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VALIDATED RP-HPLC STABILITY-INDICATING METHOD OF ANTICOAGULANT ACTIVE PHARMACEUTICAL INGREDIENT; APIXABAN

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Keywords:

Apixaban, RP-HPLC, Method development, Validation, Forced degradation

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ABSTRACT: A simple and precise stability-indicating reversed-phase (RP-HPLC) method was developed and validated for the determination of Apixaban active pharmaceutical ingredient. The chromatographic condition was carried out on Phenomenex Luna 5 µm C18 100 Å (150 × 4.6 mm) HPLC column with prepared buffer solution 1.0 ml of Orthophosphoric acid mixed with 0.2% of potassium dihydrogen phosphate solution, use as mobile phase (a), mobile phase (b) was acetonitrile. The mobile phase flow rate at 1 ml/min and the detection wavelength is 230 nm. Forced degradation study was covered acid hydrolysis, base hydrolysis, peroxide, photolytic (UV light) and thermal degradation was performed to prove the specificity of the proposed method and degradation was achieved. The developed method has been statistically validated according to ICH guidelines and found to be simple, precise, linear and accurate with the prescribed values. Thus the proposed RP-HPLC method was successfully applied for the substance in routine quality control analysis in active pharmaceutical ingredients.

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-l H-pyrazolo [3, 4], pyridine-3-carboxamide and sold under the brand name Eliquis to treat the people with atrial fibrillation (a heart rhythm disorder) to lower the risk of stroke caused by a blood clot. “Eliquis” is also used after hip or knee replacement surgery to prevent a type of blood clot called deep vein thrombosis (DVT), which can lead to blood clots in. The lungs (pulmonary embolism) [1, 6]. The development of an accurate and efficient analytical method to determine the quality of the product is a critical activity during the process of development of the drug substance in generic pharmaceutical industries.

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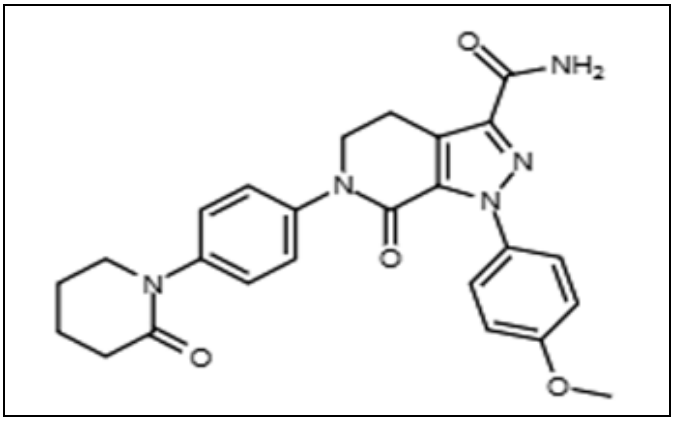

FIG. 1: THE STRUCTURE OF APIXABAN

The analytical research and development activities conducted to develop an efficient method using the C18 chromatography column for the quantitative determination [7, 8]. Further, Apixaban is not yet official in any of the pharmacopoeia. Hence, there is the need for the development of a selective, fast, and stability-indicating RP- HPLC method.

MATERIALS AND METHODS: The gradient grade acetonitrile, ammonium dihydrogen orthophosphate, hydrochloric acid, sodium hydroxide, and hydrogen peroxide were all of AR grade, procured from Merck (India). HPLC grade water obtained from Millipore system (Millipore Inc., USA) was used throughout the analysis.

Instrumentation and Analytical Condition: Chromatographic condition was carried out on WATERS HPLC Auto Sampler, separation module 2695 photodiode array detector, Empower-software version-2. Phenomenex Luna 5 µm C18 100 Å (150 × 4.6 mm) HPLC column with sample compartment temperature at 35 °C was used for separation. 1.0 ml of Orthophosphoric acid mixed 0.2% potassium dihydrogen orthophosphate solution was prepared and filtered through 0.45 µm membrane filter (0.45 µ, Millipore) and degassed in ultrasonic bath prior to use as mobile phase A. Acetonitrile was used as mobile phase B. The flow rate and injection volumes were 1.0 ml/min and 10 µl, respectively. The analysis was carried out under the gradient condition as time (min)/A (v/v): B (v/v); T0.01/74:26, T40.0/35:65, T45.0/35:65, T50.0/74:26. The data were acquired at 230 nm for 50 min. The photodiode array detector was used to determine the peak purity of the stressed sample.

