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Original Article

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPTLC METHOD FOR DETERMINATION OF APIXABAN AS BULK DRUG

MRINALINI C. DAMLE*, SWAPNIL S. WAGHMARE, PURUSHOTAM SINHA

Department of Quality Assurance, AISSMS College of Pharmacy, Savitribai Phule University, Kennedy Road, Near RTO, Pune 411001, Maharashtra, India
Email: mcdamle@rediffmail.com

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ABSTRACT

Objective: To develop and validate simple, sensitive stability indicating HPTLC (High performance thin layer chromatography) method for apixaban.

Methods: The chromatographic separation was performed on aluminium plates precoated with silica gel 60 F_{254} using toluene: ethyl acetate: methanol (3:6:1 v/v/v) as mobile phase followed by densitometric scanning at 279 nm.

Results: The chromatographic condition shows sharp peak of apixaban at Rf value of 0.38±0.03. Stress testing was carried out according to international conference on harmonization (ICH)Q1A (R2) guidelines and the method was validated as per ICH Q2(R1) guidelines. The calibration curve was found to be linear in the concentration range of 100-500 ng/band for apixaban. The limit of detection and quantification was found to be 11.66ng/bandand35.33ng/band, respectively.

Conclusion: A new simple, sensitive, stability indicating high performance thin layer chromatographic (HPTLC) method has been developed and validated for the determination of apixaban.

Keywords: Apixaban, HPTLC, Stability indicating method

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INTRODUCTION

Apixaban is an oral anticoagulant and direct inhibitor of factor Xa, which is used to decrease the risk of venous thrombosis, systemic embolization and stroke in patients with atrial fibrillation. Apixaban has been linked to a low rate of serum aminotransferase elevations during therapy and to rare instances of clinically apparent liver injury [1]. Apixaban is chemically described as 1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl)phenyl]-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide. Its molecular formula is $C_{25}H_{25}N_5O_4$ and molecular weight is 459.5 (fig. 1) [2].

Apixaban was approved for the prevention of stroke, blood clots in patients with non-valvular atrial fibrillation on December 28, 2012 [3]. On March 14 2014 it was approved for the use of preventing deep vein thrombosis in adult patients who have undergone total knee or hip replacement surgery [4].

On August 21, 2014, the food and drug administration (FDA) approved apixaban for the treatment of recurring deep vein thrombosis and pulmonary embolism [5]. Apixaban is not official in any of the Pharmacopoeia (USP, BP, EP–checked by online, IP-2014). According to literature survey,there are some HPLC, spectrofluorimetric and HPTLC methods reported for determination of apixaban [6-9] and hyphenated techniques such as LC-MS [10], UHPLC–MS/MS [11], either alone or ina combination tirofiban hydrochloride or with rivaroxaban.

To the best of our knowledge, no stability indicating HPTLC method has been reported for the determination of apixabanas bulk drug. The core-objective of this research work was to develop a simple, accurate, precise, and stability-indicating HPTLC method for the determination of apixaban as bulk drug.

MATERIALS AND METHODS

Chemicals and reagents

Apixaban was provided as a gift sample by wockhardt research and development centre, Aurangabad. Ethyl acetate AR grade purchased from FINAR chemicals ltd. methanol, toluene, and all other chemicals used in this study were of AR grade purchased from LOBA chemiepvt. ltd. Mumbai, India.

Instruments

Linomat-5 sample applicator (Camag, Switzerland), twin trough chamber (10 x 10 cm; Camag, Switzerland), TLC scanner 3 (Camag, Switzerland), WinCATS version 1.4.3 software (Camag, Switzerland), Photostability chamber (Newtronics NEC103RSPI), Shimadzu balance (Model AY-120), Camag100 µl sample syringe (Hamilton, Switzerland) were used in the study.

Preparation of standard solutions

Standard stock solution of apixaban was prepared by dissolving 10 mg of the drug in 10 ml of methanol to get concentration of 1000µg/ml. From the standard stock solution, working standard solution was prepared containing 20µg/ml of apixaban.

Selection of analytical wavelength

The standard solution of apixaban of concentration 10µg/ml was prepared using methanol and scanned over the wavelength range 200 nm to 400 nm by using UV-Visible spectrophotometer. λ_{max} was found to be 279 nm (fig. 2).

