



## A Stability-Indicating Ultra-Performance Liquid Chromatographic Method for Estimation of Related Substances and Degradants in Rivaroxaban Active Pharmaceutical Ingredient.

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### ABSTRACT

**Objective:** The objective of the study was to develop and evaluate the reverse phase ultra-performance liquid chromatography (RP-UPLC) method for the quantitative determination of potential impurities of rivaroxaban active pharmaceutical ingredient. **Method:** The method uses a water acquity BEH C8 column (100mm x 2.1 mm, 1.7 $\mu$ m) with mobile phase A consisted, 0.05M diammonium hydrogen phosphate, pH adjusted to 3.0 and acetonitrile (80:20, v/v) and mobile phase B consisted mixture of acetonitrile and water (90:10, v/v) with a gradient programme. The column temperature was maintained at 30°C and the detection was carried out at 254nm. **Results and Discussion:** Efficient and reproducible chromatographic separation was achieved on BEH C8 stationary phase in gradient elution profile. The newly developed UPLC method was validated accordingly to ICH guidelines considering five impurities to demonstrate precision, linearity, accuracy and robustness of the method. The developed UPLC method was found to be rapid (15.0min runtime), accurate and sensitive. The correlation coefficient values are greater than 0.999 for rivaroxaban and its five impurities. Detection limit and Quantitation limit were 0.0005 $\mu$ g/mL and 0.0015  $\mu$ g/mL respectively, indicating the high sensitivity of the newly developed method. Accuracy of the method was established based on the recovery obtained between 98.4% and 103.5% for all impurities. The results of robustness study also indicates that the method is robust and is unaffected by small variation in chromatographic conditions. **Conclusion:** The proposed UPLC method provides reliable, reproducible, accurate and sensitive for the quantification of rivaroxaban related substances.

**Keywords:** UPLC, Forced degradation, Rivaroxaban, Stability-indicating, validation

### INTRODUCTION

Rivaroxaban is 5-chloro-N-({ 5S)-2-oxo-3-[4-(3-oxomorpholin-4-yl) phenyl]-1,3-oxazolidin-5yl} methyl) thiophene -2-carbinamide<sup>[1]</sup>. RIV is an oral anticoagulant invented and manufactured by Bayer; it is marked as Xarelto<sup>[2]</sup>. It is an oxazolidinone derivative anticoagulant that inhibits both free Factor Xa and Factor Xa bound in the prothrombinase complex<sup>[3]</sup>. It is a highly selective direct Factor Xa inhibitor with oral bioavailability and rapid onset of action. Inhibition of Factor Xa interrupts the intrinsic and extrinsic pathway of the blood coagulation cascade, inhibition both thrombin formation and development of thrombi.

Rivaroxaban can help prevent dangerous blood clots from forming after a hip or knee replacement surgery. It also prevents blood clots and strokes in people with an irregular heart rhythm known as atrial

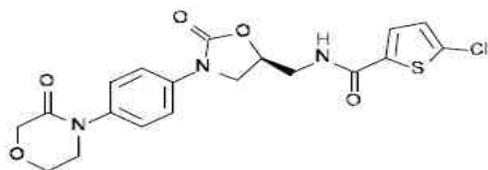
fibrillation. It comes in the form of a tablet that is taken 10mg once daily. Few methods have been reported for the simultaneous determination of rivaroxaban, High performance Liquid Chromatography, TLC Densitometry, First derivative and First-derivative ratio spectrophotometry for de-termination of Rivaroxaban and its alkaline degradates in bulk powder and its tablets<sup>[4]</sup>, Enantioselective determination of alprenolol in human plasma by liquid chromatography with tandem mass spectrometry using cellobiohydrolase chiral stationary phases<sup>[5]</sup>, RP-HPLC method development and validation for estimation of rivaroxaban in pharmaceutical dosage forms<sup>[6]</sup>, New method development and validation for analysis of rivaroxaban in formulation by RP-HPLC<sup>[7]</sup>, New spectrophotometric methods for the quantitative estimation of rivaroxaban in formulation<sup>[8]</sup>. Furthermore, there is no stability-indicating UPLC method reported in the literature that can adequately separate and accurately quantify rivaroxaban and its process related impurities. It is therefore, felt necessary to develop a new stability indicating UPLC method for the related substance determination of rivaroxaban. We intend to adopt for a faster chromatographic technique UPLC for the current