Preparation of Analytical Solutions: A mixture of water and acetonitrile in the ratio of 60:40 (v/v) was used as a diluent in the preparation of analytical solutions. The stock solutions of each impurity (Impurity-1 to Impurity-6, **Table 1.** at a concentration about 150 µg/ml was prepared in diluent and further diluted to prepare the standard solution for quantification of impurities. The specification limits used for study were 0.15% for the related substances *viz.*, Impurity-1, Impurity-2, Impurity-3, Impurity-4, Impurity-5 and Impurity-6. Apixaban standard solution (600 µg/ml) spiked with all impurities at a specification level (w/w) was used as a system suitability solution (SST).

The blank, system suitability solution and six replicates of standard and single test solutions were injected separately under the optimized chromatographic conditions.

TABLE 1: IUPAC NAME OF APIXABAN AND ITS RELATED IMPURITIES

Short Name	IUPAC Name
Apixaban	l-(4-Methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-l-yl)phenyl]-4,5,6,7-tetrahydro-lH-pyrazolo[3,4-c]pyridine-3-carboxamide
Impurity-1	N-[(1S)-2-[(1S,3S,5S)-3-Cyano-2 azabicyclo [3.1.0]hex-2-yl]-1-(3-hydroxytricyclo[3.3.1.13,7]dec-1-yl)-2-oxoethyl]carbamic acid 1,1-dimethylethyl ester
Impurity-2	4-Morpholino-1-(4-(2-oxopiperidin-1-yl) phenyl) 5, 6-dihydropyridin-2(1H)-one.
Impurity-3	1-(4-Ethoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide
Impurity-4	1-(4-Chlorophenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide
Impurity-5	Methyl1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxylate phenyl) 5, 6-dihydropyridin-2(1H)-one.
Impurity-6	1-(4-Methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl) phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxylate

RESULTS AND DISCUSSION: Development and Optimization of Chromatographic Conditions: The objective of method development was to separate Apixaban and its process-related impurities. Method development trials were conducted on different stationary phases like C8, C18, Phenyl, and Cyano along with the optimization of other chromatographic conditions like detection of wavelength, type and quantity of organic/inorganic buffer, pH of the mobile phase, sample temperature and column oven temperature. The resolution between Impurity-3 and Impurity-4 was critical and thus considered as the system suitability criteria. Satisfactory resolution achieved, and good peak shape of Apixaban was observed on Phenomenex Luna 5 µm C18 100 Å (150 × 4.6 mm) HPLC with column flow rate 1.0 ml/min, wavelength 230 nm, and column oven temperature

35 °C. The mobile phase was consisting 1.0 ml of Orthophosphoric acid mixed potassium dihydrogen phosphate buffer and acetonitrile with gradient elution mode.

The column efficiency/theoretical plates should be more than 10,000, and tailing factor should be less than 2.0 and %RSD for six replicate injections of standard solution should be less than 5.0% were finalized as system suitability criteria. The resolution between Impurity-3 and Impurity-4 with not less than 2.5 was fixed as system suitability criteria

Method Validation: Specificity: Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Stress testing of the drug substance can help identify degradation products, which help establish the degradation pathways and intrinsic stability of the molecule and validate the stability-indicating of the analytical procedures used.

