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Stress study and estimation of a potent anticoagulant drug rivaroxaban by a validated HPLC method: Technology transfer to UPLC

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ABSTRACT

This work deals with the method development and validation of Rivaroxaban in pharmaceutical dosage form on both the instruments HPLC and UPLC (technology transfer HPLC to UPLC) with forced degradation study. In present method chromatographic separation was performed using phenomenax C8 100A (250 X 4.6mm id, 5 µm particle size) HPLC column and acquity UPLC (R) BEH C8 (100 X 2.1mm id, 1.7 µm particle size) UPLC column with 30°C column oven temperature. The isocratic mobile phase was consisted 0.1% OPA: ACN (60: 40 V/V) for HPLC and (55: 45 V/V) for UPLC. The detection was monitored at wavelength of 280 nm. Flow rate consisted 1ml/min and 0.25 ml/min simultaneously for HPLC and UPLC with 20µl and 2µl injection volume. The total analysis takes 15 minutes for HPLC and 3 minutes for UPLC.

Keywords: Stress study, Estimation, HPLC, UPLC, Potent, Anticoagulant, Technology transfer

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INTRODUCTION

Rivaroxaban is chemically known as 5-Chloro-N-({(5S)-2-oxo-3-[4-(3-oxo-4-morpholinyl) phenyl]-1,3-oxazolidin-5-yl}methyl)-2thiophene carbox-mide. The molecular formula of Rivaroxaban is $C_{19}H_{18}ClN_3O_5S$ and the molecular weight is 435.89 g/mol. Rivaroxaban is an oral anticoagulant substance that prevents coagulation (clotting) of blood. It is an odorless, non-hygroscopic, white to yellowish powder and pure (S)-enantiomer. Rivaroxaban is an oral, direct Factor Xa inhibitor that has been shown to exhibit well-defined pharmacokinetic and pharmacodynamic properties, with high oral bioavailability [1]. Rivaroxaban is invented and manufactured by Bayer and it is marketed as xarelto [2]. Each xarelto tablet contains 10 mg, 15 mg, or 20 mg of Rivaroxaban. The inactive ingredients of xarelto are croscarmellose sodium, hypromellose, lactose monohydrate, magnesium stearate, microcrystalline cellulose, and sodium lauryl sulfate [3]. In the United States It is marketed by Janssen Pharmaceutica [4]. Rivaroxaban is the first available orally active direct factor Xa inhibitor from class of oxazolidinone derivative and it has been optimized for inhibiting both free factor Xa and factor Xa bound in the prothrombinase complex [5]. Rivaroxaban is indicated for the prevention of deep vein thrombosis which may lead to pulmonary embolism in patients undergoing knee or hip replacement surgery. The most common adverse reactions with Rivaroxaban are bleeding complications, including major bleeding events, fainting, itching, and muscle spasms have been reported [6]. The introduction of this drug in a surgical department represents a standard change, this drug is not administered preoperatively but postoperatively, and is not given subcutaneously but orally. Rivaroxaban inhibits free and fibrin-bound Factor Xa as well as Factor Xa in the prothrombinase complex, therefore it prevents clot formation and clot growth.

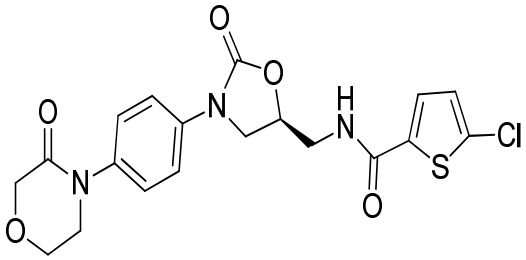

Figure 1: Structural formula of Rivaroxaban

The literature survey indicates that there are many methods are available for the estimation of Rivaroxaban as a drug substance as well as in pharmaceutical dosage form, few of the methods are also which deals the bioanalytical study. [7-12] But all of these are not stability indicating. Most of the reported methods either do not include stress degradation studies or are not completely validated and they are time consuming and expensive. Furthermore these methods are not impressionable to achieve the high throughput study which can be possible by optimizing the method in such a way which includes shortest run time with maximum selectivity.