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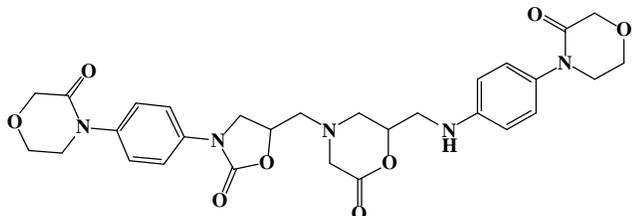
study [10]. An attempt was made to determine whether UPLC can reduce analysis times without compromising the resolution and sensitivity [11].

Hence, a reproducible stability indicating reverse phase UPLC method was developed for the quantitative determination of rivaroxaban and its five impurities, namely Impurity A, Impurity B, Impurity C, Impurity D, and Impurity E. This method was successfully validated according to the ICH guidelines. The chemical structure and names of rivaroxaban and the impurities Impurity A, Impurity B, Impurity C, Impurity D and Impurity E are depicted in Fig 1.

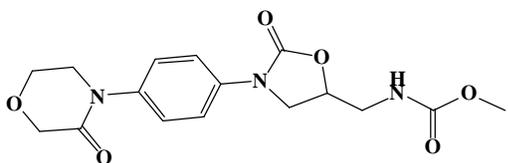


Structure of Rivaroxaban

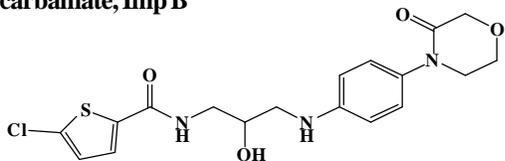
**5-Chloro-N-((5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl)phenyl]-1,3-oxazolidin-5-ylmethyl)-2-thiophene-carboxamide**



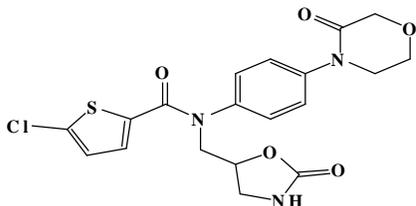
**4-(4-((2-oxo-3-((2-oxo-3-((4-(3-oxomorpholino)phenyl)oxazolidin-5-yl)methyl)oxazolidin-5-yl)methyl)amino)phenyl)morpholin-3-one, Imp A**



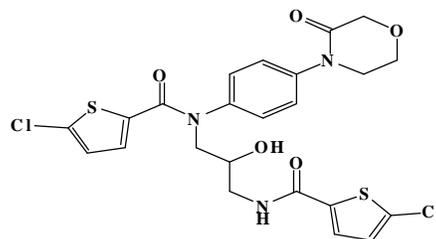
**Methyl((2-oxo-3-(4-(3-oxo-morpholino)phenyl)oxazolidin-5-yl)methyl)carbamate, Imp B**



**5-Chloro-N-[(2R)-2-hydroxy-3-[[4-(3-oxo-4-morpholinyl)phenyl]amino]propyl]-2-thiophene carboxamide, Imp C**



**5-Chloro-N-(4-(3-oxo morpholino)phenyl)-N-((2-oxooxazolidin-5-yl)methyl)thiophene-2-carboxamide, Imp D**



**5-Chloro-N-(3-(5-chlorothiophene-2-carboxamido)-2-hydroxypropyl)-N-(4-(3-oxomorpholino)phenyl)thiophene-2-carboxamide, Imp E**

Fig 1 Chemical structures of Rivaroxaban and related impurities

## Experimental

### Materials and Reagents

Rivaroxaban standards and samples were obtained from process research department Macleod's pharmaceuticals, Mumbai, India. Analytical-grade diammonium hydrogen phosphate were purchased from Merck, Darmstadt, Germany. Acetonitrile was purchased from J.T.Baker, Phillipsburg, NJ, USA. Water was prepared in-house by using a Millipore Milli-Q-water purification system (Millipore Corporate Headquarters, Billerica, MA).

