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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD FOR ESTIMATION OF APIXABAN IN PHARMACEUTICAL FORMULATIONS

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Keywords:

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ABSTRACT: A simple, accurate, precise, and rapid high-performance thin-layer chromatographic (HPTLC) method for determination of Apixaban (API) in bulk drug and the pharmaceutical formulation was developed and validated. The separation was performed on aluminum plates, pre-coated with silica gel $60F_{254}$ as the stationary phase using toluene: methanol (7:3 v/v), as mobile phase. Densitometric measurement of the API was carried out at 290 nm. The retention factor (R_f) was found to be 0.62 ± 0.04. The method was successfully validated as per International Conference on Harmonization (ICH) guidelines Q2 (R1). The calibration curve was linear in range 50-300 ng/band. The limits of detection and quantitation were 0.272 and 0.824 ng/band, respectively. Accuracy and precision of the proposed method were evaluated by recovery studies and intra-day and inter-day precision studies, respectively (standard deviation for precision studies was found to be less than 2). The developed method was used to analyze marketed formulation samples (tablet) of the API. The API was subjected to various stress conditions as per ICH Q1A (R2) guidelines. In stress studies, the drug showed significant degradation during acid, alkaline hydrolytic, and oxidative conditions. As the method could effectively separate the drug from their degradation products, it can be used as a stability indicating method.

INTRODUCTION: Apixaban (API) (1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl)-phenyl]-4, 5 dihydropyrazolo [5,4-c] pyridine-3-carboxamide) **Fig. 1** is a novel anticoagulant drug acting as a direct, selective and reversible inhibitor of the coagulation factor Xa [1, 2, 3, 4].

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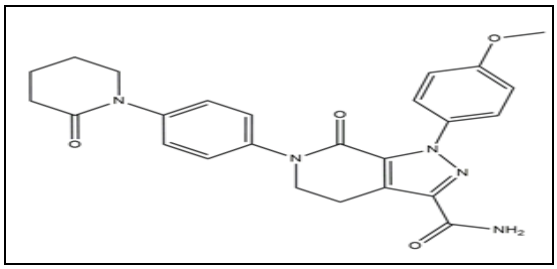

FIG. 1: CHEMICAL STRUCTURE OF APIXABAN

Apixaban is to reduce the risk of stroke and systemic embolism in patients with nonvalvular atrial fibrillation. It has also been used to lower the risk of developing venous thrombosis post-orthopedic surgical procedures [5].

LC-MS/MS methods were developed to determine apixaban alone in plasma or in the presence of its major metabolites [6, 7] and UHPLC-MS/MS [8] to support clinical uses. Assay in tablets [9, 10,] and stability-indicating assay using UV [11] and HPLC was also described [12, 13]. Characterization of degradation products using multistage mass spectrometry was also reported [14]. Most of the reported methods are based on hyphenated techniques, and overall cost of the analysis using these techniques is more as compared to high-performance thin layer chromatography [15]. In this work, a simple, sensitive, accurate, precise and economic stability-indicating high-performance thin layer chromatographic procedure was developed. The proposed method was optimized and validated as per ICH guidelines.

MATERIALS AND METHODS:

Materials and Reagents: Pure drug, Apixaban was procured from Taj Mahal labs limited, Mumbai, India. All reagents and chemicals were of analytical grade; they included, Methanol and Toluene (Merck Pvt. Ltd., Mumbai, India).

Preparation of Standard Solution: Standard stock solution of API (500 µg/ml) was prepared by dissolving accurately weighed quantity (5 mg) of the drug in 10 ml of methanol.

Chromatographic Conditions:

Selection of Mobile Phase: From the standard stock solution, 0.5 µl of the API was applied to chromatography plates in band form, and these plates were put for a run under different solvent systems. The attempts were made to achieve the preferred R_f value range 0.1-0.8. Different solvent systems were tried to determine the suitable conditions for efficient separation. From the different mobile phase combinations tested, toluene: methanol (7:3 v/v) yields a compact band which showed a symmetrical peak on chromatogram and expected R_f value of 0.62 ± 0.04 for the API.