Reason for conversation of HPLC method to UPLC method

To get faster results by applying new technology with more reliability

- Achieves more information
- Results more robust methods
- Better situational response time (stat sample faster, research decision with more information, process monitoring and product release)
- More sample analyzed per system, per scientist
- Overall increased productivity with best compatibility compare to earlier techniques [13].

EXPERIMENTAL SECTION

Instruments

The chromatographic HPLC system was used to perform, development and validation of this assay method consisted Waters 2489 equipped with UV/Visible detector, quaternary solvent manager pump and 20µl manual injector loop. UPLC analysis was performed on Waters Acquity UPLCTH system which contains binary solvent manager, auto injector with PDA detector. For data acquisition Empower 2.0 version software used for both instruments.

Materials

Reference standard of Rivaroxaban was provided as a gift sample for research purpose by Anlon Research Organization, Rajkot, India and tablet dosage form was purchased from market. HPLC grade acetonitrile was purchased from Merc India Limited, Mumbai, India and HPLC grade orthophosphoric acid was from Spectrochem Mumbai, India. Analytical grade hydrochloric acid, sodium hydroxide pellets and hydrogen peroxide were purchased from Ranbaxy Fine Chemicals, New Delhi, India. High purity deionised water was obtained from Milli-Q (Millipore, Miliford, MA, USA) purification system. 0.45 µm membrane filters were purchased from Pall Life Sciences Mumbai, India and nylon syringe filters 0.45 µm were purchased from Millex-Hn, Mumbai, India.

Chromatographic Condition

The chromatographic analysis have been performed on Phenomenax C8 100A (250 X 4.6mm id, 5 µm particle size) and Acquity UPLC (R) BEH C8 (100 X 2.1 mm id, 1.7 µm particle size) columns were used for HPLC and UPLC respectively. The HPLC mobile phase was consisted 0.1% OPA: ACN (60: 40 v/v) and for UPLC it was consisted 0.1 % OPA: Acetonitrile (55:45 v/v) with 280 nm UV detection and 30°C column oven temperature. HPLC flow rate was adjusted at 1ml/min with 20µl injection volume and for UPLC it was adjusted at 0.25 ml/min with 2µl injection volume. The total analysis time was selected 15 minutes for HPLC and 3.0 minute for UPLC.

Solution preparation for HPLC analysis
Stock solution preparation
The stock solution of 500µg/ml Rivaroxaban was prepared by dissolving accurately weighted 50mg of Rivaroxaban working standard in 100ml volumetric flask. Add 50ml of diluent (water: methanol (50:50) V/V) to dissolve the substance by sonication for about 5 minutes and then dilute to volume up to mark with diluent. For standard solution preparation pipette out 5.0ml of above stock solution into 50ml volumetric flask and dilute up to the mark with diluent. This solution contains 50µg/ml Rivaroxaban.

Test solution preparation
The preparation of 500µg/ml stock solution, twenty tablets were accurately crushed, weighted and average weight has been calculated. The portion of powder equivalent to the weight of five tablets has been taken and transferred to a 100ml volumetric flask. Add 50ml of diluent (water: methanol (50:50) V/V) to dissolve the substance by sonication about 5-10 minutes and then dilute to volume up to mark with diluent. Filter the solution with 0.45µm membrane filter. For test solution preparation pipette out 5ml of above stock solution into 50ml volumetric flask and dilute up to the mark with diluent. This
solution contains 50µg/ml Rivaroxaban.

Solution preparation for UPLC analysis
Stock solution preparation
The stock solution of 500µg/ml Rivaroxaban was prepared by dissolving accurately weighted 50mg of Rivaroxaban working standard in 100ml volumetric flask. Add 50ml of diluent (water: methanol (50:50) V/V) to dissolve the substance by sonication for about 5 minutes and then dilute to volume up to mark with diluent. For standard solution preparation pipette out 2.5ml of above stock solution into 50ml volumetric flask and dilute up to the mark with diluent. This solution contains 25µg/ml Rivaroxaban.

