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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING ASSAY METHOD OF TICAGRELOR TABLETS BY USING RP-HPLC

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ABSTRACT

A simple, precise and accurate RP-HPLC method has been developed and validated for stability indicating assay and dissolution of Ticagrelor as per ICH guidelines. An isocratic separation was achieved using a Develosil ODS UG-5 C18 (150 X 4.6mm, 5µ particle size) columns with a flow rate of 1 ml/min and using a PDA detector to monitor the elute at 280 nm. The mobile phase consisted of potassium dihydrogen phosphate buffer: acetonitrile (60:40, v/v) with pH 3.0 adjusted with phosphoric acid. The retention time 5.35 ,linearity concentration range of 20-80 µg/ml (r^2 = 0.9992) with a limit of detection and quantitation of 0.05 and 0.15 µg/ml respectively. Intraday and interday system and method precision were determined and accuracy was between 99.3-101.9 %. the RP-HPLC method was validated and showed to be specific, linear, accurate, precise and robust. Based on the results obtained from the analysis using proposed method, it can be concluded that the method has linear response in the range of 22.5-135 µg/ml for Ticagrelor respectively. The method is validated for linearity, precision, accuracy, LOD, LOQ, as per ICH Guidelines. The result of the analysis of pharmaceutical formulation by the proposed method is highly reproducible and reliable and is in good agreement with label claim of the drugs. The additive usually present in the pharmaceutical formulations did not interfere in the analysis. So the method can be used for dissolution study. The dissolution test developed and validated for Ticagrelor tablets was considered satisfactory for routine quality control analysis and to establish *in vitro-in vivo* correlation.

KEYWORDS: Ticagrelor, RP-HPLC, ICH Guidelines, Force Degradation Studies.

INTRODUCTION

Chromatography is a powerful separation method that finds application in all branches of science which is used for method development. High Performance Liquid Chromatography or High Pressure Liquid Chromatography (HPLC) is mainly a chromatography technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of a mixture of analytes by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column). Ticagrelor is newly introduced into the market, hence the data on the qualitative and quantitative estimation of this drug is limited. It is marketed as a tablet for the treatment of thrombosis. It reduces the rate of thrombotic cardiovascular events in patients with the acute coronary syndrome. It belongs to the class of triazolo pyrimidines which are polycyclic aromatic compounds containing triazole ring fused to a pyrimidine ring. Ticagrelor and its major metabolite reversibly interact with the platelet P2Y12 ADP-receptor to prevent signal transduction and platelet activation. It is a white crystalline powder with an aqueous solubility of approximately 10µg/ml at room temperature. It has a log P of 2.30, pKaof strong acidic function 12.94 and strong basic function 2.90. It prevents platelet aggregation and thrombus formation in atheroscleroticdisease which reduces chances of cardiac arrest due to blockage.[1] The marketed formulation of ticagrelor replaced clopidogrel containing formulations due to higher efficacy and lower side effectsThe clinical evidence for the effectiveness of ticagrelor was derived from Plato, a randomized double-blind study comparing ticagrelor with clopidogrel. This product is used to treat people who have had a recent heart attack or severe chest pain that happened because their heart was not getting enough oxygen or are treated with a procedure to open blocked arteries in the heart.[2-4]

A literature search revealed very few papers on analytical methods of this drug. One research paper has reported a UV method of analysis and another one has reported a HPLC method of analysis for ticagrelor using UV detector.[5-6] Hence the aim of this study was to develop a simple, accurate and precise RP-HPLC analytical method for the estimation of ticagrelor in

tablet formulation.

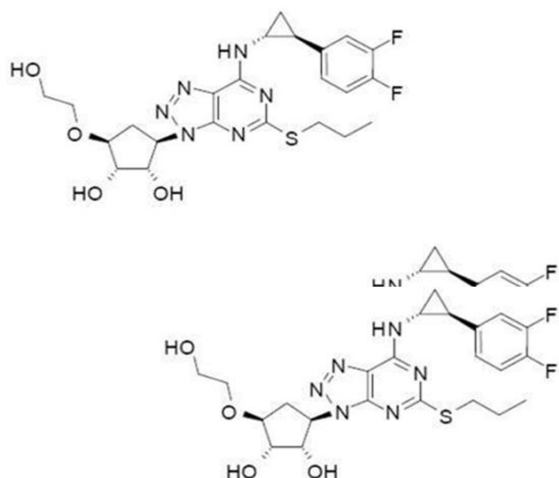


Figure. 1: structure of Ticagrelor.

