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DEVELOPMENT AND VALIDATION OF FIRST UV SPECTROPHOTOMETRIC METHOD AND RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF RIVAROXABAN AND TICAGRELOR IN SYNTHETIC MIXTURE

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Synthetic Mixture
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ABSTRACT

An accurate, precise and reproducible UV- spectrophotometric methods and liquid chromatographic assay method were developed and validated for the determination of Rivaroxaban and Ticagrelor in synthetic mixture. Spectrophotometric estimation was done by derivative spectroscopic method and methanol as solvent. In this method λ_{max} for Rivaroxaban and Ticagrelor were selected at 295 nm and 249nm. RP-HPLC analysis was carried out using Pearless C-18 column (4.6 x 250mm, 5µ particle size) and mobile phase composed of Acetonitrile: 10% Ortho-phosphoric acid in water pH 4.0 (60:40% v/v)at a flow rate of 1.0 ml/min and chromatogram was recorded at 249 nm. Linearity was evaluated over the concentration range of 2 -12 µg/ml and 9- 54 µg/ml for Rivaroxaban and Ticagrelor in UV-spectrophotometric and in RP-HPLC method Linearity was evaluated over the concentration range of 2 - 4 µg/ml and 9-54 µg/ml for Rivaroxaban and Ticagrelor (the value of r2 = 0.9977 and r2= 0.9989 found were by UV method for Rivaroxaban and Ticagrelor and the value of r2 = 0.9985 and r2= 0.9991 found were by RP-HPLC method for Rivaroxaban and Ticagrelor). The developed methods were validated according to ICH guidelines and values of accuracy, precision and other statistical analysis were found to be in good accordance with the prescribed values therefore the both methods can be used for routine monitoring of Rivaroxaban and Ticagrelor in the assay of Synthetic mixture of both drugs.

INTRODUCTION:

Rivaroxaban is a drug which belongs to class of anticoagulant. Rivaroxaban is approved for the prevention of strokes and systemic embolism in atrial fibrillation. It is useful in prevention blood clot and treatment of deep venous thrombosis. Rivaroxaban is highly selective Xa inhibitor with oral bioavailability and it inhibits both free Factor Xa inhibition of Factor Xa interrupts the intrinsic and extrinsic pathway of the blood coagulation cascade, inhibiting both thrombin formation and development of thrombi. Rivaroxaban does not inhibit thrombin (activated Factor II), and no effects on platelets have been demonstrated. Ticagrelor is a platelet aggregation inhibitor which is an antagonist of the P2Y12 receptor of thrombotic events in acute coronary syndrome or myocardial infarction with ST elevation. Ticagrelor is a P2Y12 Platelet Inhibitor. The mechanism of action of ticagrelor is as a Phenylalanine Hydroxylase Activator, and P2Y12 Receptor Antagonist, and Cytochrome P450 3A4 Inhibitor, and

Cytochrome P450 3A5 Inhibitor, and P-Glycoprotein Inhibitor. The physiologic effect of ticagrelor is by means of Decreased Platelet Aggregation. Combination of Rivaroxaban and Ticagrelor was studied under clinical trial for the Atrial fibrillation compare to Rivaroxaban and Ticagrelor monotherapy. Rivaroxaban and Ticagrelor was proved to be effective in patient with Atrial Fibrillation undergoing percutaneous coronary intervention (PCI).Atrial Fibrillation is defined as an abnormal heart rhythm which characterize by irregular and rapid beating of atria. This abnormal beating becomes longer and constant over time.

MATERIAL AND METHODS:

Instrument: Sonicator, Analytical weight balance, IR-spectrophotometer: Model-Miracle-10, single reflection ATR accessory- 8300, shimadzu, UV-Visible spectrophotometer, RP-HPLC

Chemicals: Standard API of Rivaroxaban, Standard API of Ticagrelor, Solvent (AR Grade methanol.)

Method: UV-visible spectroscopic method (First derivative method), RP-HPLC (Reverse Phase High Performance Liquid Chromatography) method.

Identification of pure API: [54-53]

Melting point determination: Melting point of Rivaroxaban API and Ticagrelor API was determined by open capillary method using Melting point apparatus in which the both drugs ware filled in capillary tubes and kept in the Melting point apparatus which shows the Melting Range Rivaroxaban and Ticagrelor.

Identification by FT-IR with ATR Spectroscopy: [56]

Identification of Rivaroxaban by FT-IR with ATR: Small quantity of Rivaroxaban was kept in the sample compartment of FT-IR (Shimadzu Miracle 10, Single Reflection ATR accessory) and it was scanned in the range of 4000-400cm-1.

Identification by Ticagrelor by FT-IR: Small quantity of Ticagrelor was kept in the sample compartment of FT-IR (Shimadzu Miracle 10, Single Reflection ATR Accessory) and it was scanned in the range of 400-4000 cm-1.

Spectrophotometric conditions: Mode: Absorption (scanning), Scan Speed: Medium, Wavelength Range: 200-400nm, Initial Baseline Correction: Methanol (AR grade)

Preparation of standard stock solution:

Preparation of standard stock solution of Rivaroxaban (100µg/ml): Accurately weight 10 mg of Rivaroxaban was transferred into a 100 ml volumetric flask and diluted with Methanol.

Preparation of standard stock solution of Ticagrelor(100µg/ml): Accurately weight 10 mg of Ticagrelor was transferred into a 100 ml volumetric flask and diluted with Methanol.

Selection of Wave length:

0.4 ml standard stock solution of Rivaroxaban (100 µg/ml) and 1.8 ml standard stock solution of Ticagrelor (100 µg/ml) was transfer in 10 ml volumetric flask and dilute up to mark with Methanol to get the 4 µg/ml of Rivaroxaban and 18 µg/ml of Ticagrelor. Each solution was scanned in the range 200 – 400 nm. The Spectra are converted to First Order Derivative. The overlain first derivative spectrums of Rivaroxaban and Ticagrelor at different concentration were recorded. The zero crossing point (ZCP) of Rivaroxaban was found to be 295 nm and ZCP of Ticagrelor was found to be 249 nm. Both this wavelength 295 nm and 249 nm were used for the determination

of Rivaroxaban and Ticagrelor respectively.

Preparation of Calibration Curve: Calibration curve for Rivaroxaban Aliquots of stock solution of Rivaroxaban (100µg/ml) 0.2,0.4,0.6,0.8,1.0,1.2 ml were pipette out in10 ml volumetric flask separately and dilute up to the mark with Methanol which will give 2-12µg/ml solution was prepared and absorbance was measured at 295 nm in U.V. Graph of Absorbance vs. Concentration was plotted.

Calibration curve for Ticagrelor: Aliquots of stock solution of Ticagrelor (100 µg/ml) 9, 18, 27, 36, 45, 54 ml were pipette out in 10 ml volumetric flask separately and dilute up to the mark with Methanol which will give 9-54 µg/ml solution was prepared and absorbance was measured at 249 nm inU.V. Graph of Absorbance vs. Concentration was plotted.

