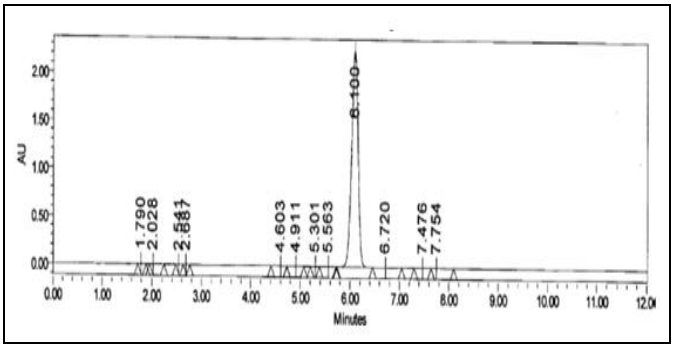

FIG. 14: APIXABAN CHROMATOGRAM IN CONDITION OF (DEGRADATION BY OXIDATION)

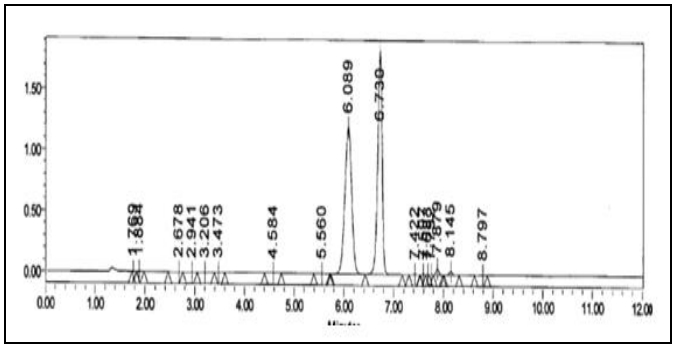

FIG. 15: APIXABAN CHROMATOGRAM IN CONDITION OF (ACIDIC HYDROLYSIS)

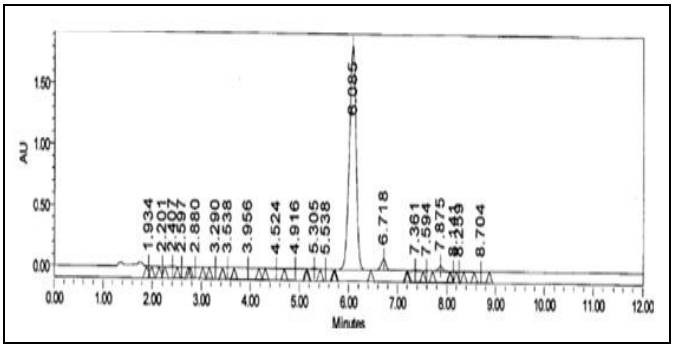

FIG. 16: APIXABAN CHROMATOGRAM IN CONDITION OF (BASICITY HYDROLYSIS)

TABLE 8: FORCED DEGRADATION STUDIES OF APIXABAN

Sample no.	Stressor condition	Retention time of API (min)	Impurities generate
1	Thermo stability	6.071	3
2	Photostability	6.076	3
3	Hydrolytic degradation	6.102	10
4	Oxidative degradation	6.100	11
5	Acid hydrolysis	6.089	13
	And 1 major impurity		
6	Alkaline hydrolysis	6.085	19

Validation Studies: The developed RP-HPLC method was validated according to the International Conference on Harmonization (ICH) Q2 guidelines for various parameters like accuracy, precision, specificity, linearity and range, LOD, and LOQ, robustness, and system suitability.

Accuracy: The accuracy of the developed method was evaluated by conducting recovery studies. The accuracy was calculated by assay method at concentrations of 50%, 100%, and 150%. The calculated amount of apixaban stock solutions were spiked to get the concentrations. The % mean recovery of spiked samples was found to be 99.82% **Table 10**.

TABLE 9: ACCURACY STUDIES OF APIXABAN

Level	Mean of concentration added (µ/mL)	Mean of concentration obtained (µ/mL)	% mean Recovery
50%	45.052	44.810	99.793
100%	89.990	89.953	99.930
150%	135.009	134.678	99.750

Precision: The intra-day precision studies were performed using apixaban reference standard solution of 90 ppm. The solution was injected at various time intervals, and the percent related standard deviation (% RSD) was determined. The inter-day precision was studied by injecting the same concentration of the standard solution on consecutive days, and the % RSD was calculated. The relative standard deviation obtained from 12 assay results was found to be 0.069% **Table 11**.

