RESEARCH PAPER

AQbD-Driven Development of a Sustainable RP-HPLC Method for Concurrent Quantification of Ticagrelor and Rivaroxaban: A White Analytical Chemistry Perspective

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ABSTRACT

Background/Objectives: A robust, sensitive reversed-phase high-performance liquid chromatographic method was developed and validated for the simultaneous estimation of ticagrelor and rivaroxaban in bulk and synthetic mixture using an Analytical Quality by Design approach.

Methods: A risk-based approach was used to develop the method in order to identify an operable design region. 3-level factorial design optimized important parameters including the ratio of acetonitrile in the mobile phase (A), flow rate (B) and column oven temperature (°C) that were auto-generated by using Design Expert 10.

Results: The separation was performed on a C18 column (250 × 4.6 mm, 5 µm) using acetonitrile: methanol (60: 40 v/v) as the mobile phase at 0.8 mL/ min with detection wavelength of in UV 245 nm. For ticagrelor and rivaroxaban, retention times were around 3.56 and 6.69 min, respectively with good resolution and minimal tailing. The method was validated as per ICH Q2(R2) guidelines and found to be linear in the range of 45-135 µg/mL (ticagrelor) and 2.5-7.5 µg/mL (rivaroxaban) having correlation coefficient (R^2) >0.999. The accuracy (98.2 to 101.4 %), precision (%RSD < 2) and robustness of the method shown in the method operable design region were excellent. Results of environmental and operational evaluations based on criterion AGREE, Eco-Scale, and the RGB model demonstrated better greenness, less use of resource materials and better user-friendly features than the reported methods.

Conclusion: The research effectively illustrates the use of analytical quality by design thoughts in developing robust, compliant and green analytical method for the routine analysis and quality control ticagrelor and rivaroxaban combinations pertinent to the treatment of thromboembolic disorders

Keywords: Ticagrelor; Rivaroxaban; RP-HPLC; Analytical Quality by Design; Design Expert 10; ICH Q2(R2)

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INTRODUCTION

Cardiac and thromboembolic diseases are the leading causes of morbidity and mortality worldwide. Current treatment options have gradually shifted toward a combination of antiplatelet and anticoagulant therapy to minimize the risk of ischemic events with an acceptable bleeding profile [1]. Ticagrelor (TICA), a reversible $P2Y_{12}$ receptor blocker, reduces adenosine diphosphate - induced platelet aggregation with strong and fast acting antiplatelet effects [1,2]. Rivaroxaban (RIVA) is a direct factor Xa inhibitor that inhibits both thrombin generation and clot formation via selective inhibition of the coagulation cascade [1,2]. Indeed, coadministration of these drugs has demonstrated clinical benefit in patients with atrial fibrillation, coronary artery disease and post-percutaneous intervention syndromes where inhibition of both platelet activation and fibrin clot formation is required [1–3].

Analytical quality by design (AQbD) has been proposed for development of robust and rugged analytical methods as a strategy supported by scientific principles and regulatory policies. In accordance with the concept of pharmaceutical QbD described in ICH Q8–Q14, AQbD contributes to achieving predefined objectives by gaining knowledge on method parameters and their influence over analytical performance [4–7]. This is based on establishing ATP (Analytical Target Profile), CMAs (Critical Method Attributes) and CMPs (Critical Method Parameters), using DoE (Design of Experiments) to set MODR (Method Operable Design Region). This allows analytical flexibility in a scientifically supported design space [8–13].

Recent developments of analytical science have brought the development and validation of analytical methods according to the principles of Green Analytical Chemistry (GAC), which has evolved towards an integrated approach, named White Analytical Chemistry (WAC): a unique concept aimed at developing more efficient environmentally friendly methods with user-friendly applications. This wider context builds on and goes beyond classical GAC, based on twelve holistic principles that cover the space between analytical performance and ecological and analytical practical considerations. The RGB (Red–Green–Blue) model is adopted for the evaluation: red implies the multiple analytical performance parameters including the method applicability, linearity

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range, sensitivity (LOD and LOQ), precision and accuracy; green means environment-friendly nature based on the core principles of Green Analytical Chemistry; while blue focuses on economically viable-running cost, ease of operation and time saving. Nowadays, more and more recently published papers followed WAC concepts demonstrating its increasing impact as a versatile approach to enable sustainable and high- performance analytical methods [8–10,14–17]. Green Analytical Chemistry (GAC) has been proposed as a structured approach for determining the environmental sustain ability of analytical methodologies. It includes assessment methods Analytical Eco-Scale and the Analytical GREEnness (AGREE) metrics to measure method greenness [12,16–19]. The focus is on achieving reduced reagent consumption and waste generation, decreased energy dissipation, and operator safety combined with preserved analytical accuracy, precision and reliability. By incorporating these concepts, GAC address to the generation of environmentally friendly tools that comply with today's safety and sustainability considerations.

Although these molecules are increasingly clinically relevant, only a few analytical methods [20,21] and UPLC-MS method [22] are available for their simultaneous determination in plasma, and bulk samples of TICA and RIVA. However, no any of the above method was based on AQbD strategy and white analytical chemistry assessment by RGB model.

Despite the fact that modern HPLC systems have not reached the ultrahigh performance level, RP-HPLC is still a method of choice in multicomponent analysis, because it provides high sensitivity and reproducibility for most matrices. In the present work, we have employed AQbD approach for the development and validation of RP-HPLC method for simultaneous estimation of TICA and RIVA in bulk as well as laboratory formed tablets. The critical parameters (acetonitrile ratio, flow rate and column oven temperature) were optimized by 3-level factorial design in Design Expert 10. The developed chromatographic conditions were validated as per ICH Q2 (R2) for linearity, precision, accuracy, sensitivity and robustness. The developed method was extensively evaluated in order to compare it with other reported analytical methods, as for instance validation; environmental impact; productivity and user-friendly. Various assessment methods have been used including the RGB model, AGREE (Analytical GREEnness) measurement and analysis with Eco-Scale Assessment (ESA). This multilayer approach guaranteed an unbiased assessment of the method's analytical quality and environmental compatibility.

