



Stability-indicating UPLC method for determining related substances and degradants in Rivaroxaban

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ABSTRACT

A simple, sensitive and reproducible method was developed on ultra performance liquid chromatography (UPLC) coupled with a photodiode array detector for the quantitative determination of Rivaroxaban (RVB) in drug substance and pharmaceutical dosage forms. The method is applicable for the quantification of related substances and assays of drug substances. Chromatographic separation was achieved on Acquity UPLC BEH HSS T3 100-mm, 2.1-mm, and 1.8- μ m columns, and the gradient elution within a short runtime, i.e., within 12.0 min. The eluted compounds were monitored at 248 nm, the flow rate was 0.45 mL/min, and the column oven temperature was maintained at 25 °C. The resolution between RVB and ten impurities was greater than 2.0. The high correlation coefficient (> 0.9995) value indicates the clear correlations between the investigated compound concentrations and their peak areas within the test ranges. The repeatability and intermediate precision expressed by the RSD were less than 6.5 %. The accuracy and validity of the method were further ascertained by performing recovery studies via a spike method. Rivaroxaban was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. Rivaroxaban was found to degrade significantly in acid and base stress conditions and stable in thermal, photolytic degradation, oxidative and hydrolytic conditions. The degradation products were well resolved from main peak and its impurities, proving the stability-indicating power of the method. The developed method was validated as per international conference on harmonization (ICH) guidelines.

Key words: UPLC, Rivaroxaban, Forced degradation, Validation, Stability-indicating

INTRODUCTION

High performance liquid chromatography (HPLC) is well recognized as regular separation technique in the pharmaceutical industry. This is preferred mostly due to the many features offered by HPLC like robustness, ease of operation, well-understood separation principles, sensitivity and tunable selectivity. However, the main constraint of HPLC since many years has been the lack of high efficiency and the analysis time. The escalating need for impurity analysis in pharmaceuticals provides the basis for evaluating rapid, simple and reliable methods of analysis. UPLC provides the speed and resolving power to develop efficient separation methods. This new category of analytical separation science retains the practicality and principles of HPLC while increasing the overall interrelated attributes of speed, sensitivity and resolution. Using a 1.7 μ m reversed-phase packing material and

operating at high pressure (up to 15000 psi), makes Ultra-performance liquid chromatography (UPLC) a unique and highly sophisticated technique to achieve separations with very high-resolution within a short period of time and with little organic solvent consumption¹⁻³ which has attracted much attention of pharmaceutical and biochemical analysts. In the present work, this technology has been applied to the method development and validation study of related substance and assay determination of RVB bulk drug and dosage forms. The chemical name of Rivaroxaban is 5-Chloro-N-((5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl)phenyl]-1,3-oxazolidin-5-yl)methyl)-2 thio phenecarboxamide (Fig.1A). Rivaroxaban used for potent anticoagulant and antithrombotic effects^{4,5}. Very few methods appeared in the literature for the determination of RVB in pharmaceutical dosage forms, determination of RVB in human plasma by using-high-performance liquid chromatography -tandem mass spectrometry (HPLC-MS/MS)⁶, determination of RVB by using

spectrophotometric methods in pharmaceutical formulations and bulk dosage forms⁷, few RP-HPLC method for determination of Rivaroxaban in pharmaceutical dosage forms⁸⁻¹⁰ and a chiral HPLC method¹¹. Furthermore, there is no stability indicating HPLC/UPLC method is available in literature for estimation of RVB along with related substances and degradants. Currently, the determination of impurities is one of the most difficult tasks for pharmaceutical analysis during method development, especially if increasing numbers of impurities are required to be determined. In RVB, separation of process related impurities and degradants are very critical and there is no HPLC/UPLC method reported in the literature that can adequately separate these process related impurities along with degradants. It is, therefore, felt necessary to develop a new stability indicating method for the related substance determination and quantitative estimation of RVB which can separate all the impurities in short run time without compromising the resolution and sensitivity. Hence a reproducible stability-indicating RP UPLC method was developed for the quantitative determination of RVB and its ten impurities namely Imp-1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 (Fig.1B-K). This method was successfully validated according to the International Conference Harmonization (ICH) guidelines (Validation of Analytical Procedures: Test and Methodology Q2).