ASSAY: Preparation of Synthetic Mixture of Rivaroxaban and Ticagrelor: [48]: Powder weight equivalent to 20mg of Rivaroxaban and 90 mg of Ticagrelor was taken and than added other excipients 4 mg Povidone, 15 mg Micro crystalline cellulose, 2 mg Magnesium- Stearate, 1 mg Talc and 88 mg dilute Lactose Monohydrate into Petri dish. Powder weighed equivalent to 4 mg of Rivaroxaban and 18 mg of Ticagrelor was taken and transferred into 100 ml volumetric flask. Added 50 ml of Methanol and sonicated it. Diluted up to the mark with Methanol. This solution was filtered through Whatman Filter paper. The mixture contains 40µg/ml of Rivaroxaban and 180µg/ml of Ticagrelor.

Preparation of sample Solution: From above synthetic mixture 1 ml was pipetted out in 10ml volumetric flask and made up to the mark with Methanol to make final concentration of Rivaroxaban 4µg/ml and Ticagrelor 18 µg/ml.

Method Validation: The developed method was validated with respect to linearity, accuracy, and precision, limit of detection and limit of quantification in accordance with the ICH guideline**.**

Linearity & Range(n=6): The linearity of Rivaroxaban and Ticagrelor was taken to be in the range of 2-12 µg/ml and 9-54 µg/ml respectively. Calibration curve of Absorbance Vs Concentration was plotted and from that slope, intercept, correlation coefficient and regression line equation for Rivaroxaban and Ticagrelor was constructed.

Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: Intermediate (Intraday) precision, reproducibility (Interday precision), repeatability.

Intraday Precision (n=3): Solutions containing 2, 4, 6 µg/ml of Rivaroxaban and 9, 18, 27 µg/ml of Ticagrelor were analyzed three times on the same day and % RSD was calculated.

Interday Precision (n=3): Solutions containing 2, 4, 6 µg/ml of Rivaroxaban and 9, 18, 27 µg/ml of Ticagrelor were analyzed three different successive days and % RSD was calculated.

Repeatability (n=6): Solutions containing 4 µg/ml of Rivaroxaban and 18 µg/ml of Ticagrelor were analyzed for six times and % R.S.D was calculated. R.S.D was not more than 2%.

- **Limit of Detection (LOD):**

Limit of Detection can be calculated using following equation as per ICH guidelines.

$$LOD = 3.3 \times (\sigma / S)$$

Where, σ = standard deviation of the Y intercept of calibration curve S = Mean slope of the corresponding calibration curve.Limit of Quantification (LOQ):

Limit of Quantification can be calculated using following equation as per ICH guidelines.

$$LOQ = 10 \times (\sigma / S)$$

Where, σ = standard deviation of the Y intercept of calibration curve S = Mean slope of the corresponding calibration curve.Accuracy (RECOVERY STUDY) (n=3):

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as aconventional true value or an accepted reference value and the value found.Accuracy of the developed method was confirmed by doing recovery study as per ICH guideline at three different concentration levels 50%, 100%, 150% and the values were measured for Rivaroxaban (**4 µg/ml**) and ticagrelor (**18µg/ml**). This performance was done intriplicate.

Application of method to synthetic mixture: Preparation of Synthetic Mixture of Rivaroxaban and Ticagrelor:

The synthetic mixture of Rivaroxaban and Ticagrelor was prepared in ratio of 1:4.5 Commonexcipients: Microcrystalline cellulose, Lactose, Magnesium stearate,Talc with drug Rivaroxaban 20 mg and Ticagrelor 90mg.Accurately weighed equivalent weight of Rivaroxaban (4 mg) and Ticagrelor(18mg)whichtransferredin100 mlvolumetric flask and makeup half mark with Methanol. Then this concentration of Rivaroxaban 40 µg/ml Ticagrelor 180 µg/ml.

Preparation of Sample Solution:

From the above synthetic mixture 1ml was pipetted out in volumetric flask and made up to mark with Methanol to make final concentration of Rivaroxaban 4µg/ml and Ticagrelor 18 µg/ml.

RP-HPLC Method:

Preparation of standard stock solution:

- **Rivaroxaban (100 µg/ml):**

  Accurately weighed Rivaroxaban (10 mg) was transferred to a 100 ml volumetric flask and diluted to the mark with Acetonitrile to obtain a standard stock solution (100 µg/ml).

- **Ticagrelor (100 µg/ml):**

  Accurately weighed Ticagrelor (10 mg) was transferred to a 100 ml volumetric flask and diluted to the mark with Acetonitrile to obtain a standard stock solution (100 µg/ml).

Preparation of binary mixture of Rivaroxaban (4µg/ml) & Ticagrelor (18µg/ml): Standard solution of Rivaroxaban (0.4 ml) and Ticagrelor (1.8 ml) were transferred to a 10 ml volumetric flask and diluted up to the mark with mobile phase ACN: water (60:40) (pH 4 with ortho phosphoric acid).

Selection of Detection Wavelength: The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. Absorbance maximum was obtained at 249 nm. So, 249 nm was selected for detection of Rivaroxaban and Ticagrelor.

Mobile phase selection: The composition and flow rate of mobile phase were changed to optimize the separation condition using combined solution. The pKa value for

Rivaroxaban and Ticagrelor is 13.6 and 12.94 respectively. After number of trial experiments, it was established that the mobile phase ACN: water (60:40) (pH 4 with ortho phosphoric acid)shows good peak shape and resolution.

Preparation of 10% Ortho phosphoric acid: 10% ortho phosphoric acid was prepared by diluting 1 ml of concentrated ortho phosphoric acid in 10 ml HPLC grade water.

Chromatographic condition:

- **Column: Thermo C-18 (250 mm × 4.6 mm, 5 µm)**
- **Flow rate:** 1.0 ml/min
- **Run time:** 10 min
- **Detection wavelength:** 249 nm
- **Detector:** U.V Detector
- **Injection volume:** 20µl

Preparation of calibration curve

Aliquots equivalent to 0.2, 0.4, 0.6, 0.8, 1, 1.2 ml working standard solution of Rivaroxaban and 0.9, 1.8, 2.7, 3.6, 4.5, 5.4 ml of Ticagrelor were transferred into a series of five 10 ml Volumetric flasks and volume was adjusted to the mark with mobile phase to get concentration 2, 4, 6, 8, 10 and 12 µg/ml of Rivaroxabanand 9, 18, 27, 36, 45 and 54µg/ml of Ticagrelor. 20 µl of each of the solution were injected into HPLC system and analyzed. Calibration curve was obtained by plotting respective peak area against concentration in µg/ml and the regression equation was computed

Method Validation: The developed method was validated with respect to linearity, accuracy, precision, limit of detection and limit of quantification in accordance with the ICH guideline.

Specificity: Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, matrix etc.

Linearity & Range: The linearity of Rivaroxabanand Ticagrelor was found to be in the range of 2-12 µg/ml and 9-54 µg/ml respectively. Plot the calibration curve of Area (µV .s) vs. Concentration (µg/ml). Linearity of both the drugs was checked in term of slope, intercept and correlation coefficient.

Precision: The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be consider at three levels: Intermediate (Intraday) precision, reproducibility (Interday precision), repeatability.

1) Intraday Precision: Solution containing 2, 4, 6 µg/ml of Rivaroxaban and 9, 18, 27 µg/ml of Ticagrelor were analyzed three times on the same day and %RSD was calculated.

