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Stability Indicating RP-HPLC Method Development and Validation for Simultaneous Estimation of Rivaroxaban and Clopidogrel Bisulphate in Pharmaceutical Dosage Form

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Abstract- A simple, specific, accurate and stability-indicating reversed phase high performance liquid chromatographic method was developed for the simultaneous determination of Rivaroxaban and Clopidogrel, using a C18 (25cm x 0.46 cm) Hypersil BDS column and a mobile phase composed of buffer (pH 4.5): methanol (70:30). The detection was carried out at wavelength 214 nm. The retention times of Rivaroxaban and Clopidogrel were found to be 3.300 min and 4.740min, respectively. Linearity was established for Rivaroxaban and Clopidogrel in the range of 2-6µg/ml and 7.5-22.5µg/ml, respectively. The percentage recoveries of Rivaroxaban and Clopidogrel were found to be 100.09% and 99.79%, respectively. Both the drugs were subjected to acid, alkali, oxidation, thermal and photolytic UV degradation. The degradation study shows that both drugs are susceptible in all parameter. Clopidogrel is more susceptible for photo and thermal degradation.

Keywords: clopidogrel bisulphate, rivaroxaban, rp-hplc, stability indicating method.

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Strictly as per the compliance and regulations of:

Stability Indicating RP-HPLC Method Development and Validation for Simultaneous Estimation of Rivaroxaban and Clopidogrel Bisulphate in Pharmaceutical Dosage Form

N. I. Majan [α], N. I. Patel [σ] & Aejaz Ahmed [ρ]

Abstract- A simple, specific, accurate and stability-indicating reversed phase high performance liquid chromatographic method was developed for the simultaneous determination of Rivaroxaban and Clopidogrel, using a C18 (25cm x 0.46 cm) Hypersil BDS column and a mobile phase composed of buffer (pH 4.5): methanol (70:30). The detection was carried out at wavelength 214 nm. The retention times of Rivaroxaban and Clopidogrel were found to be 3.300 min and 4.740min, respectively. Linearity was established for Rivaroxaban and Clopidogrel in the range of 2-6µg/ml and 7.5-22.5µg/ml, respectively. The percentage recoveries of Rivaroxaban and Clopidogrel were found to be 100.09% and 99.79%, respectively. Both the drugs were subjected to acid, alkali, oxidation, thermal and photolytic UV degradation. The degradation study shows that both drugs are susceptible in all parameter. Clopidogrel is more susceptible for photo and thermal degradation. It shows less degradation in basic environment as compare to all others. Clopidogrel shows average thirty percent degradation in UV light which is highest degradation as compare to other. Rivaroxaban is more susceptible for thermal and oxidative degradation. It shows less degradation in basic environment as compare to all others. Both drug's degradation products were well resolved from the pure drug with significant differences in their retention time values. This method can be successfully employed for simultaneous quantitative analysis of Rivaroxaban and Clopidogrel in bulk drugs and formulations. The proposed method was found to be accurate, reproducible, and consistent. The method was validated in compliance with ICH guidelines.

Keywords: clopidogrel bisulphate, rivaroxaban, rp-hplc, stability indicating method.

I. Introduction

Rivaroxaban ((S)-5-chloro-N-{[2-oxo-3-[4-(3-oxo-morpholin-4-yl) phenyl] oxazolidin-5-yl] methyl} thiophene-2-carboxamide) (fig. 1) is an anticoagulant and the first orally active direct factor Xa inhibitor [1]. Clopidogrel Bisulphate methyl (2S)-2-(2-chlorophenyl)-2-{4H, 5H, 6H, 7H-thieno [3, 2-c] pyridin-5-yl} acetate (fig. 2)is an antiplatelet agent structurally and pharmacologically similar to ticlopidine. It is used to inhibit blood clots in a variety of conditions such as peripheral vascular disease[2].

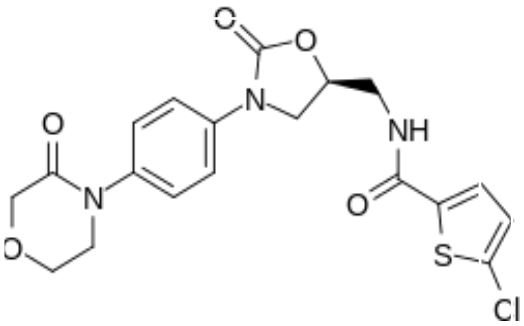

Figure 1: Structure of Rivaroxaban

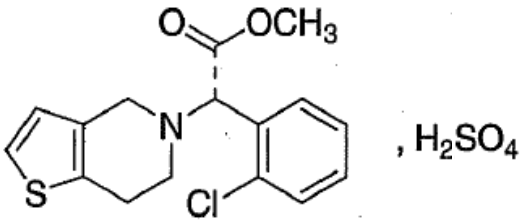

Figure 2: Structure of Clopidogrel Bisulphate

Fixed dose combination (FDC) for the probing drugs is not available commercially, yet this FDC has not been listed in any of the common pharmacopoeia. When go through the literature lead to the occurrence of various singular methods for this drug like, for Rivaroxaban RP-HPLC[3-7], RP-HPLC and UPLC (Forced Degradation study)[8], RP-UPLC (Stability indicating Method)[9], RP-HPLC (Stability indicating Method)[10], RP-HPLC (Stability indicating Dissolution Method)[11], RP-HPLC and TLC[12], HPTLC[13], UV Spectrophotometry[14] and for Clopidogrel Bisulphate Chiral Chromatography[15-16], RP-HPLC[17-20], Stability Indicating RP-

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HPLC[21-22]. In combination of this drug only RP-HPLC method were available in mobile phase and also in plasma and urine [23-24-25]. However no stability indicating RP-HPLC method was set up for this combination.

We are involved newly to conduct investigation relating to stability indicating RP-HPLC method development of Rivaroxaban and Clopidogrel for fill this information gap. It was tried to develop and validate RP-HPLC method with stability indicating properties for this combination (Rivaroxaban and Clopidogrel Bisulphate). We expect the inclusion of this knowledge in the current literature will be benefit for the pharmaceutical industries for support the quality of their products holding these active ingredients and also the execution agencies in broad to evaluate the quality of the marketed preparations.

