Quality risk assessment and DoE – Practiced validated stability-indicating chromatographic method for quantification of Rivaroxaban in bulk and tablet dosage form

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ABSTRACT

A systematic DoE and Analytical Quality by Design (AQbD) approach was utilized for the development and validation of a novel stability indicating high-performance thin-layer chromatographic (HPTLC) method for Rivaroxaban (RBN) estimation in bulk and marketed formulation. A D-optimal design was used to screen the effect of solvents, volume of solvents, time from spotting to development and time for development to scanning. ANOVA results and Pareto chart revealed that toluene, methanol, water and saturation time had an impact on retention time. The critical method and material attributes were further screened by Box-Behnken design (BBD) to achieve optimal chromatographic condition. A stress degradation study was carried out and structure of major alkaline degradant was elaborated. According to the design space, a control strategy was used with toluene: methanol: water (6:2:2) and the saturation time was 15 min. A retention factor (RF) of 0.59 ± 0.05 was achieved for RBN using chromatographic plate precoated with silica gel at detection wavelength 282 nm with optimized conditions. The linear calibration curve was achieved in the concentration range of 200–1,200 ng/band with \( r^2 > 0.998 \) suggesting good coordination between analyte concentration and peak areas. The quadratic model was demonstrated as the best fit model and no interaction was noted between CMAs. The optimized HPTLC method obtained through AQbD application was potentially able to resolve all degradants of RBN achieved through forced degradation study. The obtained results demonstrate that a scientific AQbD approach implementation in HPTLC method development and stress degradation study drastically minimizes the number of trials in experiments, ultimately time and cost of analysis could be minimized.

KEYWORDS

analytical quality by design (AQbD), stability study, Rivaroxaban, validation, high performance thin-layer chromatography (HPTLC)
INTRODUCTION

Rivaroxaban (RBN) is a novel, direct acting, target-specific, potent oral anticoagulant drug. It acts at a crucial stage in the process of blood-clotting by inhibiting potentially the free and clot-bound coagulation factor Xa (Vitamin K dependent plasma protein) and prothrombinase activity, thus effective blocking of thrombin generation leads to prolongation of clotting time [1]. It is used in treatment and prevention of stroke in adult patients suffered with atrial fibrillation, inhibition of cardiovascular events in association with acute coronary syndrome and the inhibition of venous thromboembolism in patients which undergo selective knee or hip replacement surgery [2]. RBN is an excellent alternative to low molecular weight heparins for the treatment and prevention of cancer-associated deep venous thrombosis/pulmonary embolism [3]. RBN, an oxazolidinone-based anticoagulant is small, water insoluble molecule, has molecular formula C_{19}H_{18}ClN_{3}O_{5}S and chemically it is (S)-5-chloro-N-[2-oxo-3-[4-(3-oxomorpholin-4-yl)phenyl]-1,3-oxazolidin-5-yl-methyl] thiophen-2-carboxamide (Fig. 1) [4]. It is odourless, white to yellowish powder with molecular weight of 435.882 g mol$^{-1}$ [5].

The International conference on harmonization of technical requirements for registration of pharmaceuticals for human use (ICH) guidelines on stress testing of new drug substances and product require that stress testing imperative step of drug carried out to determine the stability of the drug and active constituent. It helps to analyze the intrinsic stability of the drug. Identification of degradation products and degradation pathway have a crucial role in drug development [6]. The ICH stability guideline Q1A (R2) necessitates stress study of drugs to be performed under hydrolysis, oxidation, under the exposure to UV light and varied temperature. The degradation products formed during different stress conditions might be unsafe, toxic and results in certain physiological complications. Hence it was utmost necessary to identify and quantify the degradation product.

The Quality-by-Design (QbD) concept is thorough understanding of the process to achieve robust method along with better quality product. According to ICH Q8 guidelines begins with predefined objectives and focused on product and process understanding based on sound science and quality risk management. It is imperative to enhance the efficiency and cost-effectiveness. The principle of QbD when applied to analytical method is termed as analytical QbD (AQbD). The output of AQbD is a well understood and robust method that constantly supplies the desired performance throughout its lifecycle. Factors affecting the robustness are taken into account for the improvement of the analytical method in the QbD [7, 8]. In AQbD robustness and reproducibility of the method build while developing the method while in traditional approach quality is assured at the end of sample testing. AQbD is flexible process and allows continuous improvement [9, 10]. The ease of identification of factors affecting the method performance is enabled by AQbD approach. One of the key role of QbD is the designing of a design space that allows the analytical method to be developed and validated under a varied factor with the minimum number of experiments. Pareto chart are useful to find the critical material attributes and critical processing parameters and response surface methodology was useful to optimize these parameters. In addition, modification within the design space are acceptable by regulatory authorities [11].