2) Interday Precision: Solutions containing 2, 4, 6 µg/ml of Rivaroxaban and 9, 18, 27 µg/ml of Ticagrelor were analyzed three different successive days and %RSD was calculated.

3) Repeatability: Solutions containing 4 µg/ml of Rivaroxaban and 18 µg/ml of Ticagrelor were analyzed for six times and %RSD was calculated. %RSD was not more than 2%.

Limit of Detection (LOD): Limit of Detection can be calculated using following equation as per ICH guidelines.

$$\mathbf{LOD = 3.3 \times (\sigma/S)}$$

Where, σ = standard deviation of the Y intercept of calibration curve. S = Mean slope of the corresponding calibration curve.

Limit of Quantification (LOQ): Limit of Quantification can be calculated using following equation as per ICH guidelines.

$$\mathbf{LOQ = 10 \times (\sigma/S)}$$

Where, σ = standard deviation of the Y intercept of calibration curve, S = Mean slope of the corresponding calibration curve

Accuracy: 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. Accuracy of the developed method was confirmed by doing recovery study as per ICH guideline at three different concentration levels 50%, 100%, 150% and the values were measured at all wavelengths for (4 µg/ml) of Rivaroxaban and (18µg/ml) of Ticagrelor. This performance was done in triplicate.

Preparation of sample solution: Accurately weigh powder equivalent to 4mg of Rivaroxaban and 18mg Ticagrelor from the prepared synthetic mixture and transferred in 100 ml volumetric flask and make up to the mark with Acetonitrile. This solution was sonicated and filtered. The mixture contains Rivaroxaban 40µg/ml and Ticagrelor 180µg/ml. From the above synthetic mixture 1 ml was pipetted out in 3 different 10ml volumetric flask and spiked with standard stock solution of Rivaroxaban and Ticagrelor in 50, 100, 150 level. Made up to the mark with Acetonitrile to obtain final concentration.

Robustness: The robustness of an analytical procedure is a measure of its capacity to remainunaffected by small, but deliberate variation in method parameters and provides an indication of its reliability during normal usage. It should show there liability of an analysis with respect to deliberate variation in method parameter. In case of liquid chromatography, examples of typical variations are:

- Influence of variations of pH in mobile phase; Influence of variation in mobile phase composition;
- Different columns (different lots and/or different supplier) Flow rate

System suitability tests: A system suitability test is an integral part of liquid chromatography. They are used to verify that resolution and reproducibility of chromatography system are adequate for the analysis is to be done. The test includes the resolution, column efficiency, tailing factor and theoretical plates.

Analysis of synthetic mixture:

Preparation of synthetic mixture of Rivaroxaban and Ticagrelor

Preparation of Synthetic Mixture of Rivaroxaban and Ticagrelor: The synthetic mixture of Rivaroxaban and Ticagrelor was prepared in ratio of 1:4.5. Powder weight to 20 mg of Rivaroxaban and 90 mg Ticagrelor was taken and transferred into 100 ml volumetric flask than add polyvinyl Pyrolidine, microcrystalline cellulose, lactose, talc, mg. stearate into Petridish. Powder Weighted equivalent to 4 mg of Rivaroxaban and 18mg of Ticagrelor was taken and transferred into 100 ml volumetric flask. Added 50 ml of Acetonitrile and sonicated it. Diluted up to the mark with Acetonitrile. This solution filtered through Whatmann filter paper. This mixture contains Rivaroxaban 40 µg/ml Ticagrelor 180 µg/ml.

Preparation of Sample Solution:

From the above synthetic mixture 1ml was pipette out in 10ml volumetric flask and made up to the mark with mobile phase ACN: water (60:40) to obtain final concentration of Rivaroxaban 4 µg/ml and Ticagrelor 18 µg/ml.

RESULT AND DISCUSSION:

Selection of elution mode: Reverse phase chromatography was chosen because of its recommended use for ionic and moderate to non-polar compounds. Reverse phase chromatography is not only simple, convenient but also better performance in terms of efficiency, stability and reproducibility. C18 column was selected because it is least polar compare to C4 and C8 columns. C18 column allows eluting polar compounds more quickly compare to non-polar compounds. In addition to this, UV detector is used which allows easy detection of the compounds in UV transparent organic solvents. A 250 mm × 4.6 mm column of 5µm particles packing was for separation of Rivaroxaban and Ticagrelor. Isocratic modecolumn stability. This configuration provides a large number of theoretical plate's values for most separation.

Selection of Detection Wavelength:

- The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. Absorbance maximum was obtained was at 249 nm. So, 249nm was selected for detection of Rivaroxaban and Ticagrelor.

Optimization of Chromatographic conditions: The composition and flow rate of mobile phase were changed to optimize the separation condition using combined solution. The pKa value for Rivaroxaban and Ticagrelor is 13.6 and 12.94 respectively. After number of trial experiments, it was established that the mobile phase ACN: water (pH 4 adjusts with Ortho phosphoric acid) (60:40% v/v) shows good peak shape and resolution. Under these conditions, the eluted peaks were well defined, resolved and free from tailing. The elution order was Rivaroxaban (Rt =3.2) and Ticagrelor (Rt =5.2) at a flow rate of 1 ml /min.

Method validation: Specificity: It was prove by comparing the chromatogram of mobile phase, standard solution and test preparation solution to show that there was no peak of mobile phase and no any interference of excipients with the peak of Rivaroxaban and Ticagrelor as shown in figure 5.2, 5.3, 5.4, and 5.5

System suitability parameters: The resolution, asymmetry factor, tailing factor and number of theoretical plates are shown in table 5.2. The values obtained demonstrated the suitability of the system for the analysis of these drugs in combination.

Linearity: The linearity of Rivaroxaban and Ticagrelor was found to be in the range of 2-12 µg/ml and 9-54µg/ml respectively. Linearity data for Rivaroxaban and Ticagrelor are depicted in Table 5.3.

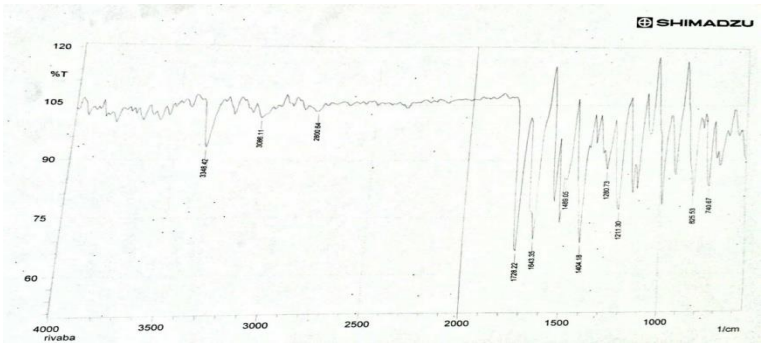


Fig 1 Sample IR Spectra of Rivaroxaba

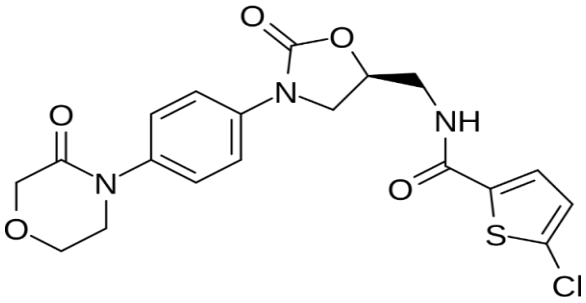


Fig.2 Structure of Rivaroxaban

S.No	Type of Validation	Standard Wave number(cm^{-1})	Observe Wave number(cm^{-1})
1	C=O	1670-1820	1728.22
2	C-H	3010-3040	3086.11
3	C-N	1350-1000	1280.73
4	N-H	3300-3500	3348.42
5	C=C	1600-1850	1643.35
6	C-Cl	800-600	825.53
7	C-S	800-600	740.67

