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STABILITY INDICATING ASSAY METHOD DEVELOPMENT AND VALIDATION OF RIVAROXABAN TABLET BY RP-HPLC

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ABSTRACT
In the present work mainly focused on the approach of the development of stability-indicating assay method for determination of Rivaroxaban in tablet formulation. The RP-HPLC separation was carried out on Agilent HPLC 1200 series, Waters HPLC with Empower software waters 2996 PDA, 2487, waters 2695 separation module PDA were used for quantitative estimation of Rivaroxaban in pharmaceutical dosage form using a Purospher star RP18 end-capped, 5µ Hiber (250×4.6mm) with mobile phase comprising water: acetonitrile (60:40 v/v)+0.1 % TEA at flow rate of 1.0mL/min and UV detection at 280.0 nm. The method was validated as per ICH guidelines for accuracy, precision, specificity, linearity and range, ruggedness and robustness. The linearity of the proposed method was investigated in the range of 5% to 200% of the target concentration; the correlation coefficient for Rivaroxaban was found to be 0.999 which indicates the linear relationship between detector response and concentration. The proposed method was found to be simple, specific, linear and rugged and can be used for routine quality control.

Keywords: HPLC, Validation, Stability Indicating method, ICH Guideline, Rivaroxaban.

INTRODUCTION

Analysis is important in every product but it is vital in medicines as it involves life. The assurance of quality is achieved through analysis of drug product. Today, absorption spectroscopy and HPLC are the most valuable analytical techniques for pharmaceuticals. They will probably remain a useful tool in the future despite further advances in analytical chemistry because of several overwhelming advantages for the solution of many problems. These advantages include speed, simplicity, specificity and sensitivity. According to ICH guidelines on impurities in new drug products, identification of impurities below 0.1% level is not considered to be necessary, unless the potential impurities are expected to be unusually potent or toxic. Impurities present in excess of 0.1% should be identified and quantified by selective methods.

Analytical method development and validation used to ensure the identity, purity, potency and performance of drug products. The majority of the analytical development effort goes into validating a stability indicating HPLC method. The goal of the HPLC method is to separate and quantify the main active drug along with any reaction impurities or any degradants. Mass balance should always be a consideration during method development.

Literature survey reveals that numerous instrumental methods are available for determination Rivaroxaban by UV only. Beside this, some methods like HPLC, HPTLC and UV spctrophotometric are also reported. But so far no related substances method has been reported for the determination of Rivaroxaban. So the scope of developing and validating a method is to ensure a suitable strategy for a particular analyte which is more specific, accurate, simple and precise.

Rivaroxaban tablets are not official in any pharmacopoeia. is a factor Xa inhibitor used to treat deep vein thrombosis (DVT) and pulmonary embolism (PE). May also be used as thrombosis prophylaxis in specific situations. Rivaroxaban is an anticoagulant and the first orally active direct factor Xa inhibitor. Unlike warfarin, routine lab monitoring of INR is not necessary.

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However there is no antidote available in the event of a major bleed. Only the 10 mg tablet can be taken without regard to food. The 15 mg and 20 mg tablet should be taken with food.

Rivaroxaban competitively inhibits free and clot bound factor Xa. Factor Xa is needed to activate prothrombin (factor II) to thrombin (factor IIa). Thrombin is a serine protease that is required to activate fibrinogen to fibrin, which is the loose meshwork that completes the clotting process. Since one molecule of factor Xa can generate more than 1000 molecules of thrombin, selective inhibitors of factor Xa are profoundly useful in terminating the amplification of thrombin generation. The action of rivaroxaban is irreversible.

Rivaroxaban is a unqiue anticoagulant for two reasons.

First of all, it is does not involve antithrombin III (ATIII) to exert its anticoagulant effects. Secondly, it is an oral agent whereas the widely used unfractionated heparin and low molecular weight heparins are for parenteral use only. Although the activated partial thromboplastin time (aPTT) and HepTest (a test developed to assay low molecular weight heparins) are prolonged in a dose-dependant manner, neither test is recommended for the assessment of the pharmacodynamic effects of rivaroxaban. Anti-Xa activity and inhibition of anti-Xa activity monitoring is also not recommended despite being influenced by rivaroxaban.

Literature survey revealed that various methods of analysis are available for the estimation of Rivaroxaban in single dosage form: Kashid et al.(2017)A simple, fast and precise high performance liquid chromatographic method was developed and validated for rivaroxaban as per ICH guidelines. Chromatographic separation of drugs was performed on a Kromasil C18 column (250 mm x 4.6 mm, 5 µ) with a mobile phase comprising of sodium acetate: acetonitrile in the ratio 50:50 (V/V) at a flow rate of 1 mL/min. The described method was linear over a concentration range of 10-50µg/mL for Rivaroxaban. Krishna et et.al. (2021) Rivaroxaban is an anticoagulant or blood thinner. It makes your blood flow through your veins more easily. This means your blood will be less likely to make a dangerous blood clot. Rivaroxaban is a selective, reversible, direct inhibitor of factor Xa indicated to reduce the risk of stroke and systemic embolism in patients with non-valvular atrial fibrillation. The present review article summarises the analytical methods so far developed for the estimation of Rivaroxaban.