MATERIALS AND METHODS

Materials and reagents

Active pharmaceutical ingredient standards and samples were supplied by Dr. Reddy's Laboratories Limited, IPDO, Hyderabad, India. Commercially available Xarelto in 20-mg tablets was used for the dosage form analysis. HPLC-grade acetonitrile and methanol, analytical-grade Ortho phosphoric acid and 1-Octane sulphonic acid sodium salt were purchased from Merck, Darmstadt, Germany. Water was prepared in-house by using a Millipore Milli-Q Plus water purification system (Millipore Corporate Headquarters, Billerica, MA).

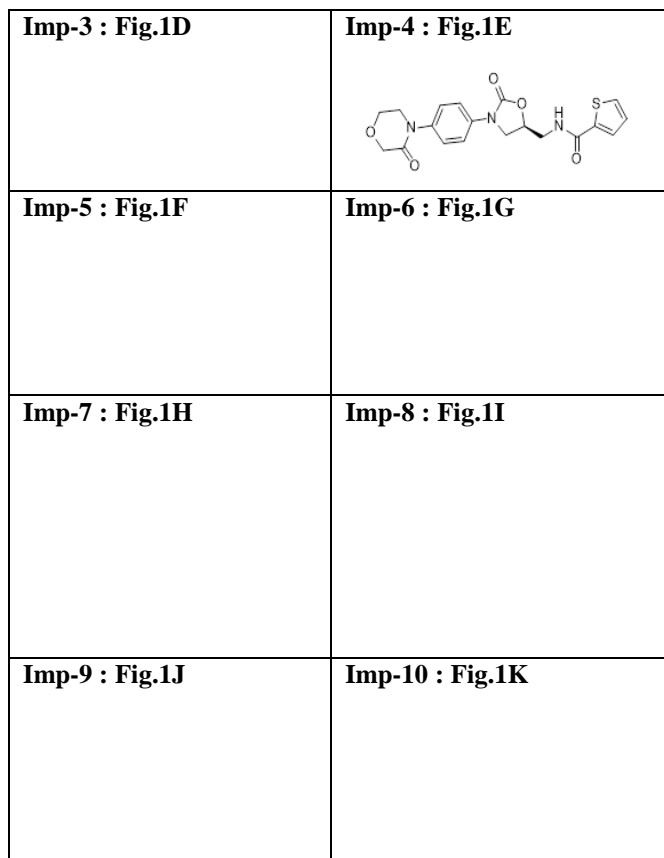
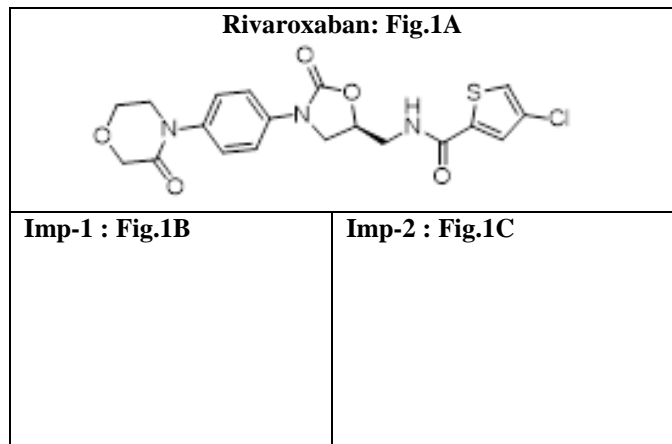


Figure – 1 (A-K): Chemical structures of Rivaroxaban and its related substances

Equipment

The LC system used for method development, forced degradation studies and method validation was Acquity UPLC system (Waters, Milford, USA) which consists of a binary solvent manager and a photodiode array (PDA) detector. The output signal was monitored and processed using empower2 software. Cintex digital water bath was used for hydrolysis studies, Photostability studies were carried out in a photostability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India).

Chromatographic conditions

The method was developed using Waters Acquity BEH HSS T3 100-mm, 2.1-mm, and 1.8- μ m particle sizes. Separation was achieved using a gradient method. Mixture of 1 mL Ortho phosphoric acid and 10 mM Octane 1-sulphonic acid sodium salt finalized as buffer. Buffer and acetonitrile in the ratio of 90:10, 20:80 was selected as mobile phase A and B respectively and wavelength was 248 nm with a flow rate of 0.45 mL/min at ambient temperature. The injection volume was 3.0 μ L. Gradient program finalized as (Time/%A/%B) 0.01/90/10, 4.5/75/25, 7.5/75/25, 9.0/50/50, 10.0/50/50, 10.01/90/10 and 12.0/90/10. 1 mL phosphoric acid in mixture of methanol and acetonitrile in a ratio of 50:50 (v/v) used as diluent.

