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Analytical quality by design (AQbD) based optimization of RP-UPLC method for determination of nivolumab and relatlimab in bulk and pharmaceutical dosage forms

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Abstract

Background The Analytical Quality by Design (AQbD) methodology extends the application of Quality by Design (QbD) principles to the management of the analytical procedure life cycle, encompassing method creation, optimization, validation, and continuous improvement. AQbD assists in creating analytical procedures that are robust, reliable, precise, and cost-efficient. Opdualag™ is a combination of Nivolumab and Relatlimab, which are antibodies that block programmed death receptor-1 (PD-1) and lymphocyte-activation gene 3 (LAG-3) receptors, used to treat advanced melanoma. This work aims to develop and validate a reversed-phase ultra-performance liquid chromatography (RP-UPLC) method using AQbD principles to determine NLB and RTB in pharmaceutical products.

Results A central composite design (CCD) comprising three factors arranged in five distinct levels was implemented via Design-expert® software to optimize the chromatographic conditions. A mathematical model was constructed and the effects of three independent factors namely flow rate (X_1), percentage of methanol in the mobile phase (X_2), and temperature (X_3) on responses including retention time (Y_{1-2}), resolution factor (Y_3), theoretical plates (Y_{4-5}), and tailing factor (Y_{6-7}) were investigated. The software determined the optimal chromatographic conditions for the separation of NLB and RTB, which were as follows: 32.80% methanol in the mobile phase, 0.272 mL/min flow rate, 29.42 °C column temperature, and 260 nm UV detection. The retention time for NLB and RTB were 1.46 and 1.88 min, respectively. The method exhibited linearity across the concentration ranges of 4–24 µg/mL for RTB and 12–72 µg/mL for NLB. The limits of detection (LOD) and limit of quantification (LOQ) for NLB and RTB, respectively, were 0.89 µg/mL, 2.69 µg/mL and 0.15 µg/mL and 0.46 µg/mL. The percentage relative standard deviation (%RSD) of intraday and interday precision for NLB and RTB was below 2. The recovery percentages for NLB and RTB were determined to be 99.57–100.43% and 99.59–100.61%, respectively. Both drugs were found to be susceptible to oxidative and photolytic degradation in forced degradation studies.

Conclusions Employing the AQbD-based methodology, a straightforward, fast, accurate, precise, specific, and stability-indicating RP-UPLC method has been established for the quantitative analysis of NLB and its RTB in pharmaceutical formulations.

Keywords Analytical quality by design, Nivolumab, Relatlimab, Central composite design, Reversed-phase ultra-performance liquid chromatography

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Background

Melanoma is the most aggressive form of skin cancer that develops from melanocytes, the skin pigment-producing cells. Although melanoma only makes up a small fraction of skin malignancies (approximately 1–2%), it is the leading cause of skin cancer-related mortality. The 5-years survival rate for melanoma is 94% when detected early, but it drops dramatically after the cancer has spread to other parts of the body. Melanoma that has progressed beyond its initial skin location to other organs, lymph nodes, or distant tissues is known as metastatic melanoma. Surgical procedures, radiation treatments, chemo, immunotherapy, and targeted therapies are all viable treatment choices [1, 2].

Among all, treatment with immunotherapy drugs such as immune checkpoint inhibitors and interleukin-2 significantly improved the outcome in patients by boosting the body's immune response to selectively target and destroy cancer cells [3]. Recently (March 18, 2022), the United States Food and Drug Administration (FDA) approved a fixed dose combination of two immunotherapy antibodies Nivolumab and Relatlimab (Opdualag™) for adults and paediatric patients (12 years or above) with unresectable and metastatic melanoma [4] (Fig. 1). Both drugs are immune checkpoint inhibitors and act by restoring the T cell's natural ability to target cancer cells by suppressing immune checkpoint proteins, which prevent excessive immune responses. Nivolumab blocks programmed cell death protein 1 (PD-1) in immune cells (T cells) and is widely used to treat melanoma and other cancers. Relatlimab blocks Lymphocyte-activation gene 3 (LAG-3) in immune cells and restores the effector function of exhausted T cells [5]. This combination therapy results in longer progression-free survival and fewer side effects in patients when compared to Nivolumab plus Iipilimumab and Nivolumab alone [6–8]. Opdualag™ is available as an intravenous injection containing 240 mg of NLB and 80 mg of RTB in 20 mL clear to opalescent, colorless to slightly yellow solution in a single-dose vial. Nevertheless, it is not included in any of the official pharmacopoeial monographs. Hence it is of utmost importance to develop an analytical method for estimation of NLB and RTB in commercially available formulations.

The analytical Quality by Design (AQbD) approach is an extension of the quality by design (QbD) concept, used in the pharmaceutical industry to ensure the quality of analytical methods. This approach emphasizes a thorough understanding of the method's critical parameters

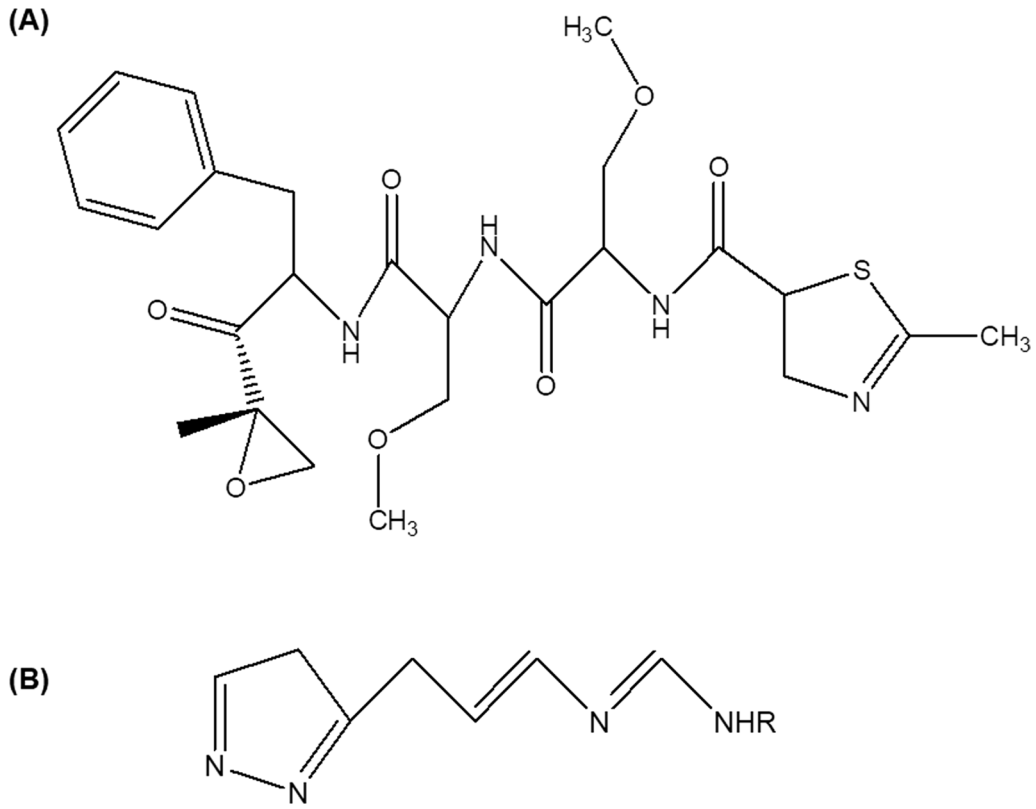

Fig. 1 Structure of Nivolumab (**A**) and Relatlimab (**B**)

and their impact on the analytical results, leading to more efficient and effective analytical development and validation processes. AQbD helps in the identification and optimization of significant factors, and their interaction effect and provides appropriate chromatographic conditions for the estimation of analyte. A central composite design is the most predominant experimental design used in response surface methodology for the optimization of chromatographic conditions. These designs provide a great deal of information with a minimum number of experimental trials and are very cost effective [9–11].

The literature study revealed that there are limited analytical methods available for the determination of NLB alone and in combination with different drugs in plasma and pharmaceutical dosage forms. These methods include RP-UPLC [12], LC–MS/MS [13], ELISA [14], LC–MS/HRMS [15], UPLC-MS/MS [16], and the UHPLC/UV-(HESI/Orbitrap™) MS approach [17]. An RP-UPLC approach was developed to simultaneously determine NLB and RTB in Opdualag™ formulations [18]. However, no analytical method has been reported for the estimation of NLB and RTB using AQbD principles.

The current work focused on the development and optimization of an RP-UPLC method using AQbD principles (central composite design) for the estimation of NLB and RTB in pharmaceutical products.

Methods

Chemicals

Reference standards of NLB (purity 99.80%), and RTB (purity 99.80%) were obtained from Akrivis Pharma Private Limited, Hyderabad. Fisher Scientific (Mumbai, India) provided HPLC-grade methanol and acetic acid. The ultrapure water was acquired from the Millipore Direct-Q®3 UV water purification equipment by Merck Millipore in India. All remaining reagents and chemicals met analytical grade standards.

Instrumentation and chromatographic conditions

The method was developed using a Waters Acquity UPLC system H-class equipped with binary pumps, a tunable UV (TUV) detector, and an autosampler. The Empower 2 software was used for data acquisition and processing. A BEH C18 column (50 × 2.5 mm i.d. particle size of 1.7 µm) was used to accomplish the chromatographic separation. The mobile phase is made up of 32.8:67.2% v/v methanol and 0.01 N phosphate buffer, which is pumped at a flow rate of 0.27 mL/min. The temperature of the column was kept at 29.4 °C, and 260 nm was used for detection. The overall chromatographic run time was 3 min and the injection volume was 5 µL. A 50/50 v/v mixture of methanol and water was utilized as the diluent.

Preparation of standard stock solutions

NLB (24 mg) and RTB (8 mg) were accurately weighed and transferred to a 50 mL clean dry volumetric flask. Add 10 mL of diluent and sonicate for 10 min to completely dissolve them. The volume was increased to 50 mL with diluent to achieve concentrations of 480 µg/mL for NLB and 160 µg/mL for RTB. To create working standard solutions, aliquots of each stock solution were diluted with the diluent to achieve concentrations of 12, 24, 36, 48, 60, and 72 µg/mL for NLB and 4, 8, 12, 16, 20, and 24 µg/mL for RTB.

Preparation of sample solution

Transfer 20 mL of Opdualag™ solution (240 mg NLB and 80 mg RTB) to a 100 mL volumetric flask. Add 50 mL of diluent and sonicate for 25 min. The volume was increased to 100 mL with diluent to achieve concentrations of 2400 µg/mL of NLB and 800 µg/mL of RTB. To acquire the final concentrations of NLB (18 µg/mL) and RTB (16 µg/mL), 0.2 mL of the aforesaid solutions was diluted to 10 mL. The resulting solution was utilized for the assay of NLB and RTB in its marketed formulation.

