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Development and Validation of Stability Indicating UPLC Method for the Determination of Rivaroxaban in Bulk and Finished Products and Identification of Degradation Products by LCMS

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ABSTRACT

The current research work reports the study on the degradation profile of Rivaroxaban, chemically known as (S)-5-chloro-N-((2-oxo-3-(4-(3-oxomorpholino) phenyl) oxazolidin-5-yl) methyl) thiophene-2-carboxamide. This work also reports the chemical stability of Rivaroxaban in different stress conditions along with the identification of degradant products by UPLC-MS. A sensitive and reproducible stability indicating ultra-performance liquid chromatography method is developed and validated for quantification of Rivaroxaban bulk drug in the presence of degradation products. The drug was subjected to various stress Conditions such as hydrolysis, oxidation, photolytic and thermal degradations. Significant degradation was observed during hydrolytic stress conditions using HCl and NaOH. It is observed that the drug is highly unstable to acidic and basic condition. Efficient chromatographic separation was achieved by using Acquity; UPLC, X-select CSH; C-18; 100 x 2.1mm; 1.7 µm column with the mobile phase consisting of 0.1% Trifluoro acetic acid in water and0.1% Trifluoro acetic acid in acetonitrilein a gradient elution mode within a short run time of 9.0minutes at a flow rate of 0.4 ml/min using PDA detector. The developed method was validated as per the current ICH guidelines with respect to specificity, precision, accuracy, linearity, robustness and solution suitability. The average recovery values of Rivaroxaban were found to be in the range of 98.10-101.92 %. The developed method was linear with the correlation coefficient value of 0.9993. The repeatability and intermediate precision were expressed by % RSD were less than 2.0% for Rivaroxaban. The test solution was found to be stable in diluent for 48 h when stored at room temperature. The developed UPLC method is superior in technology against conventional HPLC with respect to speed, resolution, solvent consumption and cost of analysis. This method is compatible to LCMS analysis which enables to identify the unknown impurities or the degradants formed in the process.

Highlights:

1. Developed a new UPLC method which is superior in technology against conventional HPLC with respect to speed, resolution, solvent consumption and cost of analysis.
2. Using this method can perform identification and quantification of Rivaroxaban drug in bulk drugs and formulation products.
3. Rivaroxaban was subjected to force degradation under acidic, basic, oxidative, photolytic and thermal conditions as per ICH guidelines. The degradant products are well resolved with main peak in this method and the method is mass compatible.

4. In hydrolytic conditions three degradant products were observed. Which is identified by LCMS and given its proposed structures.

Keywords: Rivaroxaban (RIB), UPLC, LCMS, Method Validation, stability indicating method.

INTRODUCTION

Rivaroxaban with molecular formula of $C_{19}H_{18}ClN_3O_3S$, and molecular weight of 435.88 g mol^{-1} chemically known as (S)-5-chloro-N-((2-oxo-3-(4-(3-oxomorpholino) phenyl) oxazolidin-5-yl) methyl) thiophene-2-carboxamide is an oral anticoagulant invented and manufactured by Bayer. Rivaroxaban is a recently developed first and only oral anticoagulant and direct factor Xa inhibitor which is used in the prevention of stroke and venous embolism in patients with chronic atrial fibrillation, as well as treatment and prevention of deep venous thrombosis (DVT) and pulmonary embolism (PE). DVT prophylaxis after knee or hip replacement surgery, reducing the risk of recurrence and reducing the risk of stroke in patients with nonvalvular atrial fibrillation (AF) [**1-6**]. Rivaroxaban, sold under the brand name Xarelto among others, is an anticoagulant medication (blood thinner) used to treat and prevent blood clots [**7-11**]. Specifically, it is used to treat deep vein thrombosis and pulmonary emboli and prevent blood clots in atrial fibrillation and following hip or knee surgery [**12-14**]. It works by blocking the activity of the clotting protein factor Xa. Rivaroxaban was the 105th most prescribed medication in the United States with more than 7 million prescriptions [**15-16**]. An extensive literature survey revealed that there were few analytical methods available for the determination of Rivaroxaban in plasma, biological fluids, bulk and in pharmaceutical dosage forms by liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS) techniques [**17-21**]. None of the reported methods address the drug behaviour towards various stress conditions and till date there is also no stability indicating method reported to quantify RIB in bulk drug molecule. Forced degradation studies can be a useful tool to predict the stability of a drug and the possible impurities formed when it is exposed to non-recommended conditions during various stages of development, production, analysis, shelf life, distribution and storage. Therefore, understanding drug behaviour to different stress conditions and study on the impurities formed during degradation has significant impact on the safety and efficacy of the drug [**22-29**].