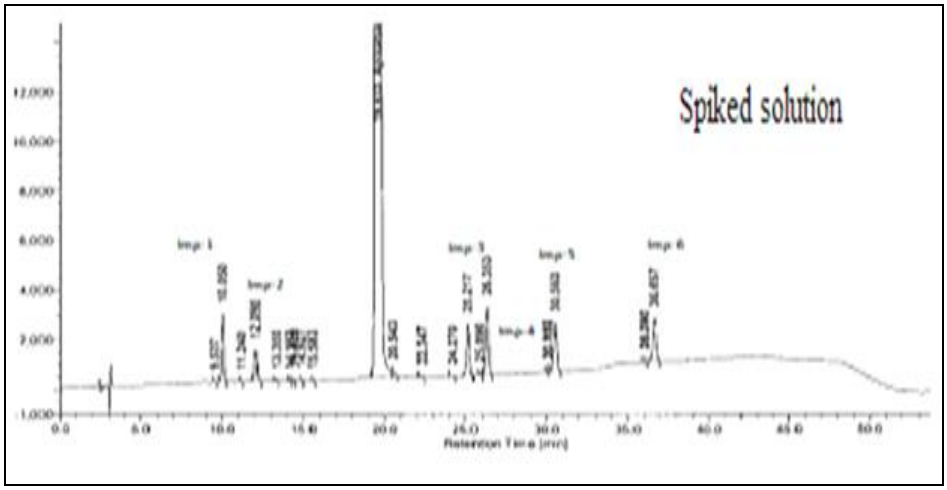

FIG. 2: HPLC CHROMATOGRAM OF SYSTEM SUITABILITY SOLUTION (SST) OF APIXABAN AND RELATED IMPURITIES OF APIXABAN

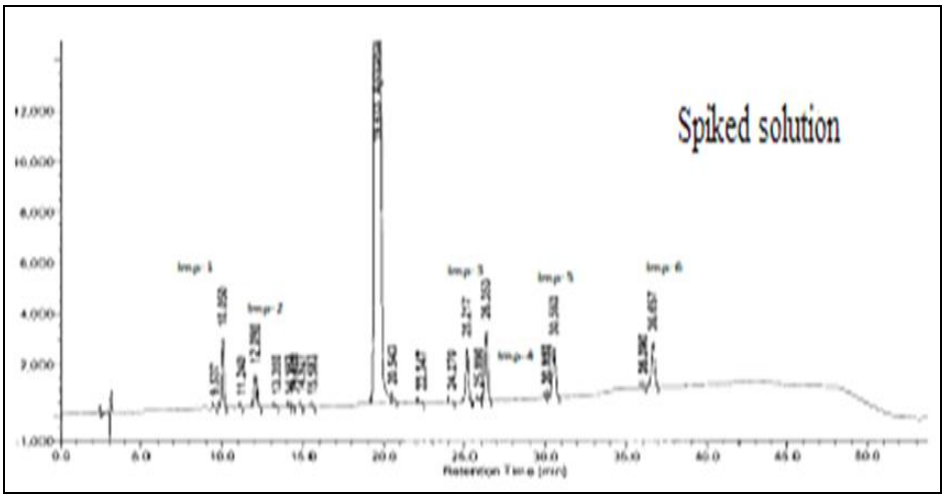

FIG. 3: HPLC CHROMATOGRAM OF TEST SOLUTION SPIKED AT SPECIFICATION LEVEL CONCENT-RATION WITH ALL IMPURITIES

The specificity of the developed RP-HPLC method for Apixaban was determined in the presence of its impurities (Impurity-1 to Impurity-6). Forced degradation studies were also performed on Apixaban to provide an indication of the stability-indicating property and specificity of the proposed method. The degradation study was performed by exposing the sample to different stress conditions such as light (1.2 million lux hours), heat (105˚C for 7 days), acid hydrolysis (2M HCl for 30 minute), base hydrolysis (2 M NaOH for 1hrs) and oxidation (30%v/v H_2O_2 for 30 min). Significant degradation was observed in acid, alkali solution, and peroxide solution, as shown in **Fig. 4, 8.** The mass balance was calculated for all the stressed samples. The mass balance is a process of adding

together the assay value, and the levels of degradation products add up to 100% of the initial value with due consideration of the margin of analytical error. A photodiode array detector was employed to check and ensure the homogeneity and purity of Apixaban peak in all the stressed sample solutions. Forced degradation study results are reported in **Table 2.**