Table 1: Interpretation of FT-IR spectra of rivaroxaban

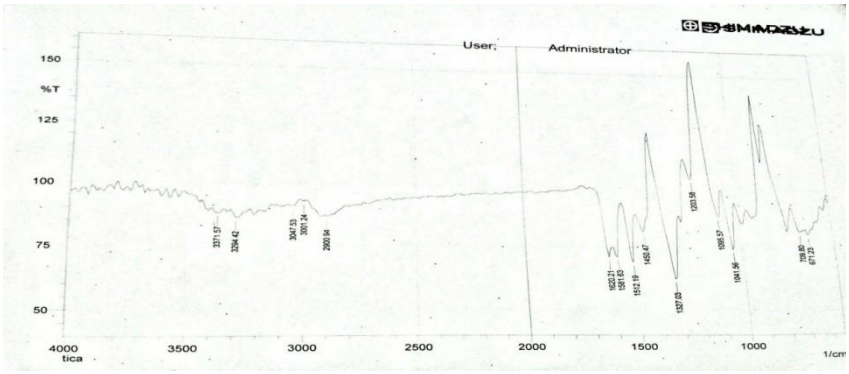


Fig. 3: Sample IR Spectra of Ticagrelor.

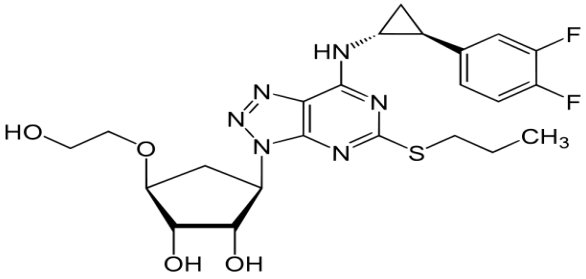


Fig 4: Structure of Ticagrelor

S.No	Type of Validation	Observe Wave number(cm^{-1})	Standard Wave number(cm^{-1})
1	C=C	1600-1850	1620.21
2	C-H	3010-3040	3001.24
3	C-N	1350-1000	1327.03
4	N-H	3500-3300	3371.57
5	O-H	3200-3600	3294.42
6	C-F	1400-1000	1041.56
7	C-S	800-600	709.8

Table 2: Interpretations of FT-IR spectra of Ticagrelor

Table 3 Wavelength of Rivaroxaban and Ticagrelor

Drug name	Observed λmax (Methanol)	Reported λmax (Methanol)
Rivaroxaban	**250**	**249**
Ticagrelor	**255**	**254**

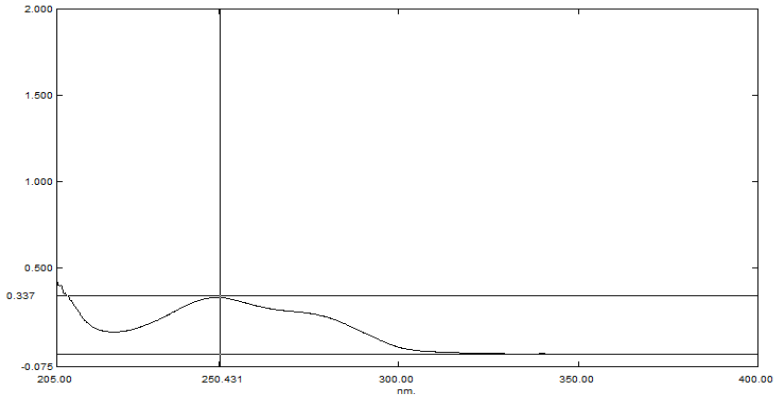


Fig 5: Rivaroxaban UV Spectra (4 µg/ml)

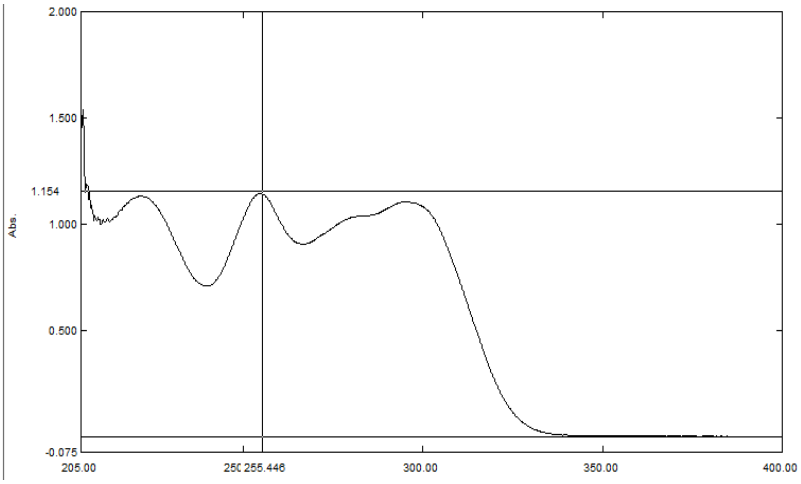


Fig 5 Ticagrelor UV Spectra (18 µg/ml)

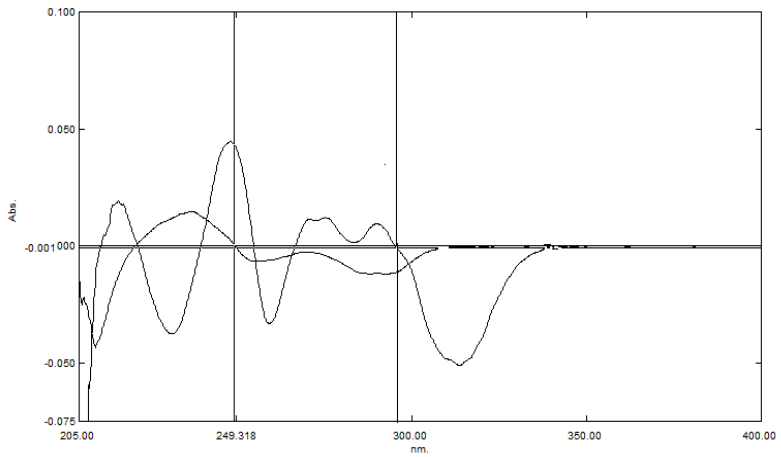


Fig 6: Zero crossing point of Rivaroxaban at 295 nm (4 µg/ml) and Ticagrelor at 249 nm (18 µg/ml)

