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Analytical Quality by Design (AQbD)-Based Development and Validation of a Robust RP-HPLC Method for Quantification of Regorafenib in Bulk Drug and Tablet Dosage Form

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ABSTRACT

Regorafenib is an orally available multikinase inhibitor which is widely used for the treatment of hepatocellular carcinoma, metastatic colorectal cancer, and advanced gastrointestinal stromal tumors. A proper and dependable quantitative determination of regorafenib is imperative to guarantee the quality, safety and efficacy of pharmaceutical formulations. An Analytical Quality by Design (AQbD) approach was used to develop and optimize a robust RP-HPLC method for quantitative estimation of regorafenib in bulk drug and tablet dosage form in the present study. The mobile-phase composition and flow rate were optimized via a 3² full factorial design, which was performed using Design-Expert® Version 13. The optimized chromatographic condition was an acetonitrile:water (75:25, v/v) mobile phase, flow rate of 1.5 mL/min, detection wavelength of 262 nm, and retention time of 3.9 min. The optimized method showed a very good chromatographic efficiency with symmetrical chromatograms and a tailing factor of 1.25 and a theoretical plate count of 12,760.

The validation of the method was carried out following the ICH Q2(R2) guidelines. The method developed showed good linearity in the range of 20–70 µg/mL with a coefficient of determination (R²) of 0.9997. The limit of detection (LOD) and limit of quantification (LOQ) were 0.25 µg/mL and 0.75 µg/mL, respectively, which indicates high analytical sensitivity. The mean recovery values of the accuracy studies were between 98.07% and 100.48% and the %RSD values of precision studies were < 0.22% indicating excellent repeatability and reproducibility. The reliability of the method was also checked through robustness testing, which showed %RSD values ≤ 0.39% after deliberate changes of the analytical conditions. The developed method was then validated by applying it to the commercial product, Regdual® 40 mg, and the obtained result was 99.43%, which fell within the acceptance criteria of ICH. The incorporation of AQbD principles such as systematic risk assessment, statistical optimization and analytical design space led to a robust, reliable and transferable RP-HPLC method for routine quality control in the pharmaceutical industry and regulatory purposes.

Keywords: Regorafenib; RP-HPLC; Analytical Quality by Design; Full-factorial design; Design space; Method validation.

INTRODUCTION

The process of tumour angiogenesis is a key factor in the development of cancer and its ability to grow, invade and metastasize. In colorectal cancer, increased angiogenic activity is closely linked to poorer clinical outcome, such as an early recurrence of the disease and a decrease in survival. The vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) signaling pathway is a very attractive therapeutic target, as it is frequently over-expressed in gastrointestinal (GI) malignancies. Regorafenib is an oral multikinase inhibitor that inhibits several receptor tyrosine kinases, including VEGFR-1, VEGFR-2 and VEGFR-3, which are involved in tumor angiogenesis and progression of the disease [1]. It has approval for the treatment of metastatic colorectal cancer, advanced gastrointestinal stromal tumors and hepatocellular carcinoma after previous systemic treatments [2]. In 2012, the U.S. Food and Drug Administration (FDA) approved regorafenib as the first multikinase inhibitor to demonstrate a benefit in the overall survival of patients with metastatic colorectal cancer (mCRC) who had failed standard treatment [3]. The FDA subsequently approved it for the treatment of advanced hepatocellular carcinoma (HCC) that has been previously treated with sorafenib, in 2017 [4]. The chemical structure of regorafenib is shown in Fig. 1.

[Insert Fig. 1 here]

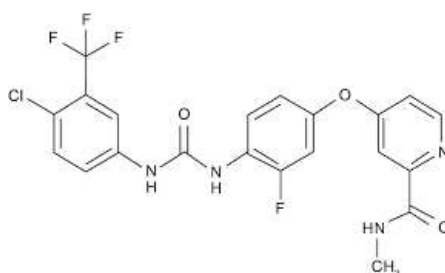


Fig. 1 Structure of Regorafenib [5]

Regorafenib, also known as 4-chloro-3,4-bis(trifluoromethyl)phenyl-4-fluorophenoxy-2-pyridinecarboxamide, is a multikinase inhibitor that is orally active. It has molecular formula $C_{21}H_{15}ClF_4N_4O_3$ and molecular weight of 482.8 g/mol. The drug comes in pale pink to white, film-coated, 40-mg tablets. Regorafenib is a Biopharmaceutics Classification System (BCS) Class II compound, has low aqueous solubility, high permeability and has a melting point of 206–210°C. It has been found to inhibit a variety of protein kinases, such as vascular endothelial growth factor receptors (VEGFRs), KIT, RAF, and platelet-derived growth factor receptors (PDGFRs), leading to the inhibition of angiogenesis, tumour proliferation, and disease progression [5]. Thus it is crucial to develop reliable and precise analytical methods for the quantitative estimation of regorafenib in bulk drug substances and pharmaceutical dosage forms for product quality, dosage accuracy, therapeutic efficacy and regulatory compliance in manufacturing and quality control process [6].