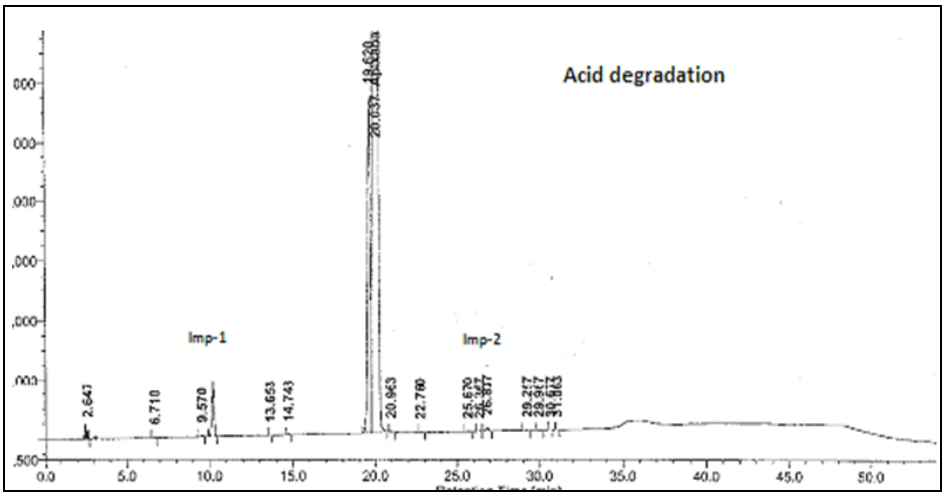

FIG. 4: CHROMATOGRAM OF ACID DEGRADATION OF APIXABAN TREATED ACID 2 M HCL, 30 MINUTES AT ROOM TEMPERATURE

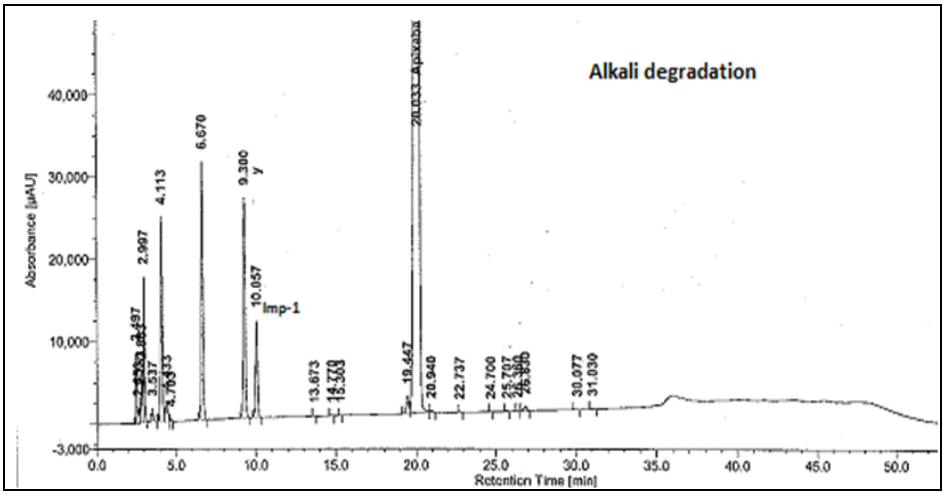

FIG. 5: CHROMATOGRAM OF ALKALI DEGRADATION OF APIXABAN ALKALI TREATED WITH 2 M NAOH, 1 HR AT ROOM TEMPERATURE

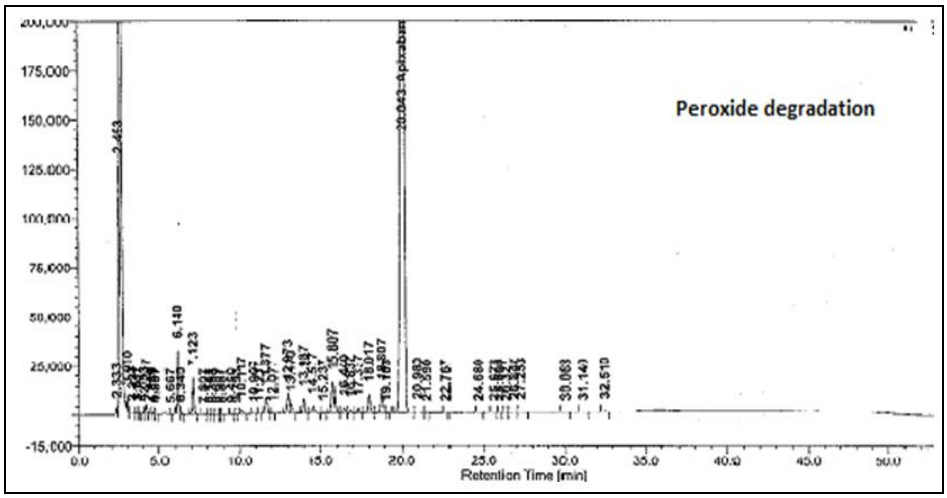

FIG. 6: CHROMATOGRAM OF PEROXIDE DEGRADATION OF APIXABAN TREATED WITH 30 % H_2O_2 FOR 30 MIN

Linearity: The Apixaban is linear over the range from 0.1455 µg/ml to 1.1218 µg/ml (About LOQ level to about 150% concentration of individual known impurity). Impurity-1 is linear over the range from 0.1469µg/ml to 1.1203 µg/ml, impurity-2 is linear over the range from 0.3062 µg/ml to1.1297 µg/ml, impurity-3 is linear over the range from 0.1462 µg/ml to 1.1025 µg/ml, impurity-4 is linear over the range from 0.1454 µg/ml to 1.1068 µg/ml, impurity-5 is linear over the rang from 0.1497 µg/ml to 1.1351 µg/ml and impurity-6 is linear over the range from 0.1504 µg/ml to 1.1397 µg/ml (Each impurity at about LOQ level to about 150% specification level). Correlation coefficient found for Impurity-1, Impurity-2, Impurity-3, Impurity-4, Impurity-5, Impurity-6 and Apixaban is 0.9999, 0.9998, 1.0000, 0.9999, 0.9999, 0.9997 and 0.9999 respectively.

