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Development and Validation of RP-HPLC Method for the Quantitation of Ticagrelor Using Box-Behnken Experimental Design

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ABSTRACT

For determination of ticagrelor, a high performance stability indicating liquid chromatographic method was developed, validated and optimized for the determination of quality in any quality control laboratory. The drug was subjected to stress alkaline, acidic, oxidative, thermal, Neutral and photo-degradation and was found to hydrolyze in acidic, alkaline conditions as well as under oxidative stress. Ticagrelor with the interferents are separated on a reversed phase Cosmosil (R) 5C8-MS (5 µm, 250 mm × 4.6 id) column using acetonitrile: 35 mM ammonium acetate buffer (pH 3.8) in the ratio of 40:60 as mobile phase at a flow rate of 1 ml min-1. The drug was detected at 254 nm over a concentration range of 5–140 µg ml−1 with mean percentage recovery 100.08% using a PDA detector. The method was validated and a 3^2 factorial was employed by using Box–Behnken experimental design for the validation of robustness. These designs have three factors such as mobile phase composition, flow rate and pH while peak area and retention time were taken as a response. This showed that little changes in the mobile phase and flow rate affect the response while pH has no effect. The compilation of precision result shows % RSD for system precision, and method precision is 0.4. The developed method was found to be precise, accurate, linear, robust and rugged. This method was successfully applied for the assay of commercially market tablet formulation; hence it can be adopted for the determination of quality in any quality control laboratory.

Keywords: Ticagrelor, Stability-indication methods, Linear regression analysis, Design of experiment

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INTRODUCTION

Environmental factors, such as temperature, pH, buffer species, ionic strength, light, oxygen, moisture, additives and excipients, can play an important role in the stability of drug substances. Stress testing can assist in identifying degradation products and furnish important info about the intrinsic stability of drug substances [1,2]. With the advent of the International Conference on Harmonization (ICH) guidelines [3], requirements for the establishment of stability indicating methods have become more clearly mandated. The guidelines explicitly require the conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. The method is expected to permit analysis of individual degradation products.

Ticagrelor (1S,2S,3R,5S)-3-[7-[(1R,2S)-2-(3,4-Difluorophenyl) cyclopropylamino]-5-(propylthio)-3H-[1,2,3] triazolo[4,5-d]pyrimidin-3-yl]-5-(2-hydroxyethoxy) cyclopen- tane -1,2-diol, (figure 1) is an inhibitor of platelet aggregation and activation mediated by $P2Y_{12}$-ADP-receptor [4]. The ticagrelor and its major metabolite are approximately equipotent and exert their action by interacting reversibly with oral $P2Y_{12}$-adenosine diphosphate (ADP) to block signal transduction. It brings down the rate of thrombotic cardiovascular events in patients with acute coronary syndrome (ACS). It may work better for patients with genetic variations regarding the enzyme CYP2C19 as it does not require hepatic activation [5–7].

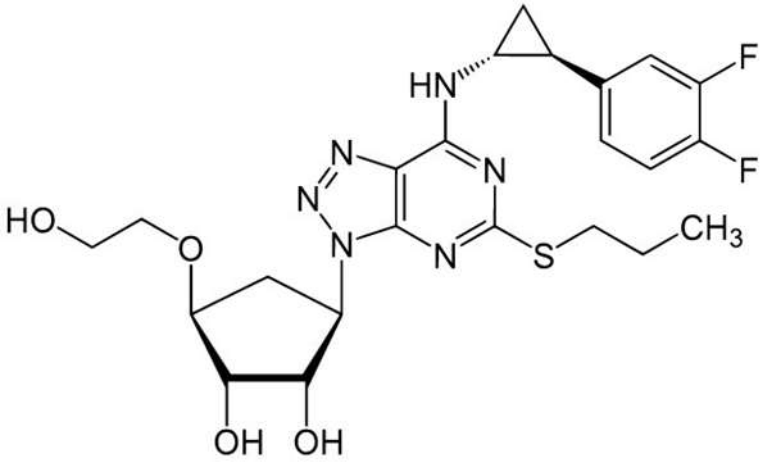


Figure 1: The Structure of Ticagrelor

Literature revels various analytical methods (high performance liquid chromatography (HPLC)) were reported for the determination of ticagrelor in biological matrices which includes, ticagrelor in human plasma [8,9], mice and rat plasma [10], ticagrelor and its metabolite deshydroxyethoxy ticagrelor in human plasma [11]. Recently chromatographic methods were developed in

pharmaceutical dosage forms [12–15]. However, a thorough literature search revealed no report of any stability-indicating analytical method for determination of ticagrelor whose robustness was tested by using design of experiment. Hence an attempt has been made to develop a sensitive, accurate, linear, precise, reproducible, repeatable and specific analytical method whose robustness was to be tested using design of experiment for the determination of ticagrelor in the presence of their degradants and also capable to separate all the major degradant peaks from each other.