The novelty of this work is reflected in the integration of AQbD and WAC principles for the first time in the development of a stability-indicating RP-HPLC method for simultaneous determination of TICA and RIVA. Unlike traditional approaches based on trial-and-error(s), the method provided here employs AQbD principles and a systematic approach for the determination of critical method attributes and the construction of a scientifically justified MODR using response surface methodology. This approach will serve to provide consistent and robust performance of the method in terms of reproducibility and compliance with the requirements of regulatory authorities. These attributes have not been demonstrated in the literature for the combination of drugs under study.

The developed method is a robust and lifecycle aligned analytical methodology that is focused on sustainability and is a first of its kind in many respects. Additionally, the methodology is reliable and will facilitate the development of formulations, and will enable quality control of the TICA and RIVA combinations used in the treatment of thromboembolic disorders.

2. Materials and Methods

2.1 Chemicals and Reagents

Pharmaceutical grade **TICA** and **RIVA** working standards were obtained as gift samples from a certified API manufacturer with a purity of 99.6 %. HPLC grade **acetonitrile, methanol,** water and disodium hydrogen phosphate anhydrous were procured from Merck (Mumbai, India). **Triethylamine** and **orthophosphoric acid** were used for pH adjustment. All solutions were filtered through a 0.45 µm nylon membrane and degassed prior to analysis.

2.2 Instrumentation

Chromatographic analysis was performed using a **Shimadzu Prominence HPLC system** equipped with a binary pump, an autosampler, and a **PDA detector.** Data were acquired and processed using **LC Solution software.** Separation was achieved on a Phenomenex **C18 column (250 × 4.6 mm, 5 µm)** maintained at a controlled column oven temperature. Analytical balance, ultrasonic bath, and pH meter were used during sample preparation.

2.3 Chromatographic Conditions

Optimal chromatographic separation of TICA and RIVA was achieved on a C18 column (250 × 4.6 mm, 5 µm) using a mobile phase comprising acetonitrile and methanol in the ratio of 60:40 (v/v). The analysis was carried out at a flow rate of 0.8 mL/min with a detection wavelength set at 245 nm, corresponding to the isoabsorptive point obtained from the overlain UV spectra of both analytes (**Fig. 1**). The injection volume was 10 µL, and the column oven temperature was maintained at 27 °C, ensuring consistent retention and peak symmetry.

2.4 Preparation of working Standard Solutions

Standard stock solutions of TICA (900 µg/mL) and RIVA (50 µg/mL) were prepared individually in methanol. Working solutions were obtained by suitable dilution with the mobile phase to yield final concentrations of TICA **45–135 µg/mL** and RIVA **2.5–7.5 µg/mL**.

2.5 Analytical Quality by Design (AQbD) Approach

AQbD focuses on the risk-based critical variables that impact the performance of the method in order to develop a novel, robust analytical method. According to International Conference on Harmonization (ICH) Q8(R2), ICH Q9guidelines, AQbD involves several steps, including a comprehensive demonstration of the quality attributes of the chromatographic method, which is known as the Quality Target Method Profile (QTMP) or Analytical Target Profile (ATP). Determining the Critical Method Attributes (CMAs) and Critical Method Parameters (CMPs) is a qualitative risk approach based on quality management theory. This chapter

describes how to analyze their impact on the determined CAAs using experimental designs, and then conducts an optimization in order to obtain MODR [23–25].

Table 1. 3^3 factorial design, 27 experimental runs selected after the risk assessment

Run	Actual Value			Coded Value		
	X1: Proportion of Acetonitrile	B: Flow rate	C: Column Oven Temperature	Factor 1	Factor 2	Factor 3
1	60	0.8	30	-1	-1	0
2	70	0.8	30	0	-1	0
3	80	1	35	1	0	1
4	80	0.8	35	1	-1	1
5	80	1.2	25	1	1	-1
6	80	1.2	35	1	1	1
7	60	1.2	30	-1	1	0
8	60	0.8	25	-1	-1	-1
9	70	1	30	0	0	0
10	60	1	35	-1	0	1
11	70	1.2	35	0	1	1
12	60	1	30	-1	0	0
13	70	0.8	35	0	-1	1
14	70	1	35	0	0	1
15	70	1.2	30	0	1	0
16	70	1.2	25	0	1	-1
17	70	1	25	0	0	-1
18	70	0.8	25	0	-1	-1
19	60	1.2	25	-1	1	-1
20	80	1	25	1	0	-1
21	80	0.8	25	1	-1	-1
22	60	0.8	35	-1	-1	1
23	80	1.2	30	1	1	0
24	80	0.8	30	1	-1	0
25	60	1.2	35	-1	1	1
26	80	1	30	1	0	0
27	60	1	25	-1	0	-1

2.5.1 Analytical Target Profile (ATP)

The ATP was defined as the development of a precise, accurate, linear, and robust RP-HPLC method capable of quantifying TICA and RIVA simultaneously in bulk and laboratory prepared tablets with resolution ≥ 2.0, tailing factor ≤ 2.0, and %RSD ≤ 2.0 across analytical runs.