Limits of Detection and Quantification (LOD and LOQ): According to ICH Q2 (R1) recommendations, the limit of detection (LOD) and the limit of quantification (LOQ) for Apixaban and its process-related impurities (Impurity-1 to Impurity-6) were estimated by calibration curve method standard deviation of the response (σ) and the slope(S), by injecting the series of dilute solutions of known concentration. The values of LOD and LOQ for impurities and Apixaban were found to be in the range of 0.016 % - 0.03% and 0.013% - 0.03%, respectively. Precision was studied at the LOQ level by injecting six individual preparations of Apixaban and its six process-related impurities, followed by the calculation of %RSD of the peaks areas. The %RSD of LOQ precision was in the range of 0.9% - 3.50%.

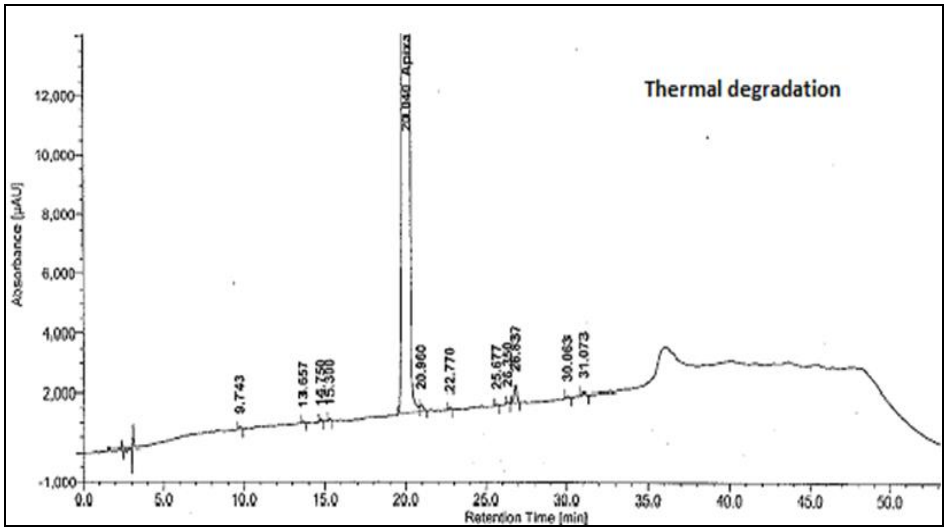

FIG. 7: CHROMATOGRAM OF THERMAL DEGRADATION OF APIXABAN KEPT AT 105˚C, 7-DAYS IN VACUUM OVEN

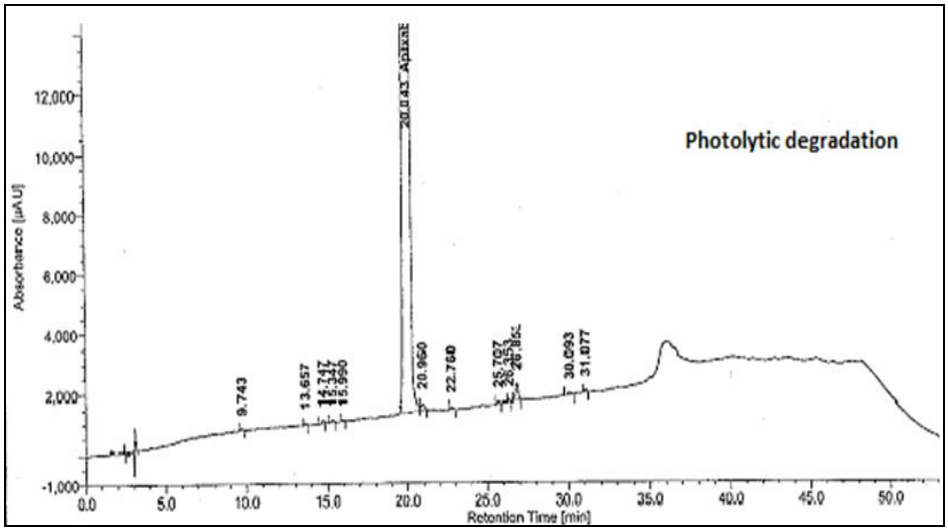

FIG. 8: CHROMATOGRAM OF PHOTOLYTIC DEGRADATION OF APIXABAN AS PER ICH GUIDE-LINES

Precision: The precision of the method is the degree of agreement between the results. The precision of the method was studied for system precision, method precision, and intermediate precision. A standard solution of Apixaban was injected six times to determine the system precision of the method, and %RSD was calculated for Apixaban and its all process-related impurities. Method precision study shows relative standard deviation of result for Impurity-1, Impurity-2, Impurity-3, Impurity-4, Impurity-5, impurity-6 and Apixaban are 0.70%, 0.72%, 0.43%, 0.41%, 0.48%, 0.30% and 0.44 respectively. Intermediate precision study shows relative standard deviation of result for Impurity-1, Impurity-2, Impurity-3, Impurity-4, Impurity-5, Impurity-6 and Apixaban are 2.07%, 3.10%, 2.66%, 0.91%, 1.96%, 2.33% and 1.03% respectively.

Accuracy (Recovery): The accuracy of the method for all the related substances was determined by analyzing Apixaban sample solutions spiked with all the related substances at four different concentration levels of LOQ, 50 to 150% of each in triplicate at the specified limit. The recovery of all these related substances was found to be in-between the predefined acceptance criterion of 80.0% - 120.0%. The average recovery found for Impurity-1, Impurity-2, Impurity-3, Impurity-4, Impurity-5, Impurity-6 and Apixaban are 101.47%, 95.78%, 99.33%, 98.53%, 104.81%, 98.69% and 105.32% respectively.