Therefore, the present study was involved in a research effort aimed at developing and validating a simple, specific, accurate and precise new stability-indicating HPLC method for determination of ticagrelor for use in stability studies and quality control applications.

MATERIALS AND METHOD

Instrumentation and Chromatographic Condition

The high performance liquid chromatography (HPLC) system consisted of two pumps (Shimadzu Corporation, Tokyo, Japan, LC-6AD/7A Pump) and a manual injector (Model 7725i, Oak Harbor, WA, USA) with 20 µL capacity per injection. The photo diode array detector (PDA) detector (Shimadzu Corporation, Tokyo, Japan, SPD-M20A) was operated at a wavelength of 254 nm and controlled using CBM-20A system controller. The software used for chromatography was Shimadzu Lab Solution Lite release 5.52 software. Chromatographic separation of ticagrelor was achieved at ambient temperature using a Cosmosil (R) 5C8-MS (5 µm, 250 mm × 4.6 id) column (Nacalai USA, Inc., San Diego, CA, USA) analytical column; the mobile phase consisted of acetonitrile–ammonium acetate (pH 3.8; 35 mM) in the ratio 40:60 v/v. The mobile phase was filtered through a 0.2 µm nylon membrane filter (Millipore, Milford, MA, USA) and sonicated for 20 min prior to use at a flow rate of 1.0 ml min-1. Injection volume was 20 µl, and the optimum wavelength selected for quantification was 254 nm. Acetonitrile was used as diluent. An Equip-Tronics EQ 621 pH meter (Mumbai, Maharashtra, India), equipped with a combined glass electrode was used for pH measurement.

Chemical and Reagents

Pharmaceutical grade ticagrelor was used and certified to have a purity of 99.8%. Acetic acid glacial, Ammonium Acetate, Sodium Hydroxide purchased from Merck Specialties Private Limited (Mumbai, India) while Hydrochloric acid, Hydrogen Peroxide solution was acquired from Loba Chemie Pvt. Ltd. (Mumbai, India) and all of these materials were of analytical grade. Acetonitrile (HPLC grade) was purchased from Merck Specialties Private Limited (Mumbai, India). Milli-Q^{TM} water (Millipore) was used in complete work.

The commercial BRILINTA® tablets (Lot no: TDEL) used was manufactured by AstraZeneca Operations, AB Sweden, Sweden. In India it is imported and marketed by AstraZeneca Pharma India Ltd. Bangalore, India and labelled to contain 90 mg per tablet.

Standard Solutions

Standard stock solutions of ticagrelor were prepared in concentrations of 1 mg/ml by transferring 10 mg portions of ticagrelor powder to 10 ml volumetric flasks, and dissolving it in with diluent; the volume was then made up to the mark with diluent. A working standard solution having a concentration of 30 µg/ml was prepared from Stock by appropriate dilution with the acetonitrile.

Preparation of Degradation Product

Samples of alkaline and acidic degradation products were prepared by accurately weighing 10.02 mg of ticagrelor powder and transferring it to a 10 mL round-bottomed flask. The drug was dissolved with acetonitrile and volume was made using the appropriate strength of NaOH/ HCl so that to obtain 0.1M NaOH/ HCl strength in 10mlL, heated at $60^{O}C$ for 3h and the solutions were neutralized appropriately. Working degradation product solutions were prepared by appropriate dilution using the diluent. Oxidative degradation was performed by dissolving 10 mg of ticagrelor powder in acetonitrile and subsequently adding H_2O_2 to make up 10mL of strength 1%, the resultant solution was kept at room temperature for 3h. For testing, 300 µL of this solution was diluted to 10 ml by the diluent.

For thermal and photo-degradation, two portions of ticagrelor powder (10 mg each) were spread as thin films in two separate Petri dishes (5 mm diameter). One portion was heated for forty-eight hours in an oven at $70^{O}C$; the other portion was exposed to a UV lamp producing UVA radiation for 7 days. Solutions for each, having concentrations of 1 mg/ml in diluent were prepared and subsequently 30 µg/ml in diluent were prepared for HPLC testing.

Method Validation

The method validation was performed in reference to the ICH method validation guideline. The parameter studied were: linearity, precision, accuracy, robustness and specificity.

Construction of Calibration Curve

Aliquots of ticagrelor working standard solutions were transferred to a series of 10 ml volumetric flasks and diluted to volume with the mobile phase to yield solutions in the concentration range of 5–140 µg/ml. A 20µl volume of each solution was injected in triplicate and chromatographed under the previously mentioned chromatographic conditions. The average peak area obtained for each concentration was plotted versus concentration.