Chromatographic conditions

Initially, mobile phase optimization trials were conducted using (Chloroform: methanol in the ratios as 5:5, 7:3, 3:7), (Toluene: ethyl acetate-5:5, 7:3, 3:7). Optimized mobile phase was toluene: ethyl acetate: methanol (3:6:1 v/v/v). TLC plates precoated with silica gel 60 F_{254}, of dimension 10 cm × 10 cm with 250 µm layer thickness were used as stationary phase. TLC plates were pre-washed with methanol and dried. The standard solution of apixaban was spotted on the dried, pre-coated TLC plate as a band with 4 mm width. The chromatographic development was carried out by using toluene: ethyl acetate: methanol (3:6:1 v/v/v) as mobile phase with 15 min chamber saturation time and run up to distance 90 mm. Densitometric scanning was performed at 279 nm.

Optimization of mobile phase

Method development for apixaban was started with the development of densitogram using neat solvents and combinations of toluene, ethyl acetate and methanol in different ratios. Toluene: ethyl acetate: methanol in the ratio of (3:6:1 v/v/v) was selected as mobile phase for apixaban which gives accepted peak parameters. The Rf was found to be 0.38±0.03 for apixaban. The standard densitogram of apixaban (200ng/band) is shown in (fig. 3).

Stress degradation studies of bulk drug

Stress testing studies were carried out to provide evidence on how the quality of drug varies under various stress conditions like oxidation, hydrolysis, photolysis and thermal, etc. Stress degradation studies were designed by referring some papers [12-15]. Optimization of stress conditions was done by changing the strength of reagent and duration of exposure to get 10-30 % degradation. The stress degradation study was carried out as per ICH Q1A (R2) and Q1 B [16, 17].

Optimization trials

Initially trials were conducted using various normalities of HCl and NaOH by keeping the sample solution overnight. For the thermal study sample was heated at 80 °C for 4 h to 8 h and for oxidation, trials were conducted using 30 % $H_2O_2$by keeping the sample solution overnight. It was observed that the drug gets degraded partially.

Optimized stress conditions

Alkaline hydrolysis

1 ml working standard solution of apixaban (200 µg/ml) was mixed with 1 ml of 1 N NaOH and volume was made up to 10 ml with methanol. The solution was kept overnight. Average 69.41 % of apixaban was recovered with no peak of degradation.

Acid hydrolysis

5 ml standard solution of Apixaban (200 µg/ml) was mixed with 5 ml of 0.5 N HCl and volume was made up to 50 ml using methanol. The solution was refluxed for 30 min and cooled to room temperature. Average 76.48 % of apixaban was recovered with no peak of degradation.

Oxidative degradation

1 ml standard solution of apixaban (200 µg/ml) was mixed with 4 ml 30% v/v H_2O_2, volume was made up to 10 ml using methanol. Average 95% of apixaban was recovered with no peak of degradation after 30 min.

Degradation under dry heat

Dry heat study was performed by keeping the drug in hot air oven at 80 °C for 8 h. Average 77.20 % of apixaban was recovered with no peak of degradation.

Degradation under neutral condition

To 5 ml of 200µg/ml solution of apixaban, 5 ml of distilled water was added. The volume was made upto 50 ml with methanol. The above solution was refluxed for 2 h and then cooled. Average 91.48% of apixaban was recovered with no peak of degradation.

Photo-degradation studies

Photolytic degradation studies were carried out by exposure of drug to UV light up to 200 watt h/m2 and subsequently to cool white fluorescent light to achieve an illumination of 1.2 million Lux h. The sample was weighed, dissolved and diluted get 20µg/ml as final concentration and was applied to TLC plate. After the photo degradation study under UV light 100% and Fluorescence light 98.78% apixaban was recovered with no peak of degradation. Spotting of 2000ng/band was done for samples at each stress condition to locate peak for a degradation product if any.

Validation parameter

The developed method was successfully validated according to the ICH Q2 (R1) guidelines [18].

Specificity

The specificity of the method was ascertained by analyzing standard drug and sample. The spot for the drug in a sample was confirmed by comparing the Rf and the spectra of the spot with that of the standard drug spot. The specificity of the method was also ascertained by peak purity profiling studies by analyzing the spectrum at peak start, middle and at the peak end.

Linearity and range

The calibration curve was obtained in the range of 100-500ng/band by applying different volumes (5-25 µl) of stock solution of (20 µg/ml) ona TLC plate. Each standard in five replicates was analyzed and peak areas were recorded. The relationship between peak area and concentration was established bythe simple regression equation method (fig. 4).