### Chromatographic Conditions and Equipment

UPLC was performed using a waters acquity system equipped with binary solvent delivery pump, an auto sampler and tunable UV detector (Waters). Compounds were separated on a waters acquity BEH C8 (100mm x 2.1 mm, 1.7 $\mu$ m) column. The mobile phase A contained a mixture of diammonium hydrogen phosphate (0.05M), pH 3.0 and acetonitrile (80:20, v/v) respectively and mobile phase B contained mixture of acetonitrile and water (90:10, v/v) respectively.

The flow rate of the mobile phase was 0.5mL/min. The UPLC gradient program was set as: time (min) / % solution B: 0.01/0, 5.0/20 6.0/80, 8.0/100, 10.0/100, 12.0/20, 15.0/0. The column temperature was maintained at 30°C, and the detection was monitored at a wavelength of 254nm. The injection volume was 5.0 $\mu$ L.

### Preparation of Stock, Standard and test solutions

The stock solutions of the five impurities of rivaroxaban were prepared by separately dissolving 10mg of each impurity in 20mL of diluent (100%). A series of dilution were made by using 100% solutions of Impurity A, Impurity B, Impurity C, Impurity D and Impurity E. The sample solution was prepared by weighing approximately 10mg of rivaroxaban into a 20mL volumetric flask, the drug was dissolved and diluted to 20mL with diluent.

### Specificity: Forced Degradation Studies

Stress studies of the drug's active pharmaceutical ingredients were utilized for the identification of the possible degradation products

and for the validation of the stability indicating analytical procedures. It is the ability of the analytical method to measure the analyte response in the presence of its degradation products. The specificity of the developed UPLC method for rivaroxaban was carried out in the presence of its five impurities. Stress studies were performed at an concentration of 0.05mg/mL of rivaroxaban in active pharmaceutical ingredients (API). Intentional degradation was attempted by the stress condition of acid (1M HCl at room temperature, 2h), base (0.5M NaOH at room temperature at 1h), hydrolysis ( water, 70°C, 1h), and oxidation (3% H<sub>2</sub>O<sub>2</sub> at 70°C, 45min), thermal degradation (60°C for 7 days) and Photolytic ( overall illumination not less than 1.2 million lux hours and integrated near ultraviolet energy not less than 200 W h/ m<sup>2</sup>) stress to evaluate the ability of the method to separate rivaroxaban from its degradation products.

The purity of the peaks obtained from the stressed samples was verified using the photo diode array (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 by comparison with reference standards. An assay was also calculated for the rivaroxaban sample by spiking all five impurities at the specification level (i.e., 0.15%).

#### Method Validation

The described method has been extensively validated for related substances by UPLC determination.

#### Precision

The repeatability of the related substance method was verified by a six-fold analysis of 50µg/mL rivaroxaban spiked with 0.15% of each of five impurities (Fig 2). The RSD (%) of peak area was calculated for each impurity at three levels 80%, 100% and 120%. Inter-day variation and analyst variation were studied to determine the intermediate precision of the proposed method. Intra-day precision was deter-

mined by a six-fold analysis of 50µg/mL of rivaroxaban spiked with 0.15% of each of the five impurities. The RSD (%) of the peak area was calculated for each impurity.

#### Accuracy

The accuracy of an analytical procedure express the closeness of agreement between the true value and the value found. For impurities recovery was determined in triplicate for 0.25, 0.50 and 0.75µg/mL (50%, 100% and 150%) of the analyte concentration (0.05 mg/mL) of the drug substance and recovery of the impurities was calculated.

#### Linearity of Response

The detector response linearity for all five impurities was assessed by injecting separately prepared solutions covering the range of LOQ-150% of the normal sample concentration (50µg/mL). The detector response linearity for rivaroxaban was assessed by injecting separately prepared solutions covering the range of 50-150% (25, 37.5, 50, 62.5, 75µg/mL) of the normal sample concentration (50µg/mL). The correlation coefficients, slopes and y-intercepts of the calibration curve were determined.

#### Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ for rivaroxaban and its impurities were estimated based on signal to noise ratio method. Precision study was also carried out at the LOQ level by injecting six (n=6) was also determined at the LOQ level, and the RSD (%) was calculated for the peak area for each impurity.