Test solution preparation
The preparation of 500µg/ml stock solution, twenty tablets were accurately crushed, weighted and average weight has been calculated. The portion of powder equivalent to the weight of five tablets have been taken and transferred to a 100ml volumetric flask. Add 50ml of diluent (water: methanol (50:50) V/V) to dissolve the substance by sonication for about 5-10 minutes and then dilute to volume up to mark with diluent. Filter the solution with 0.45µm membrane filter. For test solution preparation pipette out 2.5ml of above stock solution into 50ml volumetric flask and dilute up to the mark with diluent. This solution contains 25µg/ml Rivaroxaban.

RESULTS AND DISCUSSION

Method optimization for HPLC analysis
This study describes assay method development and validation of Rivaroxaban tablet with forced degradation study. For accurate, precise and robust method development, the analytical conditions were selected after testing the different parameters that influence LC analysis, such as analytical column, detection wavelength, aqueous and organic mobile phase, mobile phase proportion, analyte concentration, injection volume, flow rate and other factors were exhaustively studied. Phenomenax C8 100A (250 mm X 4.6 mm i.d., 5 µm particle size) column was used because of its advantages of high degree of retention, high resolution capacity, better reproducibility, ability to produce lower back pressure and low degree of tailing. For mobile phase selection, the preliminary trials using different compositions of mobile phases consisting of water and acetonitrile (50:50 v/v) gave poor peak shape.

The chromatogram obtained by this trial is indicated clearly that the peak is not symmetrical and value of theoretical plates is lower side. In focus to develop good symmetrical peak, water was replaced by 0.1% ortho phosphoric acid. Thus, better peak shape was obtained.

Further, the mobile phase proportion was optimized to retain analyte properly that provide good resolution between Rivaroxaban and its degradation impurities. Proportion of acetonitrile is finalized to 40% of the mobile phase. The detection wavelength was decided after screened the standard solution over 190- 400nm using the advantage of photo diode array detector. On the basis of peak absorption maxima and peak purity index, the 280nm was decided as the detection wavelength which was provided the maximum chromatographic compatibility to the method. As a diluent the mixture of water-methanol (50:50 v/v) was made. Injection volume was fixed to 20µl and the flow rate of the mobile phase was set to 2ml/min. On this finalized chromatographic condition, obtained chromatogram was

having of good peak symmetry and higher theoretical plates. The representative chromatogram for the same is shown as under.

The drug substance was easily extracted from pharmaceutical dosage using diluent as water methanol (50:50 v/v). Tablet was easily isolated using water and methanol. The drug substance is freely to very soluble in methanol. Extraction trials are finalized to keep sonication time for around 15 - 20 minutes. Solutions of standard preparation and test preparation were found stable in diluent. All validation parameters were performed by taking same diluent concentration.

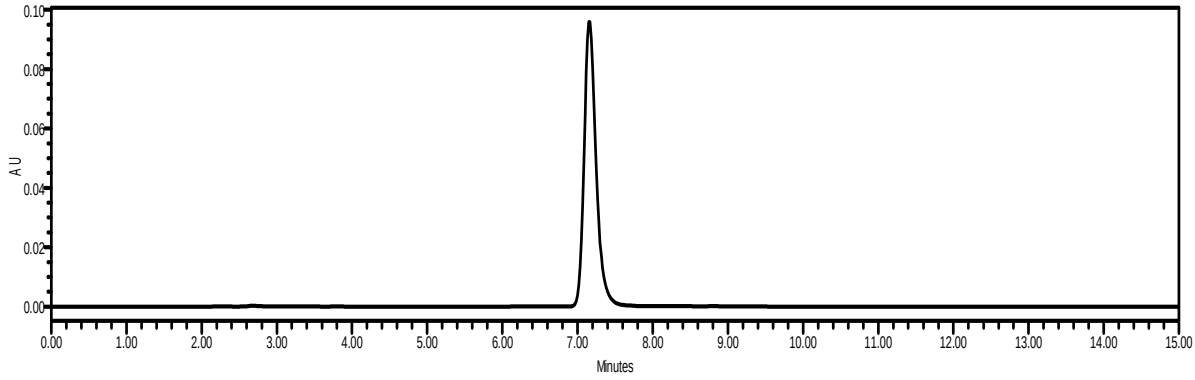

Figure 2: Chromatogram of standard preparation obtained by HPLC [0.1% OPA: Acetonitrile (60: 40v/v)]