LC–MS conditions

An LC–MS system (Agilent 1200 series LC coupled with Applied Biosystems 4000 Q Trap triple quadrupole mass spectrometer with Analyst 1.4 software, MDS SCIEX, USA) was used for to identify the unknown compounds formed during forced degradation studies. An Inertsil C8, 150 mm × 4.6 mm, 3.0 µm column (Agilent, USA) was used as the stationary phase. A 1.0 mL Ortho phosphoric acid in water and acetonitrile in a ratio of 20:80 (v/v) were used for solvent B. The gradient program (T/%B) was set as 0.01/10, 5/10, 20/35, 30/80, 42/80, 42.1/10 and 50/10. 1 mL phosphoric acid in methanol and acetonitrile in a ratio of 50:50 (v/v) used as diluent. The flow rate was 0.8 mL/min. The analysis was performed in positive electro-spray/positive ionization mode, the ion source voltage was 5000 V, and the source temperature was 450 °C. GS1 and GS2 were optimized to 30 and 35 psi, respectively. The curtain gas flow was 20 psi.

Preparation of stock solutions

A stock solution of RVB (2000 µg/mL) was prepared by dissolving an appropriate amount of the drug in diluent. Working solutions containing 500 µg/mL and 100 µg/mL were prepared from this stock solution for the determination of related substances and for the assay determination respectively. A stock solution of impurities (mixture of Imp-1 to 10) at 50 µg/mL was prepared in diluent.

Preparation of sample solution

Twenty (n = 20) RVB 20-mg Xarelto tablets were weighed, and the pellets were transferred into a clean, dry mortar. Pellets equivalent to 100 mg of the drug were dissolved in 100 mL of diluent to make a 1000-µg/mL solution. 10.0 mL of this solution was diluted to 20 mL with diluent, yielding 500-µg/mL solution. 10 mL of this solution was diluted to 50 mL with diluent, yielding 100-µg/mL solution. These solutions were filtered through a 0.22-µm nylon membrane filter. The resulting solution was analyzed by UPLC.

Stress studies

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities¹². The specificity of the developed LC method for RVB was carried out in the presence of its ten impurities. Stress studies were performed at an initial concentration of 500 µg/mL of RVB in active pharmaceutical ingredients (API) and tablets to provide the stability-indicating property and specificity of the proposed method. Intentional degradation was attempted by the stress conditions of UV light (254 & 365 nm), heat (105 °C), acid (0.1-N HCl at 70 °C), base (0.1-N NaOH at RT), hydrolysis (70 °C) and oxidation (3.0 % H₂O₂ at 70 °C) to evaluate the ability of the proposed method to separate RVB from its degradation products. For heat and light studies, the study period was 10 days; whereas for the hydrolysis, base, acid and oxidation studies, the study periods were 48 h, 1 h, 2 h, and 48 h, respectively.

The purity of the peaks obtained from the stressed samples was verified using the PDA detector. The purity angle was within the purity threshold limit obtained in all the stressed samples and demonstrated the analyte peak homogeneity. An assay of stressed samples was performed (at 100 µg/mL) by comparison with reference standard and the mass balance (% assay + % impurities + % degradation products) for each of the stressed samples was calculated. An assay was also calculated for the RVB sample by spiking all ten impurities at the specification level (i.e., 0.15 %).

METHOD VALIDATION

The described method has been validated for the assay and related substances by UPLC determination as per ICH guidelines¹³.