#### 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 rivaroxaban

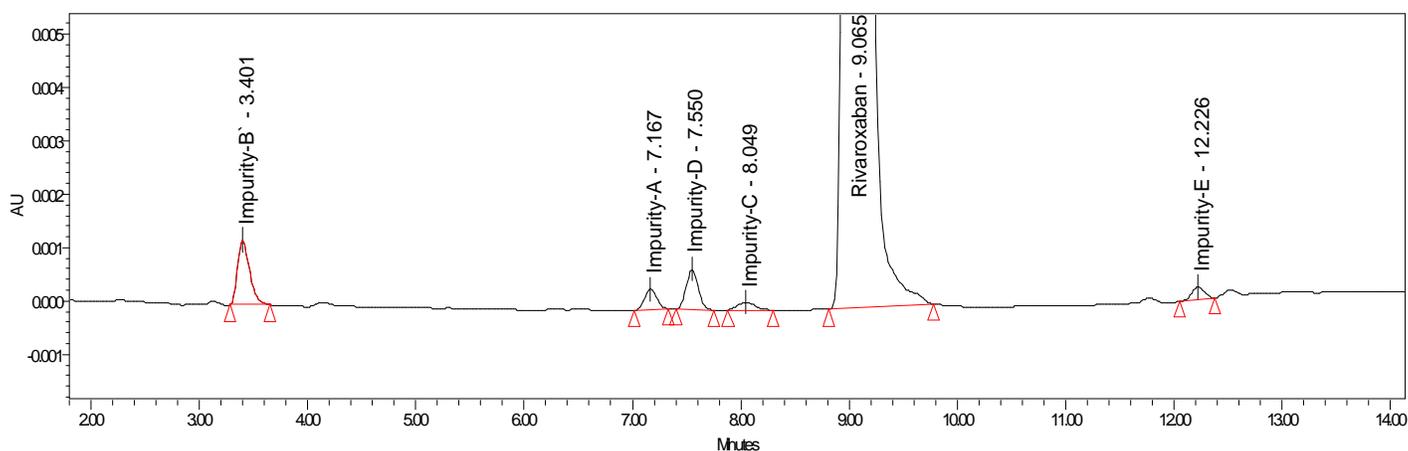


Fig 2 UPLC PDA analysis of sample solution spiked with specified impurities of Rivaroxaban column: acquity UPLC BEH C8 (100 mm x 2.1mm, 1.7µm waters), mobile phase A: diammonium hydrogen phosphate and acetonitrile (80:20, v/v) and mobile phase B: acetonitrile and water (90:10 v/v), column temperature 30°C, flow rate: 0.5mL/min. Injection volume 5µL, UV detection 254nm.

and its impurities was evaluation. The mobile phase flow rate was 0.5mL/min; to study the effect of the flow rate on resolution, the flow rate was changed to 0.45 and 0.55mL/min. The effect of the column temperature was studied at 25°C and 35°C.

#### Sample solution stability:

The stability of the drugs in solution during analysis was determined by repeated analysis of samples during the course of experimentation on the same day and also after storage of the drug solution for five days under laboratory bench conditions (25±2°C) and under refrigeration (5±3°C). From stock solution II of each drug (50µg/mL), combination working solutions were made after suitable dilution with the solvent to get a final concentration of (5µg/mL) of each drug. The samples were analyzed immediately and after a period of one, three and five days.

## RESULTS AND DISCUSSION

### Method Development and Optimization

The primary criteria for the development of a successful UPLC method for the determination of related substances and degradants in rivaroxaban was that the method should be able to determine related substances and degradants within 15 minutes of runtime and should be accurate, reproducible, robust, indicative of stability, free of interference from degradation products and impurities and straightforward enough for routine use in a quality control laboratory. One of the patents for the synthesis of rivaroxaban states a reverse phase HPLC method for the estimation of Impurities B and C. Impurities A, D, E were not considered for analysis. The total runtime for this method was approximately 60minutes and further analysis using this methodology showed that Impurities D and C were co-eluting, which clearly indicates that the method is not indicative of stability, be-