Assay

5 tablets were accurately weighed and powdered. From the powder, an amount equivalent to 5 mg of apixaban was accurately weighed and transferred to 10 ml volumetric flask. Methanol was added, sonicated for 15 min, a solution was filtered. Dilutions were made to get the final concentration20µg/ml. The assay was calculated by extrapolation from standard curve which was found to be 101.02 %

Accuracy

To check accuracy of the method, recovery studies were carried out by adding a standard drug to sample at three different levels 80, 100 and 120 %. Basic concentration of the sample chosen was 200ng/band. The drug concentrations were calculated by using the regression equation of apixaban.

Precision

The precision of the method was demonstrated by intra-day and inter-day studies. In the intra-day studies, 3 replicates of 3 standard solutions were analyzed in a same day and percentage RSD was calculated. For the inter-day, 3 standard solutions were analyzed on three consecutive days and percentage RSD was calculated.

Method sensitivity 'Limit of detection' (LOD) and 'limit of quantification' (LOQ)

LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S respectively. Where σ is the standard deviation of the lowest concentration response and S is the slope of the calibration plot. The LOD and LOQ were found to be11.66ng/band and 35.33ng/band respectively.

Robustness

The robustness of the method were studied during method development, by small but deliberate variations in chamber saturation time (13, 17 min), change in mobile phase composition, Time was changed from spotting to development and development to scanning and the effect on the peak area was noted.