Literature survey revealed variety of analytical methods employed for estimation of Rivaroxaban either alone or in combination with other drug as well as on bulk drug, marketed formulations or on human plasma like UV spectrophotometric method [12], HPLC and RP-HPLC method with UV or DAD detector [4, 13–17], LC-MS/MS [18–22], UPLC [23] etc. has been reported. To the best of our knowledge, there is no DoE practiced, AQbD based stability indicating TLC densitometric method reported for quantitative estimation of RBN in marketed formulation as well as potentially resolves the degradation products obtained in stress degradation study. Hence, present study was a successful effort, to develop a scientific, validated DoE designed, AQbD based HPTLC method for quantitative estimation of RBN in marketed formulation. Presented method potentially resolves and quantitates the degradation product identified through stress degradation studies on RBN as per ICH-stated conditions. Further degradation pathway was established for isolated alkaline degradation product after characterization with LC-MS and FT-IR technique.

EXPERIMENTAL

Materials and reagents

RBN pure drug (Purity $\sim$ 99.8%) was procured from Bayer Pharmaceutical Private Limited, (India) as a gift sample. The solvents methanol, toluene and water used were purchased of HPLC grade (Merck specialties Pvt. Ltd). Tablets of Xarelto® (Bayer HealthCare) were purchased from the local pharmacy. Pre-coated silica gel 60F$_{254}$ on aluminium sheets was procured from E. Merck Ltd, India.

Instrumentation

The instrument used for analysis was Camag HPTLC system comprising Linomat V automatic sample applicator (Camag,
Mutenz, Switzerland), Microsyringe (Linomat 5 syringe, Hamilton-Bonaduzschweiz, Camag, Switzerland), pre-coated silica gel 60F-254 glass plates (10 × 10 cm with 200 l mm thickness HPTLC; Merck, Germany), twin trough chamber 10 × 10 cm (Camag, Mutenz, Switzerland), UV chamber (Camag, Mutenz, Switzerland), TLC scanner III (Camag, Mutenz, Switzerland), and winCATS version 1.4.0 software (Camag, Mutenz, Switzerland). Other equipment used were sonicator (Imecoultrasonics, India), analytical balance (Shimadzu AUX 200, Japan) and auto pipettes (Eppendorf, Hamburg, Germany). Design expert software 12® (Trial version-Stat-Ease Inc., Minneapolis) was used for implementing QbD in a present research work.

**Preparation of solution**

**Standard solution of RBN.** Accurately weighed quantity (100 mg) of RBN was transferred to a 100 mL volumetric flask, dissolved and diluted up to the mark with methanol. From this solution, 10 mL was transferred to a 100 mL volumetric flask and diluted to the mark with methanol. The solution was mixed and filtered through a 0.45 μm membrane filter.

**Forced degradation studies.** In order to evaluate the stability indicating property of the developed HPTLC method, stress studies were carried out under ICH recommended conditions. Intentional degradation was tried by exposing the tablet sample to the following stress conditions: acid (0.1 N HCl at 80°C), base (0.1 N NaOH at 80°C), oxidation (3% H2O2 at 80°C), dry heat (80°C), and UV light (254 nm). The ability of the proposed method to measure the analyte response in the presence of its degradation products was studied [23].

**Isolation of alkaline degradation product.** An accurately weighed quantity 100 mg of RBN was dissolved in 70 mL methanol. Subsequently, 1.0 N sodium hydroxide was added and volume was made up to 100 mL in a graduated A-grade volumetric flask. The resulted solution was refluxed in round bottom flask on a temperature controlled precision water bath at 80°C for 3.0 h. The alkaline degradation of the RBN was confirmed by newly developed HPTLC method, where the major degraded form in alkaline stressed condition was isolated through preparative HPTLC technique. The peak area was determined and quantitative estimation of RBN and degradant formed in alkaline stressed condition was carried out from the corresponding regression equation.

**Preliminary trials to identify critical method and material parameters (CMPs).** Various preliminary trials were performed to identify critical method and method parameters (CMPs) for the development of a stability-indicating HPTLC method. The amount of toluene, methanol and water, volume of mobile phase, saturation time, time from spotting to development and time from development to scanning were found CMPs for retention factor of drug and its degraded products.