Technology transfer to UPLC

As the term technology transfer suggests that here the earlier method (developed and validated in previous chapter) was optimized to achieve the more speed, sensitivity and resolution. The HPLC technology was scale down to attain better chromatographic compatibility in order to using smaller particle size column and UPLC equipment. On the basis of literature survey, previous experience and chromatographic suitability achieved by several investigative efforts suggest 0.1% OPA : ACN (55: 45 v/v). Column selection is the most important part in the method development. Matching the column chemistry with Phenominex C8 which was used earlier for HPLC method, here we used the Acquity UPLC (R) BEH C8 (100mm X 2.1mm X 1.7µm) column. We have screened the standard solution over 190nm to 400nm using the advantage of photo diode array detector. On the basis of peak absorption maxima and peak purity index of analyte, the 280nm was decided as the detection wavelength which also gives the maximum chromatographic compatibility to the method.

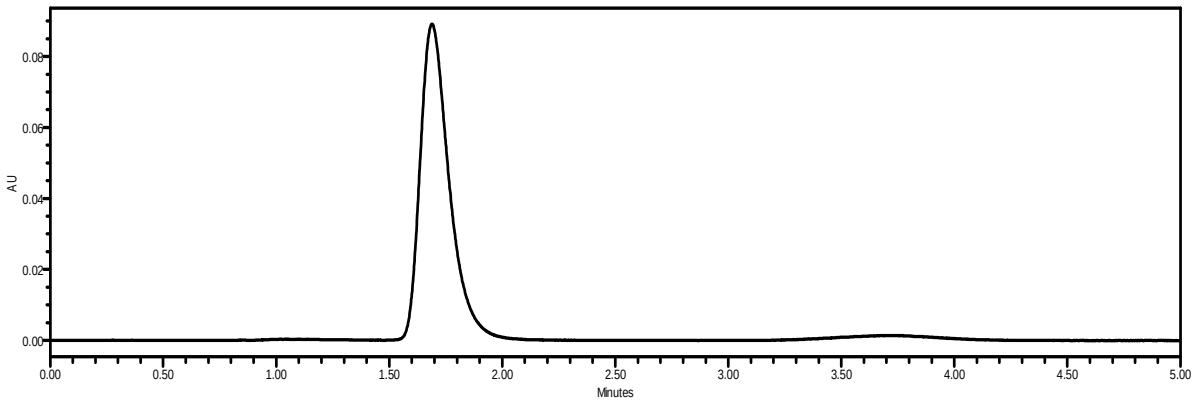

Figure 3: Chromatogram of standard preparation obtained UPLC [0.1% Ortho phosphoric acid: Acetonitrile 55: 45v/v]

Table 1: Method transfer for HPLC to UPLC

Sr. no	Experimental conditions	HPLC Method	UPLC Method
1	Mobile Phase	0.1% OPA : ACN (60: 40 v/v)	0.1 % OPA : ACN (55:45 v/v)
2	Column	Phenomenax C8 100A (250mm X 4.6mm X 5µm)	Acquity UPLC (R) BEH C8 (100mm X 2.1 mm X 1.7 µm)
3	Flow rate	1 ml/min	0.25 ml/min
4	Column oven temperature	30˚C	30˚C
5	UV detection	280 nm	280 nm
6	Run time	15 minutes	5 minutes
7	Injection volume	20 µl	2 µl
8	Stock solution (standard and test)	500 ppm	500 ppm
9	Sample solution (standard and test)	50 ppm	25 ppm
10	Diluent	Water:ACN (50:50 v/v)	Water:ACN (50:50 v/v)