2.5.2 Identification of CMAs and CMPs

Based on prior knowledge and preliminary trials, **Critical Method Attributes (CMAs)** are **retention time, tailing factor, and resolution,** and that those for **Critical Method Parameters (CMPs)** included **percentage of acetonitrile (A), flow rate (B),** and **column oven temperature (C).**

2.5.3 Risk Assessment

A risk assessment was conducted using an Ishikawa (fishbone) diagram to identify factors significantly impacting method performance. Mobile phase composition, flow rate, and column oven temperature were found critical, while injection volume and detection wavelength were less influential.

2.5.4 Experimental Design

DoE is a statistical tool for the optimization of chromatographic parameters. After the risk analysis, the high-risk level factors were chosen to be studied using a d-optimal experimental design with 3 levels and 3 central points. DoE is utilized for the evaluation of the relationships between input factors and output responses (Y1: Retention time of TICA, Y2: Retention time of RIVA, Y3: Tailing factor of TICA, Y4: Tailing factor of RIVA, Y5: Resolution) by using response surface methodology (RSM). A **3-level factorial design (3^3)** was generated using **Design Expert version 10.** The independent variables and their coded levels were: A (% Acetonitrile): 60% (−1), 70% (0), 80% (+1), B (Flow rate): 0.8 mL/min (−1), 1.0 mL/min (0), 1.2 mL/min (+1), C (Column temperature): 25 °C (−1), 30 °C (0), 35 °C (+1) (Table 1). The experimental matrix comprised 27 runs with the combinations of factors at the chosen levels. Statistical evaluation of model terms (linear,

interactive, quadratic) was done through analysis of ANOVA, and polynomial equations were generated for each response.

2.6 Method Validation

The method was validated as per **ICH Q2(R2)** guidelines for linearity, precision, accuracy, sensitivity, robustness, and system suitability [26].

2.7 Assay of laboratory prepared tablet

A Ten tablets containing TICA (900 mg) and RIVA (50 mg), Hydroxypropyl methylcellulose (5.5 mg), Microcrystalline Cellulose (177 mg), talc (13 mg), Magnesium stearate (4 mg) was prepared. A tablet powder equivalent to TICA (90 mg) and RIVA (5 mg), transferred into 10 ml volumetric flask and dissolved in few ml of methanol. The solution was ultrasonicated for 15 min and filtered with 0.45µ Whatman filter paper no. 41 and the volume was made up to the mark with mobile phase. The solution was further diluted to obtain 90 µg/mL and 5 µg/mL of TICA and RIVA, respectively. The solution was injected in to the HPLC system on optimized chromatographic condition and analyzed using optimized method.

3. Results and Discussion

During preliminary chromatographic trials for TICA and RIVA, several mobile phase compositions and flow rates were evaluated to achieve optimum peak resolution and symmetry. Initial experiments conducted with varying acetonitrile, methanol and water in different proportions and flow rates (0.8–1.2 mL/min) revealed inadequate separation and occasional peak tailing. Adjustment of the organic phase concentration and column temperature significantly improved selectivity and reduced retention time variability. Based on these observations, a mobile phase consisting of acetonitrile and methanol (70:30 v/v, pH 4.0) at a flow rate of 1.0 mL/min and column temperature of 30 °C was selected as the optimized condition for further AQbD-based optimization and validation.

3.1 Application of AQbD for Method Development

A d-optimal experimental design was applied for the evaluation of the selected risk factors based on the results of risk assessment (X1: ratio of solvent, X2: flow rates, X3: column temperature). The 27 experimental runs were performed using the 3^3 factorial design according to which responses (Y_1–Y_5) were determined (Table 2).

Table 2. 3^3 factorial design, 27 experimental runs with measured responses

Run	A: Proportion of Acetonitrile	B: Flow rate	C: Column Oven Temperature	Y1 (R_t of TICA)	Y2 (R_t of RIVA)	Y3 (T_f of TICA)	Y4 (T_f of RIVA)	Y5 (Resolution)
1	60	0.8	30	3.707	6.842	1.107	1.536	4.162
2	70	0.8	30	3.599	6.714	1.176	1.603	4.264
3	80	1	35	3.614	6.731	1.291	1.673	4.321
4	80	0.8	35	3.647	6.742	1.317	1.693	4.328
5	80	1.2	25	3.621	6.748	1.288	1.689	4.327
6	80	1.2	35	3.594	6.711	1.249	1.648	4.303
7	60	1.2	30	3.627	6.777	1.076	1.509	4.128
8	60	0.8	25	3.722	6.866	1.119	1.559	4.197
9	70	1	30	3.564	6.689	1.141	1.586	4.243
10	60	1	35	3.658	6.795	1.098	1.499	4.121
11	70	1.2	35	3.486	6.623	1.091	1.527	4.199
12	60	1	30	3.674	6.804	1.101	1.527	4.148
13	70	0.8	35	3.562	6.687	1.142	1.582	4.241
14	70	1	35	3.538	6.666	1.128	1.567	4.228
15	70	1.2	30	3.507	6.647	1.102	1.555	4.218
16	70	1.2	25	3.531	6.682	1.121	1.573	4.233
17	70	1	25	3.580	6.703	1.152	1.598	4.265
18	70	0.8	25	3.621	6.752	1.201	1.644	4.289
19	60	1.2	25	3.645	6.791	1.099	1.523	4.159
20	80	1	25	3.658	6.777	1.321	1.701	4.361
21	80	0.8	25	3.687	6.787	1.355	1.732	4.389
22	60	0.8	35	3.691	6.818	1.101	1.512	4.149
23	80	1.2	30	3.608	6.729	1.267	1.666	4.314
24	80	0.8	30	3.666	6.768	1.338	1.708	4.366
25	60	1.2	35	3.612	6.753	1.054	1.483	4.106
26	80	1	30	3.639	6.753	1.308	1.692	4.333
27	60	1	25	3.692	6.821	1.108	1.539	4.172

3.1.1 Effect of factors on the retention time of TICA (Y1)

The polynomial describing the retention times of TICA (Y1) describes the quantitative relationship between key chromatographic variables; acetonitrile ratio (A), flow rate (B), and columns temperature(C), to their combined effects on retention. Negative linear coefficients for A, B, and C are indicative that increasing any of these parameters would retract the optimal retention time; a logical deduction associated with chromatographic variables where high organic strength solvent, high temperature/accelerated flow enhance analyte elution. The flow rate was only second to compound effect which indicates the significant role of this factor on analyte elusion from the stationary phase (**Fig. 2** and Table 3).