**Screening of potential method variables by D-optimal design.** A D-Optimal factorial design (Design Expert software 12- Trial version) was applied for the screening of CMPs on retention factor as this design provides optimum combination with minimum runs. Thirty-four experimental runs were performed in the laboratory in triplicate (Table 1). TLC plates were checked for compact and sharp spots of the drug and its degradation products with a better retention factor. All the values for the retention factor were entered against the respective experimental run and analyzed for the effect of CMPs on retention factor. Statistical analysis was carried out by ANOVA and Pareto chart was employed for identification of the effect of CMPs on retention factor.

**Response surface modeling by Box-Behnken Design (BBD).** The effect of toluene, methanol, water and saturation time was found significant on retention time, hence these CMPs were varied further to achieve the optimum method parameters (Table 2). For response surface Modeling Box-Behnken design was used to study these effects on retention factor (Design-Expert software 12- Trial version). Twenty-five runs were carried out in triplicate by varying solvents and saturation time at three levels. The retention factor was calculated for each run. The retention factor values were entered against each run and analyzed for the impact of CMPs on retention factor.

**Optimized chromatographic conditions.** Aluminium plates of dimensions 10 × 10 cm, pre-coated with a 250-μm layer of silica gel 60F254 (E. Merck, Darmstadt, Germany) were used for chromatographic separation. Plates were pre-washed with methanol and dried in an oven at 60°C for 15 min. The samples were spotted on a TLC plate 15 mm from the bottom edge by a CAMAG Linomat V semi-automatic spotter using a bandwidth of 8 mm and an application rate of 0.3 μL s⁻¹. The TLC plate was developed in the twin-trough chamber using toluene: methanol: water (6:2:2 v/v) as the mobile phase at a chamber saturation time of 15 min, relative humidity of 35 ± 5%, a temperature of 25 ± 2°C and a migration distance of 80 mm. The bands were scanned using the TLC scanner 3 in the reflectance/absorbance mode at 282 nm and analyzed by win CATS software using a slit dimension of 4 × 0.30 mm and a scanning speed of 20 mm s⁻¹. Concentrations of the sample were determined from the intensity of reflected light and by comparing peak area of sample band with that of the standard band.

**Wavelength detection for drug analysis.** From both the working standard solution and the forced degraded sample of the RBN, an aliquot of 20 μL was spotted on aluminium plates pre-coated with a 250-μm layer of silica gel 60 F254. The TLC plate was developed and dried and spots were...
scanned in a range of 200–800 nm for detection of wavelength [24].

Assay of RBN marketed formulation. Twenty tablets of Xarelto® were weighed accurately to obtain the average weight and finely powdered by crushing in mortar and pestle for 10 min. An accurately weighed quantity of tablet powder equivalent to about 10 mg of RBN was transferred to a 10.0 mL volumetric flask, added 3 mL methanol and the contents of the flask were sonicated for about 15 min, volume was then made up to the mark with methanol. The solution was mixed and filtered through the Whatman filter paper no. 41. An aliquot of 1 mL of this solution was diluted to 10.0 mL with methanol. Further, resulting solution (0.3 μL) was applied on HPTLC plates in the form of bands, plates were developed, and TLC-densitometric evaluation was conducted to obtain the results.

Preparation of calibration curve. The working standard solution of 200, 400, 600, 800 and 1,200 ng were spotted on the TLC plate. The plate was developed, dried, and analyzed. The calibration curve was constructed by plotting peak area versus concentration of RBN.

Method validation. The method validation process was carried out by performing the validation parameters as per ICH recommended guidelines. The Linear relationship between peak area and concentration of the drugs was evaluated over a range of concentrations expressed in ng/band, making five measurements at 10 concentration levels in the range of 200 ng/band – 1,200 ng/band. 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 (standard addition method). Hence, 8 mg, 10 mg, and 12 mg of RBN were spiked to the pre-analyzed tablet powder containing 10 mg of RBN. The system precision was evaluated by six replicate analysis of the standard solution. The method precision was studied by analyzing six different standard solutions of same concentration. The results for method precision and system