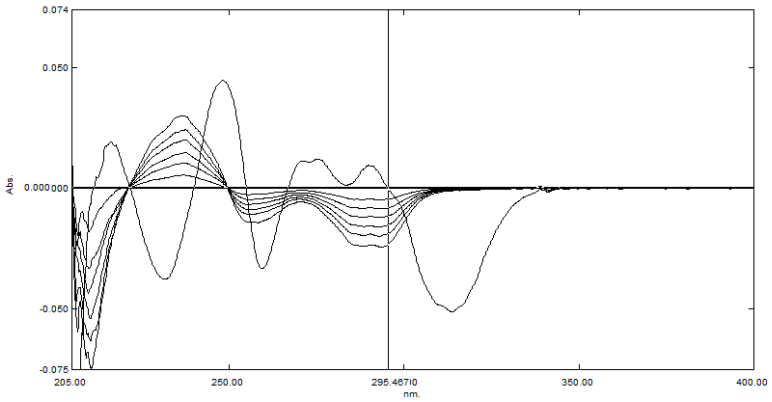


Figure 7: Linearity of 1st Derivative spectra of Rivaroxaban (295 nm)

Rivaroxaban		
Concentration (µg/ml)	**Mean Absorbance ± SD**	**%RSD**
2	|-0.005|±0.00010	1.96
4	|-0.008|±0.00014	1.65
6	|-0.012|±0.00016	1.36
8	|-0.016|± 0.00017	1.05
10	|-0.020|±0.00018	0.91
12	|-0.024|±0.00021	0.88
Rivaroxaban		

Concentration (µg/ml)	Mean Absorbance ± SD	%RSD
2	|-0.005|±0.00010	1.96
4	|-0.008|±0.00014	1.65
6	|-0.012|±0.00016	1.36
8	|-0.016|± 0.00017	1.05
10	|-0.020|±0.00018	0.91
12	|-0.024|±0.00021	0.88

TABLE: 4 Linearity data of Rivaroxaban

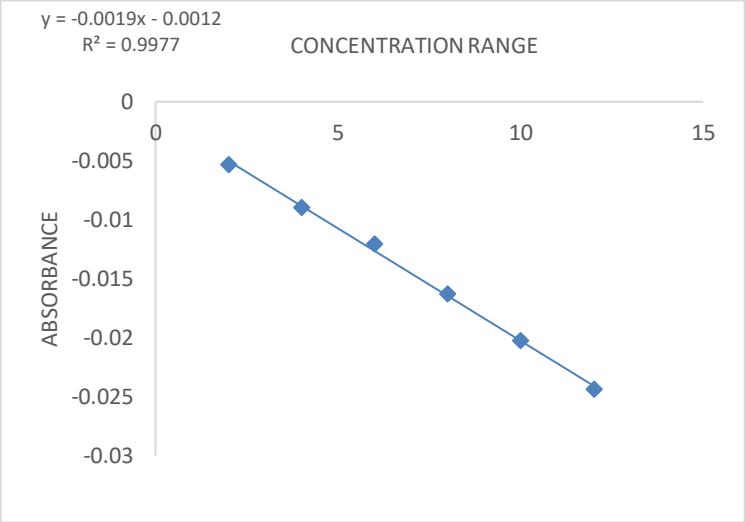


Fig 8 Calibration Curve of Rivaroxaban

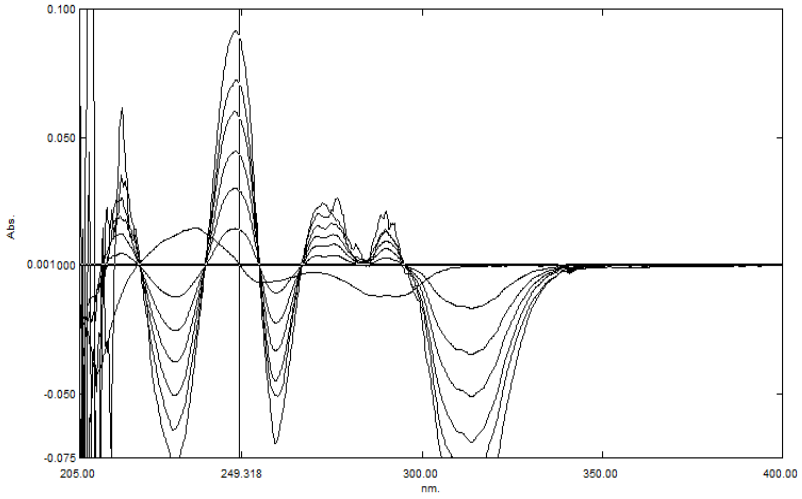
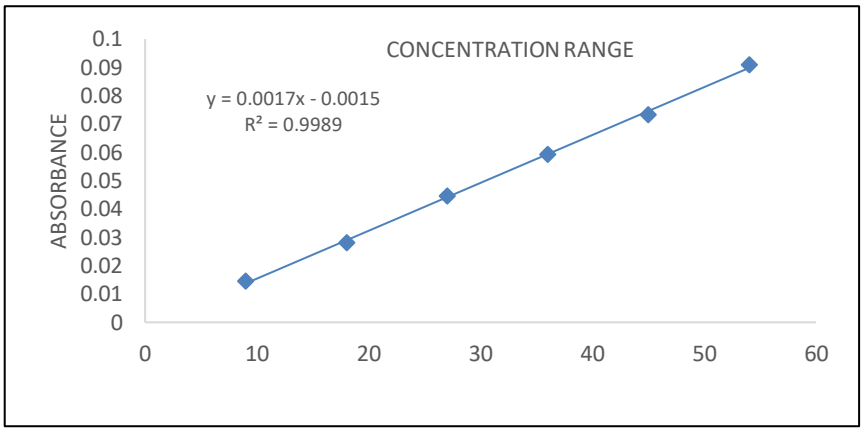


Figure 9: Linearity of 1_{st} Derivative spectra of Ticagrelor (249 nm)

Ticagrelor		
Concentration (µg/ml)	**Mean Absorbance ± SD**	**%RSD**
9	0.014± 0.00018	1.30
18	0.028± 0.00021	0.73
27	0.044± 0.00024	0.55
36	0.059± 0.00026	0.43
46	0.073±0.00028	0.38
54	0.091±0.00029	0.32

TABLE: 5 Linearity data of Ticagrelor at 249 nm

Table 5.1:- Melting Point of Rivaroxaban and Ticagrelor



S.no	Drug	Reported melting Point	Observed melting Point
1	Rivaroxaban	228-230°C	228-230°C
2	Ticagrelor	138-140 ^{O}C	136-138°C

Fig 10: Calibration Curve of Ticagrelor

Intraday Precision of Rivaroxaban (n=3)		
Conc. (µg/ml)	**Mean Absorbance ±SD (n=3)**	**% RSD**
2	|-0.0063|±0.00010	1.58
4	|-0.0086|±0.00011	1.33
6	|-0.0120|±0.00015	1.26
Interday Precision of Rivaroxaban (n=3)		

Conc. (µg/ml)	Mean Absorbance ±SD (n=3)	% RSD
2	|-0.0560|±0.00010	1.78
4	|-0.0087|±0.00015	1.74
6	|-0.0133|±0.00020	1.56
Repeatability of Rivaroxaban (n=6)		
Conc. (µg/ml)	**Mean Absorbance ±SD (n=6)**	**% RSD**
4	|-0.0090|±0.00010	1.11

