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Development of a validated RP-HPLC method for rivaroxaban quantification in pharmaceutical formulation and human blood plasma

Muhammad Jahangir*, Aymen Awan and Arooj Azhar
Department of Chemistry, Government College University, Lahore.

Abstract: Rivaroxaban is an anticoagulant used to prevent thromboembolism after hip or knee joint replacement surgery. The purpose of study was the development of an efficient, simple and economic reverse phase HPLC-PDA method for Rivaroxaban determination in pharmaceutical dosage forms and blood plasma of human beings. The separation was carried out at room temperature by using Thermo Scientific ODS Hypersil C_{18} (250×4.6mm, 5µm) and mobile phase 70:30 (%v/v) mixture of ACN / H_2O, 1.2 ml/min flow rate, detection on 253nm wavelength by PDA detector with run time of about 7 mins. The retention time observed was about 3 mins. The validation was performed on the proposed method in accordance with ICH guidelines and found that the method is linear within the range of 100-400µg/mL with the correlation coefficient 0.9996. The method was also precise, accurate, robust and rugged, and showed specificity in all applied stress conditions i.e. photolytic (200-800nm, 3h) thermal (70°C, 2h), oxidative (3% H_2O_2, 70°C, 1h), acidic (0.1 N, 70°C, 1h), and basic (0.1 N, 60°C, 1h). This method was applied successfully for the Rivaroxaban quantitative determination in pharmaceutical dosage and human blood plasma.

Keywords: Rivaroxaban, RP-HPLC, Stability Indicating, Solid dosage form, Quantitative determination

INTRODUCTION

Rivaroxaban is an anticoagulant medication, which directly inhibit factor Xa to avoid blood clotting and approved currently in patients with total replacement surgery of knee and hip joint to prevent venous thromboembolism (Duggan *et al*., 2009). It is active when taken orally. It works by binding to the factor Xa catalytic pocket directly thus blocking the amplification of coagulation cascade of both extrinsic and intrinsic pathway to prevent the thrombus formation (Perzborn *et al.*, 2010). Rivaroxaban half-life is observed to be five to nine hours in young patients and eleven to thirteen hours in older patients. Rivaroxaban is prevented for use in patients with severe renal failure and for those patients who are under 18 years old.

Its chemical name is (S)-5-Chloro-N-((2-oxo-3-(4-(3-oxomorpholino) phenyl) oxazolidin-5-yl) methyl) thiophene-2-carboxamide (PubChem, 2021) with molecular formula $C_{19}H_{18}ClN_3O_5S$ and molar mass 435.88 g·mol^{-1} and the chemical structure is shown in fig. 1. Its physical appearance is odorless white to yellowish non-hygroscopic powder.

By surveying the pharmacopeias, it was revealed that there is no available official monograph for the Rivaroxaban. So, a preliminary literature survey (Mehta and Maheshwari, 2018) to observe previous published methods was made which showed that there are some methods available for the Rivaroxaban determination through UV Spectrophotometry, RP-HPLC, UPLC, HPLC-MS, UPLC-MS, HP-TLC and TLC densitometry. Hence, HPLC is the most common technique used in pharmaceutical industries and other medical labs due to its accuracy and speed of analysis. The reported HPLC methods suffered from long retention time and some methods were using costly solvent system and diluents other than M.P which required long analysis time and require an expertise operational personal.

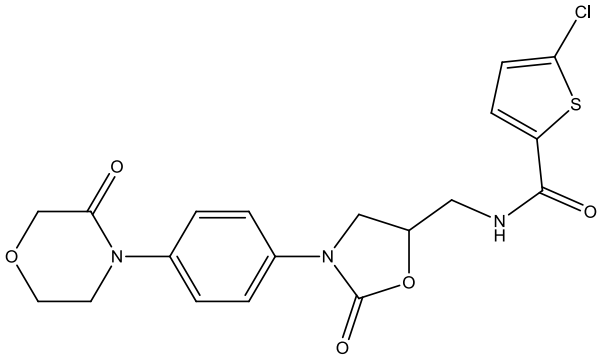

Fig. 1: Chemical Structure of Rivaroxaban

The aim of current work was to develop a validated method for Rivaroxaban quantitative analysis which is accurate, economical, simple, rapid and reliable. The parameters for the validation according to the ICH guidelines (ICH, 2005), i.e. accuracy, linearity, precision, specificity, sensitivity, ruggedness and robustness were studied.