MATERIALS AND METHODS

Chemicals & Reagents

Rivaroxaban API and tablet formulation were supplied by Glenmark Pharmaceuticals Ltd. Mumbai. Label claim of Rivaroxaban in formulation is 10 mg.

Chemicals and Reagents:

a) Acetonitrile - HPLC grade
b) Potassium dihydrogen phosphate - AR grade
c) Triethylamine - AR grade
d) Double Distilled Water
e) Methanol - HPLC grade

Instruments

The Agilent HPLC 1200 series, Waters HPLC with Empower software waters 2996 PDA, 2487, waters 2695 separation module PDA were used for quantitative estimation of RIVAROXABAN in pharmaceutical dosage form.

Experimental Works

Preparation of diluent:

Acetonitrile and HPLC grade water was mixed in the ratio of 60:40.

Preparation of mobile phase:

Mobile phase was prepared using Water and acetonitrile was mixed in the different ratios. Shaked and mixed well then sonicated for 5 min.

Preparation of Rivaroxaban Standard Solution

An accurately weighed quantity about 50.0 mg Rivaroxaban reference standard was transferred into 50.0 mL volumetric flask. About 5 mL acetonitrile was added, sonicate for 5 min to dissolved and volume was made up to the mark with diluent. A 5 mL portion of this solution was transferred into 50 mL volumetric flask and volume was made up to the mark with diluent. (Concentration: 30µg/mL)

Selection of detection wavelength

About 50.0 mg of **Rivaroxaban** reference standard was transferred to 50.0 mL volumetric flask. To it 5 mL of acetonitrile was added and sonicated to dissolve. Then volume was made up to mark with acetonitrile (1000 µg/ mL). From above solution 5 mL was further diluted to 100 mL with acetonitrile (30 µg/ mL). This solution was scanned in the UV range from 400-200 nm.

At 280 nm **Rivaroxaban s**hows sufficient absorbance. Hence it was selected as the detection wavelength for further experimentation.

Chromatographic system:

The Agilent HPLC 1200 series, Waters HPLC with Empower software waters 2996 PDA, 2487, waters 2695 separation module PDA were used for quantitative estimation of Rivaroxaban in pharmaceutical dosage form. Stationary phase consists of a Purospher star RP18 end-capped, 5µ Hiber (250×4.6mm) with mobile phase comprising water: acetonitrile (60:40 v/v) + 0.1 % TEA at flow rate of 1.0mL/min and UV detection at 280.0 nm. Mobile phase selected composed of buffer consisting triethylamine 0.1 % in HPLC grade water and pH was adjusted to 2.0 with ortho phosphoric acid. The

composition diluent can be selected as water: Acetonitrile (40:60). A reversed phase Purospher star RP18 end-capped, 5µ Hiber (250×4.6mm) with pore size of 100 A^0 was used for chromatographic studies. The column was maintained at 40°C and injection volume of 20 µl was used. The mobile phase used for the studies consist of water: acetonitrile (60:40 v/v)+0.1 % TEA. The mobile phase was filtered through 0.45µ membrane filter and degassed by sonication. The flow rate was adjusted to 1.0 mL/min and detection was carried out on wavelength 280 nm. The diluent was used for the preparation of stock and diluted solutions of standard and test samples.

Preparation of Stock and Standard Solutions:

An accurately weighed quantity about 50.0 mg Rivaroxaban reference standard was transferred into 50.0 mL volumetric flask. About 5 mL acetonitrile was added, sonicate for 5 min to dissolved and volume was made up to the mark with diluent. A 5 mL portion of this solution was transferred into 50 mL volumetric flask and volume was made up to the mark with diluent. (Concentration: 30 µg/mL)

Study of system suitability parameters

Standard solution prepared for trial four was used for present study. Five replicate injection of the standard solution of 20 µl were injected.

The RSD of peak area for five replicate injections should not be more than 2.0%. Asymmetry for peak should not be more than 2.0. Number of theoretical plates should not be less than 3000.From the above table the three criteria were found to be in the acceptance range. Hence system is suitable for analysis.

Assay of Rivaroxaban sample

The optimized chromatographic conditions as described earlier were set and mobile phase was allowed to run for equilibration of column, as indicated by steady baseline. A 20 µL injection of diluent (blank) prepared earlier was injected in system.

Sample preparation

Accurately weighed quantity of about 50 mg Rivaroxaban sample was transferred into 50 mL volumetric flask. About 5 mL of acetonitrile was added, sonicated for 5 minutes with intermittent shaking. Sample was allowed to cool at room temperature. Volume was made up to the mark with diluent and mixed. A 5 mL portion of this solution was further diluted to 50 mL with diluent. The procedure was repeated six times.