The interaction terms (AB and BC) were positive but very small, indicating a high degree of synergy when these variables changed together. Positive coefficient of the quadratic term of acetonitrile (A^2) indicates curvature in the response, that is, retention times at extremes of acetonitrile level are slightly higher than those at the mid-point. This response reflects an optimum medium organic composition providing both a balance of elution and resolution. The small negative quadratic sums of squares for flow rate (B^2) and temperature (C^2) indicates that the method is linear over their tested ranges.

Table 3. ANOVA for study of main effects, interactions, and quadratic effects of CMVs on selected CAAs using BBD

Y1: Retention time of TICA			
	F	p-value	Significance level: P-value less than 0.05
Source	Value	Prob > F	
Model	240.24	< 0.0001	Significant
A-Proportion of ACN	107.66	< 0.0001	Significant
B-Flow Rate	560.79	< 0.0001	Significant
C-Column Oven Temp	156.97	< 0.0001	Significant
AB	6.50	0.0207	Significant
AC	0.32	0.5815	Not significant
BC	1.17	0.2950	Not significant
A^2	1324.33	< 0.0001	Significant
B^2	4.24	0.0553	Not significant
C^2	0.22	0.6453	Not significant
Y2: Retention time of RIVA			
Model	202.65	< 0.0001	Significant
A-Proportion of ACN	297.91	< 0.0001	Significant
B-Flow Rate	291.09	< 0.0001	Significant
C-Column Oven Temp	176.48	< 0.0001	Significant
AB	15.17	0.0012	Significant
AC	0.42	0.5249	Not significant
BC	0.95	0.3438	Not significant
A^2	1041.18	< 0.0001	Significant
B^2	0.61	0.4437	Not significant
C^2	0.018	0.8951	Not significant

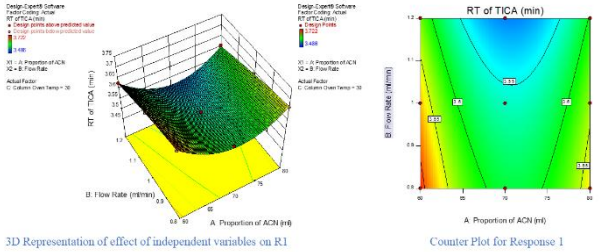

Figure 2. Effect of independent variable on retention time of TICA (Y1).

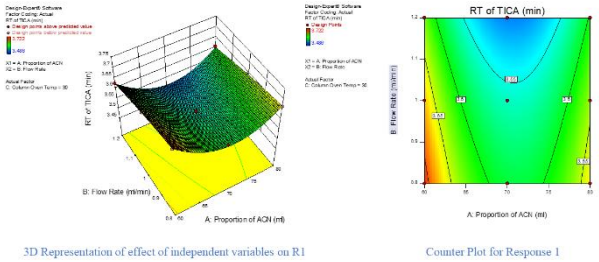

Figure 3. Effect of independent variable on retention time of RIVA (Y2).

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3.1.2 Effect of factors on the retention time of RIVA (Y2)

The Polynomial equation of the retention time RIVA is related to a combination of acetonitrile amount (% ACN; A), flow rate (F) and column temperature (°C) as follows. The negative values of the coefficients associated to the linear terms (A, B and C) showed that an increase in them resulted in a decrease of retention times, according well with physico-chemical properties of moderate lipophilic analytes when reversed-phase chromatography is used. Between the two, concentration and flow rate of acetonitrile exerted similar effects towards size separation, suggesting that they played a major role in determining elution strength and analyte migration in the column. **Fig. 3** and Table 3 show that the positive AB interaction indicates a small compensatory effect that is caused by increasing both acetonitrile and flow rate at the same time, AC and BC also as well played a negligible effect in retention factors. The positive quadratic term for acetonitrile (A^2=0.094) suggests a slight curvature, suggesting that the retention factor increases slightly at the limiting levels of organic content relative to central point and therefore there is a compromise between solubility and stationary phase affinity over the center of design space. Very low quadratic coefficient for flow rate (B^2) and almost zero positive value for temperature (C^2) reflected the small degree of curvature, evidencing stability of retention in this range.

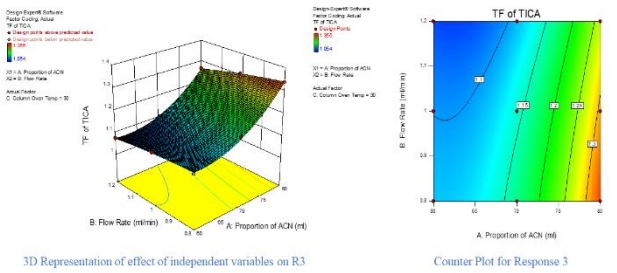


Figure 4. Effect of independent variable on tailing factor of TICA (Y3).