Method optimization by experimental design

The developed method's chromatographic conditions were optimized using Design-expert® software (Version 11.1.0.1, Stat-Ease Inc., USA) employing a central composite design (CCD) with three factors at five levels ($-\alpha$, -1, 0, $+1$, and $+\alpha$). The mobile phase was chosen based on preliminary investigation using methanol and 0.01N phosphate buffer. The three independent variables chosen were flow rate (X_1),% methanol (X_2), and temperature (X_3), while the dependent variables were retention time of NLB (Y_1), retention time of RTB (Y_2), resolution factor

Table 1 Variables and their levels used in central composite design

Name	$-\alpha$	-1	0	$+1$	$+\alpha$
Independent variables					
X_1: Flow rate (mL/min)	0.2495	0.27	0.30	0.33	0.3505
X_2: % methanol (v/v)	26.59	30.00	35.00	40.00	43.41
X_3: Temperature (°C)	24.95	27.00	30.00	33.00	35.05
Dependent variables					
Y_1: Retention time of NLB					
Y_2: Retention time of RTB					
Y_3: Resolution factor					
Y_4: Number of theoretical plates of NLB					
Y_5: Number of theoretical plates of RTB					
Y_6: Tailing factor of NLB					
Y_7: Tailing factor of RTB					

(Y_3), number of theoretical plates of NLB (Y_4), number of theoretical plates of RTB (Y_5), tailing factor of NLB (Y_6), and tailing factor of RTB (Y_7) (Table 1). The Design expert® program proposed twenty runs based on the central composite design. All experiments employed a standard concentration of 48 µg/mL of NLB and 16 µg/mL of RTB.

Method validation

Following ICH Q2 (R1) criteria, the developed RP-UPLC method was validated for system suitability, linearity, LOD, LOQ, precision, accuracy, and robustness [19]. System suitability testing involves injecting six replicates of NLB (48 µg/mL) and RTB (16 µg/mL) standard solutions, evaluating their theoretical plates, tailing factor, resolution, % RSD of retention time and peak area. The linearity of the method was verified by plotting the calibration curve of peak area against concentration for six concentrations of NLB (12–72 µg/mL) and RTB (4–24 µg/mL) working standard solutions. LOD and LOQ were determined using the standard deviation (σ) and slope (S) of the calibration curve: $LOD = 3.3 \times \sigma/S$ and $LOQ = 10 \times \sigma/S$. The method's precision was confirmed by intra-day and inter-day variance investigations. Three concentrations of NLB (12, 36, and 72 µg/mL) and RTB (4, 12, and 24 µg/mL) were tested three times per day for intra-day precision (repeatability), whereas three concentrations on three different days were assessed for inter-day accuracy (intermediate precision). Accuracy was assessed by computing the mean percentage recovery of NLB and RTB standard solutions spiked at different concentration levels (50, 100, and 150%) to the pre-analyzed NLB and RTB samples. The method's specificity was verified by comparing the representative chromatograms of the blank, placebo, and NLB/RTB standard solutions. Robustness was evaluated by purposefully modifying the ideal chromatographic parameters, including flow rate (0.1 and 0.3 mL), methanol percentage (± 5% in mobile phase), and column temperature (± 5 °C).

Sample solution stability

The stability of the NLB and RTB in the solution was determined by keeping the samples in a volumetric flask at normal ambient laboratory conditions for a period of 24 h. After 24 h, the retention time and peak area of the NLB and RTB were calculated and compared against the initial readings.

Forced degradation studies

Forced degradation studies of NLB and RTB were carried out in various stress conditions. The NLB and RTB solutions were exposed to acid (2N HCl, 60 °C for 30 min), alkaline (2N NaOH, 60 °C for 30 min), dry heat (105 °C for 6 h), oxidizing (60% H_2O_2 60 °C for 30 min), neutral (water at 60 °C for 6 h) and photolytic (UV light for 7 days) degradation. Following the exposure, the resultant solutions were diluted to obtain 18 µg/mL of NLB and 16 µg/mL of RTB. Five microliters of each solution were then injected into the system, and chromatograms were recorded to evaluate the sample's stability.

Statistical analysis

Data were reported as mean ± SD. The regression coefficient, mean, SD, and % RSD were calculated in Excel. Model and model term significance was determined using Analysis of variance (ANOVA). Model and model terms were significant if the *p*-value was less than 0.05.

Results

Method development studies

The development of the LC method involves the use of complex mobile phases with various solvents (acetonitrile, methanol, ethanol, water and phosphate buffer at a specific pH), different modes of flow (isocratic/gradient), different columns (C_{18}, and C_8) and temperature settings for the separation of compounds. The preliminary trials suggested the use of methanol and 0.01N phosphate buffer as suitable mobile phase for the separation of NLB and RTB with excellent peak shape, shorter retention time and less tailing.

Method optimization by experimental design

The present study employed a central composite design with 20 trials to examine the effect of three independent variables on seven dependent variables. The independent variables include flow rate (X_1), % methanol (X_2), and temperature (X_3). The dependent variables are retention time of NLB (Y_1), retention time of RTB (Y_2), resolution factor (Y_3), number of theoretical plates of NLB (Y_4), number of theoretical plates of RTB (Y_5), tailing factor of NLB (Y_6), and tailing factor of RTB (Y_7). The results are summarized in Table 2. The impact of the independent variables on each dependent variable was examined by fitting the collected responses to different mathematical models and then generating second-order polynomial equations. Analyzing the model and its terms using analysis of variance (ANOVA) allowed us to establish their statistical significance. To better understand the relationship between the dependent and independent variables, 3D-response surface plots and 2D-contour plots were generated. Finally, the numerical optimization technique was used to predict the optimal chromatographic conditions based on the given acceptance criteria.

Table 2 Central composite design along with the observed values for optimization of RP-UPLC method for NLB and RTB

Run no.	X_1:Flow rate (mL/min)	X_2:% MeOH (%/v/v)	X_3:Tem (°C)	Y_1:Retention time NLB (min)	Y_2: Retention time RTB (min)	Y_3: Resolution factor	Y_4: Theoretical plates NLB	Y_5:Theoretical plates RTB	Y_6:Tailing factor NLB	Y_7: Tailing factor RTB
1	0.33	40	27	1.382	1.715	2.8	3088.50	4164.70	1.39	1.21
2	0.30	35	30	1.334	1.652	3.3	3436	4305	1.30	1.28
3	0.30	35	30	1.335	1.652	3.3	3445	4359	1.30	1.28
4	0.27	40	27	1.705	2.305	3.6	4578.90	6318	1.38	1.12
5	0.27	30	27	1.654	2.207	4.4	4398.90	6053.50	1.30	1.36
6	0.33	30	33	1.118	1.676	3.4	1998	2268.60	1.25	1.29
7	0.30	26.591	30	1.414	1.764	3.8	3330.50	4364.80	1.41	1.32
8	0.30	35	35.0454	1.108	1.567	3.3	3076.90	3209.50	1.23	1.30
9	0.30	35	24.9546	1.716	2.130	4.0	4279.70	5601.70	1.26	1.22
10	0.33	40	33	1.011	1.382	2.5	2232.30	3000.40	1.21	1.40
11	0.2495	35	30	1.618	2.282	4.1	4234.70	6378.60	1.39	1.26
12	0.30	43.409	30	1.237	1.542	2.4	3817	5071.90	1.45	1.19
13	0.33	30	27	1.375	1.751	3.6	3468.90	4257.90	1.36	1.27
14	0.30	35	30	1.335	1.653	3.3	3454	4314	1.29	1.28
15	0.27	40	33	1.282	1.758	2.9	4663.50	5506.60	1.45	1.20
16	0.3505	35	30	1.055	1.451	2.8	2012.40	2540.40	1.24	1.33
17	0.30	35	30	1.334	1.652	3.3	3406	4328	1.30	1.28
18	0.30	35	30	1.335	1.652	3.4	3418	4378	1.30	1.28
19	0.27	30	33	1.453	1.880	3.9	3680.20	4815.50	1.40	1.26
20	0.30	35	30	1.334	1.652	3.2	3412	4310	1.30	1.27

Fitting of responses to the model

The best-fit model was determined by fitting the observed responses from all 20 runs to each of the mathematical models using the Design-Expert program. All models' values of SD, correlation coefficient (R^2), adjusted and predicted R^2s, coefficient of variation (CV), and predicted residual sums of squares (PRESS) are displayed in Table 3. High R^2 values, low SD, CV, and PRESS, and close proximity between adjusted and predicted R^2 values were the criteria for selecting the best-fitting model across all responses. For all the responses, (Y_1–Y_7), the quadratic model was found to be the best fit.

Effect of independent variables on the Retention time of NLB (Y_1)

The following quadratic equation describes the relationship between the independent variables and the retention time of NLB (Y_1).

$$\begin{aligned} Y_1\left(\textit{Retention time of NLB}\right) = {} & 1.33 - 0.15X_1 - 0.03X_2 \\ & - 0.16X_3 + 0.002X_1X_2 \\ & - 0.0005X_1X_3 - 0.042X_2X_3 \\ & + 0.0035X_1^2 - 0.0004X_2^2 \\ & + 0.03X_3^2 \end{aligned} \quad (1)$$

Table 3 Regression analysis for different responses Y_1 to Y_7 for fitting to different polynomial models