Stress degradation study

Degradation study was performed by transferring powdered tablets, equivalent to 50mg Rivaroxaban into 250ml round bottom flask. Then prepared samples were employed for acidic, alkaline, oxidant media and also for thermal and photolytic conditions. After the degradation treatments were completed, the stress content solutions were allowed to equilibrate to room temperature and diluted with mobile phase to attain 50 µg/ml concentrations of Rivaroxaban. The placebo was also subjected to same stress conditions to identify any response due to the forced degradation conditions. Specific conditions were described as follow.

1. Acidic condition

Acidic degradation study was performed by heating the drug content in 20ml 0.5 N HCl at 80ºC for 45 minutes. After heating equilibrate the solution at room temperature and the mixture was neutralized with 0.5 N NaOH solutions. Further the mixture was diluted with diluents to achieve 50 µg/ml drug concentration. The resulting chromatogram obtained by acidic degradation described the drug was degraded around 13%. The major impurity peak was found at 3.6 min and the degradation products resulting from the stress studies did not interfere with the detection of Rivaroxaban.

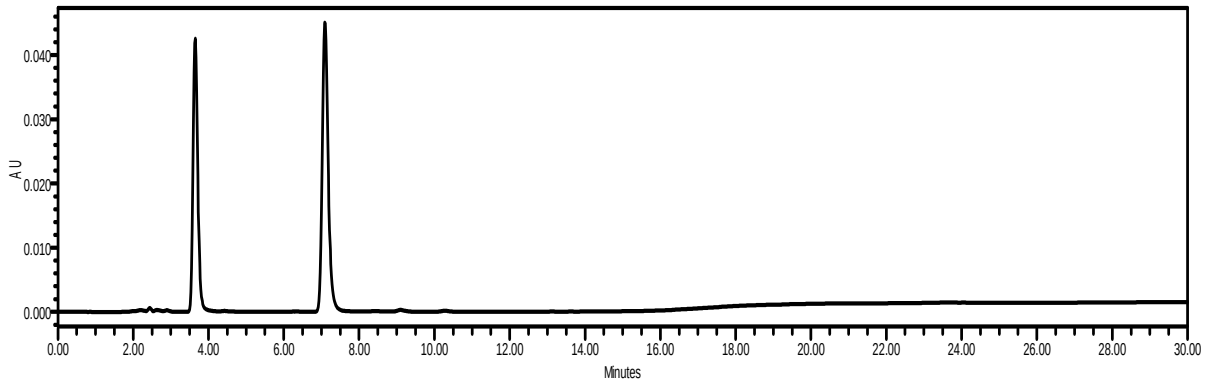

Figure 4: Chromatogram obtained by acidic stress degradation study

2. Alkali condition

Alkaline degradation study was performed by heating the drug content in 20ml 0.1 N NaOH for 60 minutes at 80ºC. After heating equilibrate the solution at room temperature and the mixture was neutralized with 0.1 N HCl solutions. Further the mixture was diluted with diluents to achieve 50 µg/ml drug concentration. The resulting chromatogram obtained by alkali degradation described the major degradation was found in alkali condition and the drug was degraded up to 50%. The major impurity peak was found at 2.5 min and the degradation products resulting from the stress studies did not interfere with the detection of Rivaroxaban.