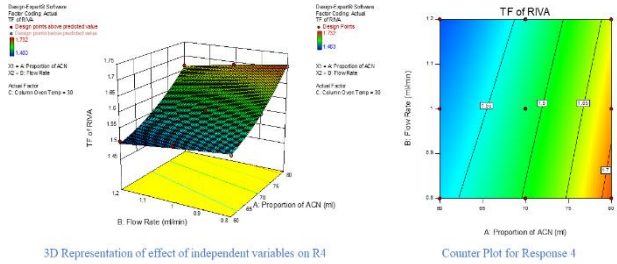


Figure 5. Effect of independent variable on tailing factor of RIVA (Y4).

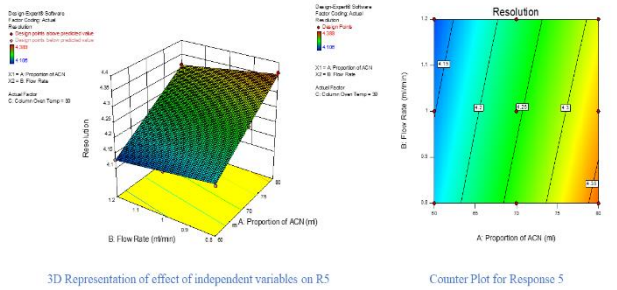


Figure 6. Effect of independent variable on resolution (Y5).

Table 4. ANOVA for study of main effects, interactions, and quadratic effects of CMVs on selected CAAs using BBD

Y3: Tailing factor of TICA			
	F	p-value	Significance level: P-value less than 0.05
Source	Value	Prob > F	
Model	375.64	< 0.0001	Significant
A-Proportion of ACN	2776.15	< 0.0001	Significant
B-Flow Rate	205.46	< 0.0001	Significant
C-Column Oven Temp	68.08	< 0.0001	Significant
AB	13.88	0.0017	Significant
AC	1.38	0.2571	Not significant
BC	1.190E-003	0.9729	Not significant
A^2	313.49	< 0.0001	Significant
B^2	2.29	0.1489	Not significant
C^2	2.379E-003	0.9617	Not significant
Y4: Tailing factor of RIVA			
Model	290.59	< 0.0001	Significant
A-Proportion of ACN	2261.65	< 0.0001	Significant
B-Flow Rate	154.52	< 0.0001	Significant
C-Column Oven Temperature	137.83	< 0.0001	Significant
AB	2.13	0.1623	Not significant
AC	0.53	0.4751	Not significant
BC	0.65	0.4306	Not significant
A^2	57.66	< 0.0001	Significant
B^2	0.16	0.6951	Not significant

C^2	0.16	0.6951	Not significant
Y5: Resolution			
Model	1050.73	< 0.0001	Significant
A-Proportion of ACN	5675.29	< 0.0001	Significant
B-Flow Rate	311.07	< 0.0001	Significant
C-Column Oven Temperature	307.95	< 0.0001	Significant
AB	1.70	0.2075	Not significant
AC	2.15	0.1584	Not significant
BC	6.23	0.0214	Significant

3.1.3 Effect of factors on the tailing factor of TICA (Y3)

The polynomial model for the tailing factor of TICA quantifies how acetonitrile proportion (A), flow rate (B), and column temperature (C) influence peak symmetry. The positive linear coefficient for A (+0.10) indicates that increasing the organic content tends to increase tailing, whereas the negative coefficients for B (−0.028) and C (−0.16) show that higher flow rate and temperature improve peak symmetry by reducing tailing. Interaction terms (AB, AC, BC) are very small and therefore exert negligible combined effects under the studied conditions. The positive quadratic term for acetonitrile (A^2 = 0.061) implies curvature such that extreme deviations in organic proportion—either lower or higher than the center point—further exacerbate tailing, while the small negative B^2 and near-zero C^2 coefficients indicate minimal nonlinearity for flow and temperature (**Fig. 4** and Table 4).

3.1.4 Effect of factors on the tailing factor of RIVA (Y4)

The polynomial model for the tailing factor of RIVA describes how acetonitrile proportion (A), flow rate (B), and column temperature (C) affect peak symmetry. The positive linear coefficient for A (+0.084) indicates that increasing organic content modestly increases tailing, while the negative linear coefficients for B (−0.022) and C (−0.21) suggest that higher flow rates and elevated column temperature reduce tailing and thereby improve peak shape. Interaction terms (AB, AC, BC) are small in magnitude, indicating only minor combined effects between factors within the studied range. The small positive quadratic term for acetonitrile (A^2 = 0.023) implies slight curvature, such that extreme deviations from the central ACN level may further increase tailing, whereas the negligible negative quadratic terms for flow and temperature (B^2 and $C^2 \approx -1.22\times10^{-3}$) indicate minimal nonlinearity for those factors (**Fig. 5** and Table 4).

3.1.5 Effect of factors on the resolution (RS- Y5)

The regression equation for resolution quantifies how acetonitrile proportion (A), flow rate (B), and column temperature (C) influence the chromatographic separation between TICA and RIVA. The positive coefficient for acetonitrile (+0.094) indicates that increasing organic content within the studied range enhances resolution, whereas the negative coefficients for flow rate and temperature (−0.022 each) show that higher flow and higher column temperature tend to reduce resolution. Interaction terms are very small (AB, AC, BC $\approx 10^{-3}$–10^{-4}), signifying only minor synergistic or antagonistic effects when two factors vary together. Overall, the model implies an operational trade-off: modestly higher acetonitrile improves peak separation but must be balanced against the opposing effects of increased flow and temperature to preserve selectivity (**Fig. 6** and Table 4).

Overall, the regression model demonstrates a good fit with high predicted and adjusted R^2 values, confirming its adequacy for describing the experimental data and predicting chromatographic performance within the studied design space.

Different regression models were selected for individual responses based on statistical significance and model adequacy. While retention time (Y1, Y2) and tailing factor (Y3, Y4) responses were best described by quadratic models, the resolution response (Y5) followed a reduced quadratic model, as only the linear terms (A, B, C) and the BC interaction were statistically significant. Non-significant terms were excluded to avoid overfitting and to improve model predictability.