| Trial No | Toluene (mL) | Methanol (mL) | Water (mL) | Volume of mobile phase (mL) | Saturation time (Min) | Time from spotting to development (Min) | Time from development to scanning (Min) | Retention factor |
|----------|--------------|---------------|------------|-----------------------------|-----------------------|----------------------------------------|----------------------------------------|-----------------|
| 1.       | 7            | 2             | 1          | 9                           | 15                    | 5                                      | 5                                      | 0.5             |
| 2.       | 6            | 2             | 1          | 9                           | 5                     | 5                                      | 5                                      | 0.53            |
| 3.       | 7            | 1             | 2          | 10                          | 5                     | 20                                     | 5                                      | 0.48            |
| 4.       | 7            | 1             | 2          | 10                          | 15                    | 5                                      | 20                                     | 0.5             |
| 5.       | 7            | 1             | 1          | 9                           | 5                     | 5                                      | 5                                      | 0.42            |
| 6.       | 7            | 1             | 1          | 9                           | 15                    | 20                                     | 5                                      | 0.45            |
| 7.       | 6            | 1             | 1          | 9                           | 5                     | 20                                     | 5                                      | 0.47            |
| 8.       | 6            | 2             | 2          | 10                          | 15                    | 5                                      | 5                                      | 0.57            |
| 9.       | 6            | 2             | 1          | 10                          | 15                    | 20                                     | 5                                      | 0.56            |
| 10.      | 6            | 1             | 2          | 10                          | 5                     | 5                                      | 5                                      | 0.56            |
| 11.      | 6            | 2             | 2          | 9                           | 15                    | 20                                     | 5                                      | 0.56            |
| 12.      | 7            | 1             | 1          | 10                          | 5                     | 5                                      | 5                                      | 0.43            |
| 13.      | 6            | 1             | 1          | 9                           | 15                    | 5                                      | 5                                      | 0.43            |
| 14.      | 7            | 2             | 2          | 9                           | 15                    | 20                                     | 5                                      | 0.53            |
| 15.      | 7            | 1             | 1          | 1                           | 9                     | 15                                     | 5                                      | 0.46            |
| 16.      | 7            | 1             | 2          | 9                           | 15                    | 5                                      | 5                                      | 0.49            |
| 17.      | 7            | 1             | 1          | 10                          | 15                    | 20                                     | 5                                      | 0.47            |
| 18.      | 6            | 2             | 1          | 10                          | 5                     | 5                                      | 5                                      | 0.49            |
| 19.      | 6            | 2             | 2          | 10                          | 5                     | 20                                     | 5                                      | 0.55            |
| 20.      | 7            | 1             | 1          | 10                          | 15                    | 5                                      | 5                                      | 0.43            |
| 21.      | 6            | 1             | 2          | 9                           | 15                    | 20                                     | 5                                      | 0.5             |
| 22.      | 6            | 1             | 2          | 10                          | 15                    | 20                                     | 5                                      | 0.5             |
| 23.      | 6            | 2             | 2          | 9                           | 15                    | 5                                      | 5                                      | 0.56            |
| 24.      | 7            | 2             | 1          | 10                          | 15                    | 5                                      | 5                                      | 0.51            |
| 25.      | 7            | 2             | 1          | 10                          | 5                     | 20                                     | 5                                      | 0.45            |
| 26.      | 6            | 2             | 1          | 10                          | 5                     | 20                                     | 5                                      | 0.49            |
| 27.      | 6            | 2             | 1          | 9                           | 5                     | 20                                     | 5                                      | 0.52            |
| 28.      | 7            | 2             | 2          | 9                           | 5                     | 20                                     | 5                                      | 0.48            |
| 29.      | 7            | 2             | 2          | 10                          | 5                     | 5                                      | 5                                      | 0.43            |
| 30.      | 6            | 1             | 1          | 10                          | 15                    | 5                                      | 20                                     | 0.5             |
| 31.      | 6            | 1             | 2          | 9                           | 5                     | 5                                      | 20                                     | 0.5             |
| 32.      | 7            | 2             | 2          | 9                           | 5                     | 20                                     | 5                                      | 0.51            |
| 33.      | 7            | 2             | 2          | 10                          | 15                    | 20                                     | 5                                      | 0.46            |
| 34.      | 7            | 1             | 2          | 9                           | 5                     | 20                                     | 20                                     | 0.47            |
Table 2. Box-Behnken design trials to optimized solvents and saturation time