Precision

The repeatability of the method for the related substances was checked by a sixfold analysis of 500 µg/mL of RVB spiked with 0.75 µg/mL of each of the ten impurities. The % RSD was calculated for each impurity % peak area. Ruggedness study for related substances of RVB method was demonstrated on different day by varying different analyst, different equipment and different column. Intermediate precision was determined by a sixfold analysis of 500 µg/mL of RVB spiked with 0.75 µg/mL of each of the ten impurities. The RSD (%) of the % peak area was calculated for each impurity. The precision of the assay was evaluated by performing six (n = 6) independent assays of a test sample of RVB and by comparison with a qualified reference standard. The RSD (%) of the six results was calculated. The intermediate precision of the assay method was evaluated on different day by varying different analyst, different equipment and different column.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the true value and the observed value. The accuracy of the assay method for RVB was evaluated in triplicate (n = 3) at the three concentrations of 50, 100 and 150 µg/mL (50 %, 100 % and 150 %) of drug product, and the recovery was calculated for each added (externally spiked) concentration. For all impurities, the recovery was determined in triplicate for 0.375, 0.75 and 1.125 µg/mL (50 %, 100 % and 150 %) of the analyte concentration (500 µg/mL) of the drug product, and the recovery of the impurities was calculated.

Linearity

Linearity test solutions for the related substance method were prepared by diluting stock solutions to the required concentrations. The solutions were prepared at eight concentration levels from LOQ – 200 % (LOQ, 0.0375, 0.075, 0.1125, 0.15, 0.1875, 0.225 and 0.30 %) with respect to the normal sample concentration (500 µg/mL). The correlation coefficients, slopes and Y-intercepts of the calibration curve were determined. Linearity test solutions for the assay method prepared from RVB stock solutions at

five concentration levels from 50 – 150 % of assay analyte concentration (50, 75, 100, 125 and 150 µg/mL). The peak area versus concentration data was treated as least-squares linear regression analysis.

Limit of detection (LOD) and limit of quantification (LOQ)

By injecting linear (0.05–0.30 % with respect to the test concentration) solutions of known concentrations, based on the standard deviation (σ) of the response and the slope (S) of the calibration plot and using the formula $LOD = 3.3 \sigma/S$ and $LOQ = 10 \sigma/S$, the LOD and LOQ for RVB and for the ten impurities were estimated. The precision (n = 6) was also determined at the LOQ level, and the % RSD was calculated for the peak area for each impurity and for RVB.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. To determine the robustness of the method, the experimental conditions were deliberately changed. The resolution of RVB and the ten impurities was evaluated. The mobile phase flow rate was 0.45 mL/min; to study the effect of the flow rate on resolution, the flow rate was changed to 0.40 and 0.50 mL/min. The effect of the column temperature was studied at 20 °C and 30 °C (instead of 25 °C). The effect of the percent organic strength on resolution was studied by varying acetonitrile by - 10 % to + 10 % from initial composition.

Solution stability and mobile phase stability

The stability of RVB in solution was determined by leaving the test solutions of the sample and reference standard in tightly closed volumetric flasks at room temperature for 48 h during which they were assayed at 12 h intervals. The stability of RVB and its impurities in solution for the related substance method was determined by leaving spiked sample (with respect to the specification, i.e., 0.15 % level) in a tightly capped closed volumetric flasks at room temperature for 48 h and measured the impurity content of ten impurities at every 12 h. Stability of mobile phase was demonstrated by analysis of freshly prepared sample solution at 12 h intervals for 48 h and comparing the results with those obtained from freshly prepared reference standard solutions. The prepared mobile phase was kept constant during the study period.

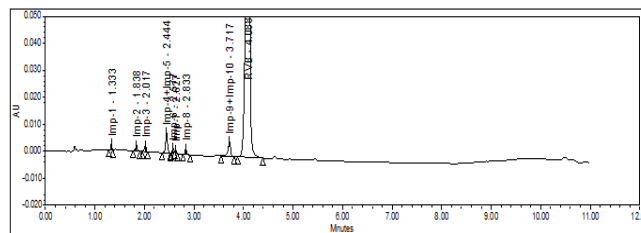
RESULTS AND DISCUSSION

Method development and optimization

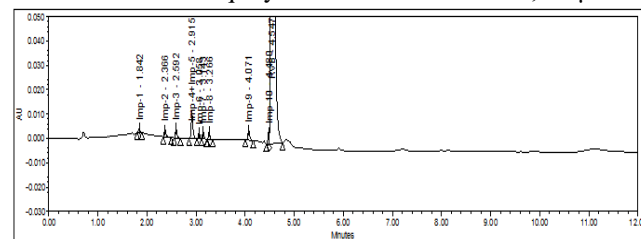
The main target of the chromatographic method is to achieve the separation of impurities (potential, bi-products and degradation impurities) from the main component of RVB. The maximum absorption wavelength of RVB and its impurities were observed at 248 nm, which is intersecting value obtained from the UV absorption spectra, hence, this particular wave length was selected for