MATERIALS AND METHODS[7-9]

Chemicals and reagents

Ticagrelor reference standard was obtained as a gift sample from Watson® Pharmaceuticals. Methanol and acetonitrile (HPLC grade), MilliQ water. hydrochloric acid (AR) 37%, sodium hydroxide (AR grade), orthophosphoric acid (HPLC grade) 85%, ammonium acetate (GR), potassium dihydrogen phosphate (AR), hydrogen peroxide (ACS) 50%, formic acid (AR), triethylamine (HPLC grade), sodium lauryl sulphate, polysorbate 80 (GR) were procured from Merck® and Fischer Scientific®.

Instruments used

The HPLC system used was an Agilent 1260 infinity equipped with a quaternary solvent pump, a thermostated autosampler, degasser, UV/VWD detector and a column oven. A double beam UV-Visible spectrophotometer (Shimadzu 1800), USP Type II-Paddle apparatus (Labindia DS2000 with Ismatec high precision multichannel pump as autosampler), photo stability chamber (Atlas Suntest XLS+), HPLC water purifier (Millipore–Merck Millipore), orbital shaker (Thermo scientific).

METHODOLOGY

Preparation of Buffer

Accurately weigh and transfer about 1.3gm of potassium dihydrogen phosphate into 1000ml of milli-Q water, dissolve completely and adjust pH to 3.0 + 0.05 with orthophosphoric acid and filter the solution though 0.45µm membrane filter and degas for10min.

Preparation of Mobile Phase

Use buffer and acetonitrile in the ratio of 60:40 as mobile phase.

Preparation of Diluent: Prepare a mixture of water and acetonitrile in the ratio of 20:80 v/v.

Preparation of stock and standard solutions

Accurately weigh and transfer about 50mg of Ticagrelor working standard into 100ml volumetric flask. Add 60ml of diluents, and sonicate to dissolve. Cool the solution to room temperature and dilute to the volume with diluent. Pipette out 5ml of above standard stock solution and transfer into 50ml volumetric flask and dilute upto the mark with diluents. The concentration obtained is 50µg/ml of Ticagrelor.

Preparation of stock and test solutions

Accurately weigh and crush 20 tablets, take powder equivalent to 50mg of ticagrelor into a 100ml volumetric flask. Add about 60ml of diluent; shake on orbital shaker for 15min. Ensure complete disintegration of tablets. Add 40ml of diluent, sonicate for 30min with occasional shakings. Take 5ml of the supernatant solution from the middle of volumetric flask and transfer to 50ml volumetric flask and dilute to volume with diluents and mix. Filter through 0.45µmNylon filter.

Optimized conditions

PARAMETERS	SPECIFICATION
Stationary phase	Develosil ODS UG-5 C18
	(150 X 4.6mm, 5µ)
Flow rate	1.0 ml/min
Wavelength	280 nm
Injection volume	10µl
Column oven temperature	35^0C
Mobile phase	Buffer (KH2PO4, pH 3.0) : ACN = (60:40)
Diluent	Milli - Q water : ACN (20:80)
Run time	12 min

Method Validation

a. Specificity

The specificity of the method was determined by checking the interference of placebo with analyte and the proposed method was eluted by checking the peak purity of Ticagrelor during the force degradation study. 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.

b. Linearity of the HPLC method

Calibration curve was constructed by plotting concentrations of Ticagrelor vs. peak areas, and the regression equations were calculated. The linearity of this method was investigated by injecting the Ticagrelor solutions in the concentration range 20-80 μg/ml (corresponded to 40% to 160%, respectively of the test solution concentration) in duplicate.

c. Accuracy (recovery)

Accuracy of the method was studied by recovery. The % recovery of the method was calculated at three different concentration levels corresponding to 50%, 100% and 150% withaddition of 25, 50, 75 μg/ml concentration softestpr eparation and amount of Ticagrelor recovered was estimated. For each level, three sets were prepared and injected in duplicate.

d. Precision (repeatability)

Precision of the assay method was demonstrated by injecting six different sample solutions containing Ticagrelor equivalent to 50μg/ml and RSD of mean assay value was calculated.

- ***Intraday Precision:*** Intraday precision was demonstrated by injecting six different sample solutions containing Ticagrelor equivalent to 50μg/ml at different time intervals within the same day and RSD of mean assay value was calculated.