Quality by Design (QbD)

Quality by Design (QbD) is a systematic and knowledge-driven approach to pharmaceutical development, which involves identifying quality goals in advance, understanding the science of the product and process in development, and managing risks throughout the product life cycle [7]. This approach is based on principles described in the International Council for

Harmonisation (ICH) guidelines, such as ICH Q8 for pharmaceutical development, ICH Q9 for quality risk management and ICH Q10 for pharmaceutical quality systems which advocate the design of robust and reliable pharmaceutical products and processes [8–10]. Analytical science is an essential part of lifecycle management: Analytical methodologies are crucial to product quality from development to commercialization [11]. In this context, Analytical Quality by Design (AQbD) refers to the application of the QbD principles to the development of analytical methods, where an analytical target profile is defined, critical method attributes and parameters identified and systematic risk assessment and optimization strategies implemented. This science and risk based approach enables analytical procedures to be developed that are robust, reliable and appropriate for routine use as well as for lifecycle management and ongoing method improvement [12,13].

Analytical gap and rationale for the present study

A thorough search in the literature revealed that limited number of methods have been reported for the quantitative determination of regorafenib such as UV–visible spectrophotometry, reverse-phase high-performance liquid chromatography (RP-HPLC), and liquid chromatography–mass spectrometry (LC–MS) [14–18]. A majority of those analytical procedures have been developed by the traditional one factor at a time (OFAT) optimization approach in which one experimental variable is changed at a time whilst all the other variables remain constant. While these methods have shown to provide acceptable analytical performance, they do not include consideration of interactions between method variables, do not have a defined analytical design space, and do not provide much information on the method robustness. Therefore, these methods might be vulnerable to minor changes of analytical conditions, which may influence their reproducibility, transferability and daily use in quality control laboratories. Moreover, there is no published RP-HPLC method for regorafenib that included a complete Analytical Quality by Design (AQbD) approach including systematic risk assessment, experimental design, statistical optimization, and a scientifically justified design space.

To meet this unmet need, the present study was conducted to develop RP-HPLC method for the estimation of regorafenib using AQbD. Unlike the previously reported OFAT based approaches, the proposed analytical strategy involves a structured AQbD workflow that includes Ishikawa (fishbone) diagram to identify the critical method parameters, preliminary risk assessment, and a 3^2 full factorial designed experiment to assess the individual and interactive effect of the critical method parameters such as mobile-phase composition and flow rate. The developed model was statistically evaluated through ANOVA and subsequently validated and established an analytical design space to guarantee the consistent and robust method performance. The inclusion of statistical optimization tools, model diagnostics and design space evaluation make it to a scientific, reliable and transferable analytical procedure for the routine quality control of regorafenib. Furthermore, the factorial and central composite experimental designs are efficient optimization approaches that allow obtaining as much information as possible from a relatively limited number of experiments, and allow systematic development of methods and management of their lifecycles [19].

Analytical method-development strategy

The basic values of Quality by Design (QbD) is to build quality into the analytical method early in its development, using a systematic, science-based and risk-oriented approach, instead of end-product testing [20]. Hence, a structured experimental approach was applied for the optimization of the analytical method. The 3^2 full factorial experimental design was chosen since it allows simultaneous evaluation of the effect of a number of critical method parameters at a minimum of 9 experimental runs. This statistically balanced design allows the estimation of main and interaction effects and of the curvature of the response surface to gain a complete understanding of the analytical system. Factorial design is more efficient than the traditional one factor at a time (OFAT) design in that it can determine the interaction between parameters, define robust operating conditions, and establish the optimized analytical design space [21]. Through the use of Design-Expert® software (Version 13, Stat-Ease Inc.), experimental design, mathematical model generation, analysis of variance (ANOVA) and response surface evaluation were used to select chromatographic conditions that consistently met the predetermined analytical performance criteria.

MATERIALS AND METHODS

Materials

Regorafenib reference standard was gifted from Hetero Pharmaceuticals Pvt. Ltd., Hyderabad, India. The acetonitrile, methanol and purified HPLC grade water used throughout the study were purchased from Variety Traders. The commercially available tablet formulation, Regdual® 40 mg (INTAS Pharmaceuticals Ltd., India) was bought from a local retail pharmacy and used for the assay and method validation studies.