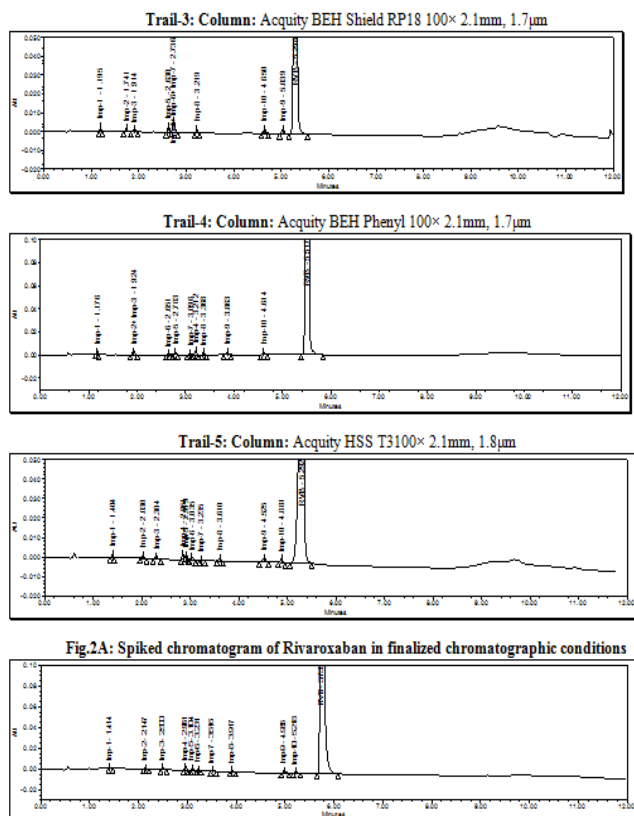
LC analysis. The blended solution containing 500 µg/mL of RVB and 0.75 µg/mL of each of the ten impurities was prepared in the diluent. For the initial trail Acquity BEH C8 (100-mm × 2.1-mm, 1.7-µm particles) column was chosen with a mobile phase composition of buffer (ortho phosphoric acid and 1-Octane sulphonic acid sodium salt as modifier) and acetonitrile in varies ratios in gradient mode. The separation of imp-4 and imp-5, imp-9 and imp-10 were found to be merge. Further trails were conducted with Acquity BEH C18 100-mm × 2.1-mm, 1.7-µm particles) by keeping the mobile phase composition same as above. The separation of imp-9 and imp-10 was found to be good (USP resolution >5). But the separation of imp-4 and imp-5 was found to be merge and imp-10 and analyte peak was found to be inadequate. Further trails were conducted with Acquity BEH Shield RP 18 (100-mm × 2.1-mm, 1.7-µm particles) by keeping the mobile phase composition same as above. Elution order changed for imp-4, imp-5, imp-9 and imp-10. The separation between imp-9, imp-10 and analyte were found to be good (USP resolution >1.5). But imp-4 separates from imp-5 and merge with imp-6 and imp-7. Further trails were conducted with Acquity BEH phenyl (100-mm × 2.1-mm, 1.7-µm particles) by keeping the mobile phase composition same as above. All impurities separated well (resolution >2) except imp-2 and imp-3 were found to be merge. Separation not achieved by changing the chromatographic conditions like gradient and column temperate Further trails were conducted with Acquity HSS T3 (100-mm × 2.1-mm, 1.8-µm particles) by keeping the mobile phase composition same as above. All impurities separated well (resolution >2) but resolution between imp-4 and imp-5 is less than 1. Further tried with different gradient. All impurities separated well (resolution >2) (Fig.2). At this mobile phase mixture and column temperature, very good separations of all the impurities were seen and the method was finalized at this particular mobile phase and column temperature (Fig.2A). The elution of analyte peak was approximately 6 min. Interference with the excipients (placebo) was also checked and no interference was observed at the impurity peaks and the analyte peak.