II. Experimental

Chemical and reagent: Pure Clopidogrel (CLP) and Rivaroxaban (RIV) were obtained as a gift sample from Remus Remedies. As Sample Clopidogrel 75mg and Rivaroxaban 20mg Synthetic Mixture is used. HPLC grade Methanol, Potassium dihydrogen, Ammonium Acetate, HPLC Grade High purity deionized water were obtained from Merck specialties Pvt Ltd., Mumbai.

Instrumentation and materials: The liquidchromatographic system was of Thermo separation Product TSP UV 2000, which consisted a gradient pump, variable wavelength, programmable UV/Vis detector, a manual injection facility with 20 µl fixed loop. The chromatographic analysis was performed using spinchrom software on a C18 Hypersil BDS column (25cm x 0.46 cm with 5µm particle size). In addition, an electronic balance (CP-124S Sartorius, Germany), a pH meter (Electroquip's Digital pH meter)were used in this study.

Chromatographic conditions: The elution of CLP and RIV was obtained by running HPLC in isocratic mode using Phosphate Buffer (pH 4.5): Methanol (70:30).Flow rate was maintained at 1.0 ml/min with run time of 6 min. The retention time for RIV was obtained 3.300 minand CLP was obtained 4.740min. Detection was performed at 214 nm. Mobile phase was previously filtered through Whatman filter paper no 41.

a) Preparation of standard solutions

CLP standard stock solution (150µg/ml): A 15 mg of CLP was weighed and transferred to a 100 ml volumetric flask. Volume was made up to the mark with mobile phase.

RIVstandard stock solution(40µg/ml): A 40 mg of RIV was weighed and transferred to a 100 ml volumetric flask. Volume was made up to the mark with mobile phase, taken 10ml from this solution and transferred to 100ml volumetric flask and volume was made up with methanol.

Preparation of standard solution of binary mixtures of CLP (15 µg/ml) and RIV(4 µg/ml): Take 1 ml from the CLP stock solution and 1ml from RIV stock solution and transferred to 10 ml volumetric flask and volume made up to the mark by mobile phase which was used in particular trials.

b) Preparation of formulation solution

Sample Stock Solution (CLP 150 µg/ml, RIV 40 µg/ml): Take Tablet powder equivalent to 15 mg of CLP and 4 mg of RIV was transferred to a 100 ml volumetric flask, Add 60 ml Mobile phase and Shake for 15 min and make up volume with Mobile phase. The solution was filtered through Whatman filter paper no. 42.

Working Sample Preparation (CLP 15 µg/ml, and RIV 4 µg/ml): Take 1 ml from standard stock solution and transferred to 10ml volumetric flask and made up volume up to the mark with the mobile phase.

c) Forced degradation study [26]

Acid degradation: Acid decomposition studies were performed by transferring 1 ml of stock solution in to 10 ml of volumetric flask. 2ml of 0.1 N Hydrochloride solutions was added and mixed well and put for 5 hrs. at room temperature. Then the volume was adjusted with diluent to get 15µg/ml for CLP and 4µg/ml for RIV.

Base degradation: Basic decomposition studies were performed by transferring 1ml of stock solution in to 10ml of volumetric flask. 2 ml of 0.1 N NaOH solutions was added and mixed well and put for 3 hrs at room temperature. Then the volume was adjusted with diluent to get 15µg/ml for CLP and 4µg/ml for RIV.

Oxidative degradation: Oxidative decomposition studies were performed by transferring 1ml of stock solution in to 10 ml of volumetric flask. 2 ml of 3% H_2O_2 solutions was added and mixed well and put for 6 hrs at room temperature. Then the volume was adjusted with diluent to get 15µg/ml for CLP and 4µg/ml for RIV.

Photo degradation: Photo degradation studies were performed by transferring 1ml of stock solution in to 10 ml of volumetric flask. The volumetric flask was kept in UV Chamber for 12 hrs. Then the volume was adjusted with diluent to get 15µg/ml for CLP and 4µg/ml for RIV.

Thermal degradation: Thermal degradation studies were performed by transferring 1ml of stock solution in to 10 ml of volumetric flask. The volumetric flask was stored in oven at 80°C for 5 hrs. Then the volume was adjusted with diluent to get 15µg/ml for CLP and 4µg/ml for RIV.

Method validation: After method development, the method was validated in compliance with ICH guidelines. The method was validated for Accuracy, Precision, Reproducibility, Specificity, Limit of Detection, Limit of Quantitation, Linearity and Range, Ruggedness and Robustness.

III. Result and Discussion

a) Optimization of mobile phase

Trial contains various mobile phase which are considered of Methanol, Water and buffer. Methanol in different proportions and different volumes at different flow rate were tried. On the basis of various trials the mixture of Buffer (pH 4.5): Methanol (70:30), at 1.0 mL/min flow rate, proved to be better than the other mixture in terms of peak shape, theoretical plate and asymmetry.

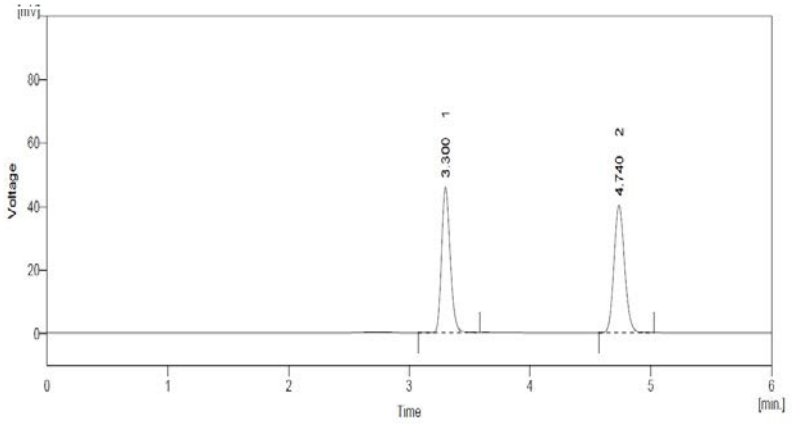

Figure 3: HPLC Chromatogram of RIV and CLP Buffer (pH 4.5): Methanol (70:30)

b) System suitability parameter

System suitability is an integral part of chromatographic method. These tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole. System suitability testing provides assurance that the method will provide accurate and precise data for its intended use. Observed values for system suitability is show in Table 1.