The optimized chromatographic parameters were applied to check the presence of unknown peaks in diluents (blank), standard solution and sample solution of

Rivaroxaban

Assay of tablets

Twenty tablets were weighed and average weight was calculated. These tablets were crushed and powdered in a glass mortar. The tablet powder equivalent to the average weight of Rivaroxaban was accurately weighed, transferred to a 50 ml of volumetric flask and diluted up to mark with water: acetonitrile (60:40v/v). The solution was filtered through Whatman filter paper no. 41. This solution was further diluted to obtain 30µg/ml with diluent and the sample solution was injected into HPLC system. This procedure was repeated in triplicate.

Forced degradation studies

To evaluate stability, Rivaroxaban was subjected to force degradation under the condition of acid, base, neutral hydrolysis and oxidation as per international conference on harmonization (ICH) guidelines.

Acid hydrolysis

100 mg of Rivaroxaban was weighed accurately and transferred to 100 ml volumetric flask containing 100 ml of 0.1N hydrochloric acid (HCl). This mixture was refluxed at 80 °C. After 2 h, 5 ml of refluxed sample was withdrawn and neutralized with 5 ml of 0.1 N sodium hydroxide. This solution was further diluted 10 times with mobile phase to obtain a concentration of 100 µg/ml.

Alkaline hydrolysis

100 mg of Rivaroxaban was weighed accurately and transferred to 100 ml volumetric flask containing 100 ml of 0.1N sodium hydroxide (NaOH). This mixture was refluxed at 80 °C. After 2 h, 5 ml of refluxed sample was withdrawn and neutralized with 5 ml of 0.1 N hydrochloric acid. This solution was further diluted 10 times with mobile phase to obtain a concentration of 100 µg/ml. The chromatogram obtained after 2 h of alkali hydrolysis is shown in fig. 6(b).

Oxidative degradation

100 mg of Rivaroxaban was weighed accurately and transferred to 100 ml volumetric flask containing 100 ml of 3% hydrogen peroxide (H2O2). This mixture was refluxed at 80 °C. After 2 h, 5 ml of refluxed sample was withdrawn. This solution was further diluted 10 times with mobile phase to obtain a concentration of 100 µg/ml. The chromatogram obtained after 2 h of oxidative degradation is shown in fig. 6(c).

Thermal degradation

100 mg of Rivaroxaban IR tablet powder sample was weighed and transferred into 100 ml volumetric flask. The contents were refluxed as such on a water bath previously maintained at 80^o C for 2 h. The sample was allowed to cool to room temperature and then the volume was made up to the mark with mobile phase and mixed

well. The solution was filtered through a 0.45µ syringe filter and analyzed. The chromatogram obtained after 2 h of thermal degradation is shown in fig. 6(d).

Photolytic degradation

Photolytic degradation of the drug was carried out by exposure of about 100 mg of Rivaroxaban IR tablet powder sample to UV radiation for 12 h. Then the sample was transferred into 100 ml volumetric flask. The sample was allowed to cool to room temperature and then the volume was made up to the mark with mobile phase and mixed well. The solution was filtered through a 0.45µ syringe filter and analyzed.

Validation of the method

The developed chromatographic method was validated for system suitability, linearity, range, accuracy, precision, LOD - LOQ and robustness parameters as per ICH guidelines

Linearity and range

Working standard solutions were injected in the range of 5-30µg/ml under the optimized chromatographic conditions and peak areas were calculated at 280 nm. The calibration curve was plotted between areas against concentrations of the drug. Linear regression data, as well as calibration curve, were shown in fig. 7.

Precision

Repeatability study was carried out with six replicates and intermediate precision studies were carried out with three concentrations of Rivaroxaban with three replicates. The values of % relative standard deviation (% RSD) of precision study are shown in table 9.

Accuracy (Recovery)

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Procedure:

Accuracy was performed by preparing sample at three levels i.e. 50%, 100% and 150% considering 100µg/mL as 100% target concentration.

Preparation of sample: Accurately weighed quantity of about 25.0 mg of Rivaroxaban sample was added into a series of six 50 mL volumetric flask and to this 25.0 mg of reference standard was added for 50% accuracy level. To each flask 5 mL of acetonitrile was added and sonicated to dissolve and volume was made up to the mark with diluent and mixed well. A 2.5 mL portion of solution from each flask was transferred into six 50 mL volumetric flask to get solution of 30µg/mL.

For the accuracy level 100%, sample was prepared by transferring accurately weighed quantity of about 25.0 mg of Rivaroxaban sample into a series of three 50 ml volumetric flask and to it 75.0 mg of reference standard was added. To each flask 5 mL of acetonitrile was added and sonicated to dissolve and volume was made up to the mark with diluent. A 2.5 mL portion of solution from each flask was transferred into three 50 mL volumetric flask to get solution of 100 µg/mL.