TABLE 6: Precision study of Rivaroxaban

Intraday Precision of Ticagrelor (n=3)		
Conc. (µg/ml)	**Mean Absorbance ±SD (n=3)**	**% RSD**
9	0.0160±0.00015	0.95
18	0.0284±0.00020	0.86
27	0.0451±0.00026	0.65
Interday Precision of Ticagrelor (n=3)		
Conc. (µg/ml)	**Mean Absorbance ±SD (n=3)**	**% RSD**
9	0.0156±0.00020	1.32
18	0.0291±0.00025	0.86
27	0.0455±0.00030	0.65
Repeatability of Ticagrelor (n=6)		
Conc. (µg/ml)	**Mean Absorbance ±SD (n=6)**	**% RSD**
18	0.0289±0.00026	0.91

TABLE 7: Precision study of Ticagrelor

- **LOD and LOQ**

Parameter	Rivaroxaban	Ticagrelor
LOD (µg/ml)	0.25	0.30
LOQ (µg/ml)	0.77	0.91

Table: 8: LOD and LOQ for Rivaroxaban and Ticagrelor

Accuracy:

Name of Drug	% Level of recovery	Test Amount (µg/ml)	Amount of drug taken (µg/ml)	Total Amt (µg/ml)	Total amount Recovered (µg/ml)	% Recovery ± SD (n=3)
Rivaroxaban	50	4	2	6	5.89	98.16±0.010
	100	4	4	8	7.96	99.50±0.020
	150	4	6	10	9.94	99.40±0.025
Ticagrelor	50	18	9	27	26.33	98.74±0.020
	100	18	18	36	35.66	99.05±0.047
	150	18	27	45	44.65	99.22±0.035

TABLE 9 Recovery Study

Name of Drug	(Amount of taken (µg/ml)	Mean Amount found (µg/ml)	% Assay ±S.D. n=3
Rivaroxaban	4	3.92	98.0±0.015%
Ticagrelor	18	17.11	98.1±0.020%

TABLE: 10: Analysis of synthetic mixture

TABLE 11: Summary of validation parameter

S.No	Parameters	Rivaroxaban	Ticagrelor
1	Wavelength (nm)	295 nm	249 nm
2	Beer's Law Limit	2-12 (µg/ml)	9-54 (µg/ml)
3	Regression equation (y = mx +c)	y = -0.0019x - 0.0012	y = 0.0015x - 0.0015
4	Correlation Coefficient (r^2)	0.997	0.9989
5	Intraday Precision (%RSD, n=3)	1.58±1.26	0.095±0.65

6	Interday Precision (% RSD, n=3)	1.78±1.56	1.32±0.65
7	Repeatability (% RSD, n=6)	1.11	0.91
8	Accuracy (% Recovery, n=3)	98.16-99.40	98.74-99.22
9	LOD (µg/ml)	0.25 (µg/ml)	0.30 (µg/ml)
10	LOQ (µg/ml)	0.77 (µg/ml)	0.91 (µg/ml)
11	Assay	98.0%	98.1%

RP-HPLC Method

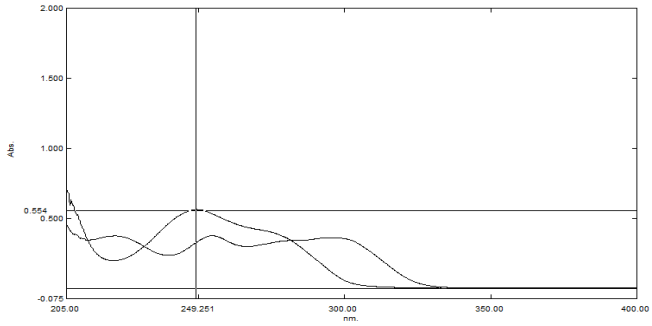


FIGURE 1 Zero order spectra of Rivaroxaban (4µg/ml) and Ticagrelor (18 µg/ml) in Methanol

Trail	Mobile phase	Ratio	Remark
1.	ACN: Water (PH 4)	60:40	One peak obtained.
2.	ACN: Water (PH-4)	70:30	Tow sharp pick, Resolution was less.
3.	ACN: Water (PH-4)	65:35	Tow sharp pick, Resolution was less.
4.	ACN: Water (PH-4)	60:40	Good resolution obtained with sharp peak, RT: Rivaroxaban- 3.4min, Ticagrelor- 5.6min

Table 1: Mobile phase optimization trial for Rivaroxaban and Ticagrelor

Method validation:

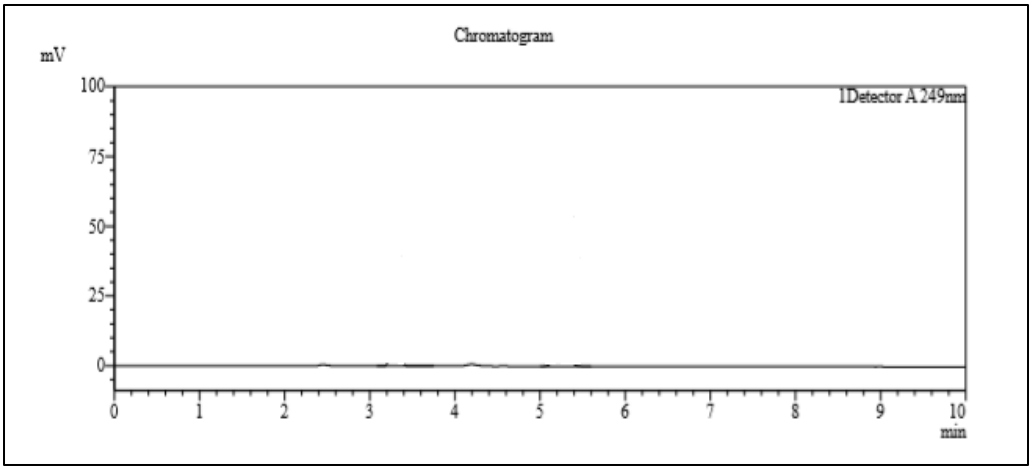


Figure 2 Chromatogram of Blank

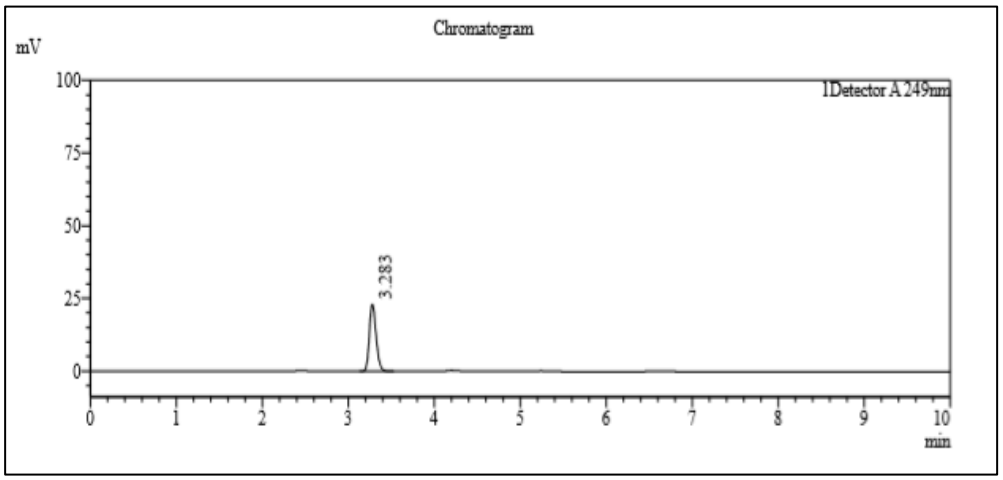


Figure3 Chromatogram of Rivaroxaban (4µg/ml) in ACN: water (60:40 % v/v)