Trail-1: Column: Acquity BEH C8 100× 2.1mm, 1.7µm



Trail-2: Column: Acquity BEH C18 100× 2.1mm, 1.7µm





METHOD VALIDATION RESULTS

System suitability

The system suitability parameters were evaluated for RVB and its ten impurities. The USP tailing factor for all ten impurities and RVB was found to be less than 1.2. The USP resolution (R_s) of RVB and the ten potential impurities was greater than 2.0 between all pairs of compounds. The USP plate count for all ten impurities and RVB was found to more than 10000 (Table 1).

Precision

The %RSD of assay of RVB during the assay method repeatability study was 0.4% and the %RSD for the %area of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9 and Imp-10 in related substance method repeatability study was within 6.5%. The %RSD of the assay results obtained in the intermediate precision study was within 0.4% and the %RSD for the area of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9 and Imp-10 were well within 5.3%, conforming good precision of the method. The %RSD values are presented in Table 2. These results confirmed that the method was highly precise.

Limits of Detection and Quantification

The determined limit of detection, limit of quantification and precision at LOQ values for RVB and its ten impurities are reported in Table 2.

Accuracy

Recovery of RVB from pharmaceutical dosage forms ranged from 99.4 to 101.1 %. Recovery of the ten

impurities from pharmaceutical dosage forms ranged from 93.9 to 107.9.

Linearity

The linearity calibration plot for the assay method was obtained over the calibration ranges tested, i.e. 50–150µg/mL and correlation coefficient obtained was greater than 0.999. The result shows that an excellent correlation existed between the peak area and concentration of the analyte. Linear calibration plot for the related substance method was obtained over the calibration ranges tested, i.e. LOQ to 0.30 % for impurities. The correlation coefficient obtained was greater than 0.9995 (Table 2). The above result shows that an excellent correlation existed between the peak area and the concentration of Imp-1, Imp-2, Imp-3, Imp-4, Imp-5, Imp-6, Imp-7, Imp-8, Imp-9 and Imp-10.

Robustness

In all the deliberate varied chromatographic conditions (flow rate, column temperature and composition of organic solvent), all analytes were adequately resolved and elution orders remained unchanged. The resolution between critical pairs, i.e. for Imp-4 and Imp-5, for Imp-5 and Imp-6 and for Imp-9 and Imp-10 was greater than 2.0 and tailing factor for Rivaroxaban and its impurities was less than 1.2.

Stability in Solution and in the Mobile Phase

Difference in % assay of RVB during solution stability and mobile phase stability experiments was within 1.0 %. No significant changes (0.02 %) in the amounts of the ten impurities were observed during solution stability and mobile phase stability experiments when performed using the related substances method. The results from solution stability and mobile phase stability experiments confirmed that standard solutions and solutions in the mobile phase were stable for up to 48 h during assay and determination of related substances.

All forced degradation samples were analyzed at an initial concentration of 500 µg/mL of RVB with UPLC conditions already mentioned, using PDA detector to evaluate the homogeneity and purity of RVB peak. Degradation was not observed when RVB was subjected to hydrolytic, oxidation, light and heat conditions. Significant degradation was observed in acid (0.1N HCl at 70 °C for 2 h) leading to the formation of an unknown impurities. Significant degradation was observed in base (0.1N NaOH at room temperature for 1 h) leading to the formation of an unknown impurity and impurity-10 and which are well separated from the analyte peak and RVB impurities (Fig.3A-B). Homogeneous Peak found for RVB in acid and base hydrolyzed samples (Fig.3C-D). LC-MS analysis was performed as per experimental conditions and mass of the unknown impurity formed in acid was 468.20 and unknown impurity formed in Base was 454.40. Known impurity formed in base hydrolysis showing the mass value at 410.30 and conformed as impurity-10 (Fig.3E-G). Results from force degradation studies have been presented in Table 3.

Table 1: System suitability parameters.

Compound name	RT(min)	RRT ^a	Resolution ^b (Rs)	Tailing factor (T)	Theoretical plates (N)
Imp-1	1.410	0.25	-	1.1	22499
Imp-2	2.144	0.37	14.1	1.0	17838
Imp-3	2.496	0.43	5.5	1.3	27490
Imp-4	2.958	0.51	6.9	1.2	28619
Imp-5	3.101	0.54	2.1	1.1	38505
Imp-6	3.227	0.56	2.1	0.9	46477
Imp-7	3.513	0.61	4.7	1.3	54168
Imp-8	3.914	0.68	6.5	1.3	68507
Imp-9	4.982	0.87	13.7	1.2	46327
Imp-10	5.215	0.91	2.7	1.2	79487
RVB	5.747	1.00	6.1	1.2	58114

^a Relative retention times (RRT) were calculated against the retention time (RT) of Rivaroxaban.