For the accuracy level 150%, sample was prepared by transferring accurately weighed quantity of about 25.0 mg of Rivaroxaban sample into a series of six 50 ml volumetric flask and to it 125.0 mg of reference standard was added. To each flask 5 mL of acetonitrile was added and sonicated to dissolve and volume was made up to the mark with diluent. A 2.5 mL portion of solution from each flask was transferred into six 50 mL volumetric flask to get solution of 150 µg/mL. The above prepared samples for different accuracy level was injected into the HPLC system with 20 µL injection volume and the area of each sample was then calculated from the chromatograms and % recovery was calculated for each level.

The accuracy of the method was determined by calculating percent recovery of the drug by standard addition method. Percent recovery of Rivaroxaban was determined at three different level 50%, 100%, and 150% of the target concentration in triplicate.

Robustness

Robustness of the optimized method was studied by changing flow rate (±0.1 ml/min), change in wavelength (±1 nm) and change in mobile phase composition (±5%) during analysis. The sample was injected in triplicate for every condition and % RSD was calculated.

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

Five sets of concentrations were prepared between 5-30 µg/ml and the corresponding areas of these sets were measured. Calibration curves were plotted for each set.

Table: 1 Optimized chromatographic conditions

PARAMETERS	CONDITION
Stationary Phase	**Purospher Star RP-18 end-capped (5 µm) Hibar 250x4.6 mm**
Mobile phase	Water: ACN (60:40) + 0.1% TEA
Flow Rate	1.0 mL/min

Detection wavelength	280 nm
Pump Mode	Isocratic
Injection Volume	20 µL
Run Time	10 min
Column Temperature	40^0C

Table: 2 Observation and result for system precision

Sr. No.	Standard Weight Taken (mg)	Std area of Rivaroxaban
1	51.15	227800
2		229426
3		228545
4		228156
5		227941
6		229036
Mean		**228484**
SD		**643**
% RSD		**0.2813**
Retention time (min)		**5.62**
Asymmetry		**1.10**
Theoretical plate		**5840**

Table: 3 Results for Forced Degradation Studies of Rivaroxaban

Sr No	Experiment	Degradation Condition	% Assay	% Degradation w.r.t. control	Peak Purity
1	Control	--	98.6	--	1.000
2	Acid Degradation	0.1N HCl - 60°C/ 1hr	87.5	8.8	1.000
3	Base Degradation	0.1N NaOH-60°C/1hr	83.5	10.5	1.000
4	Peroxide Degradation	3% H_2O_2- 60°C/ 1hr	82.7	13.1	1.000
5	Thermal Degradation	60°C – 8 hours	94.2	1.8	1.000
6	Water Degradation	60°C/ 1hr	96.1	3.5	1.000

Table 4: Results of Linearity Response Rivaroxaban

Level	Spike level in %	Conc. (µg/mL)	Response (Area)	Statistical analysis	
1	50	49.400	524468	**Slope**	**20912**
2	80	74.100	825138		
3	90	98.800	935149	**Intercept**	**-4562**
4	100	122.512	1032264		
5	110	148.200	1151785	**Correlation Coefficient**	**0.9998**

Table: 5 Result for ruggedness study of Rivaroxaban sample

Sample	% Estimation of Rivaroxaban	
	Analyst -1	**Analyst -2**
1	97.5	98.7
2	102.5	98.2
3	98.9	99.4
4	98.7	99.6
5	98.1	98.6
6	97.2	98.7
Mean	**99.2**	**98.3**
SD	**1.76**	**0.47**
%RSD	**1.5**	**0.5**
Overall Mean	**98.5**	
Overall SD	**1.17**	
Overall %RSD	**1.13**	

Table: 6 Stability of analytical solution at bench top

Sr. No.	Name	Test 1	Difference From Initial	Test 2	Difference From Initial
		%Assay		%Assay	
1	0 hr	99.7	1.2	99.5	0.7
2	After 24 h	98.5		98.8	

Table 7: Observation and result for accuracy of Rivaroxaban sample.

Sample No.	Amount found (mg)	Area (mAU)	% Recovery
Acc. 50%-1	49.4992	428744	101.5
Acc. 50%-2	50.0494	433381	101.6
Acc.50% -3	49.4148	427839	102.3
Acc. 50%-4	49.6294	429785	101.5
Acc. 50%-5	49.8107	431303	102.7
Acc. 50%-6	48.9854	424168	99.5
Acc. 100% -1	98.3251	850984	98.4
Acc. 100% -2	99.8544	864827	101.2
Acc. 100% -3	98.6013	853862	100.0
Acc. 150% -1	150.7442	1305570	102.4
Acc. 150% -2	147.4286	1276544	99.9
Acc. 150% -3	147.8804	1280888	100.3
Acc. 150% -4	149.8313	1297529	100.8
Acc. 150% -5	150.5742	1303306	101.8
Acc. 150% -6	145.7029	1261629	98.5
	Mean	**100.8**	
	SD	**1.35**	
	% RSD	**1.34**	