Stability of Analytical Solution: The solution stability study of Apixaban, Impurity-1, Impurity-2, Impurity-3, Impurity-4, Impurity-5 and Impurity-6 peak after 48 h at 25 °C temperature and no continuous increasing or decreasing trend was observed in the area of Apixaban, Impurity-1, Impurity-2, Impurity-3, Impurity-4, Impurity-5, and Impurity-6 peak.

Robustness: The method was robust for ± 10% variation in flow rate of mobile phase, ± 5 °C variation in column oven temperature, ± 10% variation in concentration of salt (Ammonium dihydrogen phosphate) in preparation of mobile phase A, ± 1% absolute organic phase variation (mobile phase-B) in gradient program and ±5nm wavelength variations. The effect of column temperature on the resolution was studied at 30 °C and 4 °C instead of 35 °C. The resolution between Imp-3 and Imp-4 was greater than 2.5 in all the deliberate varied chromatographic conditions indicating the robustness of the method.

TABLE2: RESULTS OBTAINED FROM FORCED DEGRADATION STUDY OF APIXABAN

Stressed condition	% of Apixaban	%of Degradants	Observation and mass balance	Peak purity
Undegraded	100	--	--	--
Acid degradation (2M HCl, 30 min at Room Temp.)	95.70	3.9	Major unknown degradation product (3.9%) formed (Mass balance: 95.98%)	0.9999
Alkali degradation (2 M NaOH, 1hr at Room Temp.)	93.80	6.2	Major unknown degradation product (6.2%) formed (Mass balance: 95.31%)	0.9999
Peroxide degradation(30%H_2O_2 for 30 min)	94.15	8.95	Major unknown degradation product (8.95%) formed (Mass balance: 103.10%)	0.9999
Thermal degradation (105˚C, 7-days)	100.32	0.50	No any known and unknown major degradation product formed (Mass balance: 100.32%)	0.9999
Photolytic degradation (as per ICH)	100.71	0.07	No any known and unknown major degradationproduct formed (Mass balance: 100.32%)	0.9999

CONCLUSION: A rapid, specific, sensitive and precise reverse-phase HPLC method for the quantitative determination of process-related and degradation impurities of Apixaban, an anticoagulant drug, is described. The developed RP-HPLC method was successfully applied to the analysis of Apixaban drug substances. Process-related impurities have been detected in a test sample of Apixaban by using the newly developed RP-HPLC method. A forced degradation study was carried out under acidic, alkaline, peroxide, 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 specific, precise, sensitive and robust. The developed and validated method is stability indicating method that can be used to analyze routine and stability samples of Apixaban active pharmaceutical ingredient (API).

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

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