Models	SD	R^2	Adjusted R^2	Predicted R^2	PRESS	CV (%)	Remark
Response (Y_1): Retention time of NLB (min)							
Linear	0.0464	0.9555	0.9472	0.9205	0.0615	3.42	Suggested
2FI	0.0394	0.9738	0.9618	0.9119	0.0681	2.91	
Quadratic	0.0263	0.9910	0.9829	0.9289	0.0550	1.94	
Cubic	0.0096	0.9993	0.9978	0.8443	0.1203	0.70	
Response (Y_2): Retention time of RTB (min)							
Linear	0.1250	0.8108	0.7754	0.6936	0.4050	7.08	Suggested
2FI	0.1185	0.8618	0.7981	0.6034	0.5243	6.71	
Quadratic	0.0354	0.9905	0.9820	0.9278	0.0954	2.01	
Cubic	0.0267	0.9968	0.9898	0.2868	0.9429	1.51	
Response (Y_3): Resolution factor							
Linear	0.1698	0.9110	0.8943	0.8439	0.8097	5.05	Suggested
2FI	0.1727	0.9252	0.8907	0.8548	0.7528	5.13	
Quadratic	0.0649	0.9919	0.9846	0.9618	0.1983	1.93	
Cubic	0.0593	0.9959	0.9871	0.9477	0.2714	1.76	
Response (Y_4): Theoretical plates of NLB							
Linear	283.54	0.8839	0.8621	0.7854	2.377E+06	8.17	Suggested
2FI	188.60	0.9583	0.9390	0.8961	1.150E+06	5.43	
Quadratic	98.79	0.9912	0.9833	0.9335	7.365E+05	2.85	
Cubic	42.70	0.9990	0.9969	0.8192	2.002E+06	1.23	
Response (Y_5): Theoretical plates of RTB							
Linear	207.95	0.9723	0.9671	0.9524	1.189E+06	4.64	Suggested
2FI	159.59	0.9867	0.9806	0.9636	9.087E+05	3.56	
Quadratic	61.61	0.9985	0.9971	0.9883	2.925E+05	1.38	
Cubic	27.77	0.9998	0.9994	0.9977	56,896.44	0.62	
Response (Y_6): Tailing factor of NLB							
Linear	0.0673	0.2836	0.1493	− 0.2870	0.1301	5.08	Suggested
2FI	0.0570	0.5819	0.3889	0.1164	0.0893	4.30	
Quadratic	0.0093	0.9914	0.9837	0.9371	0.0064	0.70	
Cubic	0.0086	0.9956	0.9862	0.2194	0.0789	0.64	
Response (Y_7): Tailing factor of RTB							
Linear	0.0502	0.4472	0.3436	− 0.0293	0.0751	3.95	Suggested
2FI	0.0155	0.9573	0.9376	0.8868	0.0083	1.22	
Quadratic	0.0077	0.9919	0.9846	0.9453	0.0040	0.60	
Cubic	0.0056	0.9974	0.9918	0.6801	0.0234	0.44	

Table 4 Analysis of variance (ANOVA) test results and adequate precision for various responses

Source	Responses													
	Y_1		Y_2		Y_3		Y_4		Y_5		Y_6		Y_7	
	F-value	***p*-value**	**F-value**	***p*-value**	**F-value**	***p*-value**	**F-value**	***p*-value**	**F-value**	***p*-value**	**F-value**	***p*-value**	**F-value**	***p*-value**
Model	122.69	< 0.0001	115.89	< 0.0001	135.89	< 0.0001	125.00	< 0.0001	730.29	< 0.0001	128.71	< 0.0001	136.02	< 0.0001
X_1	490.19	< 0.0001	533.21	< 0.0001	382.36	< 0.0001	791.53	< 0.0001	4608.82	< 0.0001	277.14	< 0.0001	149.68	< 0.0001
X_2	28.29	0.0003	30.86	0.0002	596.75	< 0.0001	25.27	0.0005	149.45	< 0.0001	29.68	0.0003	271.87	< 0.0001
X_3	546.15	< 0.0001	289.75	< 0.0001	144.13	< 0.0001	186.37	< 0.0001	1642.03	< 0.0001	24.59	0.0006	130.39	< 0.0001
X_1X_2	0.0721	0.7938	9.32	0.0122	0.2972	0.5976	21.96	0.0009	3.31	0.0989	28.31	0.0003	258.88	< 0.0001
X_1X_3	0.0029	0.9582	21.62	0.0009	14.56	0.0034	36.71	0.0001	40.15	< 0.0001	305.68	< 0.0001	111.79	< 0.0001
X_2X_3	20.35	0.0011	22.75	0.0008	2.67	0.1330	25.75	0.0005	51.59	< 0.0001	14.45	0.0035	258.88	< 0.0001
X_1^2	0.2496	0.6282	80.04	< 0.0001	8.27	0.0165	13.77	0.0040	8.30	0.0164	10.99	0.0078	5.34	0.0435
X_2^2	0.0037	0.9526	0.7378	0.4105	19.08	0.0014	5.79	0.0370	72.58	< 0.0001	396.36	< 0.0001	21.81	0.0009
X_3^2	18.90	0.0014	68.30	< 0.0001	49.20	< 0.0001	14.64	0.0033	2.91	0.1188	46.03	< 0.0001	14.42	0.0035
Adequate precision	39.43		38.52		45.17		38.33		93.86		37.84		50.28	

X_1, X_2 and X_3 coded levels of independent variables; X_1X_2, X_1X_3 and X_2X_3 are interaction terms; X_1^2, X_2^2 and X_3^2quadratic terms

The equation shows a negative relationship between the flow rate (X_1), percentage of methanol (X_2), and temperature (X_3) with the retention time of NLB. This indicates that when the flow rate, methanol percentage, and temperature increase, the retention time decreases. The high coefficient value of X_3 indicates that, among the three variables, temperature has the most impact on the retention time of NLB. The combined interaction terms X_1X_2, X_1X_3 and X_2X_3 have a positive effect on the retention time of NLB. Table 4 shows the results of the ANOVA for the NLB retention time data. An F-value of 122.69 indicates statistical significance for the model. The likelihood of an F-value this high being caused by random chance is 0.01%. The significance of model terms is indicated by *p*-values below 0.0500. Significant model terms here are X_1, X_2, X_3, X_2X_3, and X_3^2. There is a reasonable match between the predicted R^2 of 0.9289 and the corrected R^2 of 0.9829. The signal-to-noise ratio is a good indicator of adequate precision. Optimal ratios are greater than 4. The obtained ratio of 39.438 suggested an adequate signal. It is possible to navigate the design space using this model. The 3D response surface plots and the associated 2D contour plots in Fig. 2A, B illustrate how independent variables affect the retention time of NLB (Y_1). It was observed from the plots that an increase in flow rate, % methanol, and temperature decreased the retention time.

Effect of independent variables on the retention time of RTB (Y_2)

The following quadratic equation can be used to illustrate how the independent variables affect the retention time of RTB (Y_2).

$$\begin{aligned} Y_2\left(\textit{Retention time of RTB}\right) = {} & 1.65 - 0.22X_1 - 0.053X_2 \\ & - 0.16X_3 - 0.038X_1X_2 \\ & + 0.058X_1X_3 - 0.059X_2X_3 \\ & + 0.083X_1^2 - 0.008X_2^2 \\ & + 0.077X_3^2 \end{aligned} \tag{2}$$

The equation shows that flow rate (X_1), % methanol (X_2), and temperature (X_3) have a negative effect on the RTB retention time. As flow rate, methanol percentage, and temperature increase, the retention time of RTB decreases. The high coefficient value of X_1 shows that flow rate affects RTB retention time more than % methanol and temperature. X_1X_2 and X_2X_3 negatively affect RTB retention time, while X_1X_3 positively affects it. The ANOVA results for the data of the retention time of RTB are demonstrated in Table 4. The F-value of 115.89 indicates the significance of the model. In this case X_1, X_2, X_3, X_1X_2, X_2X_3, X_1^2, and X_3^2 are significant model terms. The predicted R^2 of 0.9278 matches the adjusted R^2 of 0.9820. Precision measures signal-to-noise ratio. A ratio over 4 is ideal. The obtained ratio of 38.524 suggests a good signal. This model can be used to navigate the design space. The 3D response surface plots and 2D contour plots in Fig. 3A, B show how independent variables affect the retention time of RTB. It was observed from the plots that an increase in flow rate, % methanol, and temperature decreased the retention time.

Effect of independent variables on the resolution factor (Y_3)

The quadratic equation below explains how independent factors affect the resolution factor (Y_3).

$$\begin{aligned} Y_3(\textit{Resolution Factor}) = {} & 3.30 - 0.34X_1 - 0.42X_2 \\ & - 0.21X_3 + 0.012X_1X_2 \\ & + 0.087X_1X_3 - 0.037X_2X_3 \\ & + 0.049X_1^2 - 0.074X_2^2 \\ & + 0.119X_3^2 \end{aligned} \tag{3}$$

The equation displays that the flow rate (X_1), % methanol (X_2) and temperature (X_3) have a negative effect on the resolution factor. This means that the resolution factor decreases with an increase in the flow rate, % methanol, and temperature. The high coefficient value of X_2 shows that % methanol affects the resolution factor more than the flow rate and temperature. The combined interaction term X_1X_2, and X_1X_3 has a positive effect and X_2X_3 has a negative effect on the resolution factor. The ANOVA results for the data of the resolution factor are demonstrated in Table 4. The F-value of 135.89 indicates the significant model. Model terms with *p*-values under 0.050 are significant. Here, X_1, X_2, X_3, X_1X_3, X_1^2, X_2^2, and X_3^2 are significant model terms. *p*-values less than 0.0500 indicate model terms are significant. The predicted R^2 0.9618 matches well with the adjusted R^2 0.9846. A precision ratio of 45.170 suggests a good signal. Hence the quadratic model can navigate design space. The 3D response surface plots and 2D contour plots in Fig. 4A, B show how independent variables affect the resolution factor. It was observed from the plots that an increase in flow rate, % methanol, and temperature decreased the resolution factor between NLB and RTB.

Effect of independent variables on the number of theoretical plates of NLB (Y_4)

The quadratic equation below explains how independent factors affect the theoretical plate of NLB (Y_4).