Thus there is a great potential for development of analytical method to monitor the levels of all possible degradation impurities and identification of the unknown impurities. The present study aimed at developing and validating an LCMS compatible UPLC method to analyse the degradation profile under various conditions and quantify the RIB in bulk drug along with identification of degradation products. Hence an accurate and reproducible stability indicating UPLC method was developed for quantitative determination of RIB. This method is LCMS compatible which enables to identify any of the unknown impurities formed in the process. This method was successfully validated as per the guidelines.

MATERIALS AND METHODS

Chemicals and Reagents: High purity ultra-pure water was obtained by using a Millipore Milli-Q water purification system. HPLC grade acetonitrile and methanol were purchased from Merck, Germany. Analytical grade Trifluoroacetic acid, HCl,H_2O_2 (30%w/w)and NaOH were purchased from Sigma Aldrich Chemicals Pvt. Ltd, Bangalore, India.

Instrumentation and Chromatographic Conditions:

Ultraperformance liquid chromatography: A prominence series Waters Acquity UPLC system equipped with a binary solvent manager pump, an auto sampler, and PDA detector with empower-3 software was used for method development, validation and stress degradation studies. The chromatographic column used was Acquity; UPLC; Xselect CSH -C18 column {(100 X2.1) mm; 1.7

µm} which was procured from waters. The mobile phase components are (A) 0.05%v/v of Trifluoroacetic acid in water and (B)0.1%v/v of Trifluoroacetic acid in Acetonitrile, separation was accomplished in a gradient elution program {time (min)/% B: 0.0/5,0.4/5,4.5/90,6.5/90,7.0/5,9/5} at a flow rate of 0.4 mL min^{-1}. The chromatographic eluents were monitored at a detection wavelength of 254 nm using a photodiode array detector (PDA), the injection volume of 1.0 µL was used for sample injections. Water and Acetonitrile in the ratio of 50:50(v/v) was used as diluent. The Structure and UV spectra of RIB shown in figure 1.

Liquid chromatography and Mass Spectrometry: Samples were analysed on the Waters Q-Tof Micro Mass with ESI ion source; other source parameters are: Capillary Voltage: 2800 V; Sample Cone Voltage: 30 V; Extraction Cone Voltage: 5 V; Source Temperature: 140°C; Desolvation Temperature: 300°C. Cone Gas: 50 L/h; Desolvation Gas: 650 L/h. Caffeine (monoisotopic mass: 194.080383 Da) was used as internal standard to calibrate the mass range and accuracy. Mass data was acquired in positive mode using Mass lynx software.

Specificity and forced degradation profile: The specificity of the developed UPLC method for RIB was determined in the presence of its degradation impurities. A forced degradation study identifies the likely DP's, and helps to establish the degradation pathways and the stability of the molecule. RIB was subjected to stress conditions such as acidic (500 mg under 10 mL of 1.0 N HCl, 60°C, 6h), basic (500 mg under 10 mL 1N NaOH, 60°C, 3h), oxidation (500 mg under 5 mL of 3% H_2O_2, 60°C, 6h) in solution state. RIB was also subjected to thermolytic (100°C, 1 day) and photolytic (UV light 254 nm, 3 days) stress in solid state. Neutralized the acid and base degradation samples before analysing the samples. For assay determination, all the degradation samples were diluted further equivalent to the test concentration. Mass balances (%assay + %impurities + %DPs) were calculated for stress samples to determine the assay of RIB. To check the purity and homogeneity of the RIB peak, the degradation samples are run through PDA detector for the peak purity estimation.

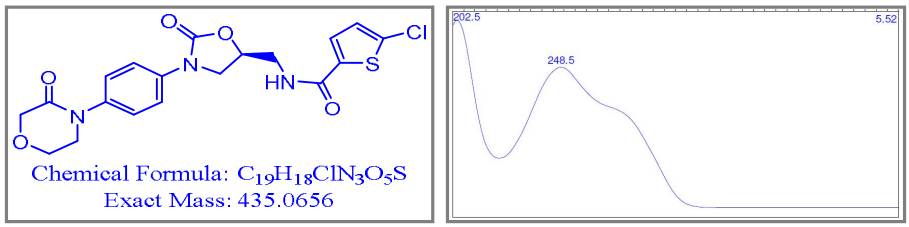

Figure 1. Structure and UV Spectra of RIB.

Method validation: Stock solution of RIB (0.5 mg mL^{-1}) was prepared by dissolving the appropriate amount in the minimum volume of acetonitrile and diluted up to the mark with the diluent. Working solutions (Assay quantification) were prepared by diluting the stock solution to different concentration for method development and validation studies.