cause Impurity D is the degradation impurity of oxidation. As a result, further trials were completed using UPLC by using an Acquity UPLC BEH C8 column (100mm x 2.1mm, 1.7µm) and 0.05M diammonium hydrogen phosphate buffer (pH 2.0) as the buffer. The pH of the buffer was found to be critical, because a pH of more than 3.0 (pH > 3.2) led to the close elution of Impurities A, C and D. Initial efforts were made to reduce the tailing factor of rivaroxaban, which is more than 2.0, by adding triethylamine or diethylamine to the buffer. However, this led to the co-elution of Impurities D and C. As a result, the addition of triethylamine or diethyl amine was avoided. Optimum separation with minimum run time was obtained by employing a linear gradient of mobile phase A and mobile phase B in the ratio of 80:20 v/v, where mobile phase A was 0.05M diammonium hydrogen phosphate (pH 3.0) and mobile phase B was acetonitrile –water in the ratio of 90:10 v/v. The flow rate was set at 0.5 mL/min. All impurities and rivaroxaban showed sufficient response at 254nm and the analytical column was maintained at 30°C during the analysis. System suitability parameters were evaluated for rivaroxaban and its five potential impurities was greater than 1.4 for all pairs of compounds. The chromatogram representing Rivaroxaban spiked with impurities A, B, C, D and E at 0.15% level is shown in Fig-3.

### Validation of the Method

#### Precision

Precision of the developed method was evaluated as instrument repeatability and method precision (intra-day and intermediate precision) and reported as %RSD on the peak areas [10, 11]. Instrument precision was evaluated by injecting six times of spiked solution of rivaroxaban at a level of 0.15%. The RSD on the peak areas of rivaroxaban was 2.5% and those impurities less than 5.0%. Intra-day precision was determined by repeatedly analyzing six independent

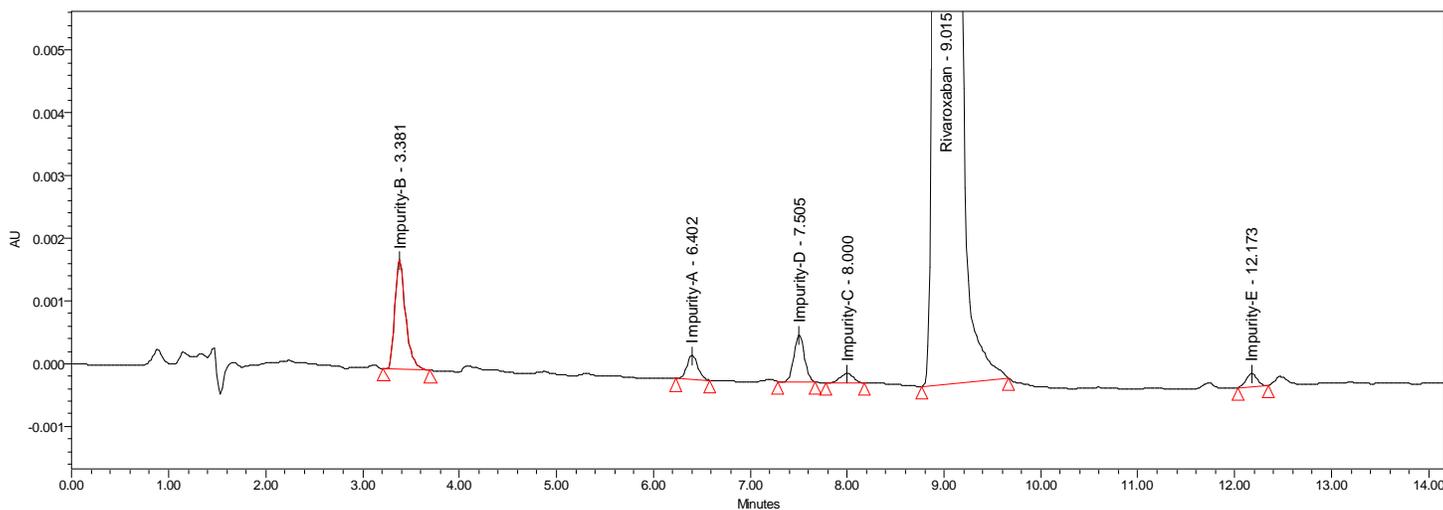


Fig 3 UPLC PDA analysis of standard solution spiked with specified impurities of Rivaroxaban column: acquity UPLC BEH C8 (100 mm x 2.1mm, 1.7µm waters), mobile phase A: diammonium hydrogen phosphate and acetonitrile (80:20, v/v) and mobile phase B: acetonitrile and water (90:10 v/v), column temperature 30°C, flow rate: 0.5mL/min. Injection volume 5µL, UV detection 254nm.