Instrumentation

Chromatographic studies were conducted with an Agilent 1100 Series HPLC system with an autosampler, UV detector and EZChrom Elite software for instrument control, data acquisition and chromatographic processing. UV-visible (UV-Vis) spectrophotometric measurements were made using a UV-3092 UV-Visible spectrophotometer made by Lab India and controlled by the UV-WIN software. Fourier transform infrared (FTIR) spectra were obtained with a Fourier transform infrared (FTIR) spectrometer (Bruker Alpha II with OPUS software for the acquisition and analysis of the FTIR spectra). Prior to analysis, sample solutions were degassed and sonicated with a Nucleus Bioscience ATS-2 ultrasonic bath. Appropriate calibration of all analytical instruments and operation under standard analytical conditions was carried out throughout the experimental study to ensure reliability and reproducibility.

Chromatographic conditions

The separation was performed on a 250×4.6 mm, $5 \mu\text{m}$ C18 analytical column at 25°C . To achieve good chromatographic performance, an aqueous–organic mobile phase was used to avoid the use of buffer salts and pH modifiers, which would complicate the analytical procedure, but also to maintain good retention and peak symmetry. Before using, the mobile

phase and diluent were passed through a 0.45 μm membrane filter and sonicated for 10-15 minutes to remove any particulate matter and dissolved gases. All analyses were performed at ambient autosampler temperature. The sequence of injections included a blank solution followed by standard and sample injections; standard injections were made periodically throughout the sequence of sample analyses to assess system suitability. The injection volume was 10 μL , the flow rate of the mobile phase was 1.5 mL/min and the wavelength of the chromatographic detection was set at 262 nm.

Organoleptic Evaluation

The organoleptic properties of regorafenib (colour, odour and physical appearance) were evaluated under standard laboratory conditions. This was a preliminary assessment to verify the identity of the drug substance and to look for any visible signs of contamination, degradation, and other physical abnormalities prior to analytical method development.

Solubility Study

Solubility of regorafenib was studied in methanol, acetonitrile and water. These solvents were chosen for use because they are suitable for chromatographic analysis and for use in the preparation of mobile phase and sample solutions in the method development process.

Selection of Detection Wavelength

Regorafenib standard solution was scanned in the UV–Visible spectrophotometer within the range of 200–400 nm to determine the maximum absorption wavelength (λ_{max}). The wavelength showing maximum absorbance was selected to achieve optimum analytical sensitivity and precision in subsequent quantitative analysis.

Preparation of Diluent

Standard and sample solutions were prepared using a mixed diluent of methanol:acetonitrile:water (55:25:20, v/v/v). The appropriate amount of each solvent was carefully measured with calibrated glassware, placed in a clean volumetric flask and thoroughly mixed to achieve a uniform solution [6].

Preparation of Mobile Phase

Mobile phase components (acetonitrile and HPLC-grade water) were prepared separately and placed in clean solvent reservoirs. The solvents were sonicated for 10-15 min before use in chromatography, to remove any gases from solution and to improve degassing to reduce baseline fluctuations during analysis.

Preparation of Standard Solution (50 µg/mL)

The stock solution of the regorafenib was prepared in the prepared diluent. Then 0.5 mL of this stock solution was accurately transferred to a 10 mL volumetric flask and diluted to the required volume with the same diluent (methanol:acetonitrile:water, 55:25:20, v/v/v) to get a working standard solution of regorafenib (50 µg/mL or 50 ppm).

Preparation of Sample Solution

Average tablet weight of twenty commercially available regorafenib tablet was accurately weighed and finely powdered. Ten milligrams of regorafenib powder was placed in a 10-mL volumetric flask. The prepared diluent (methanol:acetonitrile:water, 55:25:20, v/v/v) was added and the mixture was sonicated for 30 min to ensure complete extraction of the drug. The volume of the solution was then adjusted with the same diluent to the mark. The solution was first filtered through Whatman filter paper and then filtered through a 0.45 µm membrane filter to remove the insoluble particles. Lastly the 0.5 mL of the filtrate was diluted to 10 mL using the prepared diluent to give a sample solution of 50 µg/mL (50 ppm) of regorafenib for chromatographic analysis.

Experimental Design (3² Full Factorial Design)

A 3² full factorial design was designed and used to systematically investigate the influence of CMPs on the analytical performance of the developed RP-HPLC method. Experimental variables were chosen based on what could affect the predetermined critical quality attributes (CQAs) from the Analytical Quality by Design (AQbD) risk assessment. A nine-run experimental design was used, which resulted from two independent factors tested at three coded levels (low, medium and high). This design allowed measuring the main, interaction and quadratic effects of the chosen variables. The two independent variables selected and their coded levels are presented in Table 1, while the dependent variables (analytical responses) monitored for each experimental run are listed in Table 2.