^b Resolutions were calculated between two adjacent peaks.

^c Mean±SD.

Table 2: Method validation results

Method validation results											
Parameter	RVB	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6	Imp-7	Imp-8	Imp-9	Imp-10
Precision (RSD)	0.4	3.3	2.7	0.0	0.0	3.5	2.5	3.4	3.5	3.5	6.5
Intermediate precision(RSD)	0.4	2.5	0.0	0.0	2.6	3.3	0.0	5.3	3.5	3.8	0.0
LOQ (µg/ml)	0.125	0.165	0.135	0.130	0.125	0.155	0.130	0.155	0.145	0.120	0.125
LOD (µg/ml)	0.040	0.055	0.045	0.045	0.040	0.050	0.040	0.050	0.045	0.040	0.040
Precision at LOQ	4.4	3.6	3.3	4.1	1.7	4.7	3.2	4.3	3.2	2.5	4.8
Accuracy at LOQ	--	97.1	104.4	92.0	95.4	105.3	101.1	101.4	94.5	102.4	96.7
Correlation coefficient	0.9998	0.9995	0.9997	0.9997	0.9997	0.9996	0.9997	0.9996	0.9996	0.9997	0.9998
Slope (b)	4754	12271	23058	24352	43004	35034	15287	18880	19200	42822	21080
Intercept (a)	-4122	-20.7	-127.5	-14.5	1.0	-46.6	93.5	-19.0	15.3	-113.6	-32.7
Y-intercept at 100% level	-0.9	-1.1	-3.9	-0.4	0.01	-0.9	4.8	-0.7	0.5	-1.8	-1.0
Accuracy	---	98.2 to 101.9	99.5 to 103.8	96.3 to 107.9	104.3 to 104.8	103.0 to 105.2	99.1 to 105.0	103.1 to 106.9	103.3 to 106.7	93.9 to 99.3	96.7 to 105.4

- Linearity range is LOQ- 200 % with respect to 500 µg/ml RVB for impurities.
- Linearity range is 50–150% with respect to 100 µg/ml of RVB for assay
- Accuracy range is 50–150% with respect to specification limit.

Assay studies were carried out for stress samples (at 100 µg/mL) against qualified RVB reference standard. The mass balance (% assay + % sum of all compounds+ % sum of all degradants) results were calculated for all stressed samples and found to be more than 99 %. Thus confirms the stability-indicating power of the developed method. The results from system suitability all related substances well separated from RVB with symmetric peak shapes and shown in Table 1. Precision study evaluated by performing both repeatability and intermediate precision. %RSD

results for all related substances observed less than 6.5% indicates that the method is highly precise. The correlation coefficient was obtained greater than 0.9995 indicates linearity of RVB method. Accuracy performed at different levels from LOQ to 150% to the specification limit. Average %recovery observed between 92.0% to 107.9% indicates RVB method is highly accurate. During solution and mobile phase stability, %variation in impurity content and assay is less than 0.02% and 1.0% respectively when compared to initial results. Hence RVB spiked sample,

Assay sample and mobile phase were stable for 48 h at room temperature. Robustness study was performed by changing minute variations in column temperature, flow rate and mobile phase composition. Satisfactory results observed w.r.t resolution and tailing. Hence method is highly robust. Results for precision, Linearity and Accuracy results were shown in Table 2.