Table 1 Method Validation Data

Parameter	Rivaroxaban	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E
Limit of Detection (µg/mL)	0.0005	0.0004	0.0003	0.0004	0.0005	0.0004
Limit of Quantification (µg/mL)	0.0015	0.0012	0.0009	0.0012	0.0015	0.0012
<b>Precision (%RSD)</b>						
(Level-1) 80% (n=6)	2.8	3.2	4.2	4.6	3.7	4.8
(Level-2) 100% (n=6)	3.3	3.7	3.8	4.5	4.2	3.3
(Level-3) 120% (n=6)	2.9	4.5	3.7	2.5	2.7	3.4
<b>Accuracy (% Recovery)</b>						
(Level-1) LOQ (n=3)	-	102.4	101.7	101.2	103.5	99.5
(Level-2) 50% (n=3)	99.8	100.8	99.7	102.4	99.7	98.4
(Level-3) 100% (n=3)	99.3	98.3	99.4	102.4	99.6	102.7
(Level-4) 150% (n=3)	99.7	99.9	99.4	101.4	99.7	102.8
<b>Robustness (Resolution)</b>						
Actual flow 0.50mL/min	2.6	3.8	4.2	3.9	2.9	3.4
Different flow 0.45mL/min	4.2	3.4	4.5	2.3	2.3	2.1
Different flow 0.55mL/min	3.5	3.2	3.2	2.4	2.8	3.2
Column temperature 25°C	3.9	2.7	4.9	2.5	3.2	3.9
Column temperature 35°C	2.9	3.9	4.5	2.9	3.1	4.8

solutions prepared as described above on the same day. Precision for the main peak rivaroxaban was found to be 2.5% and not more than 5.0 for the five impurities. Results obtained are summarized in Table-1.

**Quantitative aspects**

The limit of detection (LOD) and limit of quantification (LOQ) for rivaroxaban were determined based on a signal-to-noise ratio (S/N) of 3 and 10, respectively [10,11]. An LOD value of 0.0005µg/ml, and an LOQ value of 0.0015µg/ml of the total rivaroxaban injected (100%=50µg/ml) were found. Results obtained are summarized in Table-1.

**Accuracy**

Accuracy were determined by the analysis of three concentrations chosen from the high, medium and low range of the standard curves for the selected drug (5, 15 and 25 µg/mL). Triplicates of each samples were analyzed on day 1 to determine intra-day precision and accuracy. Inter-day precision and accuracy was determined by triplicate samples of these concentrations on day 1 to 6. Accuracy (bias) is expressed as the percent difference between calculated mean concentrations relative to the nominal concentration. A precision (%CV) =5% and an accuracy (%bias) =15% are acceptable. The results obtained are summarized in Table-2.

Table 2. Recovery of the method with %Bias results and Linearity results

Amount of drug added (µg) to analyte	Mean amount (µg) (n=6)	Recovery Mean recovery %	RSD%	Bias (%)
25	25.08	100.03	0.13	0.09
50	50.07	100.02	0.24	0.14
75	75.08	99.98	0.08	-0.06

Bias (%): [(found – added)/added] x 100, RSD (%): Relative standard deviation.

**Linearity**

Linearity of the detector response was examined by analyzing a series of seven different concentrations of rivaroxaban. To assess the appropriateness of using a linear regression model to fit the data, residual plots were produced. The points in the residual plots were randomly distributed around the horizontal axis. This random dispersion of the residuals suggests that the linear model gives a good fit of the data. Correlation co-efficient (r), slope and y-intercept for the impurities were summarized in Table-2.