Selection of Analytical Wavelength for Densitometry Evaluation: After chromatographic development, the TLC plate was scanned over the wavelength range of 200-700 nm. From the spectra **Fig. 2**, it was observed that Apixaban exhibited strong absorbance at about 290 nm, which was selected as the analytical wavelength for further analysis.

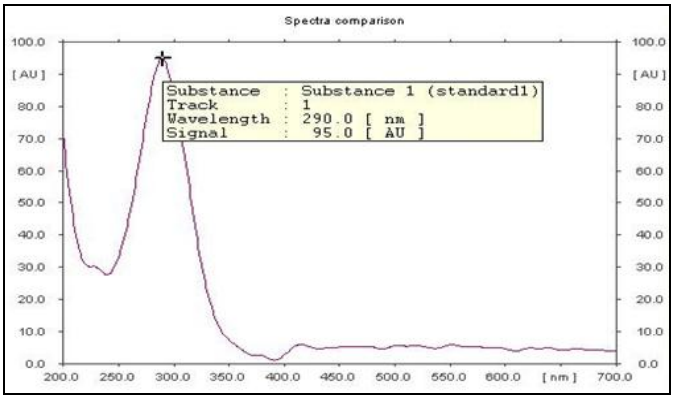

FIG. 2: SPECTRUM OF APIXABAN

Instrumentation and Optimized Chromatographic Conditions: The samples were spotted in the form of bands of width 8 mm with Camag microlitre syringe on pre-coated silica gel aluminum plate $60F_{254}$ (10 cm × 10 cm, 0.2 mm, E. Merck, Germany) using Linomat 5 (Camag, Muttenz, Switzerland) semi-automatic sample applicator. Before chromatography, the plates were pre-washed by development with methanol and activated at 60 °C for 15 min. A constant application rate of 150 nl/Sec was employed, and space between the two bands was 11.3 mm.

Linear ascending development was performed in twin trough glass chamber previously saturated with the mobile phase for 10 min. The development distance was 80 mm. After the development; TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed using Camag TLC scanner 3 in the absorbance mode at 290 nm. The slit dimensions were 6 mm × 0.33 mm. The source of radiation utilized was a deuterium lamp emitting a continuous UV spectrum in the range of 200 - 400 nm.

Analysis of Marketed Formulation:

Preparation of Sample Solution: Twenty tablets of Eliquis (manufactured by Bristol-Mayers Squibb, Frosinone, Italy) was weighed and powdered. Powder equivalent to 5 mg of apixaban was transferred to 10 ml calibrated volumetric flask; 5 ml of methanol was added and ultrasonicated for 20 min, volume was made up to the mark with methanol and then filtered using Whatman filter paper no. 42. The resulting solution was used as a sample solution. On the TLC plate two bands of the standard stock solution and four bands of the sample solution, 0.5 µl of each, were

applied, developed and scanned under optimized chromatographic conditions. Amount of drug in the sample was calculated by comparing the mean area of sample band with that of the standard band.

Forced Degradation Studies: To determine the stability-indicating ability of developed HPTLC method, the active pharmaceutical ingredient powder was stressed under various conditions as per ICH guidelines. In all cases, API (5 mg) was accurately weighed, then subjected to forced degradation conditions such as acid (0.1 N HCl at 80 ºC), base (0.1 N NaOH at 80 ºC), neutral hydrolysis (water at 80 ºC), oxidation (3% H_2O_2 at 80 ºC), heat (60 ºC), and UV light (254 nm). All stressed solutions of API were removed after stipulating time interval during the stress study and subjected to chromatography analysis after suitable dilution of stress samples [16].

Method Validation: As per the ICH guidelines, the method was validated, and included various parameters such as linearity, the limit of detection, the limit of quantitation, accuracy, precision, and robustness [17].