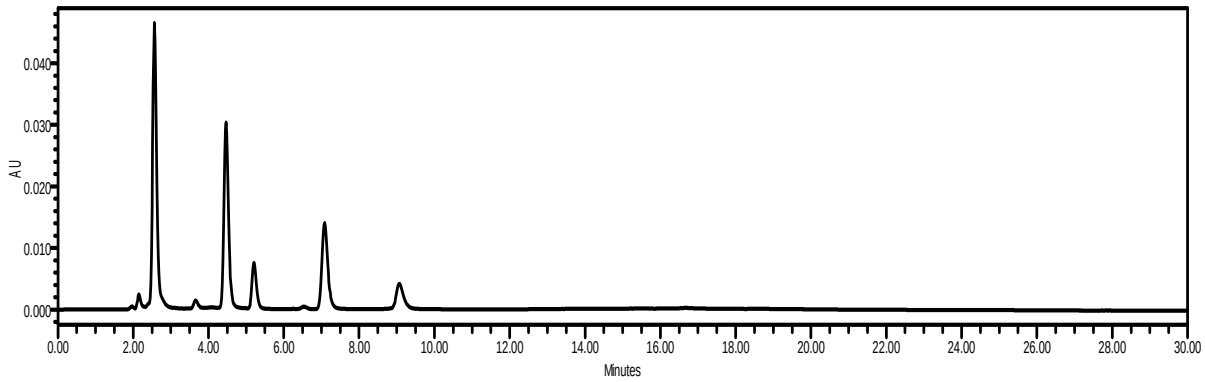

Figure 5: Chromatogram obtained by alkali stress degradation study

3. Oxidative condition

Oxidative degradation study was performed by heating the drug content in 30% v/v H_2O_2 at 60°C for 2 hour. Further the mixture was diluted with diluents to achieve 50µg/ml drug concentration. The resulting chromatogram obtained by oxidative degradation described the drug was degraded around 6% and separate degraded product was not found by thermal degradation.

4. Thermal condition

Thermal degradation was performed by exposing solid drug at 80ºC for 36 hrs in hot air oven. The resulting chromatogram obtained by thermal degradation described the drug was degraded around 3% and separate degraded product was not found by thermal degradation.

5. Photolytic condition

Photolytic degradation study was performed by exposing powder drug directly to sunlight for 72 hrs (Day hrs only). It was found that around 1.5% of the drug was degrading in photolytic condition and separate degraded product was not found by photolytic degradation.

From the above chromatogram it can be concluded that there is no interference of any degradation product to the peak of interest and impurity has been generated by each stress condition.

Method validation parameters

The developed LC method for determination of Rivaroxaban in bulk drug as well as in pharmaceutical dosage form is validated as per ICH guidelines. The method was validated by several validation parameters such as Accuracy, Precision, Linearity, Limit of detection, Limit of quantitation, Robustness and Specificity. Whole validation was performed as per ICH guideline Q2A, Q2B and Q2 (R1) [14][15][16] to ensuring that the present method was suitable for its intended purpose.

Table 2: System suitability study evaluation data

	System suitability test for HPLC			System suitability test for UPLC		
Experiment Name	**Theoretical Plates aNLT 5000**	**Asymmetry bNMT 2**	**c% RSD bNMT 2**	**Theoretical Plates aNLT 5000**	**Asymmetry bNMT 2**	**c% RSD bNMT 2**
Specificity	8302	1.16	0.32	20125	1.31	0.41
Linearity	9411	1.15	0.12	23589	1.36	0.37
LOD and LOQ	7210	1.13	0.33	21780	1.30	0.33
Method Precision	9902	1.19	0.37	25789	1.34	0.23
Int. Precision	9545	1.19	0.17	20589	1.38	0.18
Accuracy	8698	1.15	0.14	23581	1.31	0.29
Robustness	7900	1.12	0.21	23985	1.35	0.25
Solution Stability	8320	1.18	1.23	23598	1.32	0.52

a Not less than
b Not more than
c Relative standard deviation

System suitability study
A system suitability test for the chromatographic system was performed before each validation experiment. Five replicate injections of standard preparation were injected and asymmetry, theoretical plates and % RSD of peak area were determined for same. The theoretical plates should be more than 5000, asymmetry should be less than 2.0 and % RSD should be less than 2.0. As the data suggest the system suitability was within the criteria in each validation experiment. Hence the system was found suitable to perform the validation experiment which confirms the reliability of the data generated during the method validation.