Robustness study

In the present study, three factors, namely the flow rate (± 0.2 ml/min), pH of the mobile phase (± 0.2 units) and Organic phase composition (± 5 %) had been deliberately changed and robust data using Design Expert software version 9.0 (stat Ease Inc., USA), was generated and studied.

Assay for dosage form

Twenty BRILINTA® tablets were accurately weighed and two were finely powdered. An accurately weighed amount of the powdered tablets equivalent to 10 mg was transferred to 10 ml volumetric flasks, to which 5ml of acetonitrile was added. The sample was sonicated for 30 min, made up to volume with diluent, and filtered. Appropriate dilutions with the diluent were carried out to obtain solutions having concentrations of 30 µg/ml and analyzed.

RESULTS AND DISCUSSION

Ticagrelor (Figure 2) was subjected to different stress conditions and HPLC method were developed for subsequent analysis. The experimental conditions for the method was studied and adjusted to separate the drug from its degradation products. The chromatograms of ticagrelor and its degradation products obtained on stressed acidic, alkaline and oxidative decomposition are shown in Figs. 3, 4 and 5. Photo, thermal and neutral degradation chromatogram shows no degradation. The chromatogram obtained from acid degradation shows the degradant at Rt 7.751 and the main peak of ticagrelor at 7.033, while basic degradation shows degradants at Rt 3.720, 4.193, 5.122 and 8.453. In case of oxidative degradation peak at Rt 3.553 is degradation.

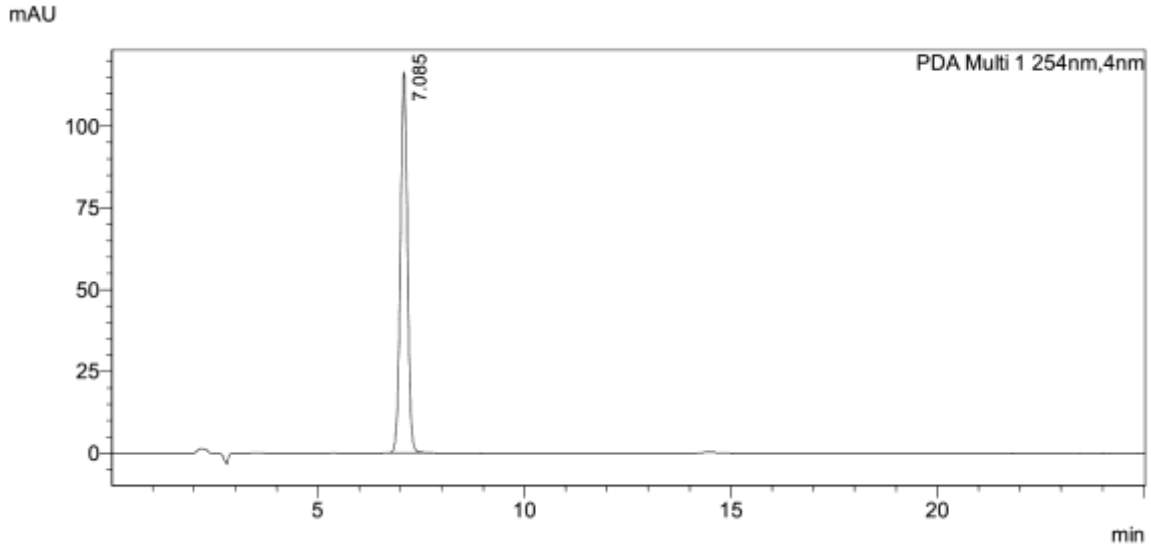


Figure 2: Typical Chromatogram Obtained form Ticagrelor

Optimization of Chromatographic Conditions

Different columns were investigated for separation of ticagrelor from it degradants; primarily the c18 column was tried with mobile phase containing methanol and water. Although the used

condition provides excellent peak for ticagrelor but they unable to separate ticagrelor from its degradants. Different salts in different ionic strength along with pH modifier at different pH strength were tried but unable to provide desired result. The use of c8 column with the acetonitrile and ammonium acetate salt at 35 mM ionic strength at pH 3.8 adjusted using pH modifier 2M acetic acid at ratio of 40: 60 v/v provided with acceptable system suitability parameter and results. The highest selectivity was obtained using 254 nm for detection.

Method Validation

The developed method shows excellent results for system suitability parameter; the value for tailing factor, capacity factor and number of theoretical plates (USP) were 1.015, 2.190 and 7999 respectively. Limit of detection and quantitation were 0.12 µg/ml and 0.38 µg/ml. The ICH guidelines for method validation was followed throughout the validation.