Table 1: Result of system suitability parameters

Parameters	RIV	CLP
Retention Time	3.300	4.740
Theoretical Plates	9427	13320
Asymmetry	1.316	1.160
Resolution	9.593	

c) Validation of RP – HPLC method

i. *Specificity*

The Chromatograms of Clopidogrel and Rivaroxaban standards and Clopidogrel and Rivaroxaban sample show no interference with the Chromatogram of Clopidogrel and Rivaroxaban Blank, so the Developed method is Specific.

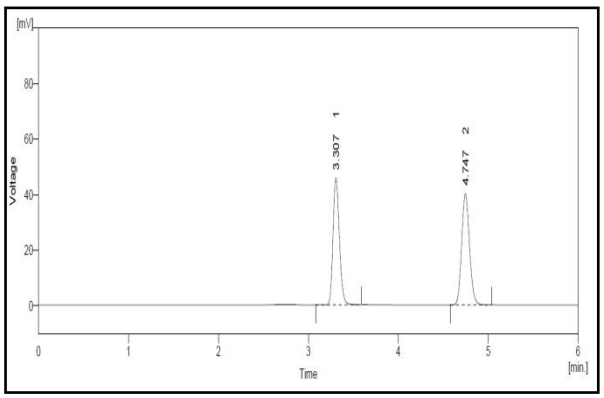

Figure 4: Chromatogram of RIV and CLP standard

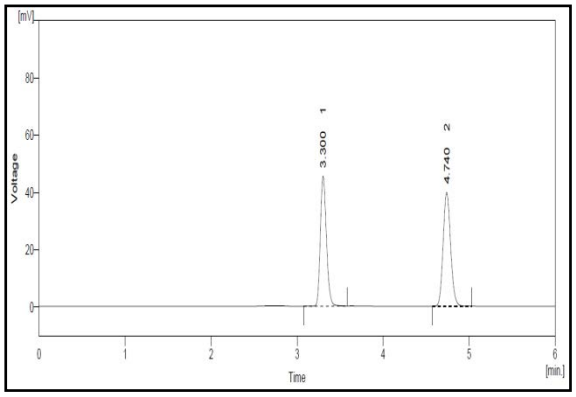

Figure 5: Chromatogram of RIV and CLP sample

d) Linearity

The linearity for RIV and CLP were assessed by analysis of combined standard solution in range of 2-6 µg/ml and 7.5-22.5 µg/ml respectively, Correlation co-efficient for calibration curve RIV and CLP was found tobe0.998 and 0.999 respectively The regression line equation for RIV and CLP are as following:

For RIVy = 55.905x + 0.2138 and for CLP y = 16.145x - 0.4796

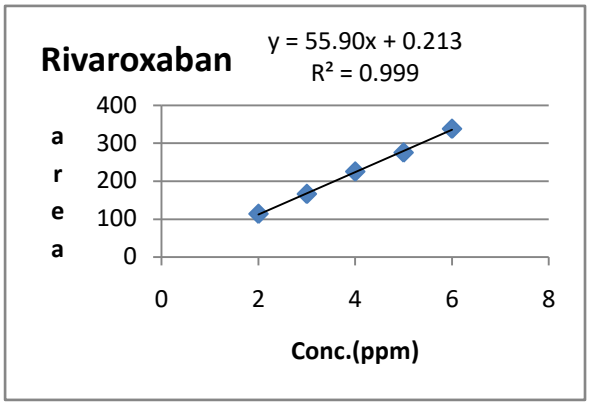

Figure 6: Calibration Curve of RIV

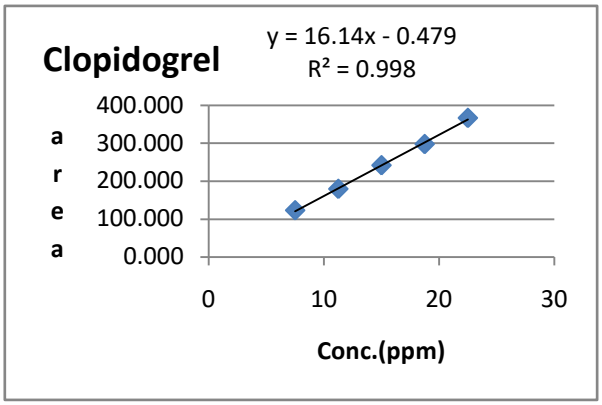

Figure 7: Calibration Curve of CLP

e) Precision

The precision of the method was demonstrated by repeatability study, inter-day precision and intra-day precision. In the repeatability study, six replicates of the same concentration of working standard solutions were prepared and injected and chromatograms were recorded. The results obtained were shown in Table 2. In inter-day precision and intra-day precision, three replicates of three different concentration of working standard solution were prepared and injected and chromatograms were recorded. The results obtained were shown in Table 3.

Table 2: Results of repeatability study

Drugs	Mean Area (n=6)	S.D	%R.S.D
RIV	224.773	1.058	0.471
CLP	242.442	1.451	0.599

n- Number of estimations

Table 3: Results of intra-day and inter-day precision

Parameter	Drug	Amount Taken (µg mL-1)	Mean Area Found (n=3)	S.D	%R.S.D
Intraday precision	RIV	2	113.066	0.556	0.451
		4	225.837	1.696	0.751
		6	335.157	1.21	0.368
	CLP	7.5	122.28	0.754	0.616
		15	243.516	1.319	0.541
		22.5	362.858	1.696	0.467
Interday precision	RIV	2	112.632	0.972	0.863
		4	224.325	1.237	0.552
		6	333.162	3.512	1.054
	CLP	7.5	122.769	0.388	0.316
		15	241.954	1.786	0.738
		22.5	360.46	4.525	1.255

n- Number of estimations

Accuracy

Accuracy of the method was confirmed by recovery study from formulation at three level of standard addition.2 µg/ml drug solution for RIV and 7.5µg/ml drug solution for CLP was taken in three different flask label A, B and C. Spiked 80%, 100%, 120% of standard solution in it and diluted up to 10ml. The area of each solution peak was measured at 214 nm. The amount of RIV and CLP was calculated at each level and % recoveries were computed. The results are shown in table 4. Percentage recovery for Rivaroxaban was 0.718%-1.357 %, while for Clopidogrel it was found to be in range of 0.649 %-1.110 %