| Trial No | Toluene (mL) | Methanol (mL) | Water (mL) | Saturation time (Min) | Retention factor |
|----------|--------------|---------------|------------|-----------------------|-----------------|
| 1.       | 6.50         | 2.00          | 3.00       | 15.00                 | 0.52            |
| 2.       | 6.50         | 2.00          | 3.00       | 5.00                  | 0.44            |
| 3.       | 6.00         | 1.00          | 2.00       | 10.00                 | 0.48            |
| 4.       | 6.50         | 2.00          | 2.00       | 5.00                  | 0.57            |
| 5.       | 6.50         | 3.00          | 3.00       | 10.00                 | 0.43            |
| 6.       | 6.50         | 3.00          | 2.00       | 15.00                 | 0.46            |
| 7.       | 6.50         | 2.00          | 2.00       | 15.00                 | 0.59            |
| 8.       | 6.00         | 2.00          | 1.00       | 10.00                 | 0.49            |
| 9.       | 6.50         | 3.00          | 2.00       | 5.00                  | 0.45            |
| 10.      | 7.00         | 2.00          | 2.00       | 5.00                  | 0.43            |
| 11.      | 6.50         | 2.00          | 3.00       | 10.00                 | 0.44            |
| 12.      | 7.00         | 2.00          | 3.00       | 10.00                 | 0.45            |
| 13.      | 7.00         | 3.00          | 2.00       | 10.00                 | 0.42            |
| 14.      | 6.50         | 1.00          | 1.00       | 10.00                 | 0.46            |
| 15.      | 6.50         | 1.00          | 2.00       | 15.00                 | 0.50            |
| 16.      | 6.50         | 2.00          | 3.00       | 5.00                  | 0.45            |
| 17.      | 6.50         | 1.00          | 3.00       | 10.00                 | 0.43            |
| 18.      | 6.50         | 3.00          | 1.00       | 10.00                 | 0.46            |
| 19.      | 6.50         | 1.00          | 2.00       | 5.00                  | 0.42            |
| 20.      | 6.50         | 2.00          | 1.00       | 15.00                 | 0.48            |
| 21.      | 6.00         | 2.00          | 3.00       | 10.00                 | 0.46            |
| 22.      | 7.00         | 1.00          | 2.00       | 10.00                 | 0.41            |
| 23.      | 7.00         | 2.00          | 1.00       | 10.00                 | 0.42            |
| 24.      | 7.00         | 2.00          | 2.00       | 15.00                 | 0.45            |
| 25.      | 6.00         | 3.00          | 2.00       | 10.00                 | 0.45            |

RESULTS AND DISCUSSION

To develop a stability-indicating HPTLC method for RBN, the retention factor and degradation products of the drug are crucially important. The current research work was aimed to identify the degradation pathway of RBN degradant, based on the development of a robust stability-indicating HPTLC assay method. To elucidate inherent stability characteristics of the drug, stress conditions were employed and retention factor of the drug was noted. While developing the method certain factors such as type of solvent, solvent volume, saturation time, time from spotting to development, time from development to scanning etc. were found important and should be optimized carefully. The validation parameters such as linearity range, accuracy and precision of the developed RP-HPTLC method were compared with existing UV, HPLC and UPLC methods. The linearity range of RBN reported using UV spectroscopy, HPLC and UPLC methods were found to be inferior to present reversed-phase densitometry, while the accuracy and precision values were comparable. The method validation parameters obtained from RP-HPTLC were found to be within the ICH recommendation limits [23]. The quantitative methods reported in literature are not as green compared to the present method. Overall, green RP-HPTLC technique was found to be reliable and superior to previously reported analytical techniques for the detection and quantification of RBN.