System suitability and Precision: The system suitability test was conducted throughout the study by injecting six replicates of RIB (500 µg mL^{-1}). %RSD values, tailing factor and theoretical plate count were studied to confirm the system suitability. The system precision was checked by analyzing five replicates of working solutions for assay (RIB-500 µg mL^{-1}). The method precision was evaluated by injecting five individual test preparations as per solution concentration of system precision. The intermediate precision was evaluated with same concentration solutions used for system precision prepared separately on a different day by different analysts. The RSD (%) of peak area was calculated.

Limits of detection (LOD) and quantitation (LOQ): The LOD and LOQ values for RIB was determined at signal-to-noise ratio of 3:1 and 10:1 respectively by injecting a series of dilute solutions

with known concentrations. Precision at LOQ level was also determined by injecting six individual preparations of RIB at its LOQ level concentration.

Linearity and Accuracy: Linearity was established by injecting RIB at six different concentration levels ranging from 25 to 150% (i.e. 25 to 700 µg mL^{-1}). Linearity was also established from LOQ to 150% of test concentration (500 µg mL^{-1}) The correlation coefficients (r^2), slopes and Y-intercepts were determined from their respective calibration plots. Accuracy of the method was evaluated by spiking known amounts of the RIB to the blank sample at three different concentrations. The recovery studies were performed in triplicate at three concentration levels (50%, 100% and 150%) to specific test concentration.

Robustness and Solution stability: The robustness study was carried out to evaluate the influence of small variations in the optimized chromatographic conditions. The factors chosen for this study were flow rate (± 0.03 mL min^{-1}) and Mobile phase composition change (±10.0%). The effect of flow rate was checked with 0.36 mL min^{-1} and 0.44 mL min^{-1} and the effect of initial mobile phase composition was checked at 4.5% and 5.5% of organic solvent mixture. System suitability parameters and assay values were checked. Solution stability was carried out by storing the system suitability working solution at room temperature for 2 days. The system suitability results were compared with initial and end of the time interval.

RESULTS AND DISCUSSION

System suitability and Precision: System suitability data was evaluated for RIB at 500 µg mL^{-1}. The results indicated that the system was suitable for use as the tailing factor for was less than 1.5 and the USP plate count for the RIB peak was greater than 10000. These values were fixed as acceptance criteria for all the remaining studies. The % RSD values for peak areas in various precision studies like system precision, method precision and intermediate precision were below 1.0% for RIB assay concentration. These results confirmed that the method was highly precise in terms of repeatability. The system suitability values are shown in figure 2 and the precision values and precision chromatogram are shown in table 1and figure 3.

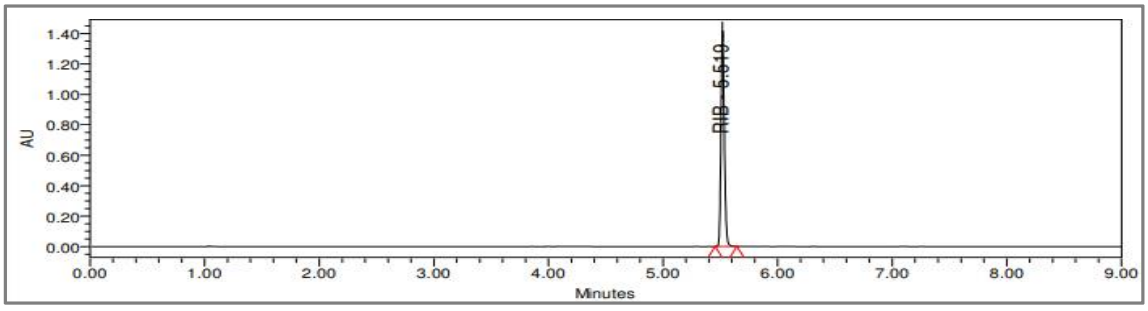

Figure 2. System suitability results for RIB.

Table 1. Precision results for RIB

RIB	System suitability	System precision	Method precision	Ruggudness-1	Ruggudness-2
No of Injs	Area	Area	Area	Area	Area
Inj-1	770246	782355	779853	769835	770322
Inj-2	786699	781698	771986	768820	776991
Inj-3	769212	782597	783659	778495	778539
Inj-4	769817	783015	787465	776394	777421
Inj-5	770445	783697	776410	776132	782194
Average	773283.80	782672.40	779874.60	773935.20	777093.40
SD	7514.20	745.50	6045.03	4319.73	4303.36
% RSD	**0.97**	**0.10**	**0.78**	**0.56**	**0.55**