The experimental planning, statistical modelling, optimization and response surface analysis were completed using Design-Expert® software (Version 13, Stat-Ease Inc., Minneapolis, MN, USA). An appropriate polynomial equation was used to fit the experimental data and thus to develop mathematical models which describe the relationship between the critical method parameters and analytical responses. Analysis of variance (ANOVA) was used to assess the significance and contribution of each of the terms of the models, as explained in the section entitled “ANOVA and Model Evaluation.” The optimum chromatographic conditions and the corresponding analytical design space was generated based on the fitted response models to ensure the robustness and reproducible performance of the method. The equation of the polynomial that was fitted is given by:

$$Y = \beta_0 + \beta_1A + \beta_2B + \beta_{12}AB + \beta_{11}A^2 + \beta_{22}B^2$$

The polynomial equation indicates that Y is the predicted analytical response, while A and B are the independent variables that correspond to the mobile-phase composition and flow rate, respectively. The intercept of the regression model (β_0) is the constant term. The parameters

β_1 and β_2 represent the individual (main) effects of the factors selected, β_{12} is the interaction effect between the selected factors and β_{11} and β_{22} are the quadratic effects included to account for the curvature of the response surface. Using the technique of backward elimination, statistically insignificant terms were eliminated one at a time to get the most suitable predictive model, which only contains the significant factors and interaction. This method enhanced model predictability, without compromising statistical validity and analytical robustness.

Risk assessment

To identify the experimental variables systematically that may affect the performance of the developed RP-HPLC method, a preliminary risk assessment was done. First, an Ishikawa (fishbone) diagram (Fig. 2) was drawn to determine possible sources of analytical variability that might occur due to instrument, analyst, method, materials, environment and measurement process. After identification of potential risks qualitatively, each risk was assessed and prioritized by a preliminary risk ranking exercise (Fig. 3) taking into account its expected effect on predefined critical quality attributes (CQAs).

Mobile-phase composition and flow rate were found to be the CMPs that most significantly affect the chromatographic performance. These variables were thus chosen to be further optimized in the 3^2 full factorial experimental design as independent variables. Other experimental variables such as sonication time, solvent degassing time, type of membrane filter, and sample preparation conditions and related operational parameters were considered as low-risk factors and kept constant during the experimental study in order to eliminate unnecessary variation and to allow for proper evaluation of the critical factors selected.

[Insert Fig. 2 here]

Figure: Ishikawa (fishbone) diagram of factors affecting the RP-HPLC method

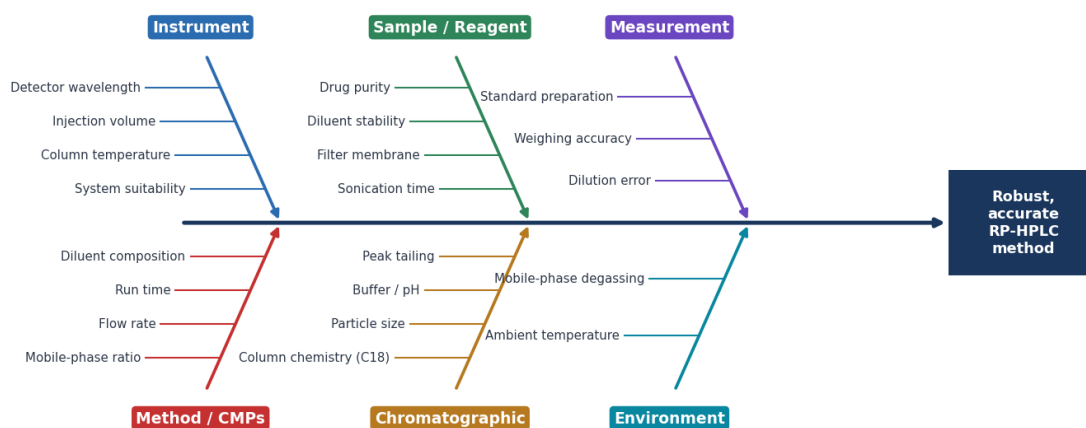


Fig. 2 Ishikawa (fishbone) diagram of factors affecting the RP-HPLC method

[Insert Fig. 3 here]

Initial risk assessment (RPN-style ranking) of method parameters

Method parameter	Severity	Occurrence / probability	Overall risk (before study)
Mobile-phase ratio (ACN:water)	High	High	High
Flow rate	High	High	High
Detection wavelength	Medium	High	Medium
Column temperature	Medium	Medium	Medium
Injection volume	Low	Medium	Low
Diluent composition	Medium	Medium	Medium
Sonication / degassing time	Low	Low	Low
Filter membrane (0.45 µm)	Low	Low	Low

High-risk factors (mobile-phase ratio, flow rate) were carried into the 3² full-factorial design; low-risk factors were held constant.