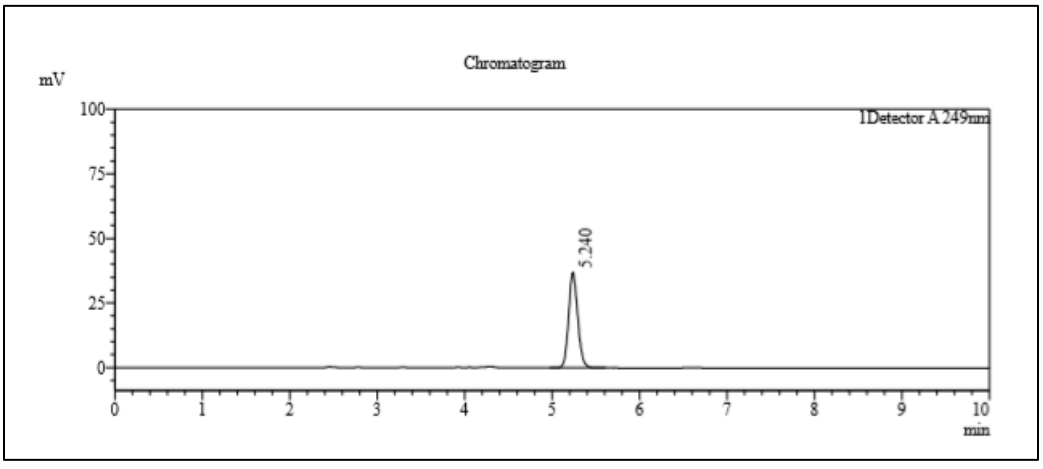


Figure 4 Chromatogram of Ticagrelor (18 µg/ml) in ACN: water (60:40% v/v)

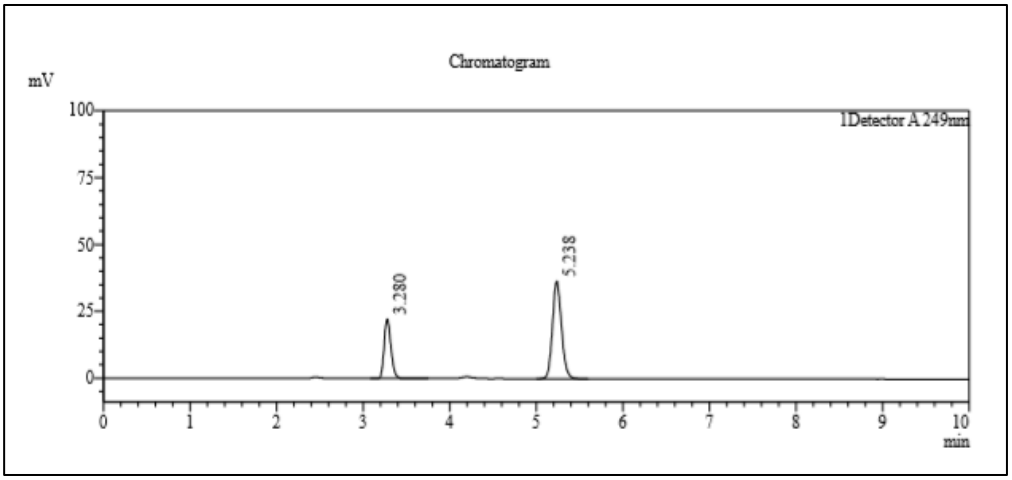


Figure 5 Chromatogram of Rivaroxaban (4 µg/ml) and Ticagrelor (18µg/ml) in ACN: water (60:40% v/v)

S.No.	System suitability parameter	Rivaroxaban	Ticagrelor
1.	Retention time	3.2	5.2
2.	Theoretical Plates	45398	71902
3.	Tailing Factors	1.216	1.131
4.	Resolution	10.856	

Table 2: System suitability parameter

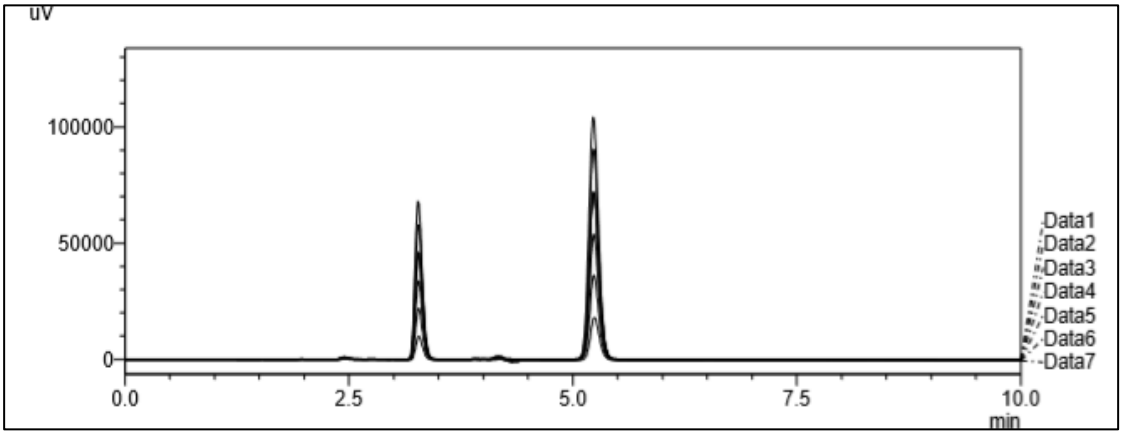


Figure 6 Overlay chromatogram of Rivaroxaban (2-12 µg/ml) and Ticagrelor (9-54 µg/ml)

Rivaroxaban			Ticagrelor		
Conc. (µg/ml)	**Mean Peak area (µV.sec)±S.D. (n=6)**	**% RSD**	**Conc. (µg/ml)**	**Mean Peak area (µV.sec)±S.D. (n=6)**	**% RSD**

2	55384.6± 484.93	0.87	9	133880.0± 1067.0	0.79
4	121340.8± 733.55	0.60	18	265791.6± 1415.19	0.53
6	191152.0± 887.94	0.46	27	396618.8± 1800.97	0.45
8	27462.2± 1049.05	0.38	36	539216.0± 2026.79	0.37
10	341684.3± 1103.03	0.34	45	689639.5± 2222.23	0.32
12	403778.5± 1214.13	0.30	54	805538.5± 2454.15	0.30

Table 3 Calibration data for Rivaroxaban (2-12 µg/ml) and Ticagrelor (9-54 µg/ml)