Linearity: The data of peak area versus drug concentration were treated by linear least-square regression analysis. The slope, intercept, and correlation coefficient (r^2) were calculated for regression analysis of API.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): The detection and quantification limits were evaluated from calibration curves. The method based on the standard deviation of the y-intercept and the slope of the calibration curves. The following equations were used to calculate LOD and LOQ.

$$LOD = 3.3 \times SD / slope$$

$$LOQ = 10 \times SD / slope$$

Accuracy: The accuracy was determined by the percent recovery method. Recovery studies were carried out by spiking three different known amounts of pure drug at 80%, 100% and 120% of label claim to the pre-analyzed tablet powder. An accurately weighed quantity of tablet powder equivalent to 5 mg API was transferred individually to nine different 10 ml volumetric flasks, to each of the flasks, add 4, 5 and 6 mg of the API in a manner like three replicates of each concentration level were analyzed. Accuracy was expressed as % recovery.

Precision: The system precision was evaluated by six replicate analysis of the standard solution. The method precision was studied by analyzing six different sample solutions of the same concentration. The results for method system precision were expressed in terms of percent relative standard deviation. For evaluating intra-day and inter-day precision, variances were determined over 1 day and 3 days, respectively.

Robustness: The effect of slight deliberate variation in the method parameters such as the composition of the mobile phase, chamber saturation time, and volume of mobile phase on R_f values and peak area, was observed one by one. The composition of the mobile phase and chamber saturation time were varied in the range of ± 0.1 ml and ± 5 min, respectively, of the used optimized conditions. The volume of mobile phase was varied by ± 1 ml. The effect of these variations on both the R_f values and peak area of the API was studied by calculating the relative standard deviation for each parameter.

RESULTS AND DISCUSSION:

Method Optimization for the Densitometric Measurements: The working standard of the drug was spotted on the TLC plates and developed in different solvent systems. Different solvents in varying proportions were tried. The optimum results were obtained with a mobile phase consisting of toluene: methanol (7:3 v/v). The chamber was saturated with the mobile phase at room temperature. The bands developed were dense and compact with the acceptable R_f value of 0.62 ± 0.04. The representative densitogram is given in **Fig. 3**.

Analysis of Marketed Formulation: The commercial tablet, Eliquis (2.5 mg) was quantitatively determined using the developed HPTLC method. The analysis of tablet formulation was based on comparing the mean peak area of the standard band with that of the sample peak area. The results obtained after tablet analysis was in good agreement with those of label claim, and the result is summarized in **Table 1**.

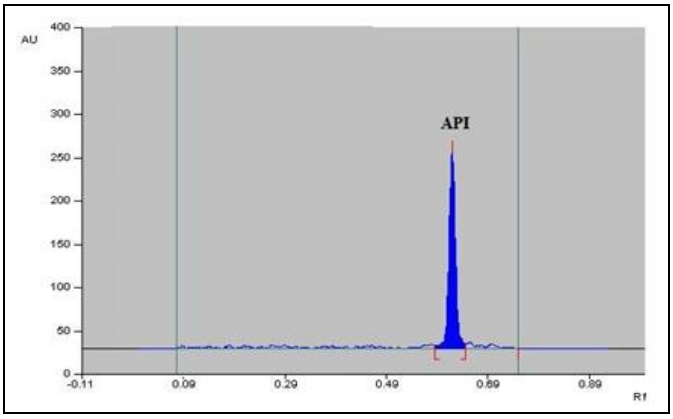

FIG. 3: TYPICAL DENSITOGRAM OF APIXABAN

TABLE 1: RESULTS OF ANALYSIS OF MARKETED FORMULATION

Brand name	Label claim (mg)	Amount estimated* (mg)	Percent label SD
Eliquis	2.5	2.49	99.74 ± 1.03

Validation of Method:

Linearity: A linear relationship established between peak area and concentration was found to be in the range of 50-300 ng/band **Fig. 4**. The correlation coefficient of the calibration curve was found to be 0.9982.