Linearity

The linear regression data for the calibration curves (n = 8 in triplicate) showed a good linear relationship over a concentration range of 5-140 µg/ml (correlation coefficient, r = 0.9994; intercept at -28358.5; slope is 49389).

Repeatability and reproducibility

The intra- and inter-day precision was evaluated by assaying freshly prepared solutions in triplicate on the same day and on three successive days, respectively. The % Recovery and RSD% in Table 2 shows the ruggedness of the method; also there was no significant difference between their reproducibility. The methods are suitable for quality control of the drug.

Table 2: Precision of Proposed HPLC Method

	System Precision [a]	Method Precision [a]	Intermediate Precision Intraday [b]	Interday [b]	Different analyst [b]
Recovery (%)	1405841.5*	100.20	100.90	99.83	99.80
SD	9058.08	0.12	0.25	0.45	0.29
RSD (%)	0.64	0.40	0.82	1.51	0.96

* Peak Area [a] Mean from six analyses (n=6) [b] Mean from three analyses (n=3)

Method precision

Twenty tablets of BRILINTA® were weighed to get the average weight and then ground. An amount of powder equivalent to about 10 mg of ticagrelor into a 10 ml clean dry volumetric flasks add acetonitrile and sonicate to dissolve it completely and make the volume up to the mark with the diluent (Stock solution). Further dilution was done to obtain 30µg/mL concentration. The above procedure used for 6 different preparations and injected, % relative standard deviation

(%RSD) was calculated. From the data obtained the developed RP-HPLC method describes the method is precise (Table 2).

Accuracy

The accuracy of the method was determined by recovery experiments. Known concentration of ticagrelor was added to the fixed concentration of the tablet sample. Percentage recovery was calculated by comparing the concentration with the pre-analyzed tablet sample. The recovery study was performed in triplicate. This standard addition method was performed at 80%, 100%, 120% level and the percentage recovery was calculated by subtracting the total concentration from pre-analyzed sample concentration. The results shown in Table 3 shows recovery was in acceptable level.

Table 3: Summary of Accuracy Analysis

Level of Addition (%)	Amount added (mg)	Recovery (%)*	Mean Recovery (%)
80	72	100.09	100.08
100	90	99.81	
120	108	100.33	

***** Value correspond to mean of three observations

Table 4: System Suitability and Regression Analysis of Linearity

System Suitability		Linearity	
Retention Time (min)	7.085±0.06	Limit of Detection (LOD)	0.12 µg/ml
Tailing Factor	1.015		
Capacity Factor (K')	2.190	Limit of Quantitation (LOQ)	0.38 µg/ml
Number of Theoretical Plate (USP)	7999		
Peak Purity Index	0.999995		

Robustness

Robustness of test using experimental design. The capacities to remain unaffected by small and measured changes in chromatographic conditions like mobile phase composition, Flow rate, column temperature etc. is known as robustness [1]. To test the robustness Box-Behnken Design was applied in which 3-independent variables mobile phase (v/v % Acetonitrile) composition (U_1), Flow rate (U_2) (ml/min) and pH (U_3) and 2-dependent variables (response) peak area (cm 2) (V_1) as well as retention time (min) (V_2) were taken as robustness parameters. 17 runs were obtained and each suggested combination were run on the system and results were obtained (Table 5).

Table 5: Experimental Design for Robustness Testing Using Factors and Obtained Responses

Run	Factor 1 A: Acetonitrile %	Factor 2 B: Flow Rate ml/min	Factor 3 C:pH	Response 1 Peak area cm2	Response 2 Retention time min
1	45	0.8	3.8	1.29096E+006	7.1
2	35	0.8	3.8	1.30286E+006	7.11
3	40	1	3.8	1.36542E+006	7.08
4	40	1.2	3.6	1.31026E+006	7.12
5	40	1	3.8	1.36542E+006	7.08
6	40	1	3.8	1.36542E+006	7.08
7	40	1	3.8	1.36542E+006	7.08
8	45	1	3.6	1.30285E+006	7.11
9	40	1	3.8	1.36542E+006	7.08
10	35	1	4.0	1.33134E+006	7.12
11	40	1.2	4.0	1.3097E+006	7.1
12	40	0.8	3.6	1.32089E+006	7.11
13	35	1	3.6	1.32505E+006	7.1
14	40	0.8	4.0	1.32849E+006	7.1
15	45	1.2	3.2	1.29952E+006	7.13
16	35	1.2	3.2	1.30205E+006	7.11
17	45	1	3.4	1.29961E+006	7.12

Table 6: Chromatographic Condition and Range Investigated During Robustness Testing

	Low Level	High Level	Optimized Level
U_1= Composition of mobile phase (Acetonitrile %)	35	45	40
U_2= Flow rate (ml/min)	0.8	1.2	1.0
U_3= pH	3.6	4.0	3.8