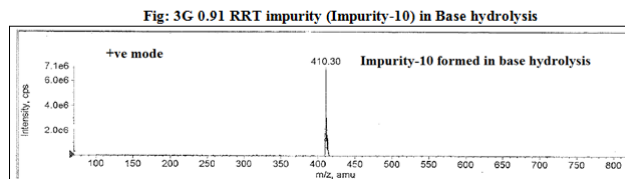
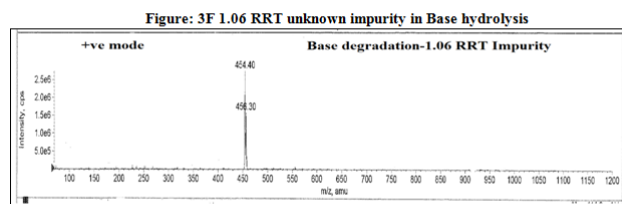
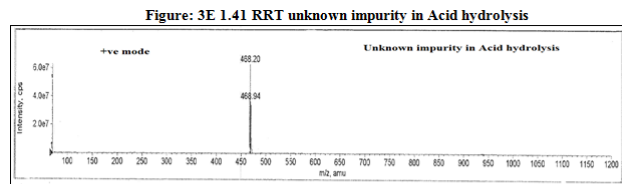
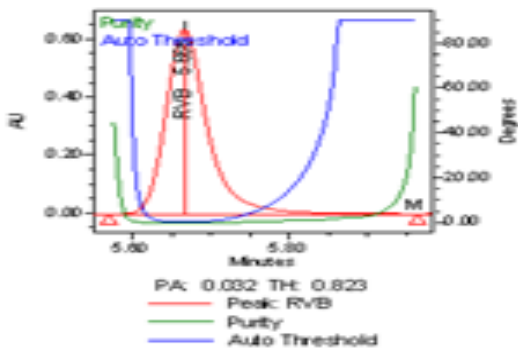
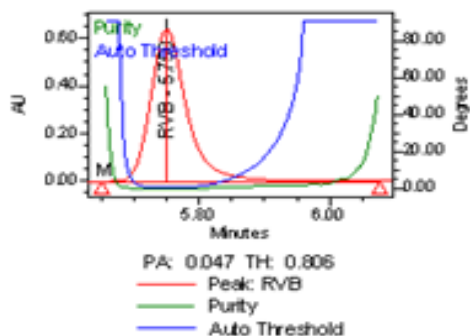
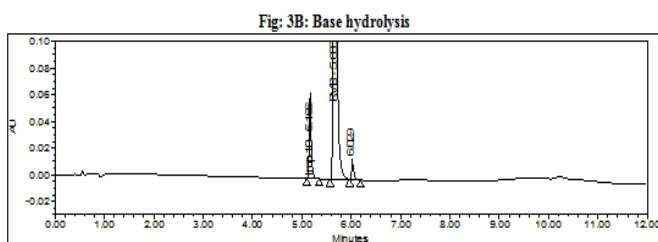
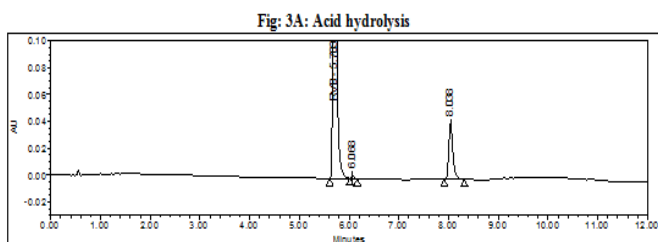


Table 3: Summary results of forced degradation

Summary of forced degradation study					
Degradation condition	Time	Assay (%w/w on dried basis)	RS by UPLC % degradation	Mass balance (%assay+Deg. Products)	Remarks/ observation
HCl, 0.1N 70°C (Acid hydrolysis)	2hrs	93.2%	6.4%	99.6	An unknown impurity was formed
NaOH, 0.1N RT (Base hydrolysis)	1hr	87.8%	11.3%	99.1	An known and unknown impurity was formed
Water hydrolysis 70°C	48hrs	99.2%	0.0%	99.2	No degradation observed
Oxidation by H2O2, 3.0%, 70°C	48hrs	99.9%	0.0%	99.9	No degradation observed
Thermal (105°C)	10days	99.4%	0.0%	99.4	No degradation observed.
UV at 254nm	10days	100.1%	0.0%	100.1	No degradation observed.

CONCLUSION

The rapid gradient RP-UPLC method developed for quantitative analysis of RVB and its related substances in pharmaceutical dosage forms is precise, accurate, linear, robust, rugged and specific. Satisfactory results were obtained from validation of the method. The retention time (12 min) enables rapid determination of the related substances and RVB. This method exhibited an excellent performance in terms of sensitivity and speed. Hence this method is cost effective with respect to time and solvent consumption. The method is stability indicating and can be used for routine analysis of production samples and to check the stability of samples of RVB.

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