Table 4: Results of recovery study

Drug	Conc. Level(%)	Sample amount added(µg/ml) (n=3)	Standard amount added(µg/ml) (n=3)	Mean of amount recovered (µg/ml) (n=3)	% Mean recovery	S.D	% R.S.D
RIV	80	2	1.6	1.601	100.081	1.358	1.357
	100	2	2	1.992	99.594	0.716	0.718
	120	2	2.4	2.415	100.623	0.734	0.729
CLP	80	7.5	6	6.001	100.028	0.649	0.649
	100	7.5	7.5	7.474	99.656	0.948	0.951
	120	7.5	9	8.975	99.728	1.107	1.11

n- Number of estimations

LOD and LOQ

Calibration curve was repeated for five times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows:

LOD = 3.3 * SD/slope of calibration curve

LOQ = 10 * SD/slope of calibration curve

Where, SD = Standard deviation of intercepts

Table 5: Results of LOD and LOQ

LOD		LOQ	
RIV	CLP	RIV	CLP
LOD=3.3x(SD/Slope) = 3.3 x(3.302/55.905) = 0.195 µg/ml	LOD=3.3x(SD/Slope) = 3.3 x(3.849/16.145) = 0.787 µg/ml	LOQ=10x(SD/Slope) = 10 x(3.302/55.905) = 0.591 µg/ml	LOQ=10x(SD/Slope) = 10 x(3.849/16.145) = 2.384 µg/ml

f) Robustness

Following parameters were changed one by one and their effect was observed on system suitability for standard preparation. (a)Flow rate of mobile phase was changed (± 0.2 ml/min) 0.8 ml/min and 1.2 ml/min. (b) Ratio of Mobile phase was changed (±2) Buffer: Methanol (72:28) and Buffer: Methanol (68:32). (c)pH of Buffer was changed (±0.2), pH 4.3 and pH 4.7.The effect of changes was found to be within the acceptance criteria as shown in table 6. The % RSD should be less than 2%.

Table 6: Results of robustness study

Parameter	Variation	RIV			CLP		
		Mean area (n=3)	S.D	%R.S.D	Mean area (n=3)	S.D	%R.S.D
Mobile phase	72:28	229.51	1.036	0.451	246.529	3.309	1.342
	68:32	217.964	0.579	0.266	234.914	0.236	0.101
Flow rate	0.8 ml/min	233.192	3.135	1.344	252.901	1.553	0.614
	1.2ml/min	213.584	0.697	0.326	230.47	0.907	0.394
pH	4.3	233.898	3.193	1.365	240.119	0.636	0.265
	4.7	222.218	1.783	0.802	252.359	1.409	0.558

g) Assay

Triturate 20 tablets, take tablet powder equivalent to 15 mg of CLP and 4 mg of RIV was transferred to a 100ml volumetric flask, Add 60 ml Mobile phase and Shake for 15 min and make up volume with Mobile phase. Take 1 ml from this stock solution and transferred to 10 ml volumetric flask and made up volume up to the mark with the mobile phase. Inject above Solution 20 µl for assay analysis. The solution was filtered through What man filter paper no. 42. The results are given in Table 7.

Table 7: Results of assay study

Tablet		
Label claim	CLP(75mg)	RIV(4mg)
Assay (% of label claim*) Mean ± S. D.	98.895±0.267	98.651±0.363