To minimise the effect of uncontrolled changes which may bias the response, experiments were designed in a randomised manner. The experimental design suggested second degree quadratic equation with highest least square regression for V_1 (r^2 = 0.9707) and V_2 (r^2 = 0.8876) as compared to others. The model was evaluated for peak area and retention time on the basis of analysis of variance (ANOVA) to and whether it was significant or not. Both responses showed that results were significant. Equations for both responses were found to be as follows

V_1 (Peak Area) $= 6.084 \times 10^{005} + 1.143 \times 10^{005} U_1 + 1.655 \times 10^{006} U_2 + 2.730 \times 10^{006} U_3 + 2340.250\ U_1\ U_2 - 2384.75000\ U_1\ U_3 - 51006.250\ U_2U_3 - 1383.885\ U_1^2 - 7.993 \times 10^{005}\ U_2^2 - 4.026 \times 10^{005}\ U_3^2$

V_2 (Retention Time) $= 11.878 - 0.0590\ U_1 - 0.763\ U_2 - 2.038\ U_3 + 7.500 \times 10^{003}\ U_1\ U_2 - 2.500 \times 10^{003}\ U_1\ U_3 - 0.0625\ U_2\ U_3 + 7.500 \times 10^{004}\ U_1^2 + 0.344\ U_2^2 + 0.344\ U_3^2$

Table 7 shows that the ANOVA results were satisfactory for response V_1 and a predicted model F-value of 25.75 and a model p -value of 0.0001 were obtained. Which suggest that the model was

highly significant and indicates only a 0.01% chance that the model F-value was larger due to noise.

Table 7: ANOVA Results for Response V_1 (Peak area) Obtained from Experimental Design

Response V_1 (Peak area)								
Parameter	**SS**	**df**	**MS**	**F-Value**	**p-Value**	**Model F-value**	**Model p-value**	**Prob>F**
Acetonitrile %	5.841	1	5.841	11.06	0.0127	25.75	0.0001	Significant
Flow rate (ml/min)	5.873	1	5.873	1.11	0.3267			
pH	1.271	1	1.271	0.24	0.6388			

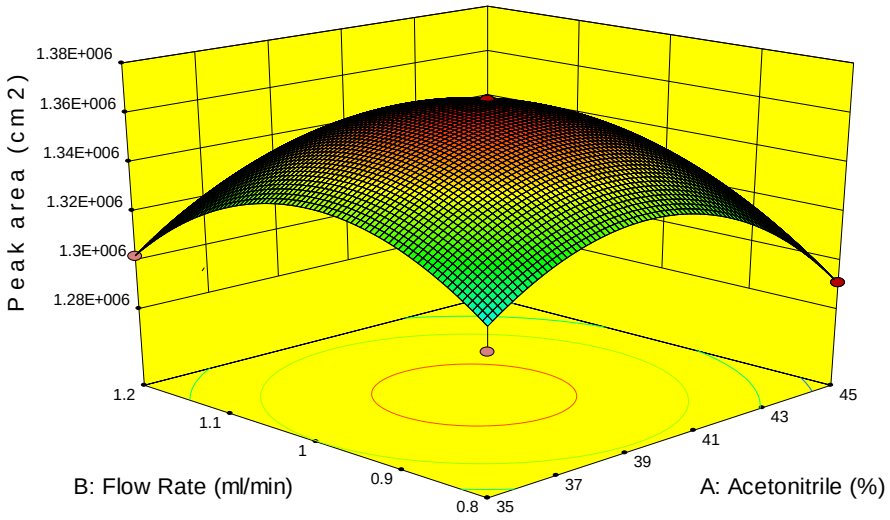
While Table 8 shows that a model F -value of 6.14 and a model p-value of 0.0129 were obtained, this predicts a 1.29% chance that the model F -value was larger due to noise. This indicates that the predicted value of both responses were in approximate to each other.

Table 8: ANOVA Results for Response V_2 (Retention time) Obtained from Experimental Design