- ***Intermediate precision (ruggedness):*** Intermediate Precision of the method was demonstrated by carrying out the experiment on different day, by different analyst and on different instrument using different C-18 column.

e. LOD and LOQ

The limits of detection and quantification were measured based on the contrast of the standard deviation of the peak area and the slope of the calibration curve of Ticagrelor. LOD and LOQ of Ticagrelor were calculated using the following equations as per International Conference on Harmonization (ICH) guidelines.

LOD = 3.3 x σ/S

LOQ = 10 x σ/S

Where, σ = Standard deviation of response,

S = Slope of regression equation

f. Robustness

Robustness of the method was demonstrated by deliberately changing the chromatographic conditions. The flow rate of the mobile phase was changed from 1.0 ml/min to 0.9 ml/min and also from 1.0 ml/min to 1.1 ml/min. The composition of mobile phase was changed from acetonitrile: phosphate buffer pH-3.0(40:60v/v) to acetonitrile: phosphate buffer pH-3.0(38:62v/v) and also from acetonitrile: phosphate buffer pH-3.0(40:60v/v) to acetonitrile: phosphate buffer pH-3.0(42:58v/v). The solutions for robustness study were applied on the column in triplicate and the responses were determined.

g. Solution stability study

Stability of solution was evaluated for the standard preparation and the test preparation. The solutions were stored at 5° C and at ambient temperature, without protection of light.

Degradation study

The degradation samples were prepared by transferring powdered 50.01mg of Ticagrelor standard into a 250ml round bottomed flask. In chemical stress degradation study, samples were employed for acidic, alkaline and oxidant media and in physical stress degradation study, samples were employed for thermal and photolytic conditions. After the degradation treatments were completed, the solutions containing stress content were allowed to equilibrate at room temperature, if it was refluxed. The solution was followed by neutralization and diluted with diluent to attain 50.10μg/ml Ticagrelor concentrations. Specific degradation conditions were defined as follows, the mentioned conditions were the initial condition, at which drug was staRting to degrade.

Acidic hydrolysis

Acidic degradation study was performed by transferring 1ml of the standard solution, sample solution and placebo separately into 50ml round bottom flasks and 1ml of 1 N HCl was added to each flask and about 35ml of diluent was added, refluxed at room temperature for 3 hrs, then the mixture was neutralized with 1N NaOH solution. The resultant solutions were diluted to obtain 50 μg/ml solutions and 10 μl solutions were injected into the system and the chromatograms were recorded to assess the stability of standard, sample and placebo.

Alkaline hydrolysis

Alkaline degradation study was performed by transferring 1ml of the standard solution, sample solution and placebo separately into 50ml round bottom flasks and 1ml of 1N NaOH was added to the flasks and about 35ml of diluent was added, refluxed at room temperature for 5 hrs, then the mixture was neutralized with 1N HCl solution. The resultant solutions were diluted to obtain 50 μg/ml solutions and 10 μl solutions were injected into the system and the chromatograms were recorded to assess the stability of standard, sample and placebo.

Oxidative degradation

Oxidative degradation study was performed by transferring 1ml of the standard solution, sample solution and placebo separately into 50ml round bottom flasks and 1ml of 3% v/v H_2O_2 was added to the flasks and about 35ml of diluent was added, refluxed at room temperature for 4 hrs. The resultant solutions were diluted to obtain 50 μg/ml solutions and 10 μl solutions were injected into the system and the chromatograms

were recorded to assess the stability of standard, sample and placebo.

Neutral hydrolysis

Neutral degradation study was performed by transferring 1ml of the standard solution, sample solution and placebo separately into 50ml round bottom flasks and 1ml of H_2O was added to the flasks and about 35ml of diluent was added, refluxed at room temperature for 4 hrs. The resultant solutions were diluted to obtain 50 µg/ml solutions and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of standard, sample and placebo.

Thermal degradation

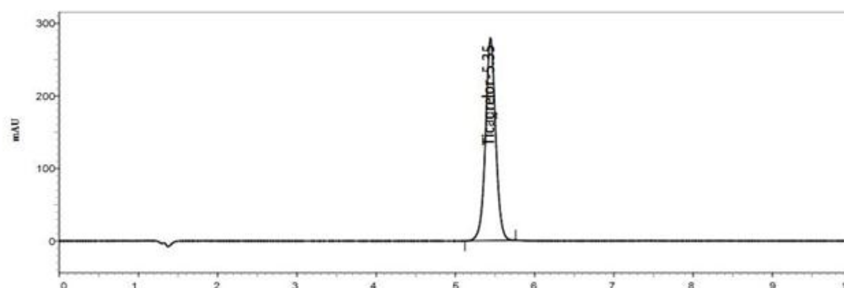
Thermal stress was performed using standard drug, test and placebo; by applying dry heat at 80˚C in a conventional oven for 10 hrs. The resultant standard drug, test and placebo solutions were diluted to obtain 50 µg/ml solutions and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of standard, sample and placebo.