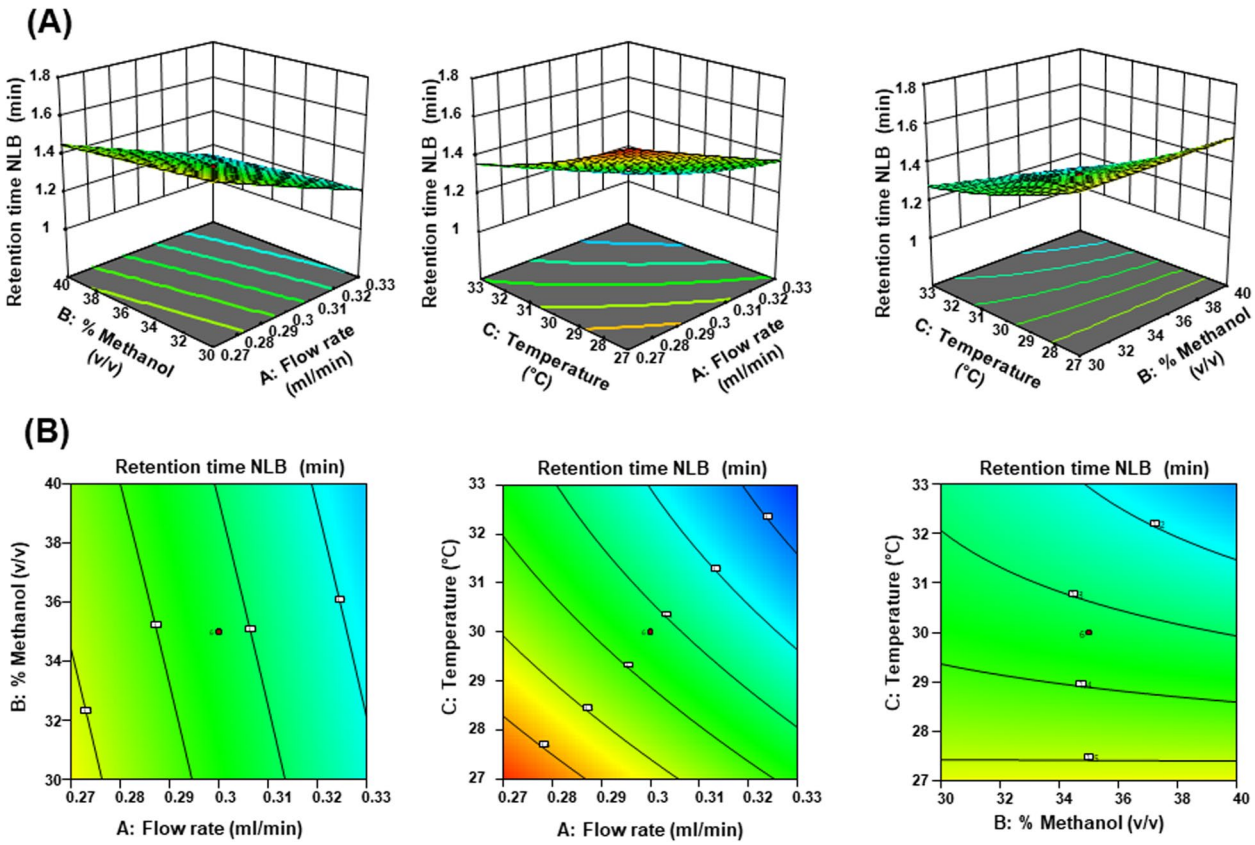

Fig. 2 The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) illustrating the effect of independent variables on the retention time of NLB (Y_1)

$$Y_4(\textit{Theoretical Plates of NLB}) = 3426.78 - 752.09X_1 + 134.39X_2 - 364.95X_3 - 163.68X_1X_2 - 211.63X_1X_3 + 177.25X_2X_3 - 96.57X_1^2 + 62.60X_2^2 + 99.56X_3^2 \quad (4)$$

The equation displays that the flow rate (X_1), and temperature (X_3) have a negative effect whereas % methanol (X_2) has a positive effect on the theoretical plates of NLB. This means that the number of theoretical plates of NLB decreases with an increase in the flow rate and temperature and increases with an increase in % methanol. The large coefficient value of X_1 shows that flow rate affects the theoretical plate of NLB more than other variables. The interaction terms X_1X_2 and X_1X_3 negatively affect NLB theoretical plates, while X_2X_3 positively affects them. Table 4 shows the ANOVA findings of the obtained data. The F-value of 125.00 indicates the model is significant. Model terms with *p*-values under 0.05 are significant. In this case X_1, X_2, X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2 are important model terms. The predicted R^2 of 0.9833 is in fair agreement with the adjusted R^2 value of 0.9335. Adequate precision measures signal-to-noise ratio. A ratio over 4 is ideal. The obtained ratio of 38.332 suggests a good signal. Hence the quadratic model can be used to navigate the design space. The 3D response surface plots and 2D contour plots in Fig. 5A, B show how independent variables affect the theoretical plates of NLB. It was observed from the plots that an increase in flow rate, and temperature decreased the theoretical plates of NLB whereas an increase in % methanol increased the theoretical plates of NLB.

Effect of independent variables on the number of theoretical plates of RTB (Y_5)

The following quadratic equation explains how independent variables affect the theoretical plate of RTB.

$$Y_5(\textit{Theoretical plates of RTB}) = 4332.06 - 1131.82X_1 + 203.81X_2 - 675.57X_3 - 39.63X_1X_2 - 138.03X_1X_3 + 156.45X_2X_3 + 46.75X_1^2 + 138.27X_2^2 + 27.69X_3^2 \quad (5)$$

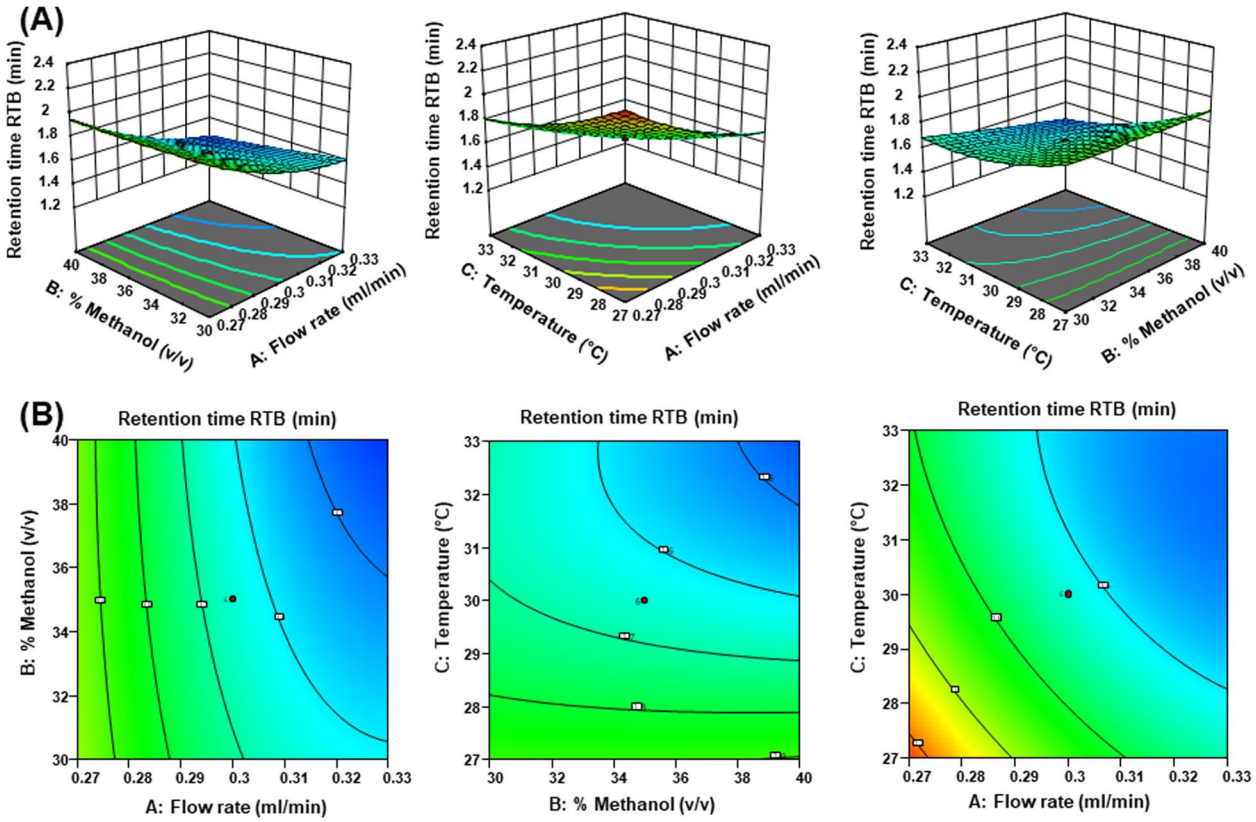

Fig. 3 The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) illustrating the effect of independent variables on the retention time of RTB (Y_2)

The equation shows that flow rate (X_1) and temperature (X_3) negatively affect RTB theoretical plates, whereas % methanol (X_2) positively affects them. This implies that theoretical plates of RTB decrease with flow rate and temperature and increase with % methanol. The flow rate affects the theoretical plates of RTB more than other variables, as shown by the large coefficient value of X_1. The interaction terms X_1X_2 and X_1X_3 negatively affect the theoretical plates of RTB, while X_2X_3 positively affects them. ANOVA findings for obtained data are in Table 4. The model F-value of 730.29 suggests the model is significant. The *p*-values (<0.05) imply X_1, X_2, X_3, X_1X_3, X_2X_3, X_1^2, and X_2^2 are significant terms. The predicted R^2 of 0.997 is consistent with the adjusted R^2 of 0.9883. The adequate precision ratio of 93.860 indicates a good signal. So, the quadratic model can navigate the design space.

The 3D response surface plots and 2D contour plots in Fig. 6A, B show how independent variables affect the theoretical plates of RTB. It was observed from the plots that an increase in flow rate, and temperature decreased the theoretical plates of RTB whereas an increase in % methanol increased the theoretical plates of RTB.

Effect of independent variables on the tailing factor of NLB (Y_6)

The following quadratic equation explains how the independent variables affect the tailing factor of NLB.

$$\begin{aligned} Y_6\left(\textit{Tailing factor of }; NLB\right) = {} & 1.30 - 0.0419X_1 + 0.0137X_2 \\ & - 0.0125X_3 - 0.0175X_1X_2 \\ & - 0.0575X_1X_3 - 0.0125X_2X_3 \\ & + 0.0081X_1^2 + 0.0488X_2^2 \\ & - 0.0166X_3^2 \end{aligned} \tag{6}$$

According to the equation, the tailing factor of NLB is positively affected by % methanol (X_2) and negatively affected by flow rate (X_1) and temperature (X_3). This means that the tailing factor of NLB decreases with an increase in the flow rate and temperature and increases with an increase in % methanol. A higher coefficient value for X_1 indicates that, relative to other variables, the flow rate significantly affects the tailing factor of NLB. The interaction terms X_1X_2, X_1X_3, and X_2X_3 have a detrimental impact on the tailing factor of NLB. The results of the ANOVA for the