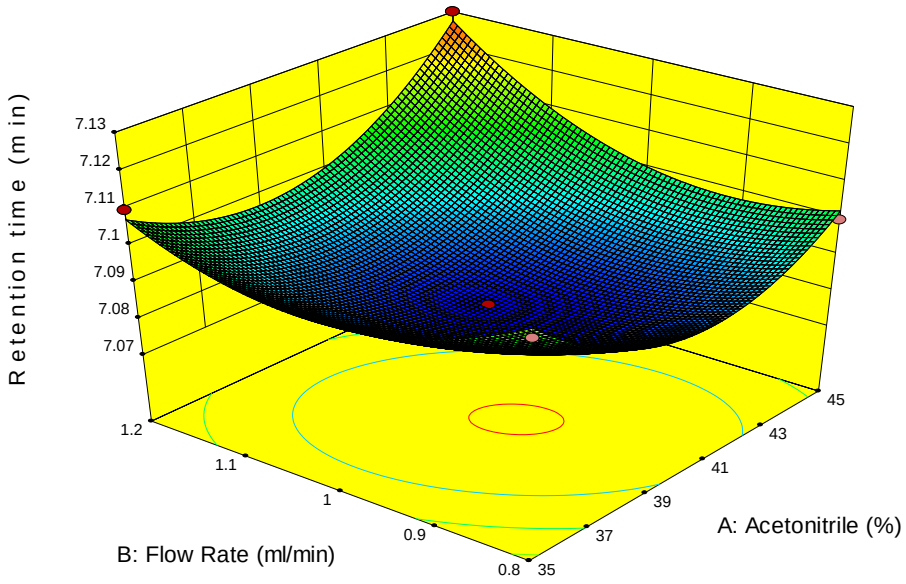
Response V_2 (Retention time)								
Parameter	**SS**	**df**	**MS**	**F-Value**	**p-Value**	**Model F-value**	**Model p-value**	**Prob>F**
Acetonitrile %	5.0	1	5.0	0.70	0.4304	6.14	0.0129	Significant
Flow rate (ml/min)	2.0	1	2.0	2.80	0.1382			
pH	0.0	1	0.0	0.00	1.000			

Figure 6 shows the effect of individual factors on responses ($V_1$and V_2). The factors U_1 and U_2 show the effect on both responses while factor U_3 has no effect. Further interaction of the factors was checked for both responses which suggest that U_1 (% Acetonitrile) and U_2 Flow rate interact and affect the responses. Figure 6 A and B

The one factor interaction study showed that the composition of the mobile phase i.e. U_1 (% Acetonitrile) was involved in interaction while the two factor interaction study revealed that both U_1 and U_2 gave a mixed response but U_3 was not involved in any interaction. Hence pH was found to be the actual factor which can be easily evaluated from the contour plot. Figure 7 shows the 3D surface responses of V_1 and V_2.

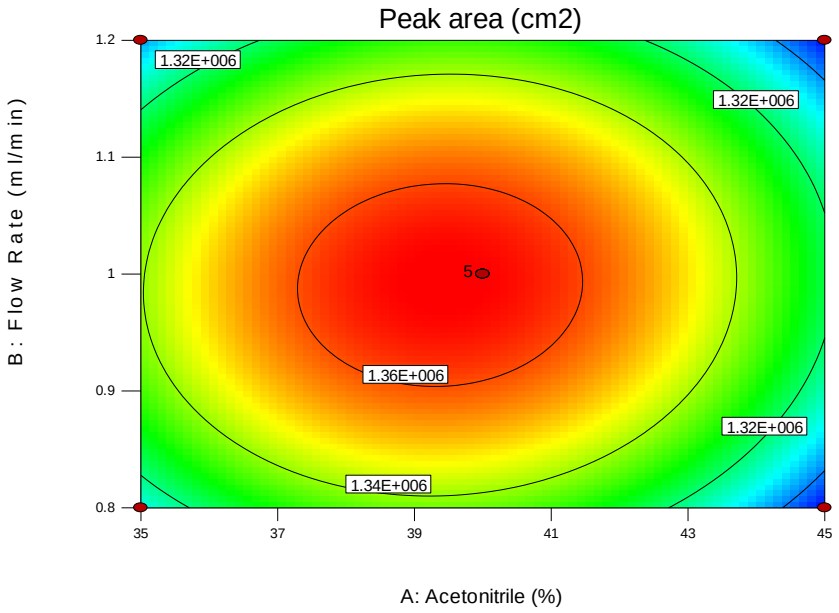


(A)