Photolytic degradation

In accordance with the ICH guidelines, the drug samples, standard and placebo were placed at a distance of 9 in. from the light bank. Both fluorescent and UV lamps were turned on simultaneously. The samples were exposed for a total of 72hrs. The resultant test drug, standard and placebo were diluted to obtain 50 µg/ml solutions and 10 µl solutions and were injected into the system and the chromatograms were recorded to assess the stability of standard, test sample and placebo.

RESULTS AND DISCUSSION

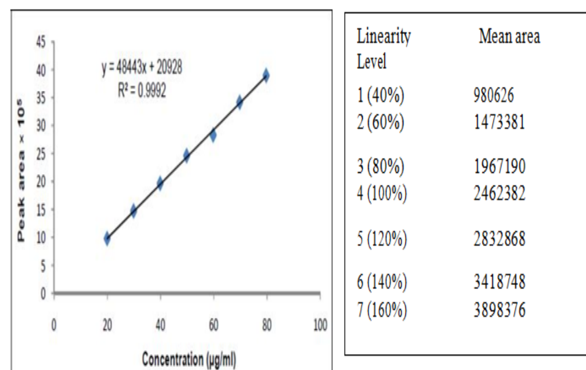
Table: System suitability.



System suitability data In-house limit	% RSD NMT 2.0	Theoretical plates NLT 3000	Asymmetry NMT 2.0
Specificity	0.52	8221	1.02
Linearity	0.43	8324	1.00
Limit of detection	0.34	8421	1.03
Limit of quantitation	0.41	8213	1.01
Method Precision	0.43	8532	1.00
Intermediate Precision	0.40	8231	1.01
Accuracy	0.34	8315	1.00
Solution stability	0.27	8231	1.01
Robustness	0.46	8328	1.02

Method validation

Linearity: Seven points calibration curve were obtained by plotting Ticagrelor concentration against peak area, in the concentration range 20-80 µg/ml. Linear regression equation was found to be y=48443x+20928, correlation coefficient R^2= 0.9992, where the x-axis and y-axis indicate the concentration (µg/ml) and the peak area (milli absorbance). The correlation coefficient (r^2) itself shows linearity was reaching throughout the linear concentration range.



Linearity Level	Mean area
1 (40%)	980626
2 (60%)	1473381
3 (80%)	1967190
4 (100%)	2462382
5 (120%)	2832868
6 (140%)	3418748
7 (160%)	3898376

Fig. 6.1.12: Linearity curve for Ticagrelor.

Accuracy Study

Accuracy was assessed by determination of the recovery of the method at three different concentrations. Known amount of Ticagrelor was added to check the accuracy of the method as well as solution preparation. The measured observations were compared with the actual value. The % recovery of the method was calculated at three different concentration levels corresponding to 50%, 100% and 150% with concentration 25, 50, 75 µg/ml of test preparation were added and amount of Ticagrelor was recovered. For each level, three sets were prepared and injected in duplicate. The mean recovery of Ticagrelor was 99.19%, 99.71% and 97.86% respectively (Table 6.1.6). The % recovery was calculated using equation.

Accuracy level	Set No.	Amount added (mg)	Amount found (mg)	Recovery (%)	Mean recovery (%)	SD	RSD (%)
50%	1	25.17	25.18	100.06	101.76	1.47	1.44
	2	25.41	26.07	102.64			
	3	25.52	26.17	102.57			
100%	1	50.51	50.25	99.48	99.32	0.14	0.15
	2	50.83	50.46	99.28			
	3	50.92	50.51	99.20			
150%	1	74.97	76.02	101.40	101.87	0.41	0.40
	2	75.02	76.55	102.04			
	3	75.21	76.84	102.16			

***Precision Study*:** Precision study was established by evaluating method precision and intermediate precision study. Method precision of the analytical method was determined by analyzing six sets of sample preparation. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation was calculated. Intermediate precision of the analytical method was determined by performing method precision on another day by another analyst under same experimental condition. Assay of all six replicate sample preparations was determined and mean % assay value, standard deviation, % relative standard deviation was calculated.