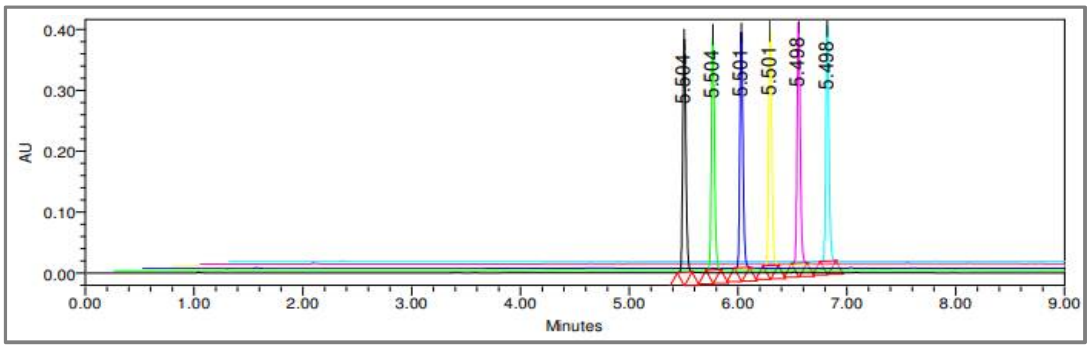

Figure 3. Precision Chromatogram.

Limits of detection (LOD) and quantitation (LOQ): The stock solutions of RIB was prepared at 1.0 mg mL^{-1} and from this solution a series of solutions at lower concentration were diluted and checked the signal to noise ratio. The determined limit of detection (LOD) and limit of quantification(LOQ) results of RIB were 0.5µg mL^{-1} and 2.0 µg mL^{-1} respectively indicating the higher sensitivity of the method. The %RSD values for precision at LOQ level of peak areas for RIB is0.37%. The LOQ precision values are tabulated in table 2 and the LOD and LOQ its precision chromatograms are shown in figure 4, 5 and 6.

Table 2. LOQ precision values for RIB

No of Injs	Area
Inj-1	45435
Inj-2	45466
Inj-3	45545
Inj-4	45269
Inj-5	45126
Average	45368.20
SD	168.66
% RSD	**0.37**

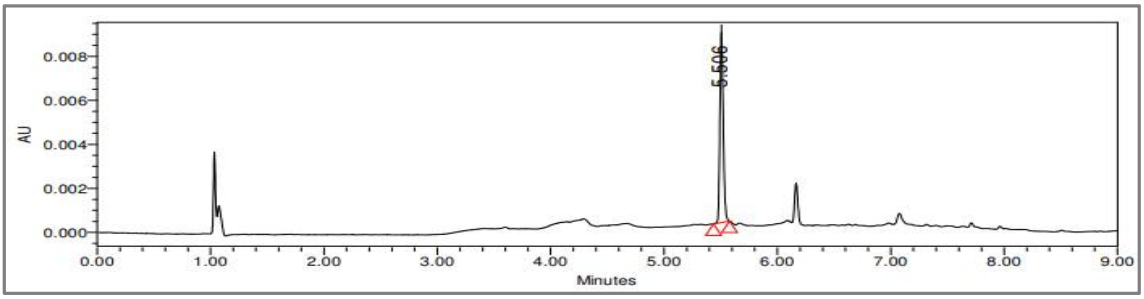

Figure 4. LOD chromatogram.

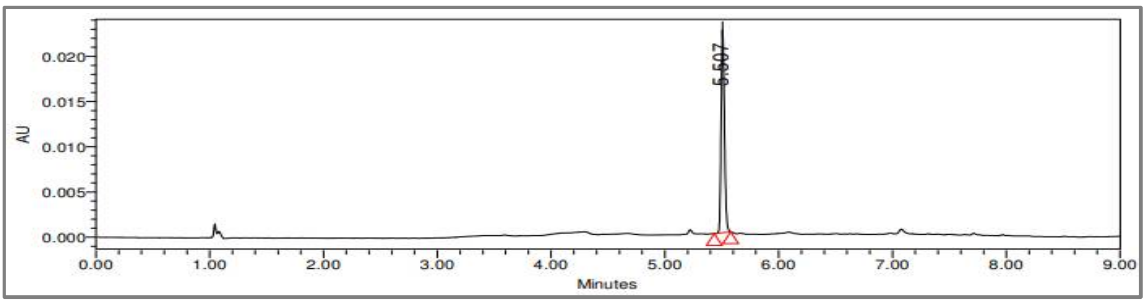

Figure 5. LOQ chromatogram.