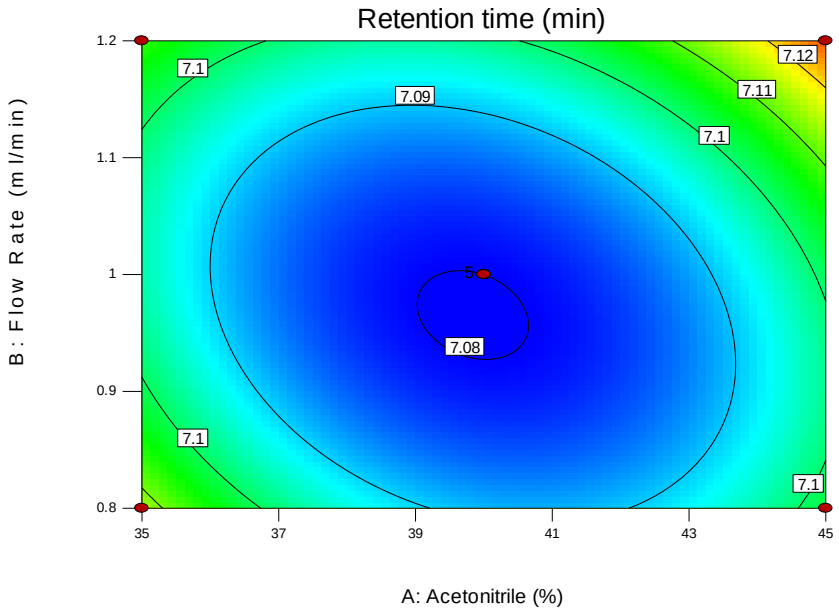


(B)

Figure 7: Three-Dimensional Response Surface for Effects of Individual Factors On Responses (V_1 and V_2); (A) for Peak Area (V_1) and (B) for Retention Time (V_2)



(A)



(B)

Figure 6: Contour Plots for Effects of Individual Factors on Responses (V_1 and V_2); (A) for Peak Area (V_1) and (B) for Retention Time (V_2)

Specificity

The specificity of the method was illustrated by the complete separation of ticagrelor from different degradation products as shown in Figures 3, 4 and 5 and Table 1.

Table 1: Results from Analysis of Samples from The Forced Degradation Study, Showing Degradant Retention Time and Percentage Degradation of Ticagrelor

Stress condition and duration	Retention time of degradants	Degradation (%)
Acidic/ 0.1M HCl/60^{O}C/ 3 h.	7.751	15.062
Basic/ 0.1M NaOH/60^{O}C/ 3 h	3.720;4.193;5.122;8.453	13.818
Oxidizing/ 1% H2O2 / 3h/ RT	3.553	10.251
Neutral/ H2O/ 60^{O}C/ 3 h	No degradation	
Thermal/ 70^{O}C/48 h	No degradation	
UV Light/ UVA / 7 days	No degradation	

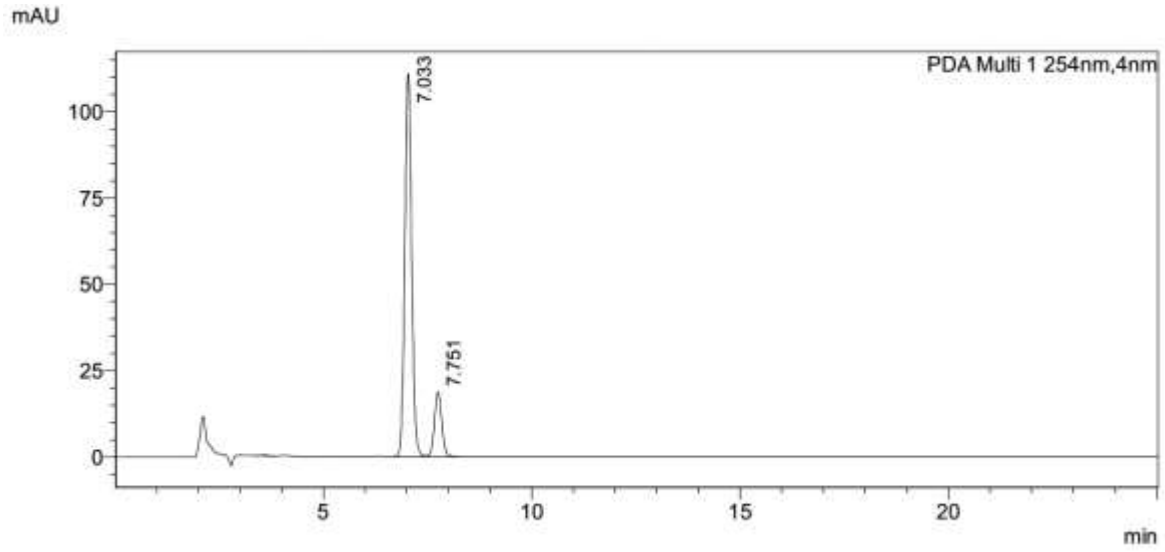


Figure 3: Typical Chromatogram Obtained After Degradation of Ticagrelor Under Acidic Condition