The ANOVA results indicated that model terms A (acetonitrile content), B (flow rate), and C (temperature) had statistically significant effects ($p < 0.05$) on retention time, tailing factor, and resolution. The predicted R^2 (0.977–0.992) was in close agreement with the adjusted R^2 (0.985–0.996), confirming model adequacy.

Polynomial equations were derived for each response; examples are shown below:

- **Retention time of Ticagrelor (Y_1) RT_1** = +3.56 − 0.016A − 0.037B − 0.020C + 0.0049AB + 0.099A^2
- **Retention time of Rivaroxaban (Y_2) RT_2** = +6.69 − 0.029A − 0.029B − 0.022C + 0.008AB + 0.094A^2
- **Tailing Factor of TICA (Y_3)** = +1.14+0.10*A-0.028*B-016*C-9.000E-003*AB+0.061*A^2
- **Tailing Factor of RIVA (Y_4)** = +1.58+0.084*A-0.022*B-021*C+0.023*A^2
- **Resolution (Y_5):** Res = +4.24 + 0.094A − 0.022B − 0.022C − 0.0038BC

All developed models exhibited adequate signal-to-noise ratios, with Adeq. Precision values exceeding 50, confirming reliable model discrimination and statistical robustness. Response surface and contour plot analysis demonstrated that increasing the proportion of acetonitrile significantly reduced the retention times of both Ticagrelor and Rivaroxaban, while its influence on resolution was comparatively moderate. An increase in flow rate further shortened retention time but marginally decreased resolution, whereas higher column temperature improved

peak efficiency by reducing tailing without adversely affecting separation.

3.2 Optimization and Establishment of MODR

The optimal chromatographic conditions were at 60% acetonitrile, flow rate of 0.8 mL min-1 and 27 °C column oven temperature was predicted by the software (Design Expert) which yielded the most desirable setup all responses within their limiting values under study. The latter conditions minimized tailing and ensured good peak reproducibility throughout the experiment runs. TICA and RIVA had retention times of 3.71 min and 6.85min, respectively. The chromatography, tested on the basis of experiments under these conditions, yielded excellent peak symmetry and resolution (**Fig. 7**). The difference between the relevant predicted and actual responses was < 5%, demonstrating the predictability of the model. AQbD led optimization successfully developed the Method Operable Design Region (MODR) and proved the robustness of particle size method for routine quality analysis.

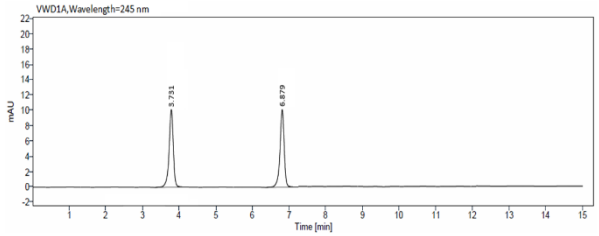


Fig. 7. The chromatogram for standard TICA and RIVA showing a peak at the retention time of 3.17 min and 6.87 min, respectively.

3.3 Method Validation

The validated method fulfilled ICH Q2(R2) requirements for all parameters.

3.3.1 System Suitability

System suitability testing was performed to confirm the reproducibility and performance capability of the developed RP-HPLC method for routine analysis. A standard solution of TICA and RIVA was injected six times under optimized conditions and critical parameter as the retention time, theoretical plate number, resolution and tailing factor were calculated. A %RSD on peak area results for each standard solution of the analytes are presented in Table 5. All system suitability results were in compliance with the requirements of USP <621>, indicating the robustness and repeatability of established method for accurate and precise quantification.

Table 5. System suitability data

Parameter	**TICA**	**RIVA**	**Acceptance Criteria**
Retention time (min)	3.59 ± 0.02	6.71 ± 0.03	–
Tailing factor (Tf)	1.15 ± 0.01	0.59 ± 0.01	≤ 2.0
Theoretical plates (N)	6408.67 ± 78.23	8552.33 ± 87.31	≥ 2000
Resolution (Rs)	–	4.28 ± 0.04	≥ 2.0
%RSD (Peak area)	0.52	0.68	≤ 2.0

3.3.2 Specificity

Specificity was evaluated to check the ability of the method for an unambiguous determination of the analytes in presence of interferences, other than test components like excipients or degradation products. Solution of blank/ placebo/ standard were injected separately and the chromatograms were screened for possible interference at the retention times of TICA and RIVA. Lack of co-eluting or overlapping peaks proved specificity of the method for both analytes.

3.3.3 Linearity

Peak areas of both analytes were linear with concentration (over the ranges of 45–135 µg/mL for TICA and 2.5–7.5 µg/mL for TIVA). The correlation coefficients (R^2) were 0.9997 and 0.9999, respectively for the calibration curves that indicated a superior linearity as well as conformity to Beer–Lambert Law in the range studied (Table 6). The chromatogram overlay was presented in Fig. 8.

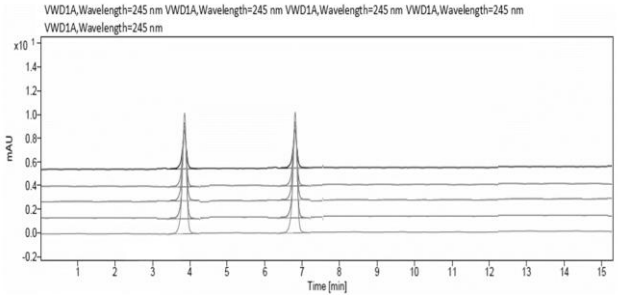


Figure 8. The overlaid chromatograms of TICA and RIVA showing linearity in the range.