h) Stability indicating method

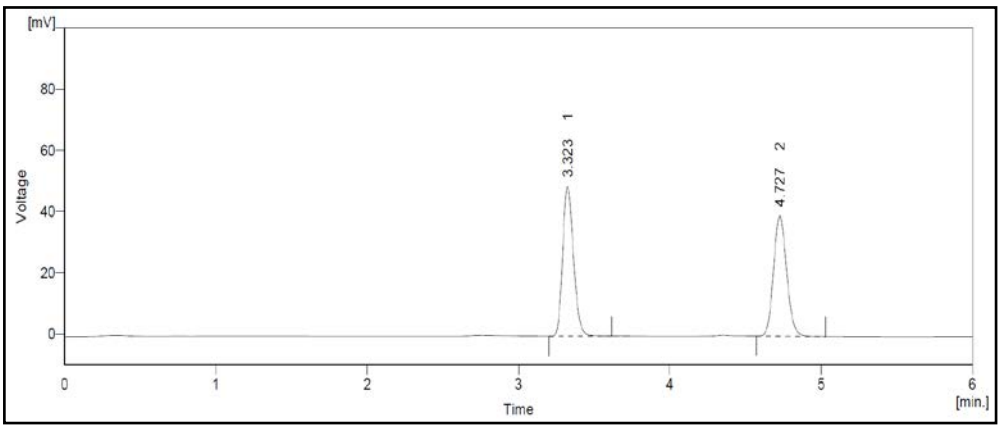

Figure 8: RIV and CLP Standard for stability

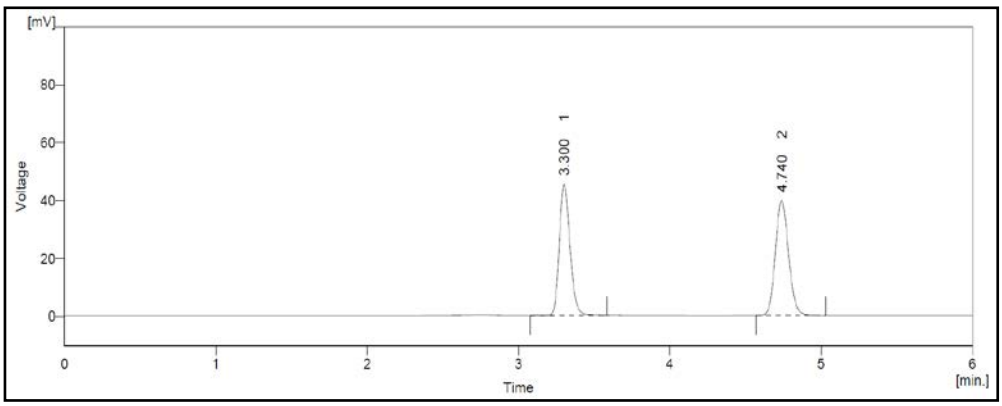

Figure 9: RIV and CLP sample

Table 8: Calculation of CLP and RIV standard for stability

Drugs	Area
Rivaroxaban	242.859
Clopidogrel	237.109

i) Acid degradation

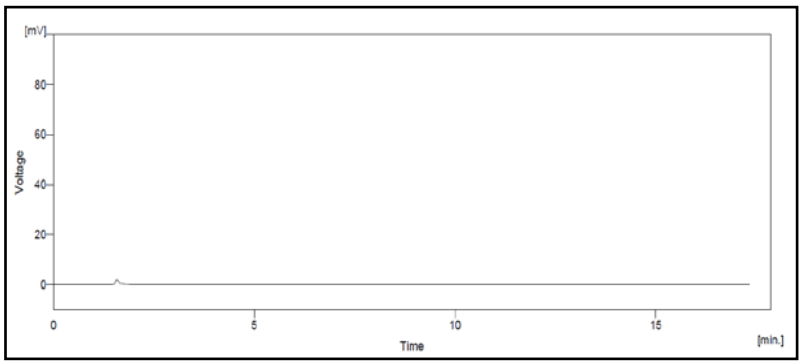

Figure 10: Acid degradation blank

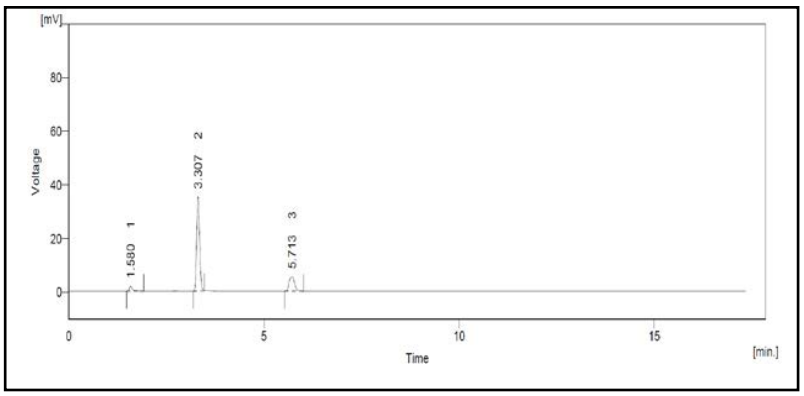

Figure 11: RIV acid degradation standard

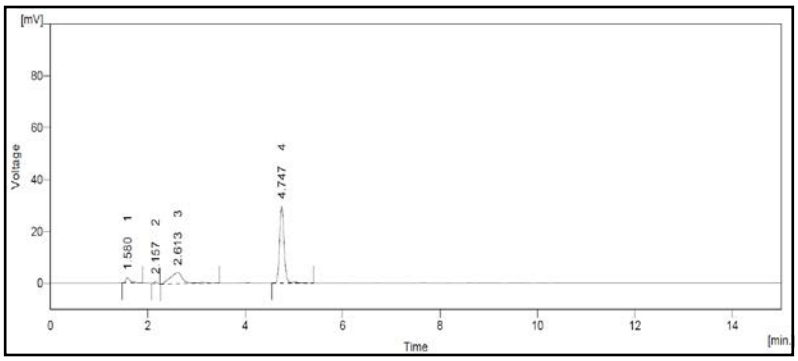

Figure 12: CLP acid degradation standard

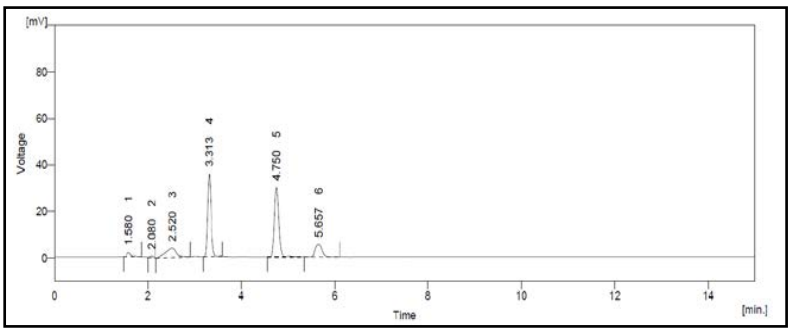

Figure 13: RIV and CLP acid degradation sample

j) Base degradation

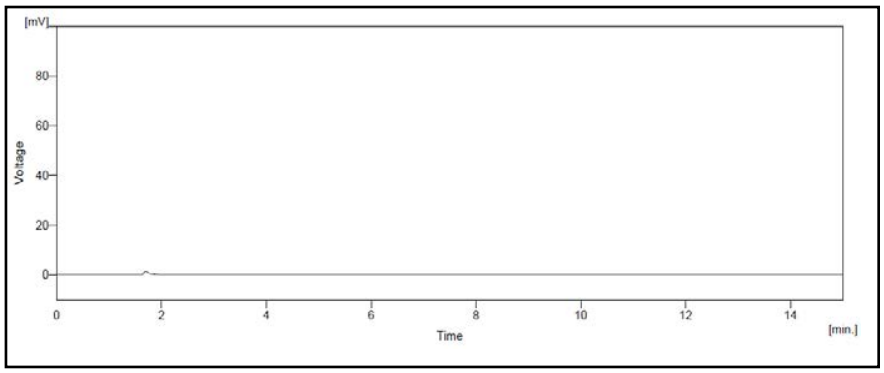

Figure 14: Base degradation blank

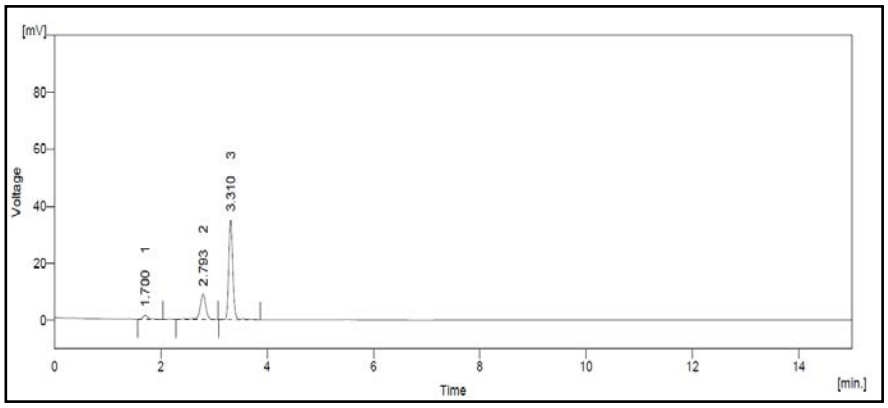

Figure 15: RIV base degradation

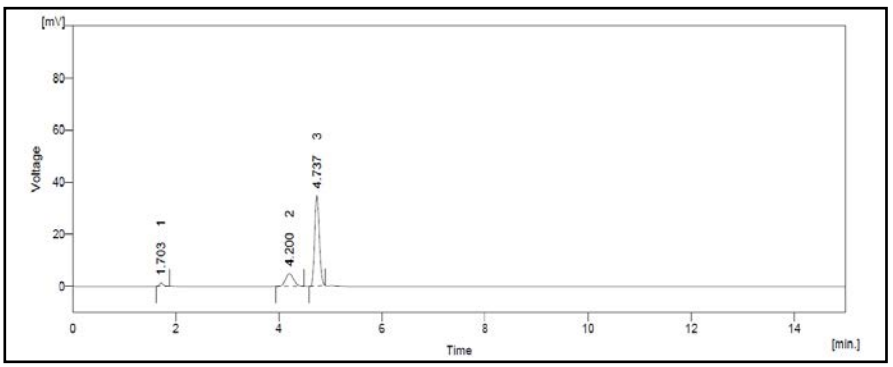

Figure 16: CLP base degradation

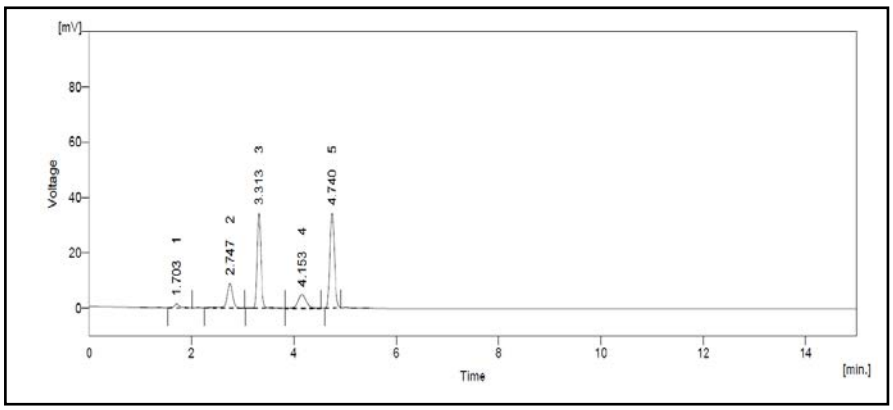

Figure 17: RIV and CLP base degradation sample

k) Oxidation Degradation