Screening of potential method variables by D-optimal design

A D-Optimal factorial design (Design Expert software 12-Trial version) was applied for the screening of CMPs on retention factor as this design provides optimum combination with minimum runs. Application of other designs resulted into too many runs; hence it was decided to choose D-optimal design for selection of significant CMPs. This design was commonly used to create fractional general factorial experiments. Thirty-four experimental runs were performed in the laboratory in triplicate (Table 1). TLC plates were checked for compact and sharp spots of the drug and its degradation products with a better retention factor. In the present study toluene was chosen as non-polar solvent, water and methanol were selected as polar solvents. The effects of different combination of these solvents on retention factors in combination with other parameters were studied. The effect of chamber saturation time was studied as incomplete saturation yields the diffused peaks of sample. It was found that the all these solvents had significant impact on the retention factor. These results were supported by Pareto chart (Fig. 2A). Pareto chart revealed that the content of toluene was beyond the Bonferroni limit, means it was very crucial factor to affect the retention factor. The amount of methanol, water and saturation was also t-value limit hence these are also significant factors to affect the retention factor. Minimum 9–10 mL of the solvent volume was used to carry out all the trials and it was found sufficient to for each
run. The design space for volume of solvent was ±1. The design space for time from spotting to development and development to scanning was varied as 5, 10, 15 and 20 min it was noted that these parameters did not exhibit any significant effect on retention factor. These results were further supported by Pareto chart; all these parameters were found below t-value, indicating that these parameters were non-significant. The D-Optimal design was statistically analyzed by ANOVA and model was found significant as F value was 11.29 and P-value was less than 0.001 (Table 3). The predicted R-Squared value of 0.5640 was found reasonable agreement with the adjusted R-Squared of 0.6859. Adequate precision measures the signal to noise ratio. A ratio greater than 4 was desirable. For the current run this value was 12.401 indicates an adequate signal.

Response surface modeling by Box-Behnken design (BBD)

From the data of D-optimal design, it was found that solvents and saturation time had significant impact on retention factor, hence these CMPs was further studied by varying at different levels. Box-Behnken design was used to screen and optimize the chromatographic conditions. Toluene was varied in the range of 6–7 mL, water and methanol was varied in the range of 1–3 mL and saturation time was varied from 5 to 15 min, total 25 trials was carried out in triplicate (Table 2). The design was statistically analyzed by ANOVA (Table 3). Quadratic model was found best fit model, F-value and P-value was 3.56 and 0.0118 respectively. These values proved that model was significant. The interaction between the variables was found insignificant (Fig. 2). The impact of variables was shown by equation

\[
\text{Retention Factor} = +0.44 - 0.038*A - 2.500 - 003*B
- 8.333 - 004*C + 0.020*D + 1.000
- 002*A*B + 0.015*A*C
+ 0.000*A*D + 0.000*B*C
- 0.018*B*D + 7.500 - 003*C*D
+ 0.019*A^2 - 0.012*B^2 + 2.917
- 003*C^2 + 0.037*D^2
\]

The response surface quadratic model was used for the desired retention factor of near about 0.5–0.6 to optimize the experimental conditions. The suggested experimental runs were performed in a laboratory to examine the validity of the model. The retention factor of all performed experiments was in agreement with the predicted responses, suggesting that the model was valid for defining design space for the development of a robust HPTLC method According to the design space, a control strategy was implemented for the development of an analytical method with a retention factor of 0.5–0.6 was 6 mL of toluene, 2 mL of methanol, 2 mL water and a

Fig. 2. A) Pareto Chart B) Contour plot C) 3D response surface graph for methanol and toluene D) Contour plot E) 3D response surface graph for toluene and water F) Contour plot G) 3D response surface graph for methanol and water H) Contour plot I) 3D response surface graph for toluene and saturation time J) Contour plot K) 3D response surface graph for methanol and saturation time L) Contour plot M) 3D response surface graph for saturation time and water on retention factor
### Table 3. ANOVA response for D-optimal factorial design and Box-Behnken design

#### D-optimal factorial design

| Source                      | Sum of Squares | df | Mean Square | F-Value | P Value | Significance |
|-----------------------------|----------------|----|-------------|---------|---------|--------------|
| Model                       | 0.044          | 7  | 6.278       | 11.29   | <0.0001 | Significant  |
| A-Toluene                   | 0.026          | 1  | 0.026       | 47.60   | <0.0001 | Significant  |
| B-Methanol                  | 4.573          | 1  | 4.573       | 8.23    | 0.0081  | Significant  |
| C-Water                     | 4.395          | 1  | 4.395       | 7.90    | 0.0093  | Significant  |
| D-Volume of mobile phase    | 4.636          | 1  | 4.636       | 8.338   | 0.9772  | Non-Significant |
| E-Saturation time           | 7.376          | 1  | 7.376       | 13.27   | 0.0012  | Significant  |
| F-Time from spotting to development | 2.259 | 1  | 2.259       | 0.41    | 0.5294  | Non-Significant |
| G-Time from development to scanning | 6.066 | 1  | 6.066       | 1.09    | 0.3058  | Non-Significant |
| Residual                    | 0.014          | 26 | 5.559       |         |         |              |
| Cor Total                   | 0.058          | 33 |             |         |         |              |
| Adj R-Squared               | 0.6859         |    |             |         |         |              |
| Pred R-Squared              | 0.5640         |    |             |         |         |              |
| Adept Precision             | 12.401         |    |             |         |         |              |