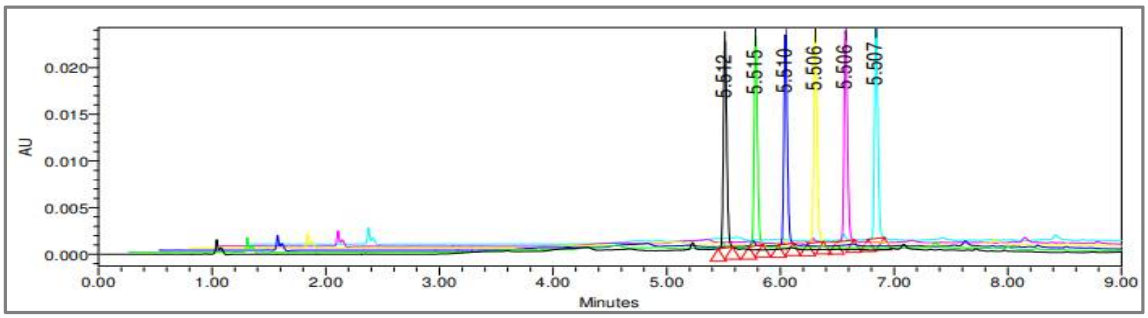

Figure 6. LOQ precision chromatogram.

Linearity and Accuracy: Linear calibration plot for the RIB assay method was obtained over the calibration ranges tested, that is LOQ to 700 µg mL^{-1} (LOQ, 50%, 75%, 100%,120% and 150%) of the target test concentration (500 µg mL^{-1}). The correlation coefficient results obtained is greater than 0.9993. The percentage recovery of RIB ranged from 98.1 to 101.9 at three spike levels (50%, 100% and 150%) assuring the accuracy of the method. The linearity plot was shown in figure 7 and recovery values are tabulated in table 3.

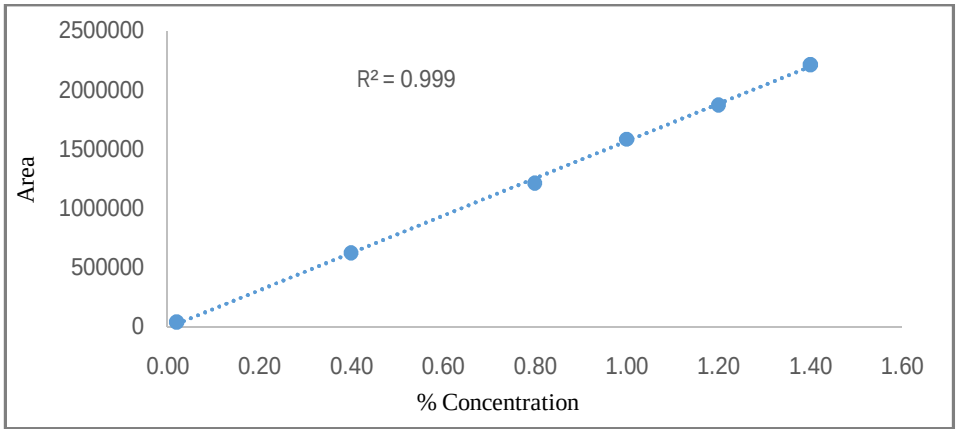

Figure 7. Linearity graph of RIB.

Table 3. Accuracy results of RIB

S.No	Conc level	% Mean recovery
1	LOQ	98.10
2	50%	101.64
3	100%	100.51
4	150%	101.92

Robustness and Solution stability: The %RSD for peak area in all the deliberately altered chromatographic conditions like flow variance and organic solvent concentration variation were precise and the result is less than 1.0%. The system suitability parameters were within the range and also the variability in the estimation of RIB assay was within ±2.0% indicating the robustness of the method. No significant changes in the retention times and area of the RIB peak observed during solution stability carried at room temperature for 2 days. All the robustness and solution stability results were summarized and tabulated in table.4 and 5.

Specificity: The specificity of the method was evaluated by verifying the peak purity of the sample. The method was found to be specific as there was no interference from blank and placebo at the

Table 4. Robustness results for RIB

RIB	Initial	Flow Decrease	Flow Increase	Organic Decrease	Organic Increase
No of Injs	Area	Area	Area	Area	Area
Inj-1	770246	783365	781265	770987	777954
Inj-2	786699	781754	776542	778645	783652
Inj-3	769212	781002	780154	780655	782971
Inj-4	769817	778985	775617	782654	777987
Inj-5	770445	777901	780897	778222	795471
Average	773283.80	780601.40	778895.00	778232.60	783607.00
SD	7514.20	2181.25	2621.63	4418.31	7153.70
% RSD	**0.97**	**0.28**	**0.34**	**0.57**	**0.91**

Table 5. Solution stability results for RIB

RIB	0hrs	24hrs	48hrs
No of Injs	Area	Area	Area
Inj-1	770246	784581	779830
Inj-2	786699	783987	773921
Inj-3	769212	784697	778560
Inj-4	769817	770940	777991
Inj-5	770445	779845	782160
Average	773283.80	780810.00	778492.40
SD	7514.20	5868.79	3016.38
% RSD	**0.97**	**0.75**	**0.39**

retention time of main peak. No degradant peaks were observed at the retention time of RIB during the thermal, photolytic and peroxide forced degradation conditions and RIB was liable to hydrolytic conditions like basic and acidic force degradation conditions. This stability study indicates that the method is stability indicating and also peak purity index for all the impurities and RIB were less than the purity threshold indicates that there was no spectral co-elution for any of the peaks in this method and also the resolution between the neighbouring peak was greater than 2.0.