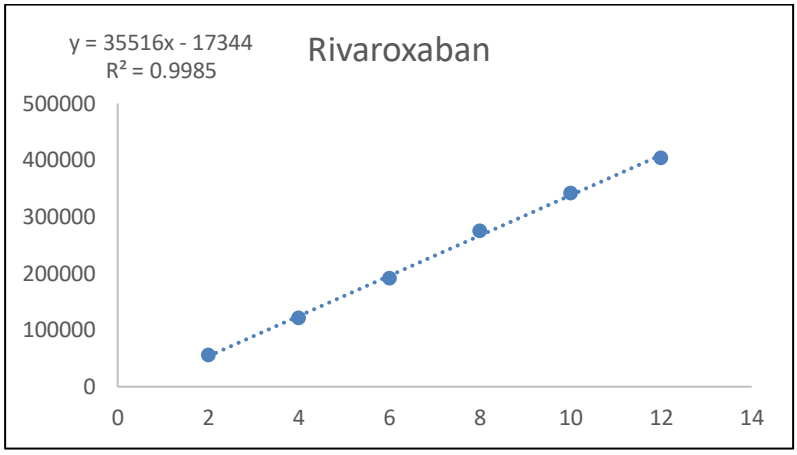


Figure 7 Calibration curve for Rivaroxaban (2-12 µg/ml)

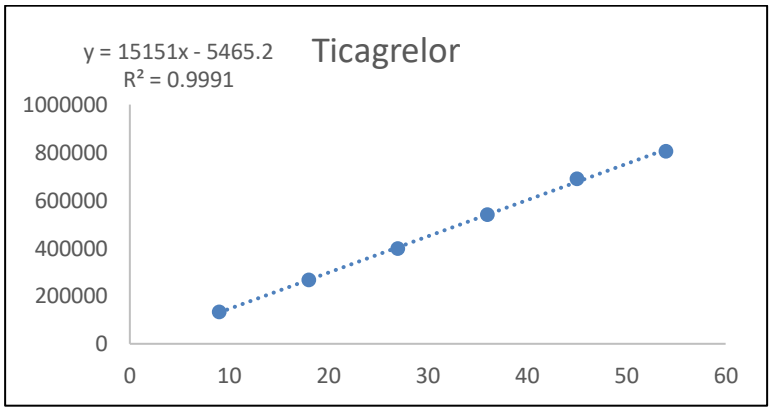


Figure 8 Calibration curve for Ticagrelor (9-54 µg/ml)

Table4: Precision study for Rivaroxaban

Rivaroxaban		
Intraday Precision of Rivaroxaban		
Conc. (µg/ml)	**Mean Absorbance ± SD (n = 3)**	**% RSD**
2	55393.0 ± 539.50	0.97
4	122543.3 ± 1080.38	0.88
6	191920.6 ± 1151.27	0.59
Interday Precision Rivaroxaban		
Conc. (µg/ml)	**Mean Absorbance ± SD (n = 3)**	**% RSD**
2	55555.0 ± 463.16	0.83
4	122883.0 ± 913.01	0.74
6	192256.3 ± 993.5	0.51
Repeatability of Rivaroxaban		
Conc. (µg/ml)	**Mean Absorbance ± SD (n = 6)**	**% RSD**
4	121950.1± 1023.1	0.83

Table 5: Precision study for Ticagrelor

Ticagrelor		
Intraday Precision of Ticagrelor		
Conc. (µg/ml)	**Mean Absorbance ± SD (n = 3)**	**% RSD**
9	134749.3± 1127.67	0.83
18	265130.3 ± 1527.85	0.57
27	399155.7 ± 1767.06	0.44

Interday Precision Ticagrelor		
Conc. (µg/ml)	**Mean Absorbance ± SD (n = 3)**	**% RSD**
9	134079.7 ± 1050.95	0.78
18	265132.3 ± 1154.70	0.43
27	397787.3 ± 1528.39	0.38
Repeatability of Ticagrelor		
Conc. (µg/ml)	**Mean Absorbance ± SD (n = 6)**	**% RSD**
27	264951.2 ± 1329.04	0.50

Table 6: Recovery study data

Drug	**% Level of Recovery**	**Test Amt. (µg/ml)**	**Amount of Drug Spiked (µg/ml)**	**Total Std Amt. (µg/ml)**	**Total Amt. Recovered (µg/ml)**	**% Recovery ± SD (n=3)**
Rivaroxaban	50	4	2	6	5.89	98.50 ±0.407
	100	4	4	8	7.98	99.75 ±0.120
	150	4	6	10	9.99	99.90 ±0.119
Ticagrelor	50	18	9	27	26.79	98.85±0.317
	100	18	18	36	35.74	99.27±0.275
	150	18	27	45	44.99	99.97 ±0.106

Table 7 LOD and LOQ

Parameter	**Rivaroxaban**	**Ticagrelor**
LOD (µg/ml)	0.090	0.274
LOQ (µg/ml)	0.186	0.565

Table 8 Analysis of synthetic mixture

Name of drug	**Amount taken (µg/ml)**	**Mean Amount found**	**%Assay ± S.D. (n=3)**

		(µg/ml)	
Rivaroxaban	4	3.94	98.50±0.243
Ticagrelor	18	17.89	99.38 ±0.320

Table 9 Robustness data

Condition	Variation	Rivaroxaban	Ticagrelor
		% Assay ± SD (n=3)	**% Assay ± SD (n=3)**
Detection Wavelength (249 nm ± 1 nm)	248	96.7±0.104	97.90±0.570
	249	**98.40±243**	**99.27±320**
	250	97.80±0.650	95.50±0.271
Mobile Phase ACN: water(60:40) (pH 4)	55:45	95.49±0.247	97.19±0.248
	60:40	**99.91±0.035**	**99.76±0.130**
	65:35	97.75±0.571	96.12±0.446

Table 10 Summary of validation parameters

S.No	Parameters	Rivaroxaban	Ticagrelor
1	Wavelength (nm)	249 nm	249nm
2	Retention time (min)	3.2	5.2
2	Beer's Law Limit	2-5	9-54

3	Regression equation (y = mx +c)	y = 35516x-17344	y = 15151x-5465.2
4	Correlation Coefficient (r^2)	0.9985	0.9991
5	Intraday Precision (%RSD, n=3)	0.59-0.97	0.44-0.83
6	Interday Precision (% RSD, n=3)	0.51-0.83	0.38-0.78
7	Repeatability (% RSD, n=6)	0.83	0.50
8	Accuracy (% Recovery, n=3)	98.50-99.90	98.85-99.97
9	LOD (µg/ml)	0.090	0.274
10	LOQ (µg/ml)	0.186	0.565
11	%Assay	98.50	99.38

CONCLUSION:

Simple, rapid, accurate and precise RP-HPLC and UV spectrophotometric methods have been developed and validated for the routine analysis of Rivaroxaban and Ticagrelor in synthetic mixture. Both methods are suitable for the simultaneous determination of Rivaroxaban and Ticagrelor in multi component formulation without interference of each other. The amounts found from the proposed methods were found in good agreement with the label claim of the formulation. Also the value of standard deviation and coefficient of variation calculated were satisfactorily low, indicating the suitability of the proposed methods for the routine estimation of combination dosage forms.

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