Accuracy study
Accuracy study was assessed by determination of the recovery of the method at three different concentrations (corresponding to 50, 100 and 150% of test solution concentration). Known amounts of Rivaroxaban (25, 50 and150µg/ml) were added to a placebo preparation and the amount of Rivroxaban recovered in the presence of placebo interference. For each concentration, three sets were prepared and injected in duplicate. % Recovery was calculated at each level. The mean recovery of Rivaroxaban was between 99 to 101% and % RSD is less than 2% for all levels. Which is between under the acceptance criteria of ICH guideline Q2(A).[14]

Table 3: Recovery study evaluation data

Recovery level	% Recovery for HPLC			% Recovery for UPLC		
	Amount added (µg/ml)	Amount found (µg/ml)	% Recovery	Amount added (µg/ml)	Amount found (µg/ml)	% Recovery
50%	25.09	25.18	100.38	12.54	12.30	98.16
	25.14	25.20	100.24	12.56	12.40	98.74
	25.10	25.21	100.42	12.57	12.57	99.97
100%	50.07	49.98	99.81	25.02	25.06	100.16
	50.12	49.98	99.72	25.05	25.12	100.29
	50.06	49.64	99.16	25.06	25.14	100.30
150%	75.11	75.00	99.86	37.54	37.46	99.81
	75.13	74.99	99.82	37.58	37.58	100.01
	75.08	75.02	99.92	37.55	37.29	99.31

Precision study
In the precision study, six different preparations of Rivaroxaban were analysed by performing multiple preparations of a single sample on the same and different day. Precision study was established by evaluating method precision and intermediate precision study.

Method precision
Method precision of the analytical method was determined by analyzing six sets of sample solution preparation. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation and % relative standard deviation for the same was calculated.

Intermediate precision
Intermediate precision of the analytical method was determined by performing same experiment as method precision on another day by another analyst using different make of raw materials under same experimental condition. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation and % relative standard deviation also calculated.

Overall assay value of method precision and intermediate precision was compared and % difference and overall % relative standard deviation was calculated. The relative standard deviation obtained from the assay value of Rivaroxaban using HPLC and UPLC have not more than 2%.

Linearity study
The linearity study was determined by analyzing seven solutions in the concentration range between 20-80µg/ml and 10-40µg/ml respectively for HPLC and UPLC. The plot of peak areas vs. concentration data were evaluated by linear regression analysis. The response of the drug was found to be linear in the investigation and the correlation coefficient of the linearity study was 0.999 for both the instruments with linear regression equation y = 21577X-42557 and y = 30742X-6515.2 respectively for HPLC and UPLC. Where y is the peak area in absorbance units; x is the concentration in µg/ml. which proves the method is highly linear over the working range between 20-80µg/ml

and 10-40µg/ml.

Table 4: Method and Intermediate precision comparison data

Sr. no.	Precision study for HPLC		Precision study for UPLC	
	Method precision	Intermediate precision	Method precision	Intermediate precision
	% Assay	% Assay	% Assay	% Assay
1	99.74	99.91	99.94	99.89
2	99.79	99.94	100.04	100.04
3	99.92	100.19	100.36	99.96
4	99.95	99.91	100.05	100.04
5	99.94	99.93	100.32	99.88
6	100.09	100.12	100.28	99.95
Mean	99.92	100	100.17	99.96
Stdev.	0.16	0.12	0.18	0.07
% RSD	0.16	0.12	0.18	0.07

Limit of detection and Limit of quantification study

LOD is the lowest amount of the drug content which can be detected by the proposed method while LOQ is the lowest amount which can be quantified by the method. The guideline suggest minimum signal to noise ratio (S/N) more than 3.3 for LOD and more than 10 for LOQ. The LOD and LOQ concentrations were found at 0.08µg/ml and 0.175µg/ml for HPLC and 0.075µg/ml and 0.150µg/ml for UPLC. It has been established by evaluating the minimum level at which the analyte could be readily detected and quantified accurately. All the results of LOD and LOQ data were within the acceptance criteria. The equations for LOD and LOQ were described below.