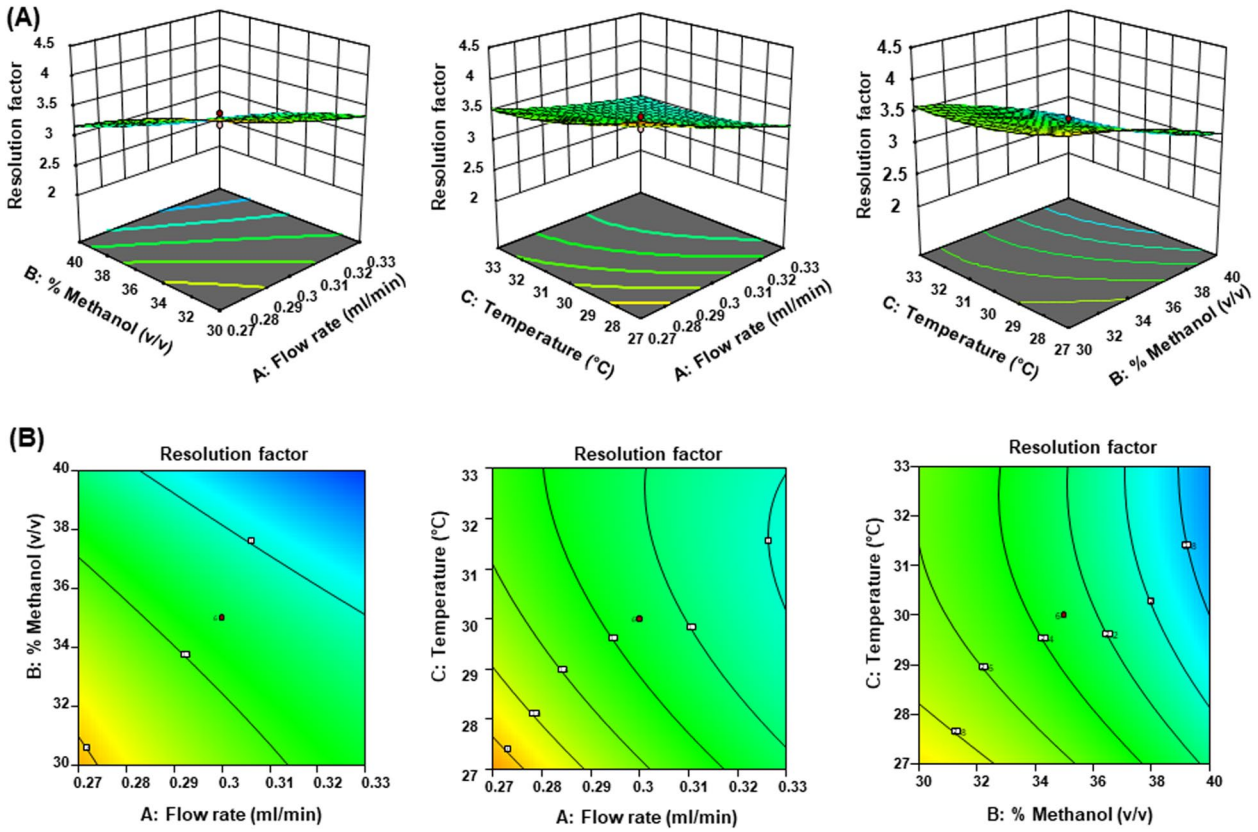

Fig. 4 The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) showing the effect of independent variables on the resolution factor (Y_3)

collected data are shown in Table 4. An F-value of 128.71 indicates statistical significance for the model. Model terms are considered significant when the *p*-value is less than 0.0500. Here, important model terms include X_1, X_2, X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^3. The predicted R^2 of 0.9837 is reasonably close to the adjusted R^2 of 0.9371. The adequate precision' ratio of 37.845 indicates a good signal. Therefore, the quadratic model serves as a useful tool for exploring the design space. Figure 7A, B show the 3D response surface plots and 2D contour plots, respectively, that illustrate the effect of the independent variables on the NLB tailing factor. It was observed from the plots that an increase in flow rate, and temperature decreased the tailing factor of NLB whereas an increase in % methanol increased the tailing factor of NLB.

Effect of independent variables on the tailing factor of RTB (Y_7)

The impact of the independent variables on the tailing factor of RTB can be elucidated by the subsequent quadratic equation.

$$\begin{aligned} Y_7\left(Tailing\ factor\ of\ RTB\right) = {} & 1.28 + 0.025X_1 - 0.034X_2 \\ & + 0.023X_3 + 0.043X_1X_2 \\ & + 0.028X_1X_3 + 0.043X_2X_3 \\ & + 0.004X_1^2 - 0.009X_2^2 \\ & - 0.007X_3^2 \end{aligned} \quad (7)$$

The equation indicates that the flow rate (X_1) and temperature (X_3) positively influence the tailing factor of RTB, whereas the percentage of methanol (X_2) has a negative impact. This means that the tailing factor of RTB increases with an increase in the flow rate and temperature and decreases with an increase in % methanol. The large coefficient value of X_2 shows that % methanol affects the RTB tailing factor more than other factors. The tailing factor of RTB is positively affected by the combined interaction term X_1X_2, X_1X_3 and X_2X_3. Table 4 displays the ANOVA results for the collected data. An F-value of 136.02 indicates a significant model. Model terms are considered significant when the *p*-value is less

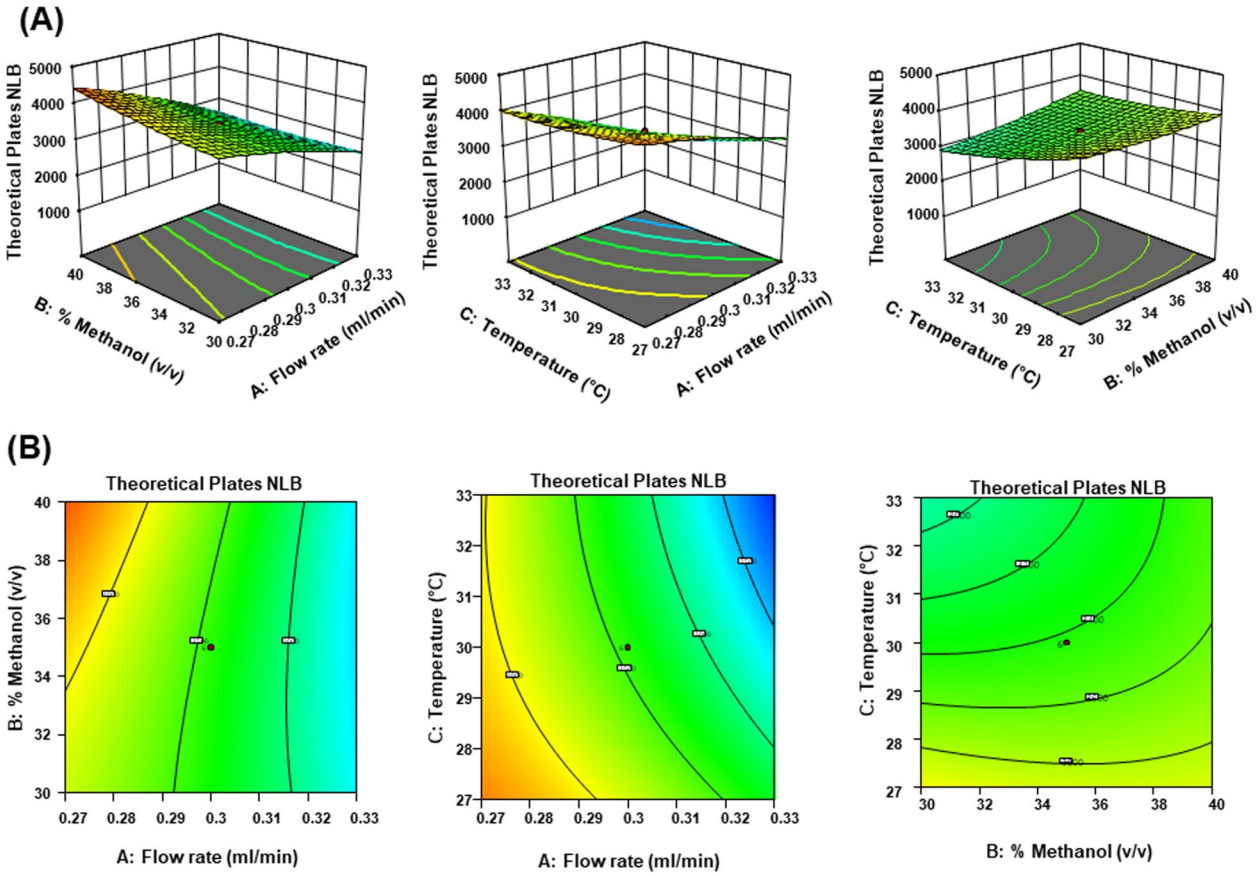

Fig. 5 The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) showing the effect of independent variables on the theoretical plates of NLB (Y_4)

than 0.05. X_1, X_2, X_3, X_1X_2, X_1X_3, X_2X_3, X_1^2, X_2^2, and X_3^2 are significant terms in this model. Both the predicted R^2 of 0.9453 and adjusted R^2 of 0.9846 are within reasonable range. The adequate precision' ratio of 50.283 specifies an adequate signal. Hence the quadratic model useful tools for exploring design space. Figure 8A, B shows the 3D response surface plots and their related 2D contour plots that demonstrate the impact of independent factors on the tailing factor of RTB, respectively. According to the graphs, the tailing factor of RTB increased with increasing temperatures and flow rates, while it reduced with increasing methanol concentrations.

Selection of optimized chromatographic condition

To choose the best chromatographic parameters, the Design expert® software's numerical optimization method was employed. The program gave the optimal chromatographic parameters for the separation of NLB and RTB, which included a mobile phase consisting of 32.80% methanol, a flow rate of 0.272 mL/min, a column temperature of 29.42 °C, and ultraviolet detection at 260 nm. A standard concentration of 48 µg/mL of NLB and 16 µg/mL of RTB were injected into the UPLC system with optimized chromatographic conditions and their responses were recorded. Table 5 displays the anticipated and observed values for the responses, as well as their percentage residual values**.** The residual values ranged from − 0.320 to 2.53, indicating that the QbD design used for selecting optimum chromatographic conditions for separating NLB and RTB was valid. The validation of the developed method was conducted using the optimized chromatographic conditions. The validity of the central composite design used was confirmed by the low % residual error values obtained for the observed values.