Forced degradation studies: Forced degradation studies were performed to establish the stability indicating power of the method. In this study RIB raw material, finished product and placebo were subjected to acidic, basic, peroxide, thermal and photolytic stress studies on sample concentration of 0.5 mg mL^{-1} in diluent. Sample equivalent to 50 mg of RIB was placed into 100 ml volumetric flask added 60 mL of diluent and sonicated for 10 min with intermediate shaking and then added respective degradant (Acid, Alkali, Oxidant) and performed the stress study. Samples were neutralized after degradation and then diluted to the volume with diluent and injected to verify the stability indicating power of the analytical method. Stress conditions under which the study was performed, the amount of RIB remains, % impurities generated and % Assay (Mass balance) results were tabulated in table 6. The chromatograms for forced degradation study were summarized figure 8.

The degradants were formed after specific time after each stress study conditions of acidic and basic. To determine the results of the solutions from all the stress studies, samples were analyzed individually in mass spectrometry. Samples were analyzed on the Waters Q-T of Micro Mass with

Table 6. Degradation profile of RIB.

S.No	Stress condition	% Drug remained	% impurities	%Assay
1	1N HCl_60°C_6 Hrs	83.98	16.02	85.78
2	1N NaOH_60°C_3Hrs	80.41	19.59	80.99
3	3% H_2O_2_60°C_6 hrs	95.76	4.24	94.15
4	100°C_24 Hrs	99.84	-	100.11
5	Photolytic stability	99.86	-	99.75

ESI ion source; Mass data was acquired in positive mode using Mass lynx software. The RIB-API and its three significant degradation products formed in the study were identified by LCMS and proposed structures are shown in figure 9 and its LCMS data shown in figure 10.