3.3.4 Precision

Precision was confirmed by intraday and interday analysis at three concentration levels (45 + 2.5, 90 + 5, and 135 + 7.5 µg/mL), %RSD were calculated. The %RSD values for both intra-day (0.70–1.37% for TICA and 0.73–1.33% for RIVA) and inter-day (0.88–1.45% for TICA and 1.05–1.50% for RIVA) precision studies were below 2%, indicating good repeatability and reproducibility of proposed method (Table 6).

3.3.5 Accuracy

The mean recoveries of TICA and RIVA were 99.12-100.58% and 99.82-100.27%, respectively, when recovery experiments at three concentration levels (50, 100 and 150%) were conducted. These results definitely demonstrated the accuracy and reliability of the method in quantitative analysis without interference from matrix compounds.

3.3.6 LOD and LOQ

The limits of detection and quantitation were determined using regression statistics. The LOD and LOQ of TICA were found to be 4.14 µg/mL and 12.56 µg/mL, while the corresponding values for RIVA were 0.25 µg/mL and 0.76

µg/mL which revealed good sensitivity of the developed method (Table 6).

3.3.7 Robustness

Minor deliberate variations in chromatographic parameters, including flow rate (±0.2 mL/min), temperature (±2 °C), and acetonitrile proportion (±2%), did not produce significant changes in peak area. The %RSD values remained below 2% for both analytes, confirming the robustness of the method under small operational variations.

Table 6. Validation Summary for TICA and RIVA

Parameter	Limit	Result		Inference
		TICA	RIVA	
Linearity range (µg/mL)		45–135	2.5–7.5	
Correlation coefficient (R^2)	R^2 >0.999	0.9997	0.9999	Method was Linear
Repeatability	%RSD < 2	0.95	1.08	Method was repeatable
LOD (µg/mL)	-	4.14	0.25	-
LOQ (µg/mL)	-	12.56	0.76	-
Inter-day precision	%RSD < 2	0.88-1.45	1.05-1.50	Method was Precise
Intraday precision	%RSD < 2	0.70-1.37	0.73-1.33	Method was precise
% Recovery	-	99.12-100.58	99.82-100.27	Method was Accurate
Robustness	%RSD < 2	%RSD < 2		Method was robust
Assay (% w/w)	-	100.64 ± 0.70	99.87 ± 0.90	-

3.4 Assay of Laboratory prepared tablets

The developed RP-HPLC method was successfully applied to the quantitative determination of TICA and RIVA in laboratory-prepared tablets. The assay results demonstrated that the percentage content of TICA and RIVA was 100.64 ± 0.70% and 99.87 ± 0.90%, respectively, which fall within the acceptable range of 98–102% as per pharmacopeial standards. To establish statistical equivalence, the assay results obtained using the proposed method were compared with those reported in the literature using Student's F-test at a 95% confidence level (Table 7). The calculated F-values were lower than the corresponding critical values, demonstrating that no significant difference exists between the proposed and reported methods in terms of accuracy and precision. The chromatograms exhibited well-resolved and symmetrical peaks without interference from excipients, confirming the suitability of the method for routine quality control of combined dosage forms.

Table 7. Assay of Laboratory prepared tablets

Particulars	Proposed method	Reported method [21]	Proposed method	Reported method [21]
Mean	100.64	99.38	99.878	98.5
Variance	0.4962	0.11995	0.18967	0.0566
Observations	5	5	5	5
df	4	4	4	4
F	4.13		3.35	
P(F<=f) one-tail	0.098		0.13	
F Critical one-tail	6.38		6.38	

Assessment of the white analytical chemistry of the developed analytical method using RGB model

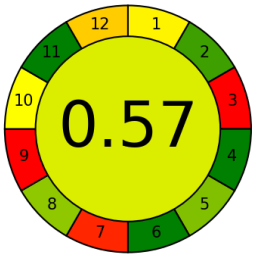
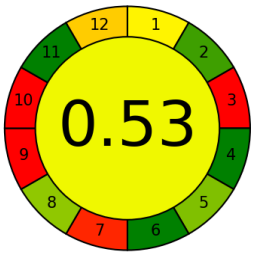
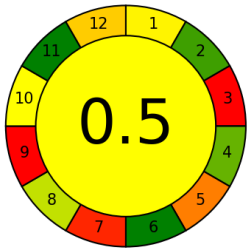
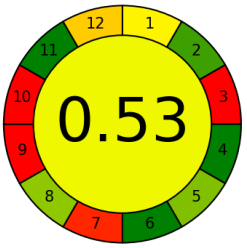
Red model–based scoring for validation efficiency

The validation results of the developed RP-HPLC methods were found to be comparable through Red model (R1–R4) guidelines as per ICH Q2(R2). All the previously reported methods obtained score less than 100 values for all validation parameters in point of concurrent determination of TICA and RIVA because of none of the method used the AQbD approach as well as lower sensitive [20–22]. The proposed method showed a wider range of analytical possibility as it allowed simultaneous determination of both analytes utilizing a single optimized chromatographic condition and AQbD-based development approach. Since this expanded scope of application, the proposed method also received 100/100 which was a strong validation efficiency in the Red model.

Green model–based assessment for procedure greenness

The Green model (G1–G4), with several GAC tools such as the AGREE metric and the Eco-Scale Assessment (ESA), was applied to evaluate the environmental burden of analysed flows. The AGREE and Eco scale score of proposed HPLC and reported analytical methods shown in table 8. The scores were 0.50-0.53 for AGREE and 68-72 for ESA, representing moderate greenness based on the published methods [12,20–22]. The developed RP-HPLC method presented AGREE score of 0.57 and ESA score of 74/100 indicating good solvents usage, waste development and overall environmental fate. These findings illustrate the improved compliance of the method with Green Analytical Chemistry.