#### Box-Behnken design

| Source                      | Sum of Squares | df | Mean Square | F-Value | P Value | Significance |
|-----------------------------|----------------|----|-------------|---------|---------|--------------|
| Model                       | 0.038          | 14 | 2.721       | 3.56    | 0.0118  | Significant  |
| A-Toluene                   | 0.018          | 1  | 0.018       | 23.09   | 0.0003  |             |
| B-Methanol                  | 7.500          | 1  | 7.500       | 0.098   | 0.7586  |             |
| C-Water                     | 8.333          | 1  | 8.333       | 0.011   | 0.9183  |             |
| D-Saturation Time           | 4.800          | 1  | 4.800       | 6.29    | 0.0251  |             |
| AB                          | 4.000          | 1  | 4.000       | 0.52    | 0.4812  |             |
| AC                          | 9.000          | 1  | 9.000       | 1.18    | 0.2960  |             |
| AD                          | 1.388          | 1  | 1.388       | 1.817   | 1.0000  |             |
| BC                          | 6.939          | 1  | 6.939       | 9.086   | 1.0000  |             |
| BD                          | 1.225          | 1  | 1.225       | 1.60    | 0.2260  |             |
| CD                          | 2.250          | 1  | 2.250       | 0.29    | 0.5958  |             |
| A²                          | 2.383          | 1  | 2.383       | 3.12    | 0.0991  |             |
| B²                          | 9.471          | 1  | 9.471       | 1.24    | 0.2842  |             |
| C²                          | 5.518          | 1  | 5.518       | 0.072   | 0.7920  |             |
| D²                          | 8.721          | 1  | 8.721       | 11.42   | 0.0045  |             |
| Residual                    | 0.011          | 14 | 7.637       |         |         |              |
| Lack of Fit                 | 0.011          | 10 | 1.069       |         |         |              |
| Adj R-Squared               | 0.3617         |    |             |         |         |              |
| Pred R-Squared              | 0.2623         |    |             |         |         |              |

**Fig. 3.** A) Densitogram of RBN  B) Standard calibration curve of RBN  C) 3D Linearity spectrum of RBN
saturation time of 10 min. By application of these condition densitogram of drug was obtained as shown in Fig. 3.

Wavelength selection for analyte detection

Wavelength selection study was carried out by spotting 20 μL of working standard solution and the forced degradation sample of RBN on aluminium plates pre-coated with a 250-μm layer of silica gel 60F254. The spots were scanned between the range 200–800 nm for detection of wavelength. The scanned samples showed maximum absorbance at 282 nm. Hence, 282 nm was selected as a detection wavelength for scanning the samples.

Method validation

HPTLC method was validated according to the ICH guidelines. The parameters such as linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy and specificity were studied (Fig. 4).

Linearity. Appropriate volume of aliquot from standard RBN stock solution was filled in the syringe and under nitrogen stream by a semiautomatic sample applicator; it was applied in form of band on a single plate having concentration 200–1,200 ng/spot of RBN (Table 4). Plate was developed using toluene: methanol: water (6:2:2 v/v) at

Fig. 4. HPTLC Densitogram of RBN in (A) Acidic condition (B) Alkaline condition (C) Neutral condition (D) Thermal condition (E) Oxidation condition (F) Photolytic condition
Table 4. Summary of linear regression and validation

| Parameters                          | RBN               |
|------------------------------------|-------------------|
| Linearity range (ng/band)           | 200–1,200         |
| Linear regression equation         | Y = 8.845x – 50.13|
| Correlation coefficient (r²)       | 0.999             |
| Standard error of slope            | 0.085             |
| Standard error of intercept        | 0.075             |
| Standard error of residual         | 0.092             |
| System suitability parameters -     |                   |
| Symmetry Factor (As)               | 1.01              |
| Capacity Factor (K)                | 0.83              |
| Selectivity factor (α)             | 1.44              |
| Limit of detection (ng/band)       | 1.12              |
| Limit of Quantitation (ng/band)    | 3.40              |
| Intraday precision (% mean ± SD)   | 99.86 ± 0.305     |
| Interday Precision (% mean ± SD)   | 99.74 ± 0.124     |
| Recovery (mean ± SD)               | 99.8 ± 0.5        |
| Amount of drug quantified in       |                   |
| formulation (%)                    | 99.94             |

Limit of detection and quantitation. In order to estimate the limit of detection (LOD) and limit of quantitation (LOQ), determinations were carried out based on the standard deviation (r) of the response and the slope (S) of the calibration curve and using the formula LOD = 3.3 r/S and LOQ =10r/S, the LOD and LOQ for RBN were estimated. Where, r was the standard deviation of the y-intercepts and S was the slope of the calibration curve. The limits of detection and quantification were found to be 1.12 and 3.40 ng/band, respectively as shown in Table 4.