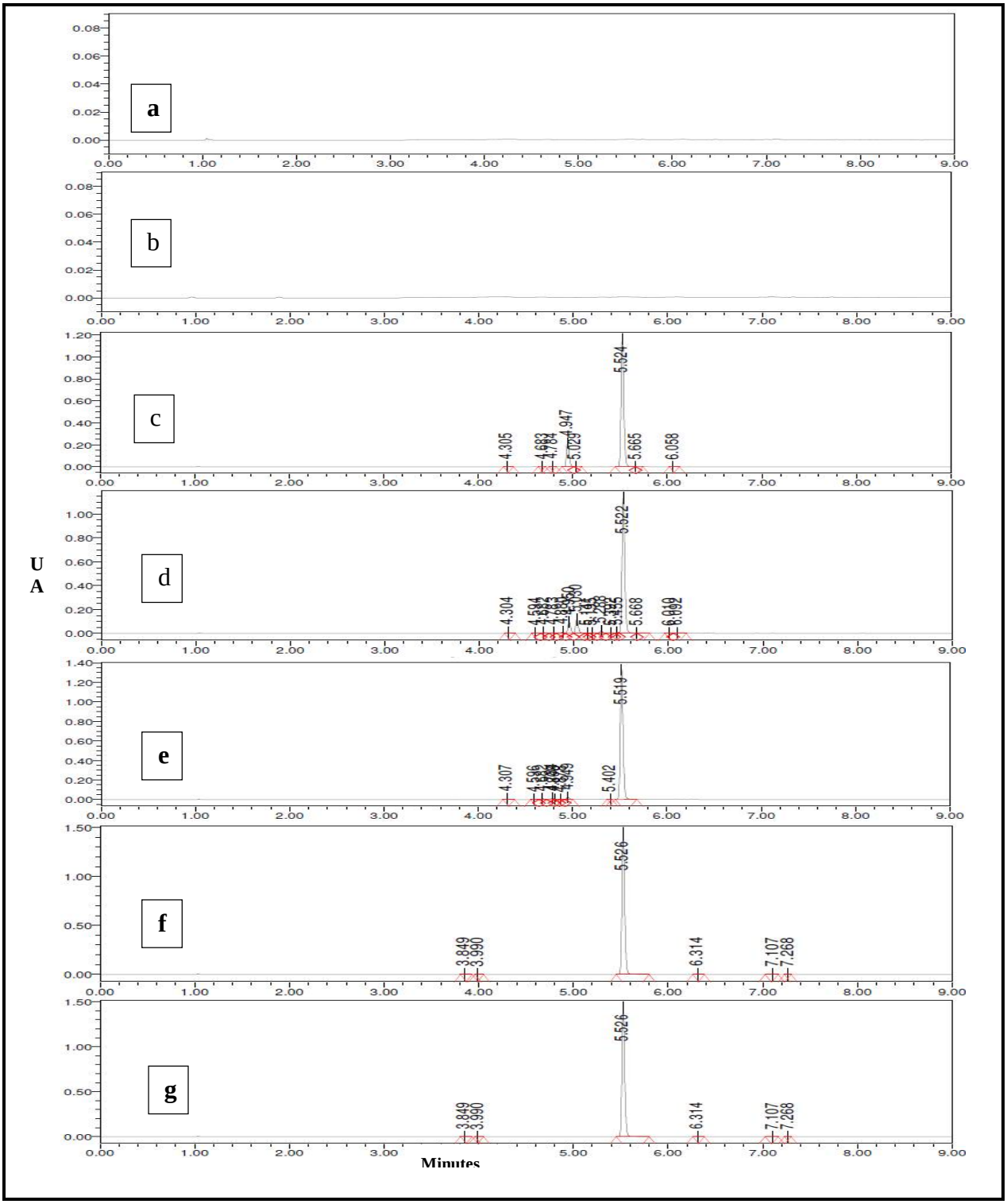

Figure 8. a) Blank, b) Placebo, c) Acidic, d) Basic, e) Oxidative, f) Thermal and g) Photostability degradation chromatograms for RIB.