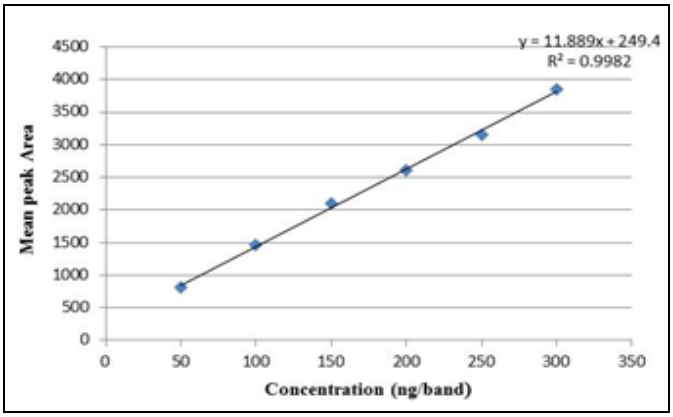

FIG. 4: CALIBRATION CURVE OF APIXABAN

Limit of Detection and Limit of Quantitation: The sensitivity of the developed method was determined in terms of the limit of detection and quantification. The limit of detection and limit of quantification were found to be 0.272 and 0.824 ng/band, respectively.

Accuracy: To check the accuracy of the method, recovery studies were carried out by the standard addition method and results of recovery studies obtained within the range of 99-101% indicates the accuracy of the proposed method. The data obtained from the recovery study are summarized in **Table 2**.

TABLE 2: RESULTS OF ACCURACY STUDIES

% Level of recovery	Percent recovery ± SD	% RSD	Mean % recovery ± SD
80	100.583 ± 1.328	1.320	100.141 ± 0.681
100	100.197 ± 0.585	0.584	
120	99.643 ± 0.138	0.139	

Precision: Repeatability or reproducibility of the proposed method was determined by intra-day and inter-day precision study. The drug was assayed three times on the same day (intra-day) and three consecutive days (inter-day). The results of the precision study were expressed in terms of standard deviation (SD) **Table 3**. The % RSD for both intra-day and the inter-day precision study was found to be less than 2 indicating the repeatability and reproducibility of the method

TABLE 3: RESULTS OF PRECISION STUDIES

Precision %	Label claim ± SD	% RSD
Intra-day precision	100.003 ± 0.98	0.98
Inter-day precision	99.467 ± 0.822	0.826

Robustness: Robustness of the proposed method were studied to measure the reliability of developed HPTLC method by deliberate variations in the optimized method parameters such as the composition of the mobile phase (± 0.1 ml), chamber saturation time (± 5 min) and volume of mobile phase (± 1 ml). The effect of such deliberate variations was studied on R_f values and peak area of drug which are indicated in **Table 4**. Each sample was studied in triplicate (n=3) and the obtained peak areas were utilized to calculate % RSD, which was found to be less than 2. Hence, the method was found robust for the determination of API in tablets.

TABLE 4: RESULT OF ROBUSTNESS STUDIES AND ITS STATISTICAL VALIDATION

Factor	Level	Peak area	R_f
Mobile phase composition (± 0.1 ml)			
6.9 : 3.1	– 0.1	3178.4	0.60
7 : 3	0	3142.5	0.62
7.1 : 3.9	+ 0.1	3176.1	0.63
	R.S.D.	0.0936	
Duration for chamber saturation (± 5 min)			
5 min	- 5	3098.6	0.58
10 min	0	3106.4	0.62
15 min	+ 5	3116.2	0.60
	R.S.D.	0.0532	
The volume of mobile phase (± 1 ml)			
9	– 1	3150.1	0.59
10	0	3120.8	0.62
11	+ 1	3145.8	0.60
	R.S.D.	0.700	

Degradation Behavior of API: The degradation behavior of the API was investigated using HPTLC underemployed hydrolytic (acid, alkali, neutral), oxidative, thermal, and photolytic stress conditions. The drug showed extensive degradation upon acid, base hydrolytic, and oxidative stress condition, whereas API was found to be stable in neutral hydrolytic, photolytic, and thermal stress conditions. Various densitogram obtained for API under different stress conditions are shown in **Fig. 5, 6,** and **7**. The obtained results of forced degradation studies are indicated in **Table 5**. The developed HPTLC method could effectively separate the API from their degradation products, which indicate the potential of stability indicating method.