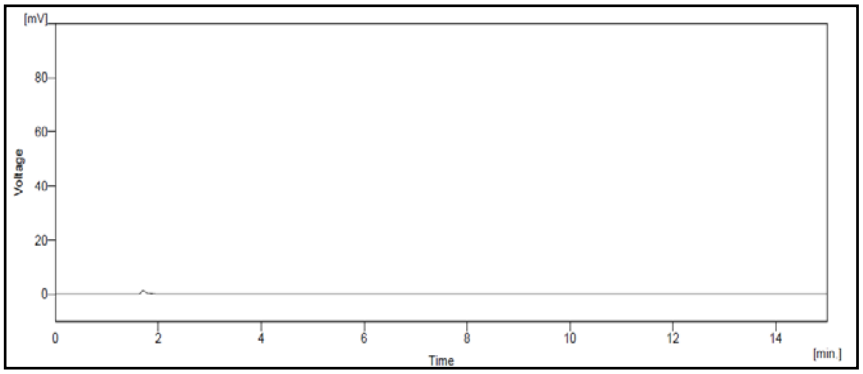

Figure 18: Oxidation degradation blank

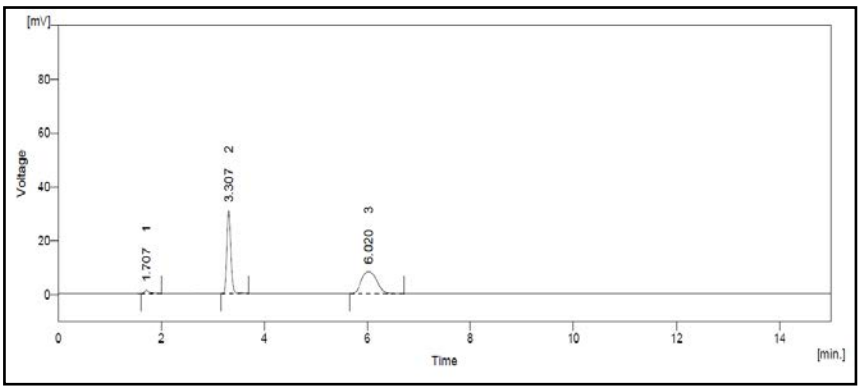

Figure 19: RIV oxidation degradation

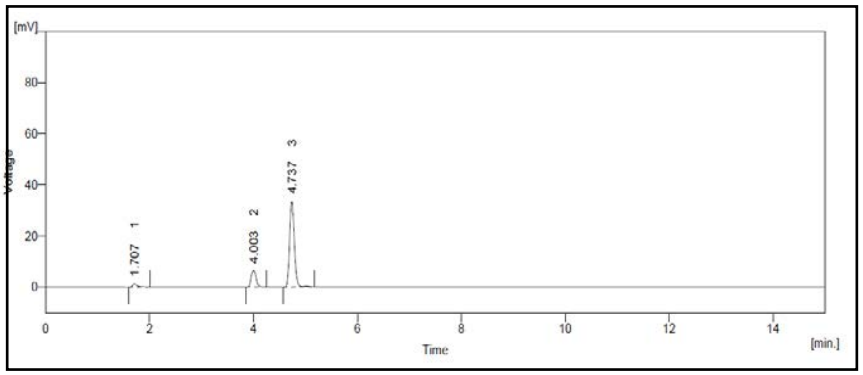

Figure 20: CLP oxidation degradation

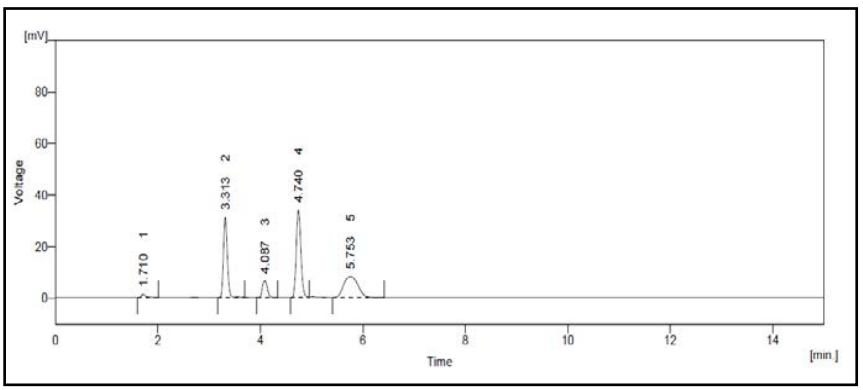

Figure 21: RIV and CLP oxidation degradation sample

I) Photo degradation

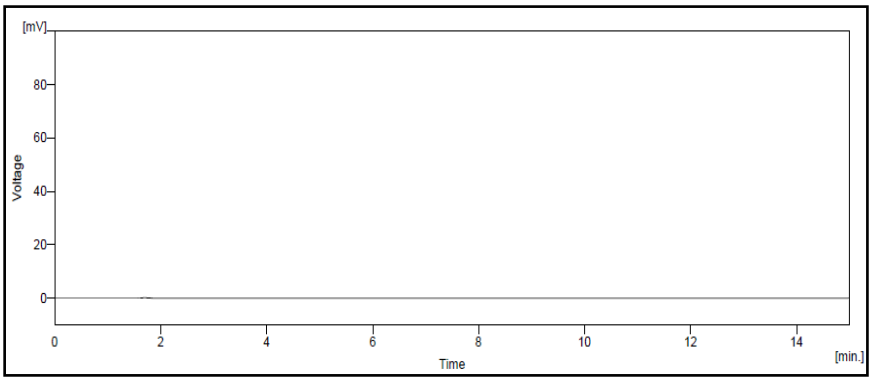

Figure 22: Photo degradation blank

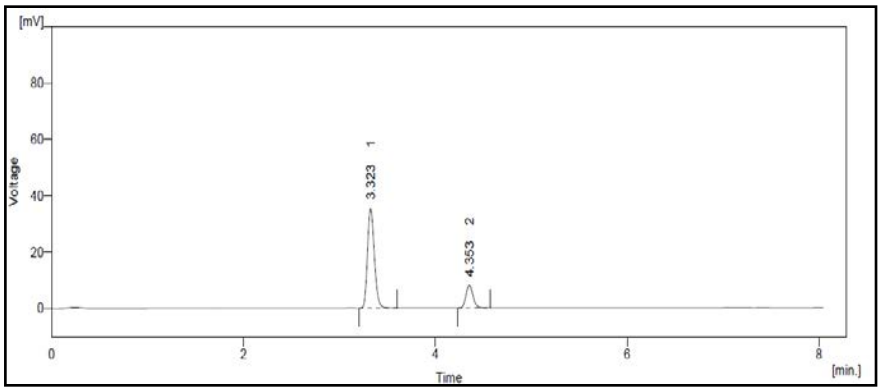

Figure 23: RIV photo degradation

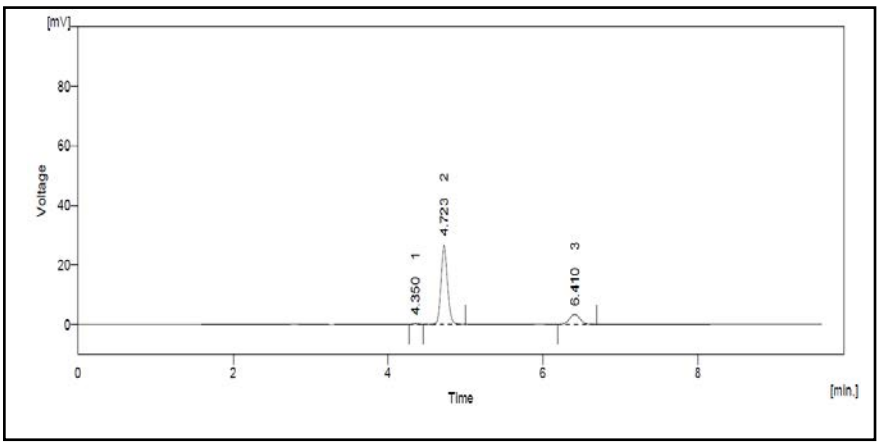

Figure 24: CLP photo degradation

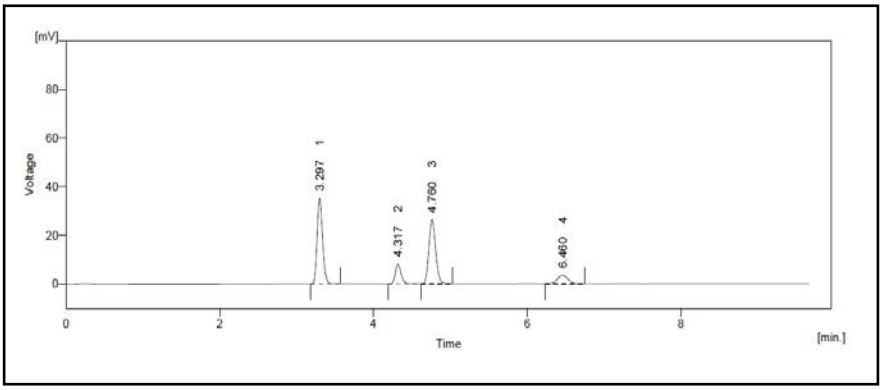

Figure 25: RIV and CLP photo degradation sample

m) Thermal degradation

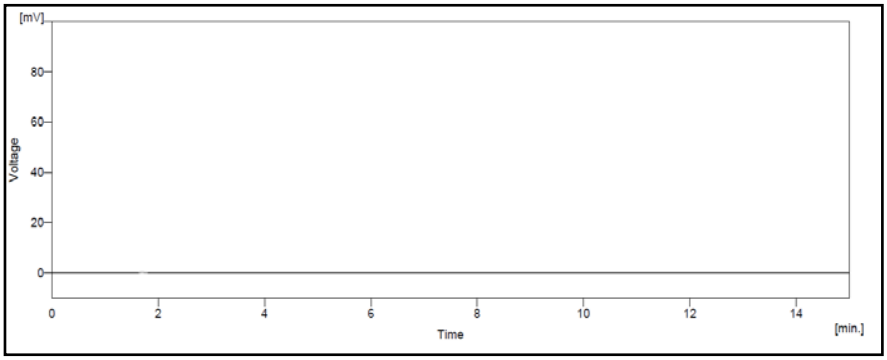

Figure 26: Thermal degradation blank

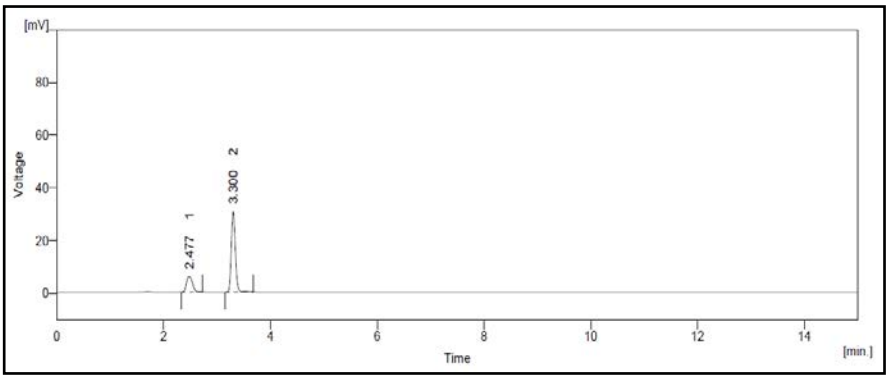

Figure 27: RIV thermal degradation

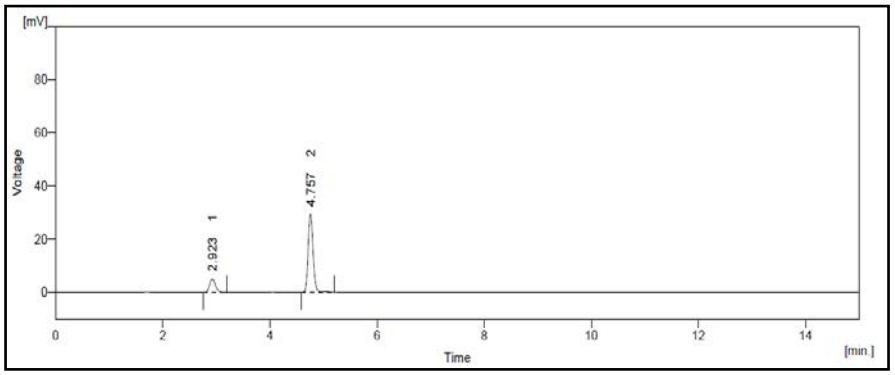

Figure 28: CLP thermal degradation

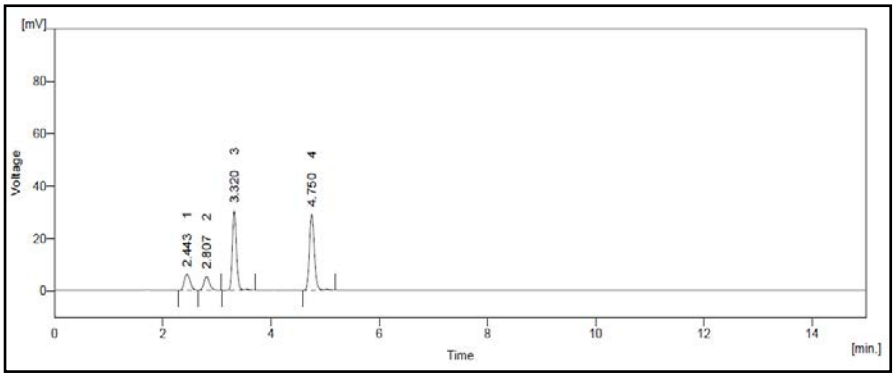

Figure 29: RIV and CLP thermal degradation sample