Figure 1: Structure of Rivaroxaban

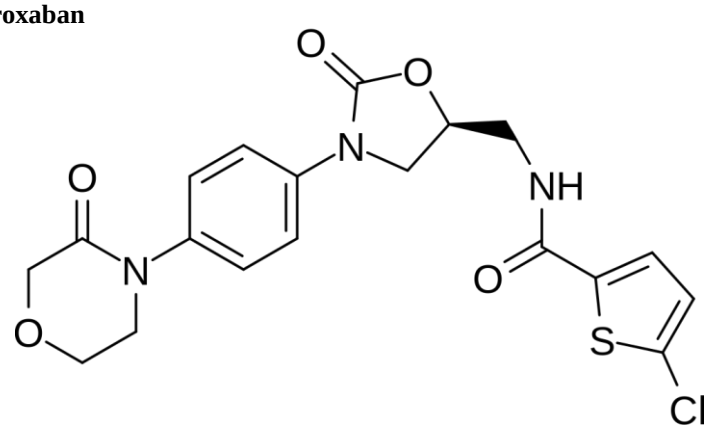


Figure 2: UV spectrum of Rivaroxaban.

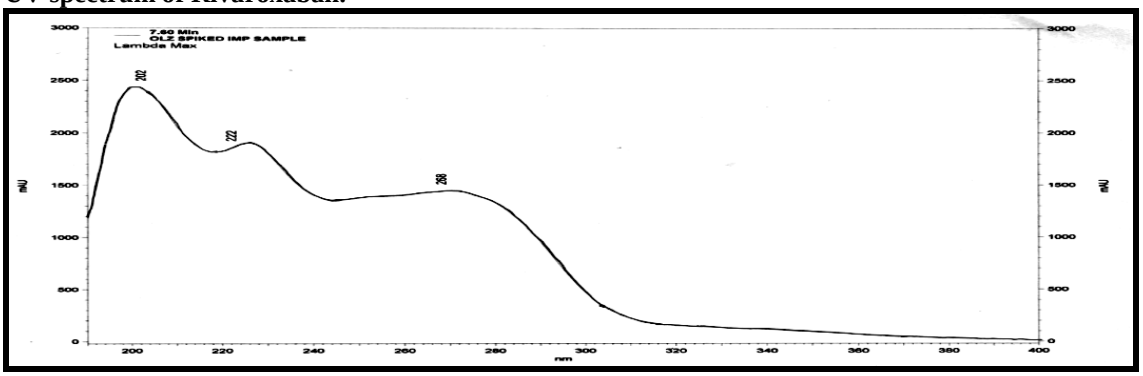


Figure 3: Standard Chromatogram of Rivaroxaban.

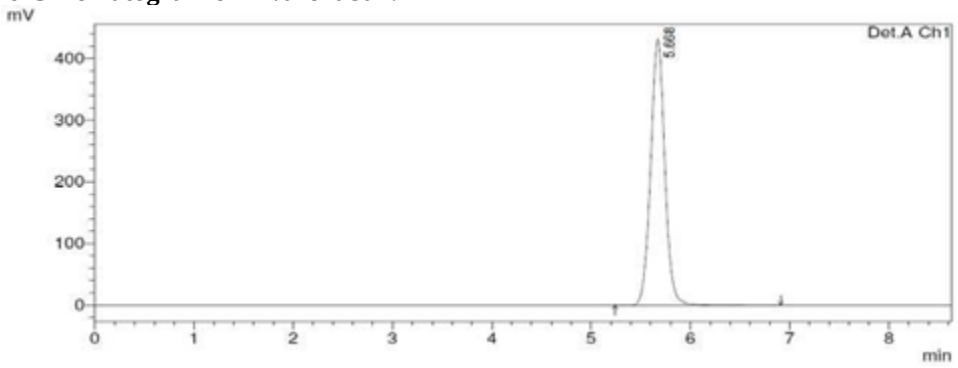


Figure 4: Chromatogram of acid treated sample.

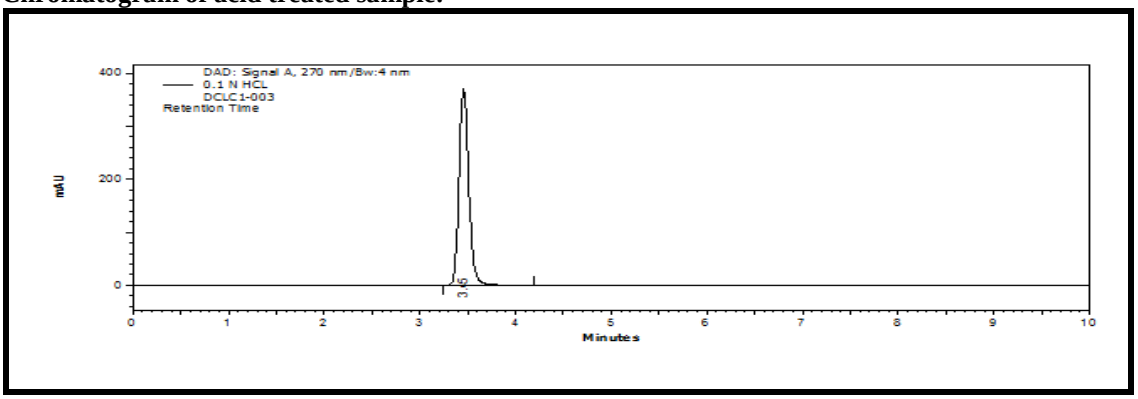


Figure 5: Chromatogram of alkali treated sample.