TABLE 5: RESULTS OF FORCED DEGRADATION STUDIES

Stress condition	Percent assay of the active substance	R_f value of the degraded product
Acid (0.1 N HCl, 80 ºC for 3 h)	62.8	0.83
Alkali (0.1 M NaOH, 80 ºC for 3 h)	85.191	0.24
Oxide (3% H_2O_2 80 ºC for 3 h)	87.656	0.79, 0.83
Neutral (Distilled water, 80 ºC for 3 h)	99.157	-
Heat (60 ºC for 24 h)	98.415	-
UV-Exposure (254 nm for 24 h)	99.544	-

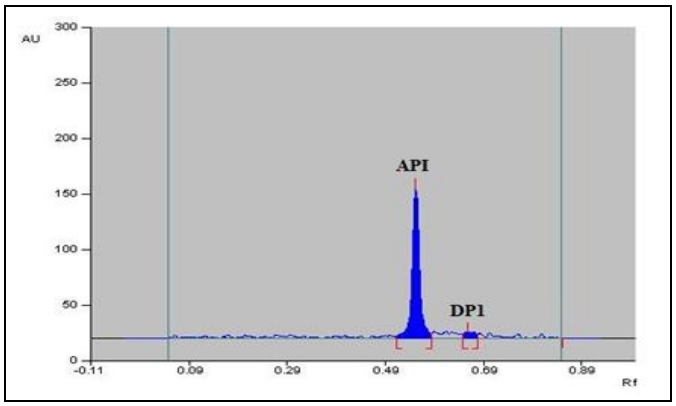

FIG. 5: DENSITOGRAM OF ACID (0.1 M HCl) TREATED SAMPLE

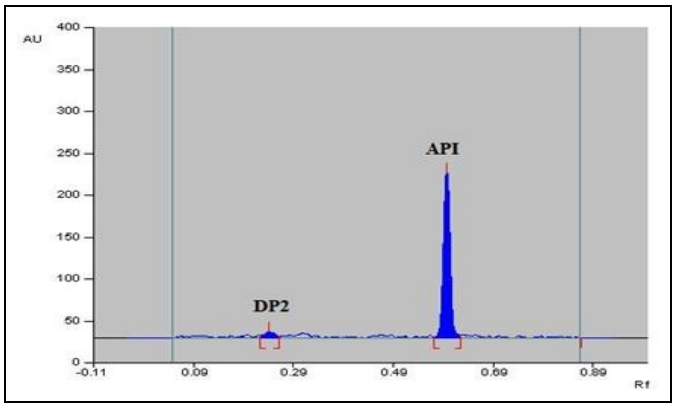

FIG. 6: DENSITOGRAM OF ALKALI (0.1 M NaOH) TREATED SAMPLE

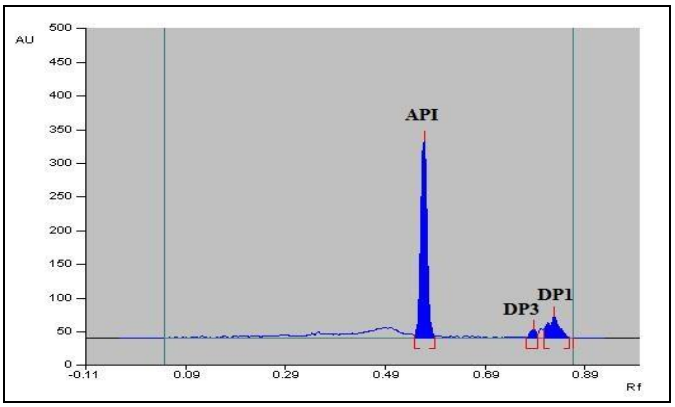

FIG. 7: DENSITOGRAM OF OXIDE (3% H_2O_2) TREATED SAMPLE

CONCLUSION: The developed method describes simple, sensitive, and selective stability indicating HPTLC method for estimation of Apixaban in pharmaceutical tablet dosage form. Method development and validation were performed as per ICH guidelines. The suggested method was found to be less time consuming and cost effective and may be more advantageous for routine analysis of drug in marketed formulation. Moreover, the method was validated as stability indicating assay for estimation of Apixaban in the presence of its degradation products. Therefore, the method can be employed as a stability-indicating one. The abovementioned study was able to explore the useful information which has not yet been reported in the literature of the API.

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CONFLICT OF INTEREST: The authors declared that there is no conflict of interest.

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