TABLE 10: PRECISION STUDIES OF APIXABAN

Precision parameter	Peak area			Mean	SD	%RSD
	Run 1	Run 1	Run 1			
Reproducibility	1389984	1389953	1388300	1389412	963.433	0.069
Intermediate precision	1389986	1389958	1388303	1389416	963.699	0.069
Repeatability	1389983	1389956	1388309	1389416	958.785	0.069

Specificity: No interferences were observed in the chromatogram of apixaban standard due to the presence of excipients and blank **Table 12**.

Linearity and Range: A graph of peak area versus concentration (in ppm) was plotted for apixaban at a concentration range between 20-90 ppm. The linear regression equation and correlation coefficient (R^2) were y = 8268.2x + 41452 and 0.995 respectively **Fig. 17**.

TABLE 11: SPECIFICITY STUDIES OF APIXABAN

Sample no.	Standard (Area AU)	Sample (Area AU)
1	138668	138650
2	138764	138758
3	138599	138596
Mean	138677	138668
SD	82.86736	82.48636
%RSD	0.059756	0.059485

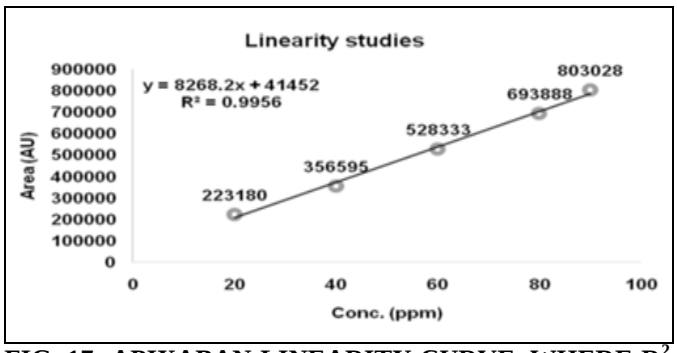

FIG. 17: APIXABAN LINEARITY CURVE, WHERE R^2 = 0.9956

LOD and LOQ: The results of signal to noise ratio was compared with the response of Apixaban standard. The LOD and LOQ were found to be 0.2887 ppm and 0.8749 ppm, respectively.

System Suitability: System suitability tests were performed using Apixaban standard and test solutions to check for compliance with specified parameters in **Table 13**.

TABLE 12: SYSTEM SUITABLE STUDIES OF APIXABAN

Sample no.	Parameter	Apixaban
1	Retention time	3.305
2	Number of the theoretical plates	5538
3	Tailing factor	0.85
4	Peak area	1134680
5	Peak height	190457

Robustness Studies: The changes were applied, and system suitability parameters were checked, found to be within the acceptable limits. It was noted that trivial changes in temperature and flow rate does not affect the method and produces results, which passes system suitability. Hence, the method was robust, as indicated in **Table 14**.

TABLE 13: ROBUSTNESS STUDIES OF APIXABAN

Factor	Level	T_R	Tailing factor
Parameter : flow rate			
1.2 ml/min	-1	3.53	0.86
1.3 ml/min	0	3.31	0.92
1.4 ml/min	+1	3.20	0.74
Mean		3.34	0.84
Parameter :column temperature			
25 ºC	-5	3.48	0.75
30 ºC	0	3.32	0.88
35 ºC	+5	314	0.81
Mean		3.31	0.79

CONCLUSION: A new RP-HPLC method for the determination of apixaban was developed and validated. The developed method was accurate, precise, specific, and sensitive for the quantitative analysis of apixaban both in bulk drugs and tablets. Validation studies indicated the method to be robust on minor variations in the chromatographic parameters. No attempt was made to quantify the degradation of products in this research project.

This method can be used for routine quality monitoring of apixaban and its tablets. This method can be extended for application in LC-MS for quantitative estimation of known and unknown impurities generated during forced degradation and related substance studies both for API and finished products of apixaban.

ACKNOWLEDGEMENT: The authors wish to thank the University of Petra-Amman, Faculty of Pharmacy and Medical Sciences, and Lucian Blaga University-Sibiu, Faculty of Medicine, for supporting this work. The authors are also thankful to colleagues in the management of their cooperation in carrying out this work.

CONFLICTS OF INTEREST: The authors mention no conflict of interest.

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How to cite this article:
Al-Ani I, Hamad M, Al-Shdefat R, Mansoor K, Gligor F and WA Dayyih: Development and validation of a stability indicating RP-HPLC method of apixaban in commercial dosage form. Int J Pharm Sci & Res 2021; 12(1): 241-51. doi: 10.13040/IJPSR.0975-8232.12(1).241-51.