Table 9: Calculation of CLP and RIV% degradation study

Parameter	CLP				RIV			
	Standard		Sample		Standard		Sample	
	Area	% Degradation	Area	% Degradation	Area	% Degradation	Area	% Degradation
Acid	190.078	19.835	193.057	18.579	186.274	23.3	189.389	22.017
Base	205.276	13.425	204.673	13.68	189.57	21.942	186.95	23.021
Thermal	188.234	20.613	184.765	22.076	163.553	32.655	162.639	33.032
Oxidation	203.616	14.126	184.765	22.076	165.6	31.812	167.695	30.95
Photo	161.942	31.701	161.186	32.02	175.273	27.829	173.045	28.747

IV. Conclusion

The Combined dosage form of Rivaroxaban and clopidogrel are not available commercially. But individually rivaroxaban is used as an anticoagulant and it is the first orally active direct factor Xa inhibitor and clopidogrel is used as an antiplatelet agent. Various methods are reported for the analysis of individual drug and in combination with other drugs but no stability indicating HPLC method reported for these two drugs in combined dosage form. Therefore, a novel RP- HPLC method has been developed for the simultaneous estimation of Rivaroxaban and Clopidogrelin combination. The optimized chromatogram was run for appropriate minutes with mobile phase Phosphate buffer (Ph 4.5): Methanol (70:30). Data related to peak like area, height, retention time, resolution etc. were recorded using software. Thermo scientific, C_{18}(25cm×0.46cm) Hypersil BDS, Mobile Phase Phosphate buffer, pH 4.5: Methanol (70:30) with Flow Rate 1.0 ml/min and Runtime 6 min Injection volume of 20.0 µl. The detection was carried out at wavelength 214 nm. It was found to be simple, precise and accurate. In this stability indicating RP-HPLC methods were developed by degradation of sample and compared with standard. The % RSD was also less than 2 % showing high degree of precision of the proposed method. The proposed method can be used for routine analysis of Rivaroxaban and Clopidogrel in combined dosage form. It can be also used in the quality control in bulk manufacturing.

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