Tableprecision results

Study	Set No.	Assay (%)	Mean assay (%)	SD	% RSD	95 % Confidence Interval
Method precision	1	101.4	101.5	0.28	0.28	0.30
	2	101.7				
	3	101.4				
	4	101.6				
	5	100.9				
	6	101.7				
Intermediate precision	1	101.5	101.4	0.47	0.46	0.49
	2	101.7				
	3	101.6				
	4	100.4				
	5	101.4				
	6	101.3				
Overall	Mean	101.4	Absolute difference between mean %			
	Stdev	0.38	assay values of Method precision and			
	RSD %	0.38	Intermediate precision= 0.1			

Limit of detection and Limit of quantitation study

S. NO	Ticagrelor	
1	LOD	0.05 ppm
2	LOQ	0.15 ppm

Robustness Study

The robustness of the method was evaluated by assaying test solutions after slight but deliberate changes in the analytical conditions. The factor chosen for this study were the flow rate (±0.1 ml/min), mobile phase composition [acetonitrile-phosphate buffer (38: 62 and 42: 58), v/v], and using different lots of HPLC column.

Table: Robustness results.

Robustness Parameter	Mean Area(n=6)		Standard Deviation	%RSD
	Standard	Test	(SD)	
Flow rate (0.9 ml/min)	2690130	2752312	10415.03	0.43
Flow rate (1.1 ml/min)	2164035	2223689	8060.53	0.34
KH_2PO_4 : ACN (62:38V/V)	2492386	2477019	9679.92	0.40
KH_2PO_4 : ACN (58:42V/V)	2486997	2475109	7999.48	0.33
Column: 1	2491837	2473538	6838.18	0.43
Column: 2	2486416	2478631	17117.05	0.52

***Solution Stability Study*:** Stability of solution was evaluated for the standard preparation and the test preparation. The solutions were stored at 5° C and at ambient temperature, without protection of light and tested after 12, 24, 36 and 48 hrs. The responses for the aged solution were evaluated by comparison with freshly prepared solutions. During study of the stability of stored solutions of standards and test preparations for assay determination the solutions were found to be stable for up to 36 hrs. Assay values obtained after 36 hrs were statistically identical with the initial value without measurable loss. Table 6.1.9 shows the summary of solution stability study.

Table solution stability study

Time intervals	Absolute difference in assay for standard solution,		Absolute difference in assay for test solution, %	
	At 5°C	At room temperature	At 5°C	At room temperature
After 12 hrs	1.63	1.66	0.01	0.21
After 24 hrs	1.51	1.71	0.26	0.36
After 36 hrs	1.69	1.92	1.50	1.70
After 48 hrs	2.02	2.45	2.06	2.26

DISCUSSION

A simple, precise and accurate RP-HPLC method has been developed and validated as per ICH guidelines. An isocratic separation was achieved using a Develosil ODS UG-5 C18 (150 X 4.6mm, 5μ particle size) columns with a flow rate of 1 ml/min and using a PDA detector to monitor the elute at 280 nm. The mobile phase consisted of potassium dihydrogen phosphate buffer: acetonitrile (60:40, v/v) with pH 3.0 adjusted with phosphoric acid. The method was validated for specificity, linearity, accuracy, precision, limit of detection and limit of quantitation, robustness, solution stability and system suitability. The specificity of the method was determined by assessing interference from the placebo and by stress testing of the drug (forced degradation). The method was linear over the concentration range of 20-80 µg/ml (r^2 = 0.9992) with a limit of detection and quantitation of 0.05 and 0.15 µg/ml respectively. Intraday and interday system and method precision were determined and accuracy was between 99.3-101.9 %. The method was found to be robust and suitable for assay of Ticagrelor in a tablet formulation. Degradation products resulting from the stress studies did not interfere with the detection of Ticagrelor and the assay is thus stability-indicating. The developed liquid chromatography method was stability indicating which suggest least storage condition of Ticagrelor drug substance as well as a drug product. Stability of Ticagrelor in present dosage form and validation procedure was recognized through the employment of ICH suggested stress condition. Hence, the method is useful for routine quality control analysis and also for determination of stability.

CONCLUSION

The developed RP-HPLC method for estimation of Ticagrelor in tablet dosage form is precise, accurate, specific, and sensitive. The protocol was written for stability indicating assay and dissolution testing, and then a formal method validation was performed. The formal method validation included specificity, linearity, accuracy, precision, intermediate precision, robustness of extraction procedure, filter study, force degradation, solution stability, and robustness testing for assay and dissolution. The robustness testing included alterations in wavelength, pH of buffer, mobile phase ratio, flow rate, and column change. The newly developed method has a faster analysis time.

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