**Corresponding author:* e-mail: mjahangir.gcu@gmail.com

MATERIALS AND METHODS

Experimental

Standard sample of Rivaroxaban ($C_{19}H_{18}ClN_3O_5S$, purity = 99.7%) was gifted from Global Pharmaceuticals pvt. Ltd. The solvents used in this analysis were analytical grade solvents i.e., acetonitrile used in the analysis was of HPLC-Isocratic grade by Panreac (Barcelona, Espana). The water used was of HPLC grade. Tablets of Rivaroxaban were by PharmEvo and purchased from local pharmacy. For method development, an HPLC system (Shimadzu) consisting of photodiode array (PDA) detector was used. Other instruments include electronic balance for sample weighing (Shimadzu - AY220), sonicator (Elmasonic E_{30H}) for sonication of samples, incubator for dry heat (IRMECO GmbH Incubator Model B-35), vortex mixer (SCILOGEX; Model: MX-S) and centrifuge (Digisystem laboratory instruments Inc.). The mobile phase was prepared by mixing HPLC grade acetonitrile and deionized water in the ratio of 70:30% (v/v) and filtered through 0.45µm vacuum filtration assembly and degassed by sonication. Sample dilutions were prepared using the same mobile phase.

Preparation of stock and sample solutions

Prepared the stock standard solution by transferring 10mg accurately weighted Rivaroxaban into 10mL volumetric flask. About 7mL of M.P was added and the solution was allowed to sonicate at room temperature for 20 minutes until clear solution obtained and made the volume up to mark to make the concentration equals to 1mg/mL and filtered through 0.45µm nylon-membrane syringe filter. Then working solutions equivalent the concentration range 100-400µg/mL were prepared by further stock dilutions with M.P and sonicating the mixture for further 10 mins.

Method development

Several trials were made for the Rivaroxaban determination. The resolution of peaks and tailing factor were observed by adding an accurately measured known quantity of the standard drug in each trial. Mobile phase selection was finalized by composing different proportions of acetonitrile and water and obtained enhanced peak symmetry with 70:30% (v/v) of acetonitrile and water respectively. The mobile phase was pumped into the system with 1.2 mL/min flow rate with run time 7 mins. The volume of sample taken by injector was 20µl, which then pass through ODS column. The eluents then monitored by preinstalled PDA detector at optimized wavelength (253nm). The whole procedure takes place at the ambient temperature.

Method validation

After the establishment of the conditions for chromatography the validation was performed following the guidelines prescribed by ICH. To check the linearity of method over 100-400µg/mL concentrations different linear dilution (100, 150, 200, 250, 300, 350 and 400µg/mL) were prepared in triplicate to construct a calibration curve (area vs. conc) and calculated the regression equation, coefficient, slope and intercept. The accuracy was calculated in terms of % recovery of standard from placebo by the comparison of calculated and true value. The recovery was done by spiking of the Rivaroxaban in the known amount of placebo at three levels of concentrations which are 80%, 100% and 120%. The whole procedure is also known as standard addition method. The tablet excipients were mixed in approximate quantities for the placebo preparation i.e., microcrystalline cellulose (35%), lactose monohydrate (35%), mannitol (20%), croscarmellose sodium (2%), Hypromellose (2.5%), sodium lauryl sulphate-SLS (1.5%), magnesium stearate (1%), talc (2%), and titanium oxide (1%). (European Commission, 2008) (European Medicines Agency, 2008). An analytical procedure is said to be precise if it represents the closeness of agreement during multiple sampling by a measurement series of the same sample under all the provided analytical condition similar. It was performed in two categories. To study Intraday precision or repeatability, injected the six replicates of 10µg/mL concentration one after the other by same analyst, under same operating conditions over short time period and the results were evaluated by their %RSD. Inter-day precision is also known as Ruggedness (USP, 2020) and intermediate precision. It is the test results degree of precision obtained by the same samples analysis under various of test conditions and performed by variation of days and analysts i.e., by two analysts and three replicates per analyst per day. Triplicates of three standard concentrations were injected per day equally by the two analysts. The %RSD was calculated to express the results.