LOD = 3.3(SD/S) LOQ = 10(SD/S)
LOD = 0.08 µg/ml (HPLC) LOQ = 0.175 µg/ml (HPLC)
LOD = 0.075 µg/ml (UPLC) LOQ = 0.150 µg/ml (UPLC)

Where, SD = Standard deviation of the response
S = Slope of the calibration curve

Specificity study

The specificity of the method was evaluated by checking for interference with drug from placebo components. Further the specificity of the method toward the drug was established by means of the interference of the degradation products against drug during the forced degradation study. There was no interference of any peak of degradation product with drug peak.

Robustness study

The robustness of the method was evaluated by assaying test solutions after slight but deliberate changes in the analytical conditions flow rate (± 0.1 ml/min), the proportions of OPA: Acetonitrile (± 2 ml) and changing the column temperature (±5°C). For each different analytical condition the standard solution and test solution were prepared separately. The results were observed in terms of assay value and chromatographic compatibility (System Suitability Test), the result obtained from assay of the test solution was not affected by varying the conditions and was in accordance with the true value. System suitability data were also found to be satisfactory during variation of the analytical conditions. The analytical method therefore remained unaffected by slight but deliberate changes in the analytical conditions.

Table 5: Robustness study evaluation data

Robustness Data for HPLC					Robustness Data for UPLC				
Conditions		% Assay	Theoretical plates	Asymmetry	Conditions		% Assay	Theoretical plates	Asymmetry
Mobil phase (OPA: ACN)	62:38	99.66	8950	1.13	Mobil phase (OPA: ACN)	53:47	100.04	20145	1.30
	58:42	99.58	6158	1.14		57:43	100.07	19856	1.35
Flow rate ml/min	0.9	100.19	7275	1.17	Flow rate ml/min	0.24	99.91	25541	1.37
	1.1	99.98	7359	1.13		0.26	99.86	23485	1.35
Column temp ºC	25	100.53	9520	1.15	Column temp ºC	25	100.14	22478	1.34
	35	100.15	8358	1.12		35	100.32	20789	1.37

Table 6: Comparison of HPLC and UPLC method by validation results

Sr. no.	Validation parameters	HPLC method	UPLC method
1	Accuracy (% Recovery)	99.16% – 100.42%	98.31% – 100.30%
2	Linearity (Concentration range)	20 – 80 µg/ml	10-40 µg/ml
3	Regression equation	y = 21577 x - 42557	y = 30742 x - 6515.2
4	Co-relation coefficient (R2)	0.9998	0.9997
5	Intermediate precision (% RSD)	0.12	0.07
6	Method precision (% RSD)	0.16	0.18
7	Robustness (% RSD)	0.21	0.25
8	Specificity (% RSD)	0.32	0.41
9	Limit of detection (µg/ml)	0.08 µg/ml	0.075 µg/ml
10	Limit of quantitation (µg/ml)	0.175µg/ml	0.150 µg/ml
11	Total analysis time (Minute)	15 minute	5 minute

APPLICATION OF CURRENT METHOD:

1. This method can be used for the quantitation of Rivaroxaban in bulk drug as well as in pharmaceutical dosage form in routine or as a special test.
2. This method has also application over the chromatographic purity of Rivaroxaban.
3. By optimizing certain chromatographic parameter it can be also used for the reaction monitoring of preparation of Rivaroxaban and its related compound.
4. Since the suggested method is based on fastest liquid chromatographic technology, it is useful for the high throughput screening of Rivaroxaban in bulk drug as well as in pharmaceutical combination dosage form.

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CONCLUSION

By use of UPLC we can increase resolution, speed and sensitivity in liquid chromatography. The main advantage of UPLC is reduction of analysis time which also helps to reduce solvent consumption as compare to others. Therefore due to UPLC new chemistry and instrumentation technology, we can provide more information per unit of work. The observation and results obtained from each validation experiment lies well inside the acceptance criteria of ICH guideline. Since, all the results are with-in the limit. So the developed analytical method is considered as validated and suitable for possible use.

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