Fig. 3 Initial risk assessment (RPN-style ranking) of the method parameters

[Insert Table 1 here]

Table 1 Independent variables and their levels

Independent variable	Low (−1)	Medium (0)	High (+1)
A = Mobile-phase ratio, ACN:water (v/v)	65:35	70:30	75:25
B = Flow rate (mL/min)	1.0	1.2	1.5

[Insert Table 2 here]

Table 2 Dependent variables (responses)

Response	Type
Y1	Tailing factor (peak asymmetry)
Y2	Theoretical plates (N)
Y3	Peak height / response (mAU)

RESULTS AND DISCUSSION

Organoleptic characteristics

The regorafenib sample as per organoleptic assessment (Table 3) was found to be white to pale-pink crystalline powder with no characteristic odour. These results correspond with the reported physicochemical parameters of regorafenib, and no sign of contamination or other macro impurities were observed in the drug sample.

[Insert Table 3 here]

Table 3 Organoleptic properties of regorafenib

Sr. no.	Parameter	Observation
1	Colour	Pale pink to white solid/powder
2	Odour	Odourless
3	Appearance	Crystalline powder

Solubility study

The solubility profile of regorafenib in the different solvents investigated is given in Table 4. It was sparingly soluble in both methanol and acetonitrile and virtually insoluble in water, which is indicative of its Biopharmaceutics Classification System (BCS) Class II property. From these observations, it was decided that mixed solvent system of methanol and acetonitrile was used as diluent to achieve complete dissolution of the drug. The acetonitrile – water mobile phase also provided suitable retention and good column performance on the C18 stationary phase, which led to good separation efficiency and peak characteristics.

[Insert Table 4 here]

Table 4 Solubility of regorafenib in different solvents

Sr. no.	Solvent	Solubility
1	Methanol	Slightly soluble
2	Acetonitrile	Slightly soluble
3	Water	Practically insoluble

Selection of detection wavelength (UV analysis)

According to the UV scan, maximum absorbance of regorafenib was 262 nm (Fig. 4) and this λ_{max} was chosen for the regorafenib quantification.

[Insert Fig. 4 here]

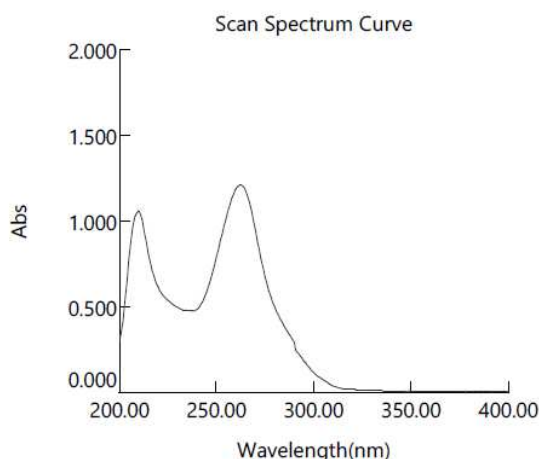


Fig. 4 UV absorption spectrum of regorafenib ($\lambda_{\text{max}} = 262 \text{ nm}$)

HPLC trials by 3^2 full-factorial design

Nine chromatographic trials were performed according to the 3^2 full factorial design, in which the mobile-phase ratio (acetonitrile:water) and the flow rate were each varied at three coded levels. The tailing factor, theoretical plate count and peak height recorded for every experimental run are summarised in Table 5. Across the experimental domain the tailing factor ranged from 1.11 to 1.29, the theoretical plate count from 12,760 to 19,737 and the peak height from 412,152 to 777,651 mAU, confirming that both critical method parameters exerted a measurable influence on chromatographic performance. Trial 2 (acetonitrile:water, 75:25, v/v; flow rate 1.5 mL/min) provided the most favourable overall balance of peak symmetry, column efficiency and detector response and was therefore selected for further statistical evaluation and optimisation.

[Insert Table 5 here]

Table 5 Factorial design runs and measured responses

Trial	Mobile-phase ratio (ACN:water, v/v)	Flow rate (mL/min)	Tailing factor	Theoretical plates	Peak height (mAU)
1	70:30	1.5	1.26	13,751	508,612
2 *	75:25	1.5	1.25	12,760	607,785
3	65:35	1.5	1.19	14,917	412,152
4	70:30	1.2	1.21	16,536	596,619
5	75:25	1.2	1.29	15,790	721,793
6	65:35	1.2	1.11	16,957	482,496
7	70:30	1.0	1.17	18,973	622,397
8	75:25	1.0	1.24	18,264	777,651
9	65:35	1.0	1.15	19,737	513,984

* Trial 2 was selected as the optimised run. The chromatograms of all nine trials are provided as Supplementary Information (Online Resource 1, Figs. S1–S9) to keep the main text concise.