Specificity test is performed to check the capacity of an analytical method for the differentiation and quantification of analyte in the presence of complex mixtures like degradants, impurities and matrix. (Ravisankar *et al.,* 2013). The specificity test to validate the proposed analytical procedure was performed by stress degradation studies. A Rivaroxaban stock solution of 1mg/mL was prepared in mobile phase. Then 1mL of stock was transferred in three 10 mL volumetric flasks. Then in one flask 2 mL of 0.1N HCl was added for acid degradation studies. In other flask 2 mL of 0.1 N NaOH was added for base degradation studies. And in the third flask 2 mL of 3% H_2O_2 was added for oxidative degradation. These three flasks were then sonicated for 20 mins. After sonication these mixtures were placed in dry oven at 70°C for 1 hour. Then these solutions were allowed to stable at room temperature for 15 mins. After stabilization the acidic and basic mixtures were neutralized and then all mixtures were filled up to mark with the help of diluent. For thermal degradation studies the 25mg Rivaroxaban was transferred to a washed and dried petri dish and then sample was kept in dry air oven

at 70°C for 2 hours. Then transferred in 25mL volumetric flask and filled with M.P to the mark. Then these solutions were sonicated until all the content dissolved. After that 1mL of this stock was transferred to 10mL volumetric flask filled with M.P. In photolytic degradation the 25mg Rivaroxaban samples were transferred as thin layer in petri dishes, then irradiated with UV light and sunlight separately for 3 hours. Then these samples were transferred to 25mL volumetric flasks and filled with M.P. Then these solutions were sonicated until all the content dissolved.

After that 1mL of each stock was transferred to 10mL volumetric flasks and diluted with M.P. Three replicated of all these final solutions were analyzed by the system and from the results obtained visualized the changes in peaks and calculated the % degradation and % recovered by comparing with true values.

The robustness of proposed method was investigated by varying chromatographic conditions i.e., wavelength (±2nm). Triplicate of this variation was analyzed by the system and the results were expressed as % RSD. The sensitivity was estimated by the limit of detection (LOD) and limit of quantification (LOQ) with the help of following formulas;

$$LOD = 3.3\frac{\sigma}{S} \quad (1)$$

$$LOQ = 10\frac{\sigma}{S} \quad (2)$$

where sigma (σ) is y-intercept standard deviation and S is calibration curve slope.

Solution preparation of pharmaceutical formulations

For the analysis of pharmaceutical formulation, six Rivaroxaban containing tablets were weighted. The average weight of these tablets was calculated. Then these tablets were grinded by mortar and pestle and transferred the quantity of grounded tablet which is equivalent to the 10mg of std drug into 10mL volumetric flask. Mobile phase was added and the sample was sonicated and filtered and then 300µg/mL concentration was prepared from this sample stock and injected to the system.

wt of 6 tablets = 615.5mg

Avg wt = 615.5/6

Avg wt of each tablet = 102.58mg ≈ 103mg

So, 103mg of rivaroxaban tablets contains 10mg Rivaroxaban

wt to be taken =103mg in 10mL diluent to make volume 1mg/mL

Solution preparation of plasma samples

Fresh human blood was collected into the tubes containing EDTA which then centrifuged at 3000 rpm for about 10 mins. After 10 mins three layers were formed into the tube. The first yellow colored layer of plasma and EDTA below it and other cellular components at the bottom. Carefully separated the plasma with the help of pipette. (Bhavyasri *et al*., 2020)