Table 8. AGREE and Eco scale score of proposed HPLC and reported analytical methods

Proposed HPLC method		**HPLC**		**UPLCMS**		**UV and HPLC**	
Reagents	PP *	Reagents	PP *	Reagents	PP *	Reagents	PP *
Acetonitrile-m	8	Acetonitrile-m	12	Methanol	9	Acetonitrile-m	12
Methanol-m	6	Acetonitrile-s	12	Formic acid	4	Acetonitrile-s	12
Methanol-s	6	Phosphate buffer	0	Methanol-s	6	Orthophosphoric acid	2
Energy used for LC	1	Energy used for LC	1	Energy used for LC-MS	2	Energy used for LC	1
Waste	5	Waste	5	Waste	5	Waste	5
Occupational hazard	0	Occupational hazard	0	Occupational hazard	2	Occupational hazard	0
Total penalty points	26	Total penalty points	30	Total penalty points	28	Total penalty points	32
ESA score	**74**	**ESA score**	**70**	**ESA score**	**72**	**ESA score**	**68**
AGREE Score							
							

* Penalty points

Blue model–based assessment for cost and time efficiency

The Blue model was employed to evaluate both sets of methods in terms of operational cost, analysis time, and user-friendliness. Published techniques—such as UPLC–MS methods—incurred greater expenses, higher energy consumption, and longer operational demands due to specialized instrumentation and resource-intensive workflows [22]. While the present proposed RP-HPLC method is developed with a single rugged chromatographic condition using an economical mobile phase, which could accomplish both drugs estimation together with lesser consumption of solvents and less run time. Based on the results, the proposed model achieved a perfect score of 100/100 in the Blue model, further confirming its cost-effectiveness and time-efficient character.

White analytical chemistry assessment

The concept of white analytical chemistry (WAC) integrates the Red, Green, and Blue model assessments, much like the formation of white light through the combination of the three primary colors. Accordingly, WAC scores for both the published and proposed methods were calculated by averaging their respective Red, Green, and Blue scores. The published methods for the simultaneous estimation of TICA and RIVA obtained WAC values below 78 out of 100, reflecting moderate analytical performance, environmental compatibility, and operational efficiency [20–22]. Contrarily, the developed RP-HPLC method a WAC score of 82.75/100, reflecting to a higher balance between validation quality, greenness and applicability preferences. These results demonstrate that the proposed approach is superior and sustainable for analysis. The comparative RGB and WAC results for all evaluated methods are depicted in Fig. 9.

White Analytical Chemistry (RGB model)								
	HPLC Method (Eswarudu et.al.)		UPLC MS Method (Moffid et.al)		UV and HPLC Method (Maheshwari et.al.)		Proposed HPLC Method	
R1- Linearity and Range	100		100		100		100	
R2- LOD, LOQ	100		100		80		100	
R3- Accuracy and Precision	100		100		100		100	
R4- AQbD and Robustness of Method	50		50		50		100	
Average Red Score	87.5		87.5		83.33		100	
	AGREE	ESA SCORE	AGREE	ESA SCORE	AGREE	ESA SCORE	AGREE	ESA SCORE
G1- Environmental Consequences of the Method	53	70	50	72	53	68	57	74
G2- Amount of Waste Produced by the Method	53	70	50	72	53	68	57	74
G3- Energy Requirements of the Method	53	70	50	72	53	68	57	74
G4- Risks to Human and Animal Health and Safety	53	70	50	72	53	68	57	74
Average Green Score	53	70	50	72	53	68	57	74
B1- Economic viability of the approach	100		80		100		100	
B2- Ease of implementation	100		80		100		100	
B3- Accessibility and usability for end-users	100		80		100		100	
B4- Time required for Sample analysis	100		100		100		100	
Average Blue Model Score	100		85		100		100	
Overall WAC Score	77.63		73.63		75.25		82.75	
WAC Status	Excellent		Good		Good		Excellent	

Figure 9. White analytical chemistry-based assessment and comparison of proposed HPLC method and published methods for estimation of TICA and RIVA using RGB model.

Conclusion

The proposed study combined WAC and AQbD concepts for the development of simple, economical and eco-friendly RP-HPLC method for simultaneous quantification of TICA and RIVA. Throughout method development, AQbD principles were built into the process to guarantee a robust analytical performance, by consistently considering critical method parameters and establishing scientifically justified MODR. The method performance characteristics

in terms of specificity, precision, accuracy and sensitivity for both analytes were excellent validating the suitability of the method to achieve consistent and reliable quantification. The Red, Green and Blue model assessments received positive scores, indicating good analytical efficiency, environmental compatibility and operational validity. It is worth mentioning that the global WAC score of 82.75/100 illustrated a relatively good linearity of power for the approach in analytical-performance, greenness and practical-applicable aspects. In view of these attributes, the developed RP-HPLC method appears to be more promising for routine quality control analysis of combined dosage forms and laboratory-prepared mixtures containing TICA along with RIVA as an economic, green, user friendly and rapid analytical tool. Its suitability is not limited to laboratory applications but also benefits companies, operating in large-scale pharmaceutical environments, as it can adhere to the new analytical requirements and bring compliance with green and regulatory specifications.

Author Contributions

Rashmi Rammilan Shukla: Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection, Formal analysis, software, and writing the original draft;

Ankit Chaudhary: Project administration, Project supervision, methodology,

Pinak Patel, Krunal Detholia: Writing, reviewing and editing

Data Availability: Data will be made available on request.

Ethics statement: Not applicable.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data sharing statement: Data available on request from the authors.

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Ethical approval: Not applicable

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