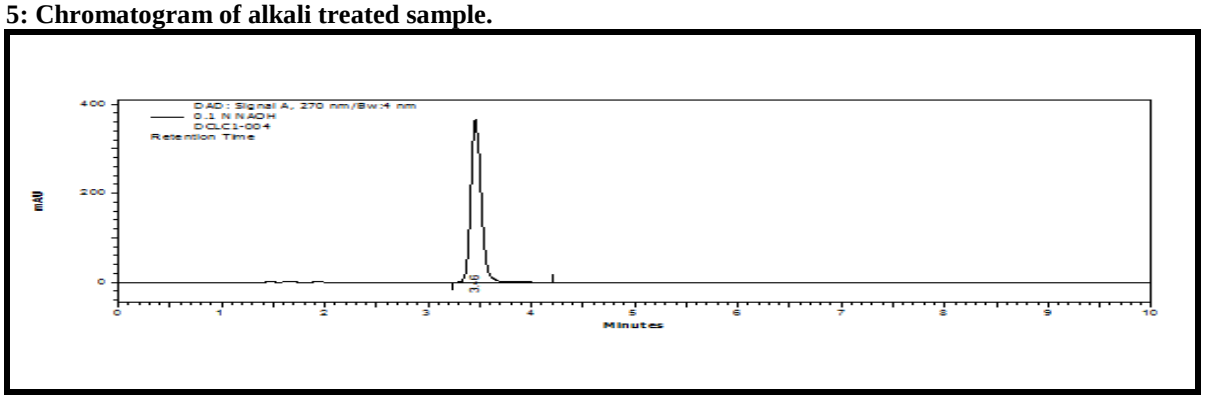


Figure 6: Chromatogram of peroxide treated sample.

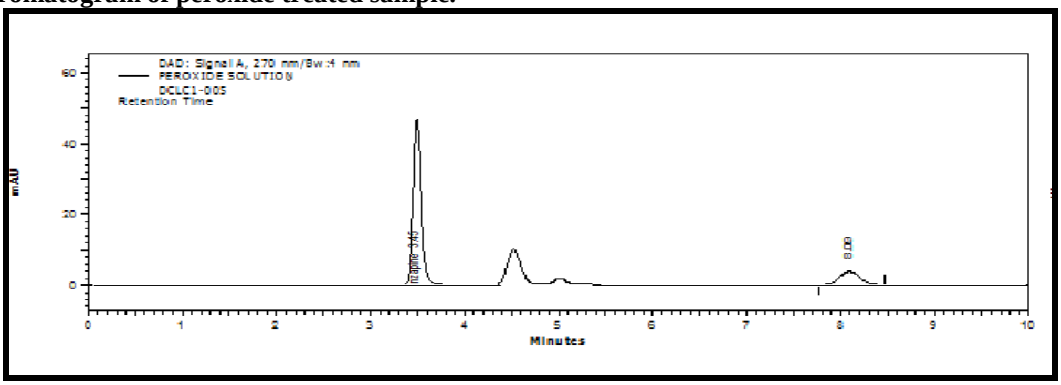


Figure 7: Chromatogram of heat treated sample

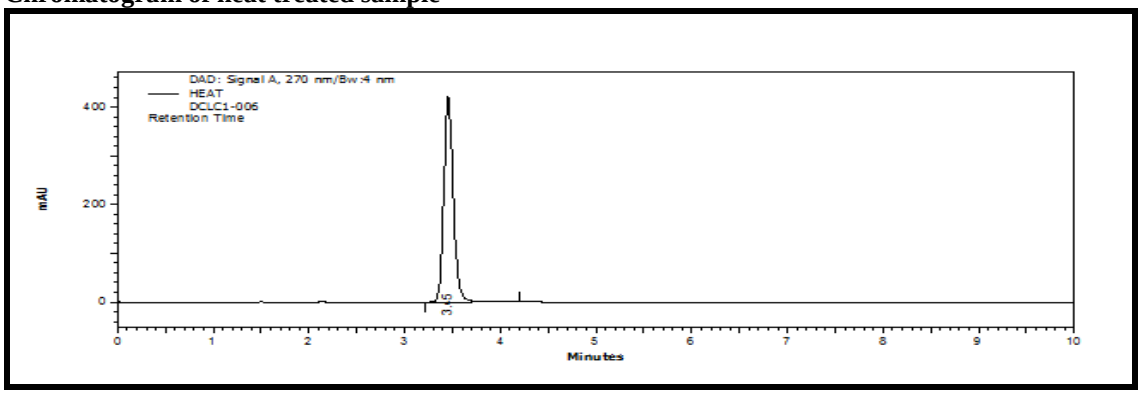


Figure 8: Chromatogram of water treated sample.