The sample preparation for this bioanalysis was performed by protein precipitation technique to deproteinate the sample before detection. In this procedure 1mL of plasma was taken in 15mL polypropylene centrifuge tubes (Falcon Tubes), to this 3mL of Rivaroxaban std stoke was added and vortexed for 1 min. In this mixture 1mL of acetonitrile was added and again vortexed for 1 min. Then centrifuged this solution at 1000 rpm for 10 mins. The supernatant was transferred accurately into 10mL volumetric flask and filled volume by mobile phase. Then the required concentration of 300µg/mL was prepared by diluting this stock and injected to the system.

STATISTICAL ANALYSIS

Microsoft Excel is used for analyzing obtained data. Percentage recovery, percentage RSD and line equantion was calculated.

RESULT

Method optimization

The selection of analytical conditions was according to the drug nature. The stationary phase or column selection criteria was based on the no. of theoretical plates, shape of peak, back pressure of column and reproducibility of method. This parameter evaluation resulted the selected the C_{18} column (250×4.6mm, 5µm). Under isocratic conditions mixture of 70:30 (%v/v) ACN: H2O was selected as the M.P. Trials by varying flow rate resulted the 1.2ml/min as optimum flow rate. Then after applying these all optimized conditions the obtained chromatogram is shown in fig. 2. The method validation acceptance criteria proposed by ICH-guidelines is given in table 1.

For the selection of wavelength, the standard sample injection was observed at different wavelengths and Rivaroxaban standard shown maximum absorption at 253nm. The overlay given in fig. 3 shows the peaks obtained at 240nm, 253nm and 270nm.

Before the validation of an analytical method, the parameter of performance (System suitability) was checked. By using current method, the results obtained by six replicates showed %RSD = 1.78%, which revealed the system is precise. And the results are mentioned in table 2.

After the establishment of the conditions for chromatography the validation was performed following the guidelines prescribed by ICH. The parameters were calculated and found the results within the acceptance criteria. Then the validated method was applied to solid pharmaceutical dosage and human blood plasma for Rivaroxaban quantification. All the results obtained by following analysis are given below.

Table 1: ICH acceptance Limits

Method Validation Parameters	Acceptance criteria
Precision, Repeatability	% RSD ≤ 2.0%
Precision, Intermediate	% RSD ≤ 5.0%
Accuracy	Recovery between 95.0% - 105.0%
Linearity	r^2 >0.9995
Retention Time (%RSD)	%RSD ≤ 2%
Theoretical Plates (N)	> 2000
Tailing Factor (T)	USP t_f ≤ 2.0
Peak Area (%RSD)	< 2

Table 2: System Suitability results

PARAMETERS	RESULTS (n=6)
Retention Time	3.04
%RSD for Retention time	0.26%
Column Efficiency	6449
USP Tailing	1.15
%RSD for replicate injections	1.78%

Table 3: Linearity Parameters of proposed HPLC method

PARAMETERS	RESULTS
Correlation Coefficient	0.9996
Y-Intercept	2936400
Slope of Regression Line	51081.1
Line Equation	y = 51081.1 x + 2936400

Table 4: Result of accuracy at three levels of concentration

SAMPLE #	% RECOVERY
80 %	100.1
100 %	102.9
120 %	98.5
AVERAGE RECOVERY	100.5

Table 5: Intra-day Precision of Rivaroxaban (300µg/mL)

SAMPLE #	% LABEL CLAIM	%DIFFERENCE FROM MEAN
1	18191062	-0.41
2	18173824	-0.32
3	18587868	-2.61
4	17936871	0.98
5	18191062	-0.41
6	17613466	2.77
MEAN	18115692.2	
SD	323078.5	
%RSD	1.78	
CONFIDENCE INTERVAL	258511.8905	

Table 6: Inter-day Precision of Rivaroxaban (300µg/mL)