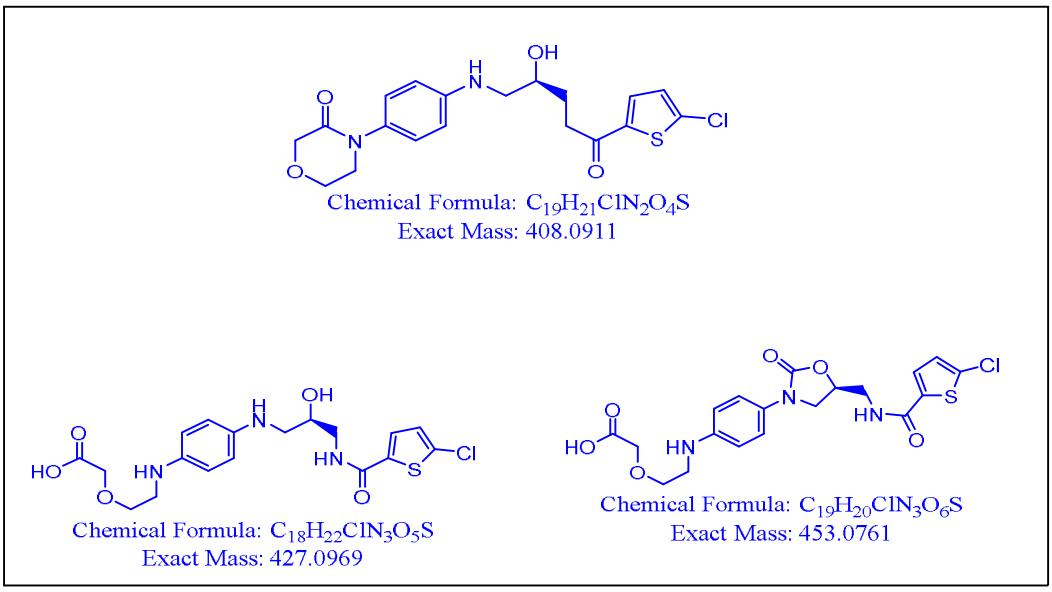

Figure 9. Possible structures of degradation products in RIB stress study**.**

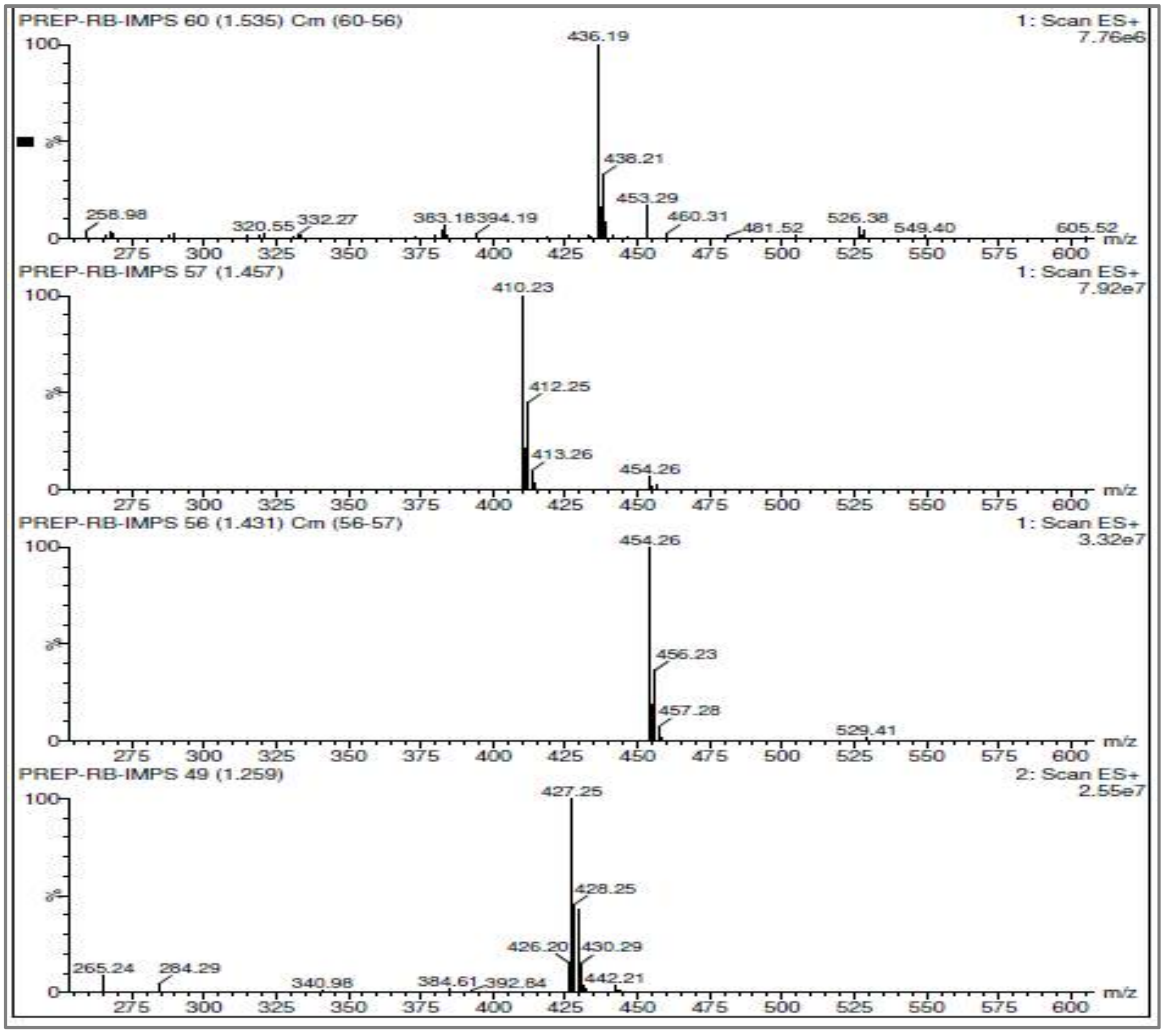

Figure 10. LC-Mass data of RIB degradant products.

APPLICATION

A step by step systematic procedure is designed to validate the stability indicating quantification method by UPLC and identification of degradant products by mass compatible LCMS method of Rivaroxaban.

CONCLUSION

A validated stability indicating RP-UPLC method has been developed to evaluate the degradation profile of RIB under various stress parameters. RIB was stable in oxidative, thermal and photolytic conditions. However, RIB was catalyzed more rapidly in hydrolytic conditions. This study gives key information that RIB drug substance needs to be handled carefully and should avoid per acid or basic reagents during synthesis, storage and all the flow through transit process. The developed UPLC method has been found to be simple, selective, precise, linear, accurate, and sensitive with a short run time of 9.0 min. The proposed UPLC method obeys linearity within the concentration range of 0.02-150% for RIB assay with correlation coefficient of not less than 0.9993. LOD and LOQ values are 0.5µg mL^{-1} and 2.0 µg mL^{-1} respectively. Inter and intraday precision with cumulative % RSD for RIB assay were found to be 0.56 % to 0.55 % respectively. % Recovery values for the RIB assay were found to be between 98.10 % and 101.92 % for the concentration range between LOQ and 150 % of the test concentration (0.5 mg mL^{-1}). The method is found to be specific and there is no interference of degradation impurities with RIB peak. This method is compatible to LCMS analysis, the degradant products which is formed under hydrolytic conditions are shown 410,454,428 m/z mass in positive ESI mode. The developed method was validated as per ICH guidelines. All the validation parameters were found to be well within the acceptance criteria. We concluded that the method is accurate, precise, linear and robust. The developed method can be successfully applied for the analysis of RIB bulk and pharmaceutical dosage form in quality control laboratories.

Conflict of interest: The authors declare that they have no conflict of interest.

Compliance with the Ethical Standards: The present manuscript does not involve any human participants and/or animal studies and complies with the ethical standards.

Informed Consent: The manuscript is being submitted with the informed consent of all the co-authors.

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