ANOVA and model evaluation

The experimental data obtained from the 3^2 full factorial design were statistically analysed for all the three analytical responses, with analysis of variance (ANOVA). Backward elimination

was used to remove statistically insignificant regression terms and enhance model simplicity without reducing the model's predictive power. Tables 6-8 show the final reduced polynomial models along with the model ANOVA results and model adequacy parameters. The model F-values were statistically significant ($p < 0.05$) for all the developed models, which indicated regression. Moreover, the difference between the adjusted R^2 and the predicted R^2 was less than 0.20 in each case, indicating good agreement between model fitting and prediction. Adequate precision > 4 for all the responses, which is considered to be good signal to noise ratio, and this confirmed that the models developed were robust and reliable enough for the response prediction, optimization and construction of analytical design space.

[Insert Table 6 here]

Table 6 ANOVA for the tailing-factor model (Y1)

Source	Sum of squares	df	Mean square	F-value	p-value
Model (A)	0.01815	1	0.01815	14.43	0.0067
A – Mobile-phase ratio	0.01815	1	0.01815	14.43	0.0067
Residual	0.00881	7	0.00126	—	—
Total	0.02696	8	—	—	—

Model summary (Y1): The developed regression model of tailing factor had R^2 , Adjusted R^2 and Predicted R^2 of 0.6733, 0.6267 and 0.4743, respectively, which were acceptable. The model showed a satisfactory signal to noise ratio with a precision value of 6.58, which is good enough for navigation in the experimental design space. The standard deviation (SD) and the coefficient of variation (CV) were 0.035 and 2.94 %, respectively, showing good analytical precision. Based on the statistical analysis, the flow rate and the interaction and quadratic terms turned out to be statistically non-significant and thus not included in the final regression equation after backward elimination. Model results showed that the most important factor controlling peak tailing was the mobile-phase composition. The relatively small differences in the experimental range of the tailing factor (1.11–1.29) observed may explain the moderate R^2 value obtained for the model. All experimental values were well below the generally accepted value of 2.0, highlighting acceptable peak symmetry across the investigated design space.

[Insert Table 7 here]

Table 7 ANOVA for the theoretical-plates model (Y2)

Source	Sum of squares	df	Mean square	F-value	p-value
Model	4.412×10^7	2	2.206×10^7	469.9	< 0.0001
A – Mobile-phase ratio	3.835×10^6	1	3.835×10^6	81.7	0.0001
B – Flow rate	4.028×10^7	1	4.028×10^7	858.1	< 0.0001
Residual	2.816×10^5	6	4.693×10^4	—	—
Total	4.440×10^7	8	—	—	—

Model summary (Y2): Excellent predictive performance was obtained for the regression model developed for the theoretical plate count with a value of R^2 of 0.9937, an adjusted R^2 of 0.9915 and a predicted R^2 of 0.9832 showing an excellent agreement between the experimental and predicted data. The model also had a satisfactory precision of 54.21, much higher than the minimum value of 4, which indicates that the signal-to-noise ratio is very

good. In addition, the standard deviation (SD) and coefficient of variation (CV) for the analysis were 216.7 and 1.32%, respectively, indicating high precision of the analysis and low experimental variation. According to the statistical analysis, the flow rate was found to be the most affecting parameter to the theoretical plate count. Column efficiency was improved, and the number of theoretical plates was significantly increased with a decrease in flow rate, which is an indicator of the better performance of chromatography. This is in line with the theory of chromatography, which states that the slower the linear mobile-phase velocity, the better the mass transfer is likely to be, so the better the separation efficiency and higher the theoretical plate value.

[Insert Table 8 here]

Table 8 ANOVA for the peak-height (response) model (Y3)

Source	Sum of squares	df	Mean square	F-value	p-value
Model	1.079×10^{11}	4	2.698×10^{10}	259.8	< 0.0001
A – Mobile-phase ratio	8.134×10^{10}	1	8.134×10^{10}	777.8	< 0.0001
B – Flow rate	2.477×10^{10}	1	2.477×10^{10}	236.8	0.0001
AB – Interaction	1.157×10^9	1	1.157×10^9	11.07	0.0292
B ² – Curvature	1.409×10^9	1	1.409×10^9	13.47	0.0214
Residual	4.183×10^8	4	1.046×10^8	—	—
Total	1.083×10^{11}	8	—	—	—

Model summary (Y3): The regression model obtained for the peak response was very well statistically with $R^2 = 0.9962$, R^2 (adj.) = 0.9923 and R^2 (pred.) = 0.9742, which showed that the model was in excellent agreement with the experimental measurements. The model also presented an acceptable precision value of 47.41, which revealed a good relation between the signal and the noise, and the model could be used to predict the signal within the experimental domain. The standard deviation (SD) and coefficient of variation (CV) were 1.02×10^4 and 1.76%, respectively which indicate good analytical precision and reproducibility.