INTERDAY (ANALYST 1) n=9		INTERDAY (ANALYST 2) n=9		ANALYST-TO-ANALYST n= 18	
MEAN AREA	RSD %	MEAN AREA	RSD %	MEAN AREA	RSD %
18103007	1.93	18167475	1.76	18135241	0.25

Table 7: Stress Degradation of Rivaroxaban

Stress condition	Peak Area	% Recovered	%Degradation
Base	8089114	44.02	55.98
Acid	9281724	50.5	49.5
UV	14476955	78.8	21.2
Dry Heat	15379052	83.7	16.3
Peroxide	17019845	92.6	7.4
Sunlight	17115313	93.2	6.8

Table 8: Robustness of proposed method over wavelength variation

No. of Replicate	251	255	253
1	18098275	18174978	18191062
2	18000444	18103784	18173824
3	18601076	18553589	18587868
Mean (n=3)	18233265	18277450	18317584
SD (n=3)	322268	241778	234231
% RSD (n=3)	1.77	1.32	1.28
Mean (n=9)	18276100		
SD (n=9)	42176		
%RSD (n=9)	0.23		

Table 9: Assay of Rivaroxaban in Commercial dosage and human plasma

SAMPLE	%RSD OF AREA	FOUND (µg/ml)	% RECOVERY
TABLET (n=3)	0.67	10.06	100.6
PLASMA (n=3)	0.77	9.86	98.6

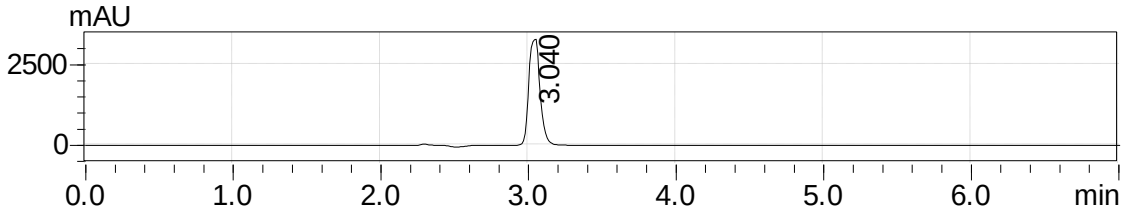

Fig. 2: Chromatogram of Rivaroxaban standard (300µg/mL)

