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 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 C25H25N5O4 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 in a combination with other analytical methods. 

To the best of our knowledge, no stability indicating HPTLC method has been reported for the determination of apixaban as 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 chemieptvt. 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 (Neutronics NEC103RSPI), Shimadzu balance (Model AV-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. λmax 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 F254, 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 10-50 % 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 % H2O2 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 76.40 % 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 H2O2, 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 2000ng/ml solution of apixaban, 5 ml of distilled water was added. The volume was made up to 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 200µg/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 200ng/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) on a TLC plate. Each standard in five replicates was analyzed and peak areas were recorded. The relationship between peak area and concentration was established by the 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 be 11.66ng/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](image-url)
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)
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 |
|-------------------------------------|------------|---------------|-------------|
| Acid (0.5 N HCl) reflux for 30 min  | 76.48      | 23.52         | 0.999       |
| Base (1 N NaOH, overnight)          | 69.41      | 30.59         | 0.999       |
| Oxidation (30% v/v H₂O₂ for 30 min) | 95         | 5             | 0.999       |
| Neutral reflux 2 h                  | 91.48      | 8.52          | 0.999       |
| Dry heat (80 °C 8 h)                | 77.20      | 22.8          | 0.999       |
| Photo stability UV 200 watt h/m²    | 100        | -             | 0.999       |
| Fluorescence 1.2 million lux. h     | 98.78      | 1.22          | 0.999       |

n = number of determinations for each conditions

Method validation

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

| Concentration (ng/band) | Amount added | 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                     | 6662.99±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
The stress conditions were optimized to achieve 10-30% degradation. In the current work, 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%. The results of validation are summarized in Table 6.

### 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 |
| Time from application to development (Immed)     | 17 min           | 4363.8±29.84   | 0.68 |
| Time from development to scanning (Immediate)    | Toluene: ethyl acetate: methanol (3:6:1) | 4449.4±49.34   | 1.10 |
| Mobile phase composition toluene: ethyl acetate: methanol (v/v) ± 0.2 methanol | 4694.6±74.40     | 1.16 |
| Time from application to development (Immed)     | Toluene: ethyl acetate: methanol (3:6:0.8) | 2872.67±34.29  | 1.19 |
| Time from development to scanning (Immediate)    | After 30 min     | 3081.43±54.52  | 1.76 |
|                                                | After 1 h         | 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

### Table 6: Summary of validation parameter

| Validation parameter | Results |
|----------------------|---------|
| Linearity            | Y = 15.05x + 1.795 R² = 0.984 |
| Range               | 100-500 ng/band |
| Precision           | 1.27 |
| A) Intra-day         | 1.30 |
| B) Inter-day         | 1.27 |
| Accuracy            | 100.42 |
| 80%                 | 100.73 |
| 100%                | 100.51 |
| 120%                | 11.66 ng/band |
| LOD                  | 35.33 ng/band |
| LOQ                  | Specific |
| Specificity          | Robust |
| Robustness           | Validated spectrofluorimetric methods for the determination of apixaban and tirofiban hydrochloride in pharmaceutical formulations. Spectrochim Acta A Mol Biomol Spectrosc 2017;174:326–30. |

### 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 was 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. 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 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.

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### AUTHORS CONTRIBUTIONS

All authors have contributed equally to this manuscript.

### CONFLICT OF INTERESTS

Declared none

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