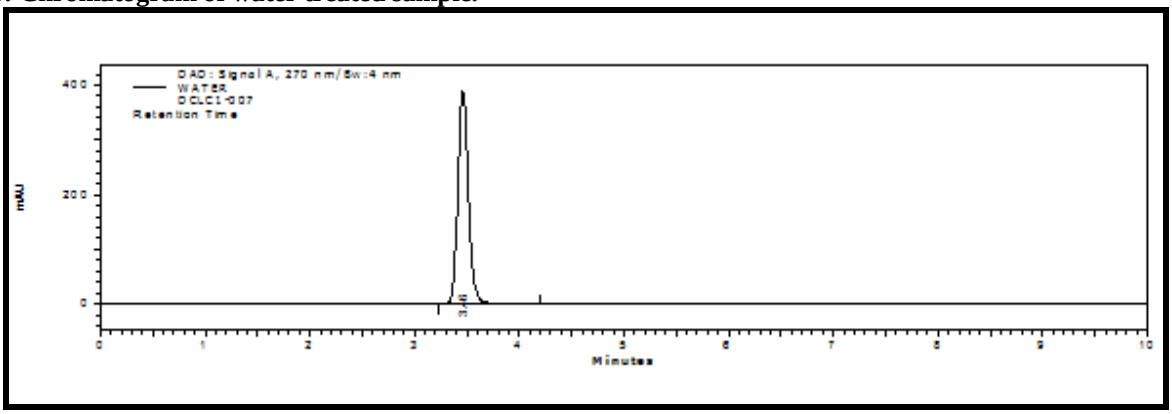


Figure 9: Chromatogram of control sample.

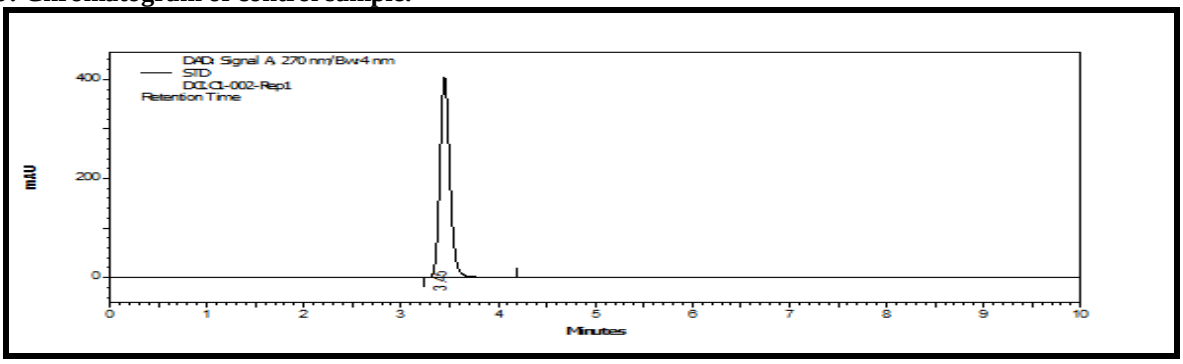


Figure 10: Study of linearity of Rivaroxaban.

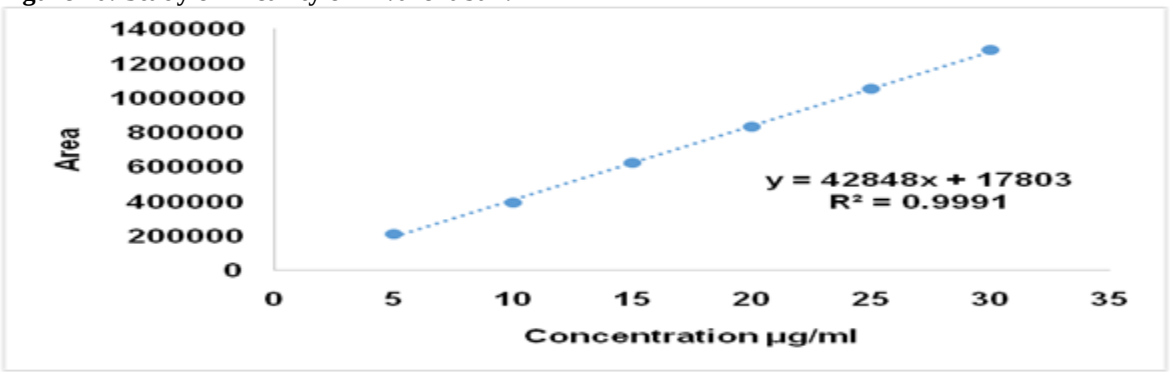


Figure 11: Chromatogram of sample 150 % level for Accuracy.