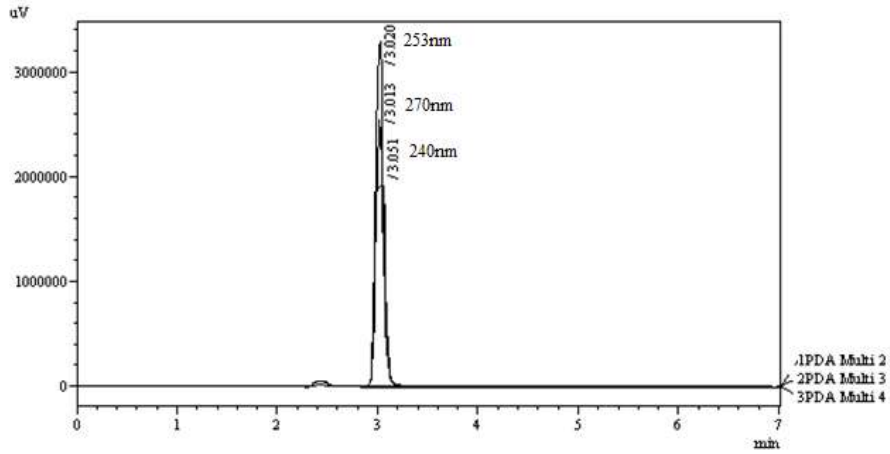

Fig. 3: Overlay at Different wavelengths

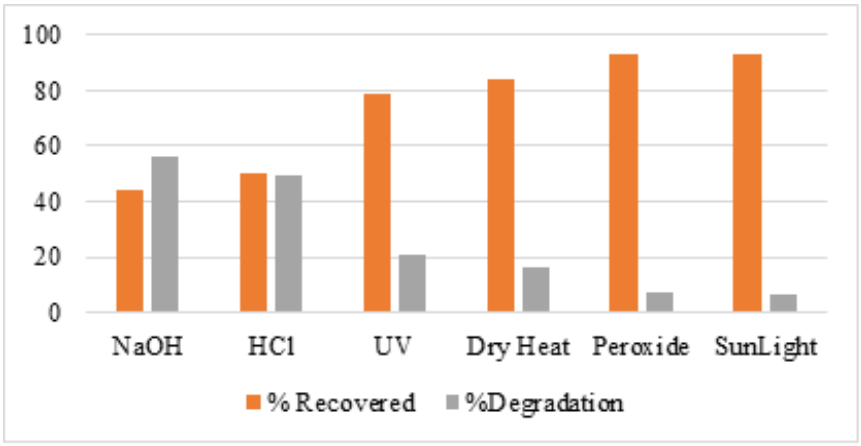

Fig. 4: Degradation effects on Rivaroxaban

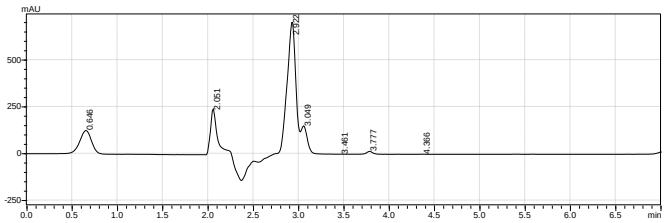

Fig. 5: Alkaline degradation chromatogram of Rivaroxaban

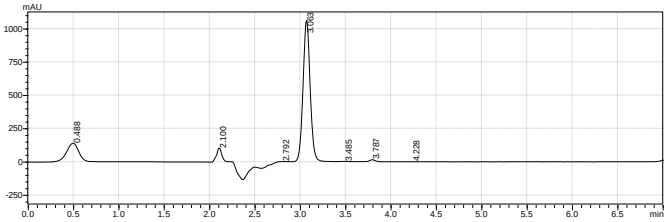

Fig. 6: Acid degradation chromatograms of Rivaroxaban

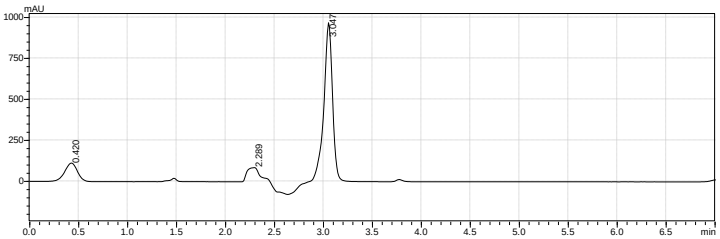

Fig. 7: Thermal degradation chromatograms of Rivaroxaban

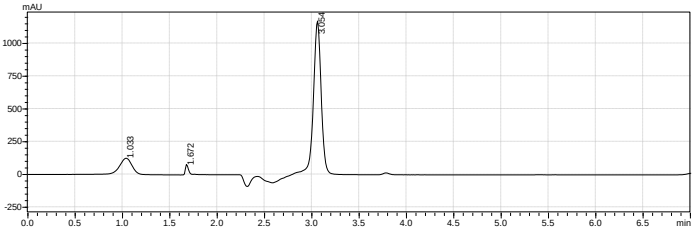

Fig. 8: Oxidative degradation chromatograms of Rivaroxaban

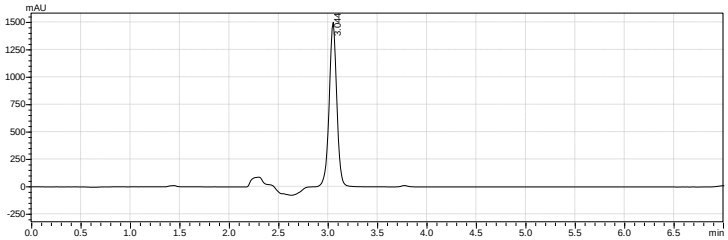

Fig. 9: Sunlight degradation chromatograms of Rivaroxaban

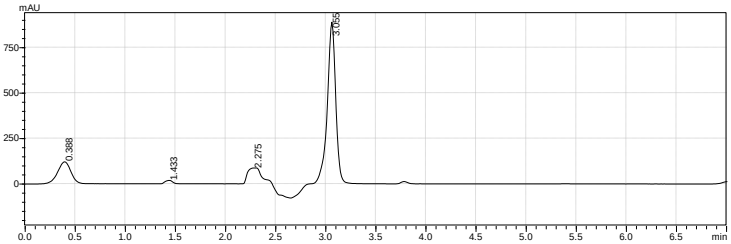

Fig. 10: UV degradation chromatograms of Rivaroxaban

DISCUSSION

Method validation

The method was linear within the range of 100-400µg/mL with the correlation coefficient (R^2) was equals to 0.9996 and the standard regression equation y = 51081.1 x + 2936400 (y = absorbance; x = drug concentration in µg/mL). The data obtained is shown in table 3.

The accuracy of this method was estimated by three levels of concentrations. Three replicates of each were injected and calculated the % recovery, which was found 98.5-102.9 % and confirmed the accuracy of method and shown in table 4.

By using current method, the results obtained by six replicates for intraday precision (repeatability) showed %RSD = 1.78%, which reveal that for this method the system is precise and results are shown in table 5.

The Results obtained for Inter-Day precision are also within acceptance criteria i.e., <5% such as, 1.93% and 1.76%. For analyst-to-analyst variation the percentage RSD was found 0.25%, which is < 2 % shown in table 6 which proved the ruggedness of method.

Specificity or stress degradation was observed by thermal degradation, photolytic degradation, oxidative degradation, acidic degradation and basic degradation at different temperature. The results obtained are shown in table 7 and demonstrated in fig. 4. Considerable degradation was observed by applying all the stress conditions except sunlight degradation. Which means that the drug is stable under sunlight and there is no as such need for specific storage condition to avoid sunlight degradation. The chromatograms obtained after the degradation of Rivaroxaban by applying different stress conditions are given in fig. 5 to 10.