Method validation

Table 6 displays the system suitability parameters and their corresponding acceptance criteria. The number of theoretical plates (3956 ± 34 for NLB and 5436 ± 19 for RTB), tailing factor (1.33 for NLB and 1.28 for RTB), resolution factor (3.90) and % RSD for the retention time and peak area (< 1 for NLB and RTB) meet the specified

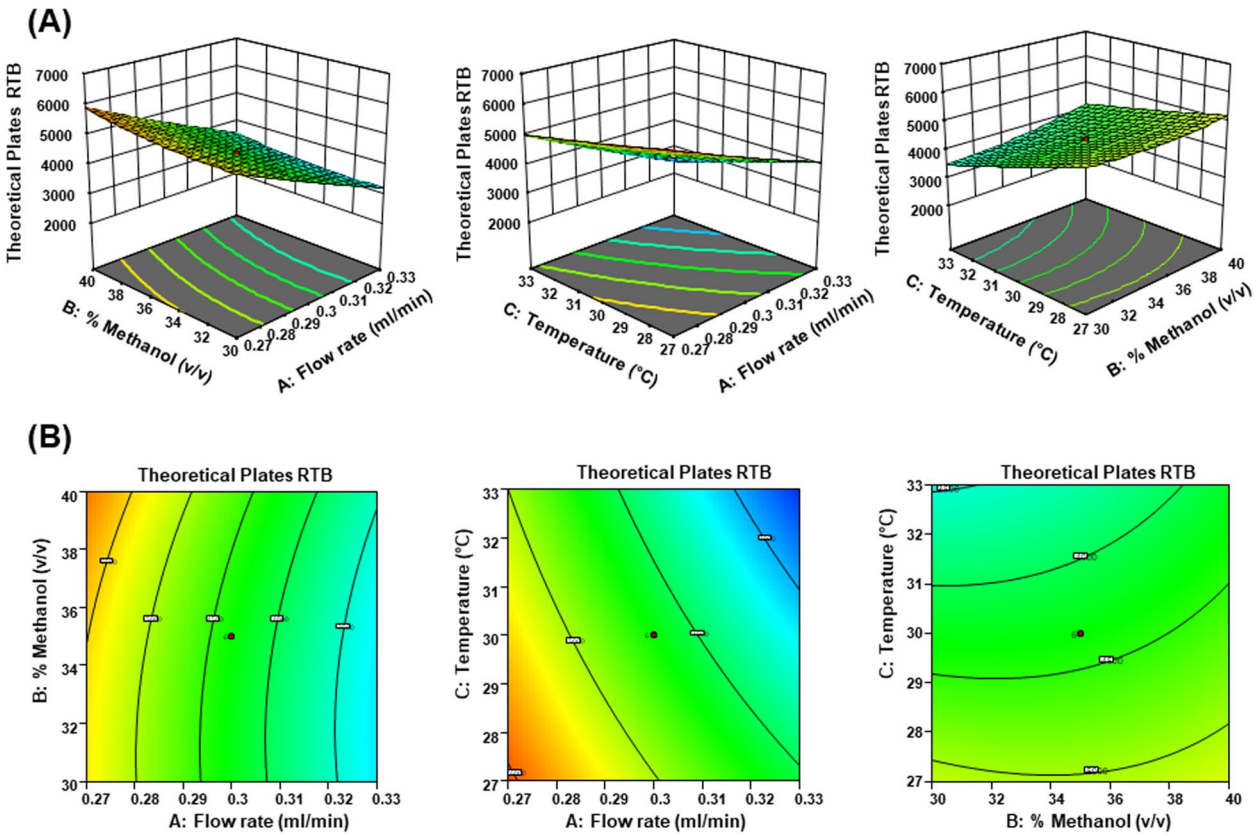

Fig. 6 The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) showing the effect of independent variables on the theoretical plates of RTB (Y_5)

reference values (theoretical plates > 2000, tailing factor < 2, resolution factor < 2, and % RSD < 2). The standard calibration curves were linear within the concentration ranges of 12–72 µg/mL for NLB and 4–24 µg/mL for RTB, as depicted in Fig. 9. The regression coefficient (r^2) values for NLB were found to be 0.9998 with its regression equation y = 22997x + 2505.9. In the case of RTB, the regression coefficient (r^2) was found to be 0.9997 with regression equation y = 21594 + 1344.2. The LOD and LOQ values were determined to be 0.89 µg/mL and 2.69 µg/mL for NLB and 0.15 µg/mL and 0.46 µg/mL for RTB respectively. The % RSD of intraday and interday precision for NLB and RTB was found to be less than < 2 (Table 7). The % recovery was found to be in the range of 99.57–100.43% for NLB and 99.59–100.61% for RTB (Table 8). The specificity of the developed method is shown in Fig. 10. No co-eluting interfering peaks from the formulation excipients were found at the retention time of NLB and RTB in the placebo and blank samples indicating the specificity of the established method. The results of the robustness data are shown in Table 9. The % RSD values for peak area and retention time were both below 2%, indicating minimal variance. System suitability characteristics including theoretical plates, tailing factor and resolution factor showed no significant variation. The percentage assay of drug content was found to be 99.46 for NLB and 99.98 for RTB in the marketed formulation. The negligible changes in the peak areas of the NLB and RTB before and after keeping the solutions at room temperature for 24 h indicate the solution stability of both drugs.

Forced degradation studies

The results of the forced degradation studies of NLB and RTB under different stress conditions are shown in Fig. 11 and Table 10. Both NLB and RTB undergo a negligible extent of degradation (less than 2%) under the specified acid, alkali, thermal and neutral conditions. Under oxidative degradation, NLB showed 5.81% degradation whereas RTB showed 6.19% degradation with one degradation peak at 1.71 min. In the case of photolytic degradation, both NLB and RTB showed a degradation of 8.79 and 7.27% with three degradation peaks at 1.25, 2.21 and 2.67 min. Further, it was observed that there was no interference from the degradation peaks.

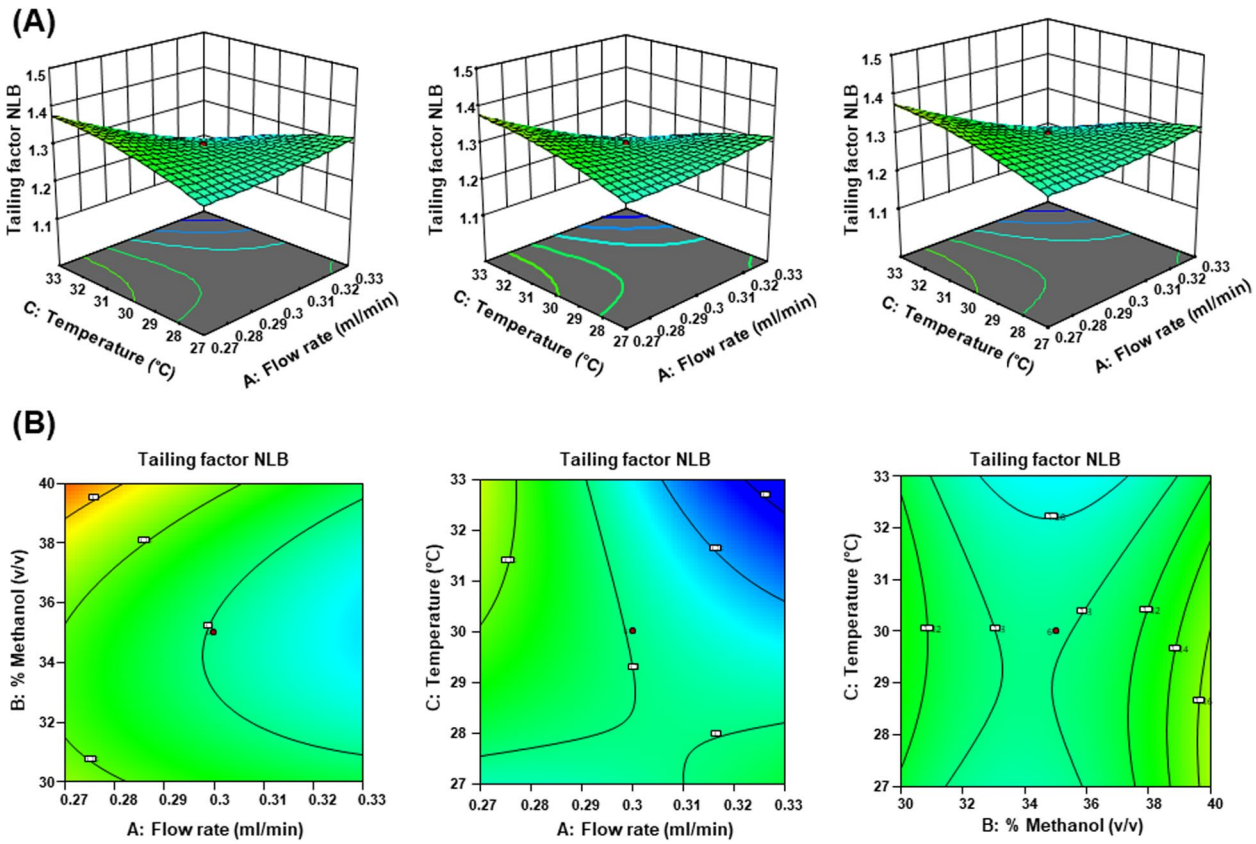

Fig. 7 The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) showing the effect of independent variables on the tailing factor of NLB (Y_6)

Discussion

The current research utilized AQbD to develop an RP-UPLC method for estimating NLB and RTB. A central composite design (CCD) with three components at five levels was employed to optimize chromatographic conditions for the developed method utilizing Design-expert® software (Stat-Ease Inc., USA). Polynomial equations, 3D response plots, and 2D contour plots were created to analyze the impact of three independent factors (flow rate, % methanol, and temperature) on dependent variables (retention time, resolution factor, number of theoretical plates, and tailing factor). ANOVA was employed to assess the statistical significance of the model and its terms. Subsequently, a numerical optimization technique was utilized to predict the ideal chromatographic conditions. According to the polynomial equations and 3D-response plot, temperature and flow rate were the most significant factors affecting the retention time of NLB and RTB respectively. The resolution factor was highly influenced by % methanol in the mobile phase in comparison to flow rate and temperature. The flow rate was found to have a more pronounced effect on the number of theoretical plates for both drugs. In the case of the tailing factor, flow rate was found to be the more influential factor for NLB whereas % methanol was the more decisive factor for RTB. The QbD model effectively selected optimum chromatographic conditions for estimating NLB and RTB, as shown by the minimal residual error and close agreement between actual and anticipated values.