System suitability. System suitability is an important parameter of chromatographic method development and validation. It was prominently utilized to identify the resolution, accuracy and reproducibility of the chromatographic system. Parameters such as peak symmetry, resolution, capacity factor and selectivity factor were analyzed. All obtained results were within the range and represent in Table 4.

Analysis of commercial tablet

The commercial tablet dosage form, Xarelto® (10 mg) was analyzed using the developed HPTLC method. The analysis was based on comparing the mean peak area of sample band with that of the standard band. The results of tablet analysis were in good agreement with the label claims. The statistical result analysis of the commercial dosage form was presented in Table 4.

Degradation behaviour

In the forced degradation studies, RBN was found sensitive and susceptible to degradation under employed acidic, alkaline, oxidative, neutral, thermal and photolytic stress conditions. The results of forced degradation studies were included in Table 5. Typical densitograms obtained for RBN under different stress conditions were shown in Fig. 3. The developed HPTLC method could effectively resolve the drugs from their degradation products which confirmed the stability indicating power of the developed method. Our reversed-phase HPTLC method for the RBN quantification was compared with analytical methods reported in the literature (Table 6).

Forced (Stress) degradation study

Identification of alkaline degradation of RBN. RBN was hydrolysed smoothly with 0.1 N sodium hydroxide after 1 h at 80 °C. Further, alkaline degradant was isolated using preparative TLC technique. The scheme of major alkaline

Table 5. Result of stress degradation study of RBN

| Sr. No. | Stress Condition | Temperature and Time | Percent degradation | Rf Value of degraded product |
|---------|------------------|----------------------|---------------------|-----------------------------|
| 1.      | Acid (0.1 N HCl) | 80 °C for 1 h        | 11.88%              | 0.47                        |
| 2.      | Alkali (0.1 N NaOH) | 80 °C for 1 h      | 13.09%              | 0.92                        |
| 3.      | Neutral (H₂O)   | 80 °C for 1 h        | 6.76%               | 0.20                        |
| 4.      | Thermal          | 80 °C for 1 h        | 4.49%               | 0.72                        |
| 5.      | Oxide (3% H₂O₂) | 80 °C for 24 h       | 13.09%              | 0.24, 0.38                  |
| 6.      | Photolytic Degradation | 24 h            | 2.90%               | 0.31                        |
degradation product (DP) achieved of RBN were represented in Fig. 5A, which was supported with analytical data. The structural elucidation of the degradation product was confirmed by the IR and mass spectral data. The IR spectrum Fig. 5C of DP was characterized by the absorption frequency of NH stretch at 3,354, CH stretch at 2,937, C=O stretch at 1,653, C=C aromatic stretch at 1,512, C-O stretch at 1,070, C-Cl stretch and C-S stretch band at 831 and 738 respectively confirms the isolated alkaline degradant when compared with RBN standard in Fig. 5B. The mass spectral data of degradant (Fig. 5D) showed tallest signal (base peak) at 327.07 characterized by the breaking of the amine group of drug in marketed formulation. The above mentioned study was able to explore the useful information which has not yet been reported in the literature of RBN. The various degradation products of RBN along with the nature of degradant and fragmentation pathway of degradant formed under alkaline stress studies were also not reported earlier.

Conflict of interest: The authors do not have any conflict of interest to declare in present research work and manuscript preparation.

Data availability statement: All the important data generated has been utilized in writing the manuscript and there is no any other additional data.

CONCLUSION

A simple, sensitive and selective AQbD based stability indicating, validated HPTLC method was developed as per ICH guidelines for estimation of RBN in the presence of its degradation products, which provided useful information regarding degradation behaviour of RBN using different stress conditions. As method was based on application of systematic, scientific analytical tool, it reduces the time and cost of analysis and ultimately become a cost effective and less time consuming, it may be more advantageous for routine analysis of drug in marketed formulation. The above mentioned study has been utilized in writing the manuscript and there is no any other additional data.
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