Robustness in procedure was calculated by small change in wavelength i.e., from 251 to 255nm. The results are mentioned in table 8 which shows %RSD < 2% and proved the robustness of method.

LOD and LOQ were calculated on basis of SD of intercept and the slope (S) obtained from calibration curve. The values obtained for LOD=0.22µg/mL and LOQ=0.67µg/mL. These all results obtained points towards the sensitive nature of method.

Analysis of pharmaceutical formulations

The proposed method for RP-HPLC was applied for Rivaroxaban analysis in pharmaceutical dosage form. The results showed recovery about 100.6% and %RSD<2%, which indicated that the method is suitable and highly precise for the determination of Rivaroxaban in solid dosage form. The results are shown in table 9.

Analysis of plasma samples

The proposed method for RP-HPLC was applied for Rivaroxaban analysis in Human Plasma samples separated from blood and spiked with drug. The results showed recovery of 98.6% and %RSD<2%, which indicated that the method is suitable and highly precise for the determination of Rivaroxaban in Human plasma. The results are shown in table 9. (Hadagali, 2015)

CONCLUSION

A reliable, specific, rapid, simple and economical method for RP-HPLC has been developed for the determination of Rivaroxaban. The proposed method was validated as per ICH-guidelines and found precise, linear, accurate, sensitive, robust, rugged for Rivaroxaban detection and quantification. This method can be used with good reproducibility in quality control analysis of Rivaroxaban for solid pharmaceutical dosage form and pharmacokinetics studies and plasma protein binding studies for human plasma samples. And this method is also stability indicating because degradation products did not show that much interference and we can quantify Rivaroxaban in the presence of its degradation products.

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