RESULTS

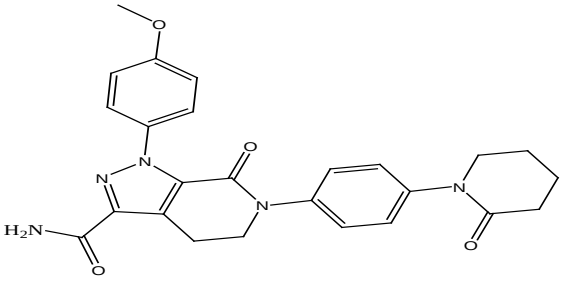

Fig. 1: Chemical structure of apixaban

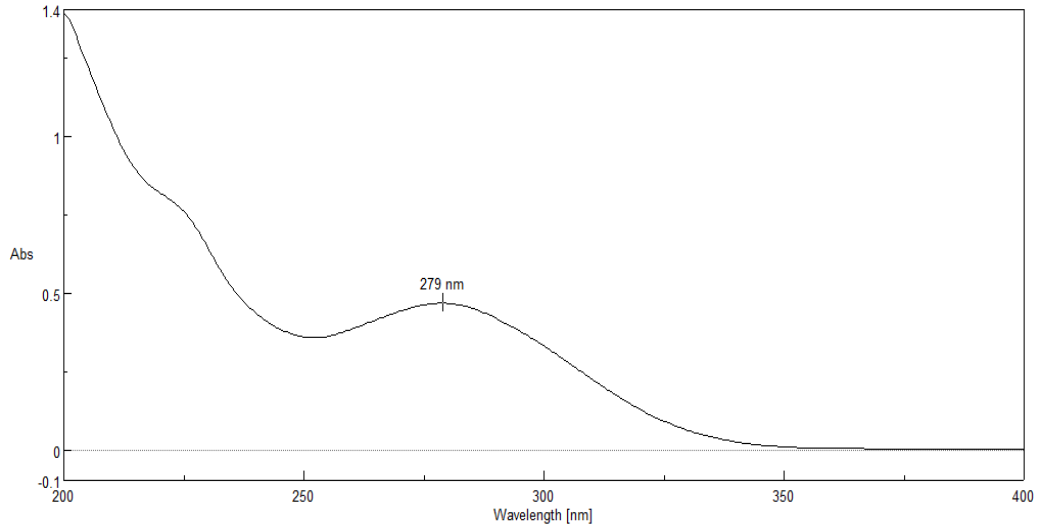

Fig. 2: UV spectrum of apixaban between 200-400 nm

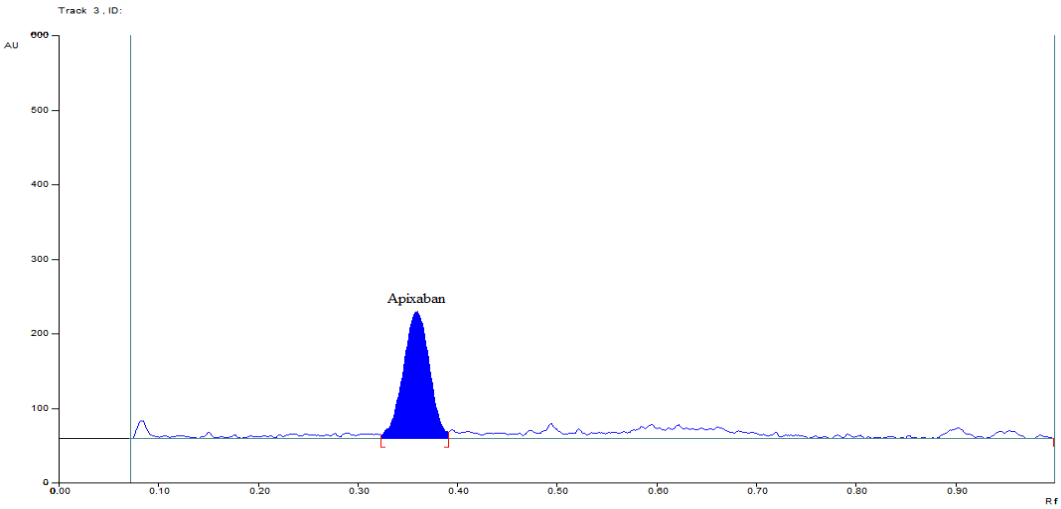

Fig. 3: Densitogram of standard solution of apixaban 200 ng/band Rf(0.38±0.03)

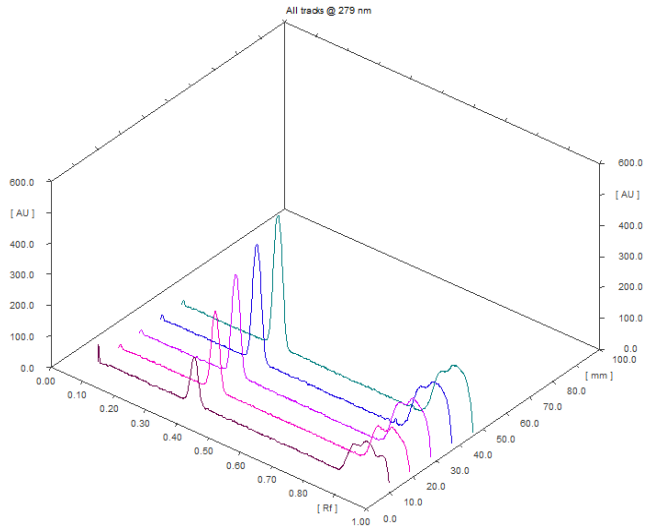

Fig. 4: Densitogram of standard solution of apixaban (100-500ng/band)

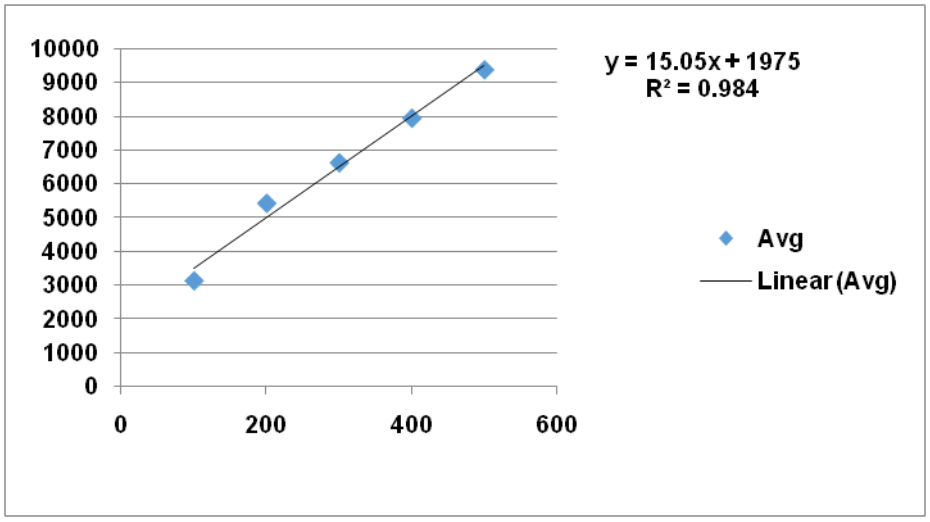

Fig. 5: Calibration curve of apixaban

Stress degradation

Initially the drug was subjected to various forced degradation conditions. The conditions of stress were optimized with respect to the strength of the reagent and exposure period so as to achieve 10 to 30 % degradation. During optimization, degradation condition was adjusted by the increase and decrease in concentration and strength of reagent and its time of exposure.