Statistically significant evaluation of the results indicated that the mobile-phase composition was the most significant factor affecting the chromatographic peak response and a lower flow rate also improved detector response. Also, the significance of the interaction term (AB) and quadratic term (B²) suggested that there were significant interaction effects and response surface curvature. These results clearly indicate that the critical method parameters are not linearly related and that the conventional one-factor-at-a time (OFAT) optimization approach is not suitable for describing this relationship, which illustrates the merits of the factorial experimental design.

The Pareto charts (Fig. 5) also confirmed the results of the ANOVA by showing the relative contribution of the model terms towards the analytical responses. The charts showed that the flow rate is the most important factor affecting the theoretical plate count while the most important factor affecting the peak response is the composition of the mobile phase. These observations confirm that both of these variables are important to be considered as method parameters for the RP-HPLC method optimization using the AQbD approach.

[Insert Fig. 5 here]

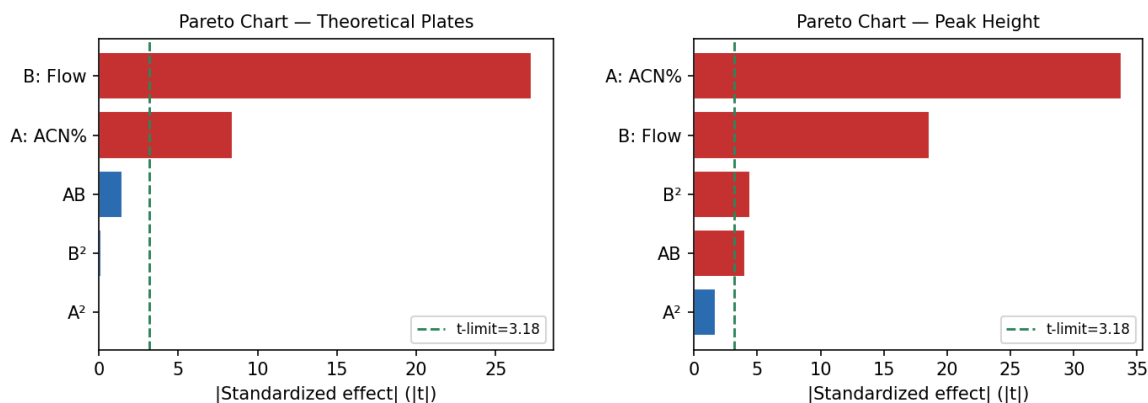


Fig. 5 Pareto charts of standardised effects for theoretical plates (left) and peak height (right)

Diagnostic plots of regression models revealed that the models fitted the data in the experiments well and met the statistical assumptions. In Fig. 6a, the residual errors were plotted on the normal probability plot, and the residuals fell closely on the reference line, suggesting that the residual errors were approximately normally distributed. Prediction-observation plots (Fig. 6b) revealed good agreement between the values from the experimental observation and the regression model predictions and suggested that the prediction models were highly capable. In addition, the plots of residuals against predicted values (Fig. 6c) showed a random distribution of the residuals with no apparent trend or systematic pattern, thus showing homoscedasticity (constant error variance) and the absence of model bias. The overall diagnosis outcome is a proof of the developed statistical models, which is satisfactory to show the adequacy, reliability, and predictive accuracy of the method optimization using RP-HPLC.

[Insert Fig. 6 here]

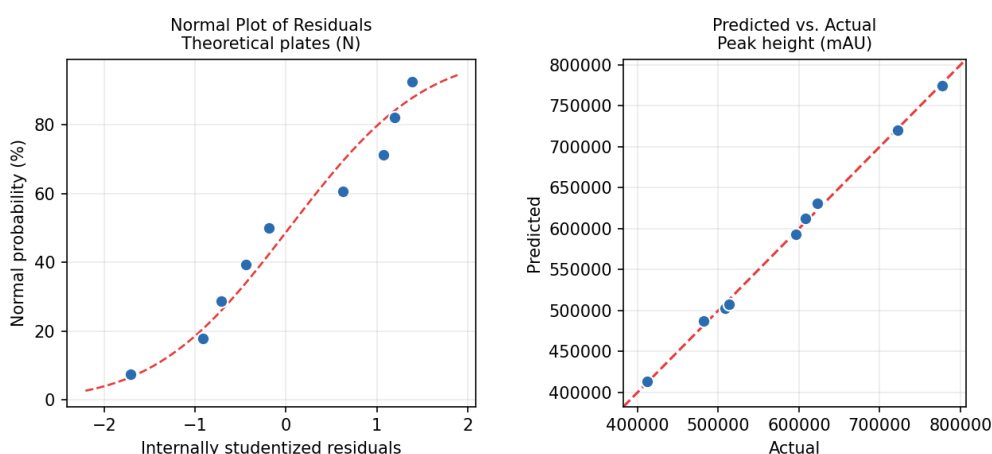


Fig. 6 (a) Normal probability plot of residuals (theoretical plates) and (b) predicted-vs-actual plot (peak height)