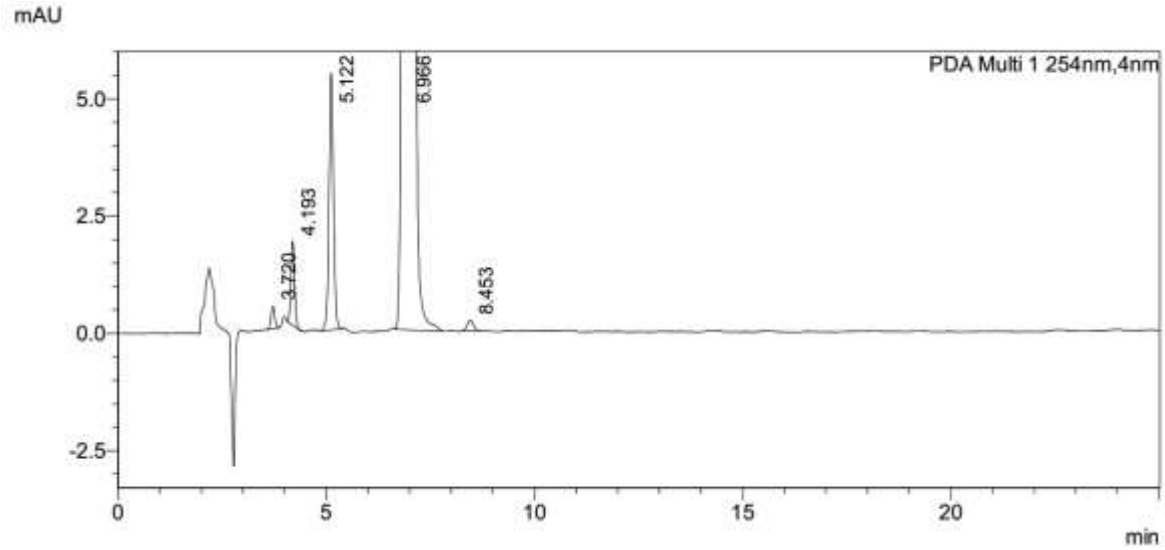


Figure 4: Typical Chromatogram Obtained After Degradation of Ticagrelor Under Basic Condition

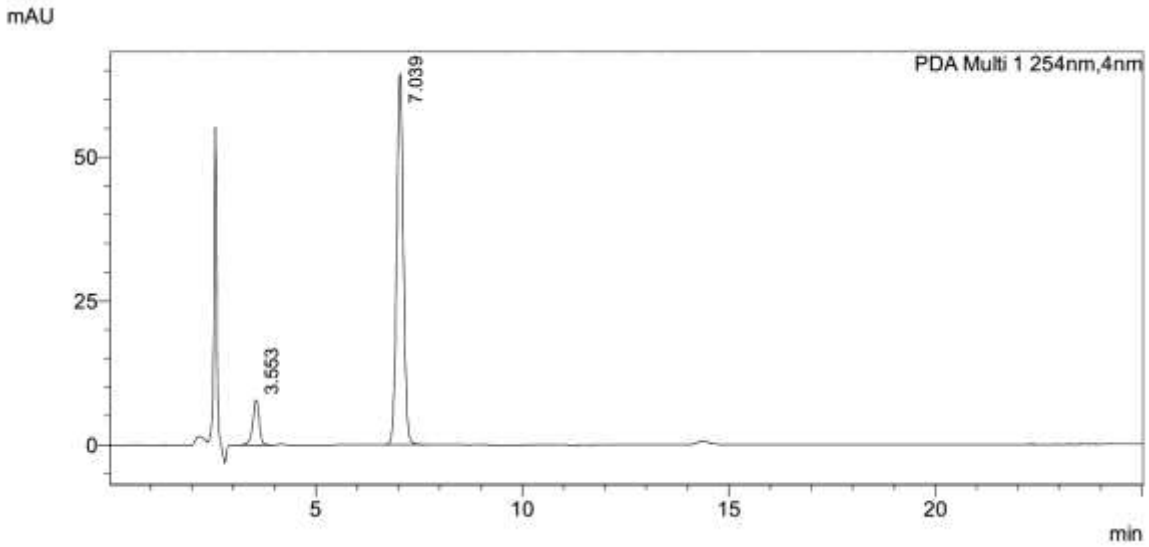


Figure 5: Typical Chromatogram Obtained after Degradation of Ticagrelor Under Oxidizing Condition

Assay of pharmaceutical formulations

The proposed method was applied to the determination of ticagrelor in commercial tablets. The results were satisfactory and were in good agreement with the labelled amount (99.1 %w/w).

CONCLUSION

The method is stability indicating and it can be used for the routine of ticagrelor in pharmaceutical dosages form and bulk drug. The results obtained from the validation of method were satisfactory. The regulatory requirements for accuracy, precision, sensitivity, selectivity, stability and ruggedness were followed and accomplished. The applied Box-Behnken design (3^2-Factorial) for optimization of robustness parameters shows that a small change in flow rate and mobile phase composition dose not significantly affect responses i.e. peak area and retention time and hence the method is robust.

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