Summary of stress degradation results is given in (table 1).

Table 1: Summary of stress degradation study of apixaban (n=2)

Stress degradation condition	% Recovery	% Degradation	Peak purity	
			r(s, m)	r(m, e)
Acid (0.5 N HCl) reflux for 30 min	76.48	23.52	0.999	0.993
Base (1 N NaOH, overnight)	69.41	30.59	0.999	0.995
Oxidation (30% v/v H_2O_2) 30 min	95	5.0	0.999	0.997
Neutral reflux 2 h	91.48	8.52	0.999	0.995
Dry heat (80 °C 8 h)	77.20	22.8	0.999	0.998
Photo stability UV 200 watt h/m2	100	-	0.999	0.992
Flourescence 1.2 million lux. h	98.78	1.22	0.999	0.994

n = number of determinations for each conditions

Method validation

Table 2: Result of accuracy (recovery) study of apixaban (n=3)

Concentration (ng/band)		Amount recovered	%Recovery mean±SD	%RSD
Sample conc	Amount added			
200	160	361.22	100.42±1.32	1.31
200	200	402.95	100.73±1.06	1.05
200	240	437.85	99.51±0.85	0.86

n = number of determinations, SD = Standard Deviation, %RSD = %Relative Standard Deviation

Table 3: Intra-day precision (n=3)

Concentration (ng/band)	Area (mean±SD)	%RSD
100	3120.07**±39.82**	1.27
200	5409.03**±56.67**	1.04
300	6629.97**±64.78**	0.97

n = number of determinations

Table 4: Inter-day precision (n=3)

Concentration (ng/band)	Area(mean±SD)	%RSD
100	3128.73**±40.76**	1.30
200	5443.3**±49.74**	0.91
300	6534.03**±56.37**	0.86

n = number of determinations

Table 5: Robustness study of apixaban (n=3)

Parameters	Robust condition	Area (mean±SD)	% RSD
Chamber saturation time (15 min)±2 min	13 min	4568.67±75.51	1.65
	17 min	4363.8±29.84	0.68
Mobile phase composition toluene: ethyl acetate: methanol (3:6:1 v/v)±0.2 methanol	Toluene: ethyl acetate: methanol (3:6:0.8)	4449.4±49.34	1.10
	Toluene: ethyl acetate: methanol (3:6:1.2)	4694.67±54.40	1.16
Time from application to development (immediate)	After 30 min	2872.67±34.29	1.19
	After 1 h	3081.43±54.52	1.76
Time from development to scanning(immediate)	After 30 min	3068.17±53.61	1.74
	After 1 h	3062.93±57.66	1.88

n = number of determinations, SD = Standard Deviation, %RSD = %Relative Standard Deviation

The method validation results were satisfactory as per ICH Q2R1 guidelines. The peak area was found to be linear over the concentration range of 100-500 ng/band with a correlation coefficient of 0.984. Method specificity can be proved using peak purity parameter in WinCATS software of HPTLC. There is a provision to compare the UV spectrum at the start, middle and end of any peak. Inter and Intra-day precision was less than 2%. Percent recovery in an accuracy study was within the limit of 98 to 102 %. The results of validation are summarized in table 6.

Table 6: Summary of validation parameter

Validation parameter	Results
Linearity	Y = 15.05x+1975 R^2 = 0.984
Range	100-500 ng/band
Precision	(%RSD)
A) Intra-day	1.27
B) Inter-day	1.30
Accuracy	(%Recovery)
80%	100.42
100%	100.73
120%	99.51
LOD	11.66 ng/band
LOQ	35.33 ng/band
Specificity	Specific
Robustness	Robust

DISCUSSION

While developing stability indicating method, in the current work, the stress conditions were optimized to achieve 10-30% degradation. In the literature survey, it observed that the degradation pattern under alkaline hydrolytic conditions reported in work by landge *et al.* [7] and prabhune *et al.* [6], do not match at all. Our results fairly match the ones reported by landge *et al.* except for the degradation product. Apixaban was found to be fairly stable to photo-degradation. Both the reported papers do not have mention of neutral hydrolysis. These papers report Apixaban to be thermally stable but we have observed degradation to an extent of 22.8%

CONCLUSION

The developed method is simple, sensitive, and precise. No degradation product was observed with the optimized stress conditions. Non-interference has been proved by peak purity studies. Since there is no interference, this method can be used routinely for estimation of apixaban.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally to this manuscript

CONFLICT OF INTERESTS

Declared none

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