Spiked Conc (µg/ml)	Found <sup>a</sup> (µg/ml)	Intra day		Found <sup>a</sup> (µg/ml)	Inter day	
		Precision %CV	Accuracy <sup>b</sup> %Bias		Precision %CV	Accuracy <sup>b</sup> %Bias
25	25.04	0.57	+0.14	24.97	0.53	-0.17
50	49.97	0.49	-0.09	50.03	0.34	0.11
75	74.97	0.74	+0.04	74.96	0.29	-0.09

a – Mean ±standard deviation, n=3

b- Bias % = [(found-Spiked)/spiked] x 100

Table 2 (continued) Linearity results

Impurity Name	Slope	Y-intercept	Correlation coefficient (r <sup>2</sup> )
Rivaroxaban	12252886.6	-208.0	0.9995
Impurity A	22268049.3	173.9	0.9992
Impurity B	17649373.9	327.1	0.9991
Impurity C	19912724.0	197.6	0.9997
Impurity D	18554743.2	83.4	0.9994
Impurity E	19327214.7	154.2	0.9991

**Robustness**

In all the deliberately varied chromatographic conditions (flow rate, column temperature), all of the analyte were adequately resolved, and the order elution remained unchanged. The resolution between all the components was greater than 2.0, and the theoretical plates of rivaroxaban was more than 3000 and tailing factor of rivaroxaban for the varied conditions of flow rate ( 0.45, 0.55mL/min) and column temperature (25°C, 35°C) was less than 2.0.

**Table 3 Summary of Forced Degradation Results**

Degradation Condition	% Degradation from Related substances by UPLC Remarks/Observation Degradation	Assay (%w/w)	Mass Balance (%Assay+% Degradation products of Related substances)	Remarks
1M HCl at room temperature for 2h (Acid hydrolysis)	3.6	95.6	98.8	Significant degradation observed
0.5M NaOH at room temperature for 1 h (Base hydrolysis)	6.3	92.7	99.0	Significant degradation observed
3% H <sub>2</sub> O <sub>2</sub> at 70°C for 45min (Oxidation hydrolysis)	5.8	92.4	98.2	Significant degradation observed
Water at 70°C for 1h (Water hydrolysis)	5.1	93.2	98.3	Significant degradation observed
<b>Thermal degradation</b>				
Heat at 60°C for 7 days	1.4	98.4	99.8	No Significant degradation observed
<b>Photodegradation</b>				
UV light (NLT 200 Watt/hr)	1.9	97.4	99.3	No Significant degradation observed
White light (1.2 million lux hours)	1.3	97.5	99.8	No Significant degradation observed

**Samples solution stability**

From stock solution II, stored under refrigeration (5±3°C) and at laboratory temperature (25±1°C) combination working solutions were made after suitable dilution with the solvent, on each day of analysis. There was no significant change in analyte composition (5µg/ml) until a period of 5 days. The mean %RSD between peak areas, for the samples stored under both refrigeration and ambient conditions were found to be less than 3%, suggesting that the individual drug stock solution II (50µg/ml), can be stored without any degradation for the time interval studies.

**Specificity: Forced degradation studies**

Stress studies of the drug’s active pharmaceutical ingredients were utilized for the identification of the possible degradation products and for the validation of the stability-indicating analytical procedures. If the ability of the analytical method to measure the analyte response in the presence of its degradation products. UPLC conditions using a PDA detector to monitor the homogeneity and purity of the rivaroxaban peak. Degradation was not observed when rivaroxaban was subjected to light and heat conditions. Significant degradation was observed when the drug was subjected to hydrolysis, oxidative, base and acid degradation.

Peak purity of these samples under stressed condition was verified using a PDA detector. The purity of the principle and other chromatographic peaks was found to be satisfactory. Results from force degradation studies are presented in Table 3.

**CONCLUSION**

The rapid gradient RP-UPLC method developed for quantitative analysis of rivaroxaban and related substances in active pharmaceuticals ingredients is precise, accurate, linear, robust and specific. Satisfac-

tory results were obtained from validation of the method. The run time (15.0min) enabled rapid determination of the drug and its potential impurities. This method exhibited excellent performance in terms of sensitivity and speed. The method can indicate novelty, stability and can be used for the routine analysis of production samples and to check the stability of rivaroxaban samples.

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