Analytical methods are validated to confirm their reliability and suitability for their purpose. The developed RP-UPLC method was validated as per ICH Q2 R1 guidelines. An essential component of method development is the system suitability test, which has been used to guarantee that the selected chromatographic system is operating properly throughout the analysis. All evaluated parameters such as theoretical plates, tailing factor, resolution factor and %RSD for peak area and retention time were well within the suggested ranges proving the suitability of the chromatographic system. The correlation coefficient (r^2) values > 0.999 for both drugs indicate a good correlation and excellent linearity over the proposed concentration ranges. Low LOD and LOQ values

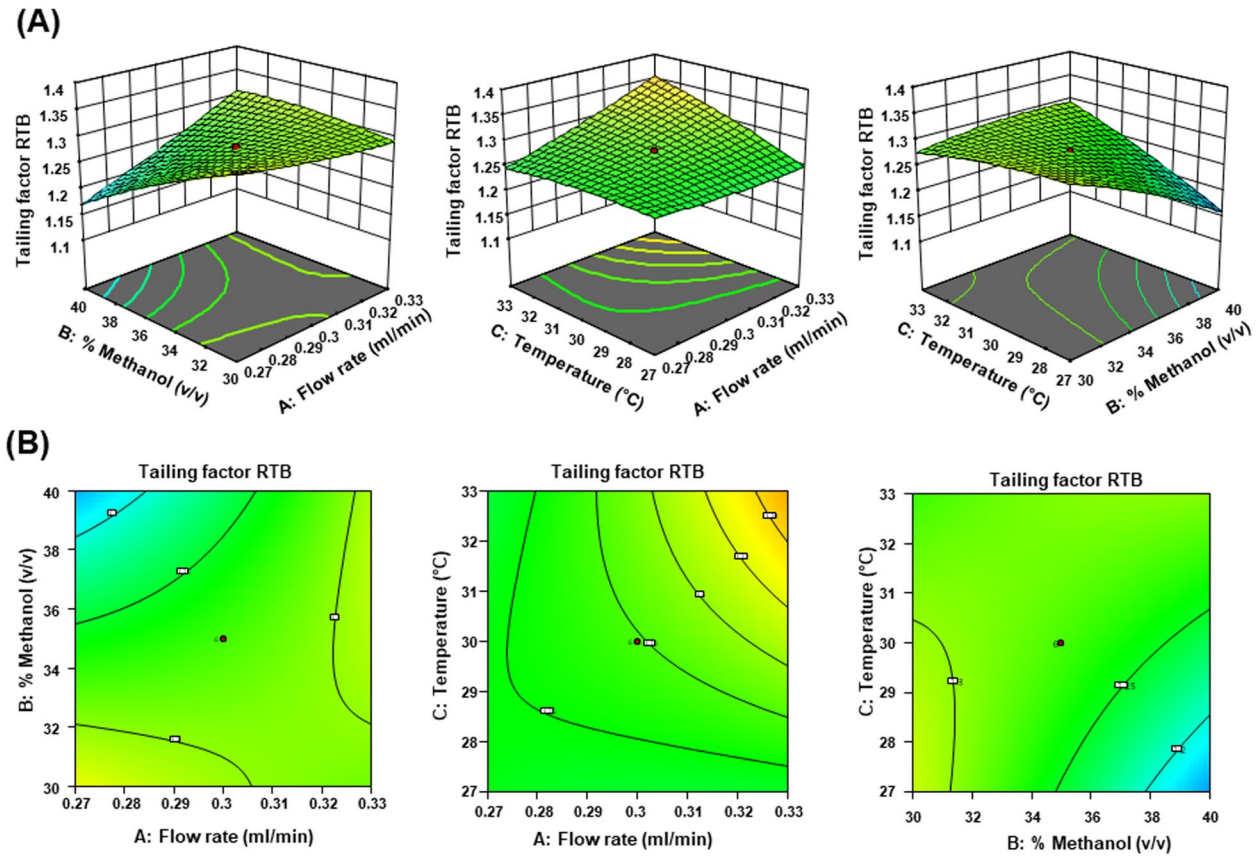

Fig. 8 The 3D response surface plots (**A**) and the associated 2D contour plots (**B**) showing the effect of independent variables on the on the tailing factor of RTB (Y_7)

Table 5 The predicted and observed values of the responses obtained from the optimized chromatographic conditions

Response	Predicted value	Observed value	Residual values (%)
Retention time NLB (min)	1.53	1.50	1.96
Retention time RTB (min)	1.97	1.92	2.53
Resolution factor	3.90	3.90	0.00
Theoretical plates NLB	3980	3993	−0.32
Theoretical plates RTB	5468	5451	0.31
Tailing factor NLB	1.33	1.33	0.00
Tailing factor RTB	1.29	1.29	0.00

showed that the proposed method was very sensitive for estimating RTB and NLB in pharmaceutical dosage forms. Analytical precision refers to the degree of agreement between measurements taken from multiple samples under similar conditions. The % RSD values for both intraday and interday precision studied at three different levels were less than 2% demonstrating the good precision of the developed method. Recovery studies assess how closely the experimental result matches the true value. The % recovery values for NLB and RTB ranged from 99.57 to 100.43% and 99.59 to 100.61% respectively demonstrating minimal variability and high agreement between experimental and actual values. An analytical method's specificity is defined as its ability to assess the target analyte even when interferences from contaminants, degradation agents, and excipients are present in commercially available formulations. The developed method is specific for the quantification of NLB and RTB from pharmaceutical dosage forms, as there are no co-eluting interference peaks at the retention time of both drugs. Robustness refers to a method's ability to remain consistent despite small changes in chromatographic parameters, demonstrating the method's reliability in everyday use. The low % RSD values, and lack of significant changes in system suitability parameters following deliberate changes in experimental conditions demonstrated good robustness of the developed method. The percentage assay results (99.46–99.98% for label claim)

Table 6 System suitability test parameters (n = 6)

Parameter*	NLB (48 µg mL^{-1})	RTB (16 µg mL^{-1})	Acceptance criteria
Retention time (t_R, min)	1.46 ± 0.005	1.88 ± 0.005	–
RSD % of retention time	0.35	0.27	< 1 for n ≥ 5
Peak area	1,110,380 ± 3844	346,204 ± 1968	–
RSD % of peak area	0.34	0.56	< 1 for n ≥ 5
Theoretical plates (N)	3956 ± 34	5436 ± 19	> 2000
Tailing factor (T)	1.33 ± 0.004	1.28 ± 0.005	≤ 2.0
Resolution (R_s)		3.90 ± 0.001	> 2.0

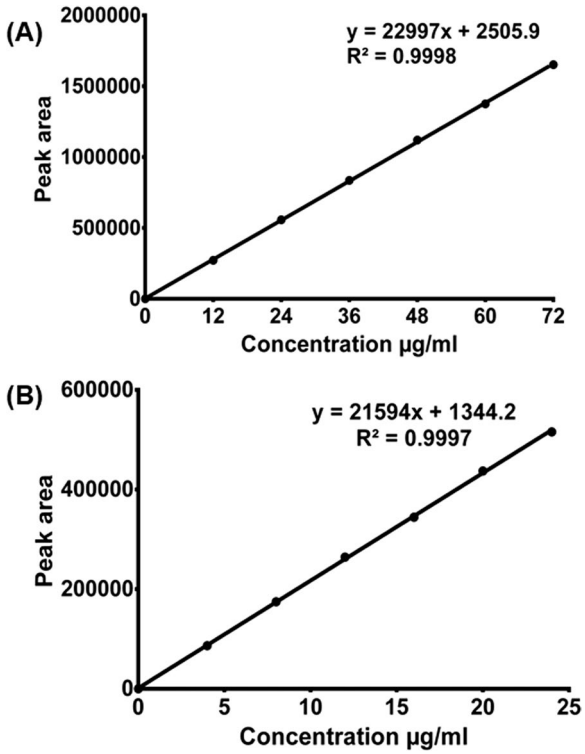

Fig. 9 Calibration curves of Nivolumab (**A**) and Relatlimab (**B**)

indicate the applicability of the developed RP-UPLC method for the estimation of NLB and RTB in marketed formulations. The stability investigation confirmed that NLB and RTB remained stable in solution at room temperature for 24 h.

Stability studies are crucial for evaluating the quality of pure drugs and drug products. The method's ability to evaluate the stability of NLB and RTB is demonstrated by assessing their degradation following exposure to different stress situations. Both drugs were unstable under oxidative and photolytic conditions but exhibited great stability under acidic, alkaline, thermal, and neutral conditions. These findings indicate the stability indicating the nature of the developed method.

Conclusions

An analytical quality by design methodology, specifically central composite design, was effectively utilized to optimize the RP-UPLC method for quantifying NLB and RTB. The optimized chromatographic conditions for the separation of NLB and RTB consisted of 32.80% v/v methanol in the mobile phase, 0.272 mL/min flow rate, column temperature of 29.42 °C, and UV detection at 260 nm. The method showed well-resolved peaks at

Table 7 Recovery studies of NLB and RTB

Compound	Contents (µg)	Quantity added (µg)	Recovered amount (µg)	Recovery (%)	% RSD
NLB	48	24	23.89	99.57	0.43
	48	48	48.13	100.28	0.54
	48	72	72.31	100.43	0.44
RTB	16	8	7.96	99.59	0.39
	16	16	16.09	100.61	0.26
	16	24	23.93	99.74	0.78

Table 8 Intraday and interday precision of NLB and RTB

Compound	Content (µg/mL)	Intraday (n = 6)		Inter day (n = 3)					
				Day 1		Day 2		Day 3	
		Found (µg/mL)	% RSD	Found (µg/mL)	% RSD	Found (µg/mL)	% RSD	Found (µg/mL)	% RSD
NLB	12.00	11.60	0.69	11.56	0.58	11.55	0.69	11.69	0.59
	36.00	36.22	0.58	36.00	0.64	36.42	0.97	36.25	0.12
	72.00	71.74	0.46	71.71	1.09	72.08	0.78	71.42	0.73
RTB	4.00	3.94	0.46	3.92	0.63	3.95	0.85	3.95	0.74
	12.00	12.33	0.97	12.42	1.10	12.19	0.98	12.38	0.86
	24.00	23.82	0.13	23.79	1.12	23.82	0.40	23.85	0.75