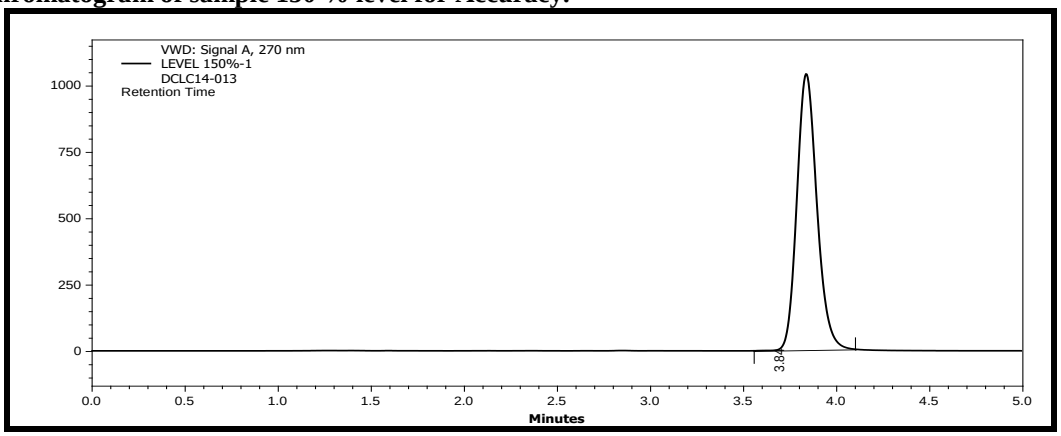
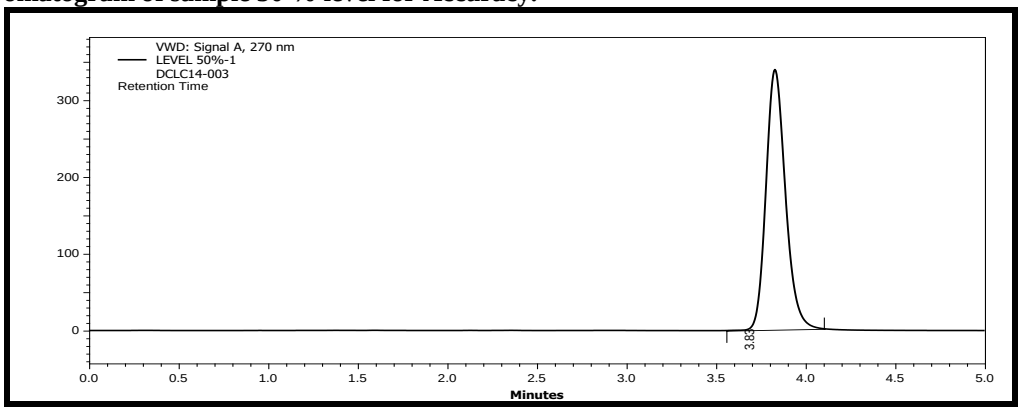


Figure 12: Chromatogram of sample 50 % level for Accuracy.



RESULTS & DISCUSSION

The present research pursuit aims at the development and validation of stability- indicating assay method for determination of Rivaroxaban in tablet dosage form. During development of the method, number of mobile phases in different compositions were attempted to elute Rivaroxaban. Method development was started with water: ACN (70:30) used in different proportions and with different pH. Various stationary phase column were also used for the development process such as ACE, C_{18}, 250x4.6mm, 5µ , Xterra RP C_{18}, 150x4.6mm, 5µ and finally Purospher Star RP-18 end-capped (5 µm) Hibar 250x4.6 mm was successfully resolved the sharp peak of Rivaroxaban. The stationary phase selected for analysis of Rivaroxaban was Purospher Star RP-18 end-capped (5 µm) Hibar 250x4.6 mm. The ratio of buffer and acetonitrile was selected on the basis of elution of Rivaroxaban with sharp well defined peak with reasonable retention time. A mobile phase containing Buffer: ACN (60:40) v/v was selected which gave reasonable retention time of 3.6. The system suitability studies were carried out which showed that all the parameters were found to be within range which passes the system suitability test and can be used for further analysis.

The developed method was also applied for routine analysis and stability studies of tablet formulation.

CONCLUSION

From all above studies, we can conclude that the proposed HPLC method can be used successfully for determination of Assay and Related substances in Rivaroxaban and can also be useful in detecting the degradation of unknown impurities. Further this method may be applied to preparative HPLC for qualification of unknown impurities which might be generated during forced degradation studies. The results obtained by HPLC method for determination of Rivaroxaban are reliable, accurate and precise. The method can be employed for routine quality control analysis of Rivaroxaban in tablet dosage form.

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