n, number of replicates; RSD, relative standard deviation

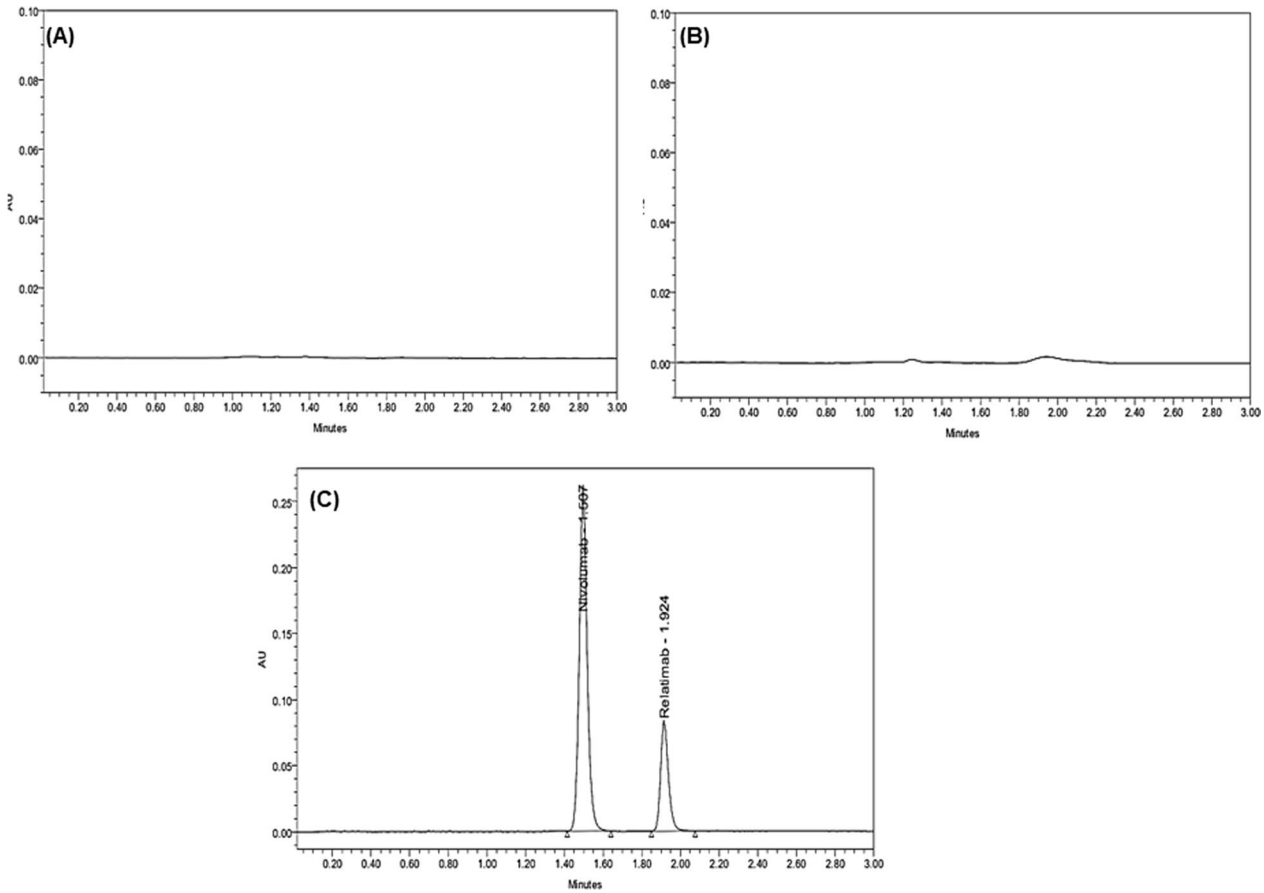

Fig. 10 Chromatograms of Blank (**A**), placebo (**B**) and standard NLB and RTB (**C**) showing the specificity of the established UPLC method

the retention time of 1.46 and 1.88 min for NLB and RTB respectively. The method was verified according to ICH criteria and demonstrated linearity, accuracy, precision, sensitivity, specificity, and robustness. The forced degradation studies showed well-resolved peaks of NLB and RTB along with degradation peaks. The findings of the present study conclude that AQbD is an effective methodology for method optimization and the established method is suitable for the accurate determination of NLB and RTB in pharmaceutical dosage forms.

Table 9 Results of robustness study (n = 6)

Parameter	Modification	Observed value							
		% RSD of peak area		Theoretical plate(N)		Tailing factor (T)		Resolution factor	
		NLB	RTB	NLB	RTB	NLB	RTB	NLB	RTB
Flow rate	0.1 mL/min	1	0.7	3920	5506	1.33	1.28	–	3.33
	Optimized	0.35	0.27	3993	5451	1.33	1.28	–	3.9
	0.3 mL/min	1.8	1	3923	5546	1.32	1.28	–	3.8
% methanol in mobile phase	31.16% MeOH	1	0.9	3961	5523	1.33	1.28	–	3.78
	Optimized	0.35	0.27	3993	5451	1.33	1.28	–	3.9
	34.44% MeOH	1.3	0.8	3922	5266	1.33	1.28	–	3.84
Column temperature	27.95 °C	1.8	0.4	4488	5309	1.32	1.28	–	3.74
	Optimized	0.35	0.27	3993	5451	1.33	1.28	–	3.9
	30.89 °C	1.3	0.6	4063	5408	1.31	1.27	–	3.8

n, number of replicates; RSD, relative standard deviation

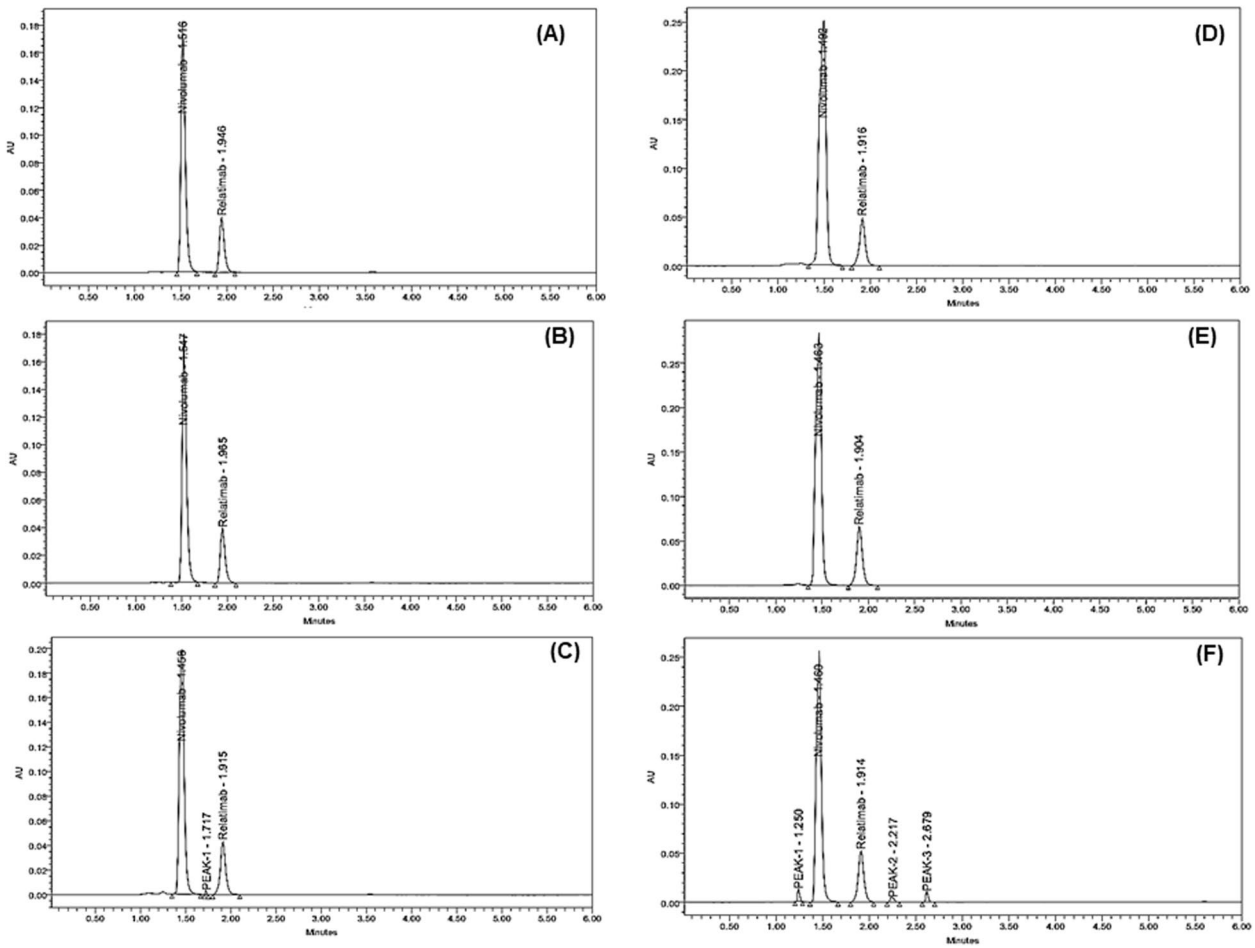

Fig. 11 Chromatograms showing the degradation profile of NLB and RTB under different stress conditions acid (**A**), alkali (**B**), oxidative (**C**), dry heat (**D**), neutral (**E**), and photolytic degradation (**F**)

Table 10 Stability studies of NLB and RTB

Stress condition	Treatment	% Degradation	
		NLB	RTB
Acid hydrolysis	2N HCl, 60 °C for 30 min	0.43	0.59
Alkaline hydrolysis	2N NaOH, 60 °C for 30 min	1.72	1.87
Oxidative degradation	60% H_2O_2 60 °C for 30 min	5.81	6.19
Thermal degradation	105 °C for 6 h	0.93	0.48
Photolytic degradation	UV light for 7 days	8.79	7.27
Neutral hydrolysis	Water at 60 °C for 6 h	0.31	1.05

Abbreviations

AQbD	Analytical quality by design
QbD	Quality by design
PD-1	Programmed death receptor-1
NLB	Nivolumab
LAG-3	Lymphocyte-activation gene 3
RTB	Relatlimab
RP-UPLC	Reversed-phase ultra performance liquid chromatography
CCD	Central composite design
LOD	Limit of detection
LOQ	Limit of quantification
FDA	Food and drug administration
LC–MS/MS	Liquid chromatography tandem mass spectrometry
ELISA	Enzyme linked immunosorbent assay
LC–MS/HRMS	Liquid chromatography–mass spectrometry/High resolution mass spectrometry
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry
UHPLC/UV-HESI	Ultra-high-performance liquid chromatography/ultraviolet-heated electro spray ionization
UV	Ultra-violet
TUV	Tunable ultra-violet
BEH	Bridged ethylene hybrid
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
H_2O_2	Hydrogen peroxide
SD	Standard deviation
RSD	Relative standard deviation
ANOVA	Analysis of variance
3D	Three dimensional
CV	Coefficient of variation
PRESS	Predicted residual error sum of squares

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Author contributions
MVN carried out the experimental work and prepared the manuscript. RS interpreted the analytical data and helped in handling of Design expert software. AKT designed and supervised the experimental work. All authors read and approved the final manuscript.

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Availability of data and materials
The data that support the findings of the present study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
The authors declare no conflict of interest.

Human and animal rights
Studies involving plants must include a statement specifying the local, national or international guidelines and legislation and the required or appropriate permissions and/or licences for the study: Not applicable.

Competing interests
The authors declare that they have no competing interests.

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