[Insert Fig. 6c here]

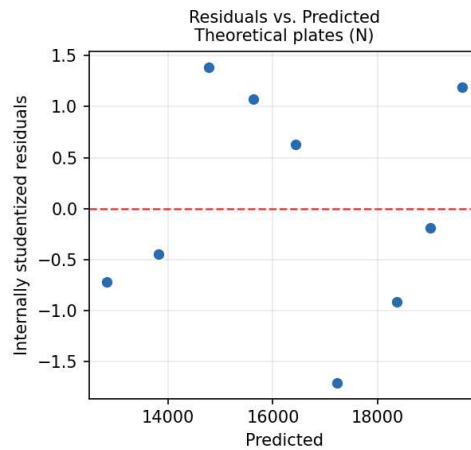


Fig. 6c Residuals-vs-predicted plot (theoretical plates)

Response-surface and contour analysis

The contour and three-dimensional response surface plots (Figs. 7–9) show the combined effect of the mobile-phase ratio and the flow rate on each response and provide confirmation of the trends indicated by ANOVA.

[Insert Fig. 7 here]

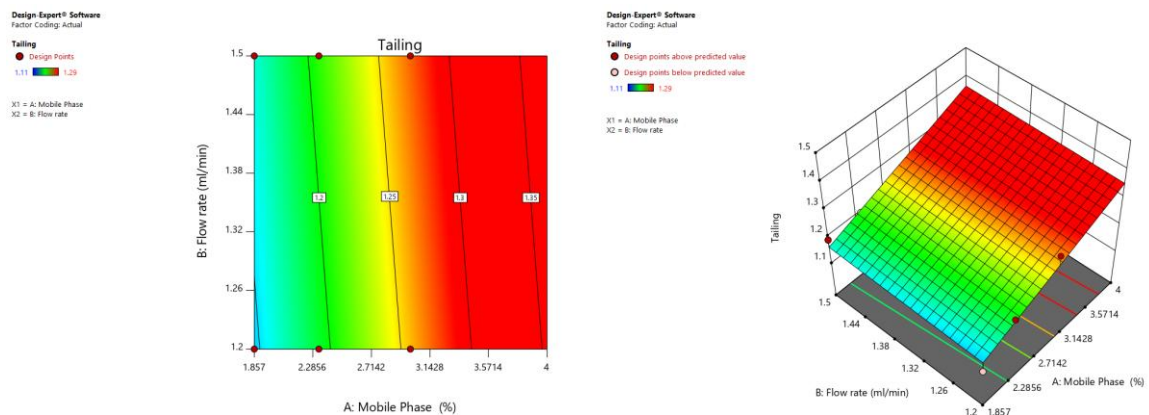


Fig. 7 Contour plot (left) and 3D response surface (right) showing the effect of the critical method parameters on the tailing factor

[Insert Fig. 8 here]

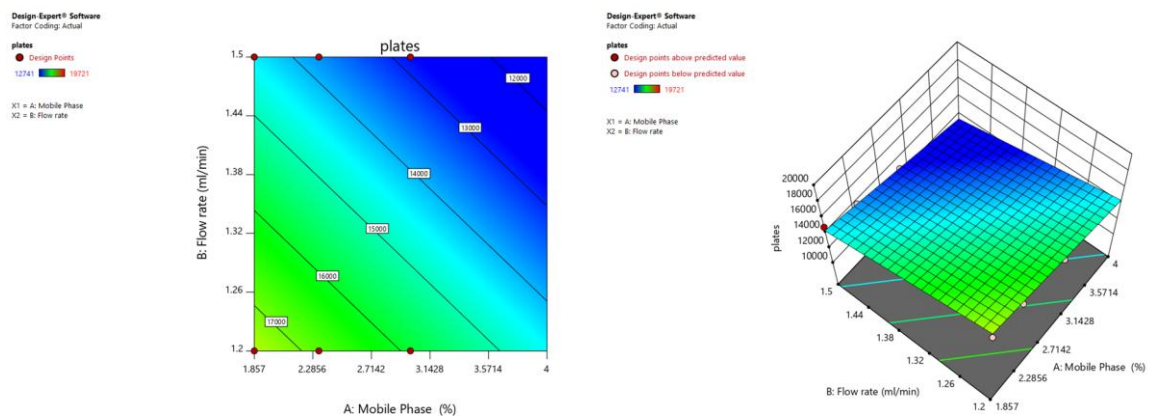


Fig. 8 Contour plot (left) and 3D response surface (right) showing the effect of the critical method parameters on theoretical plates

[Insert Fig. 9 here]

[Insert Fig. 10 here]

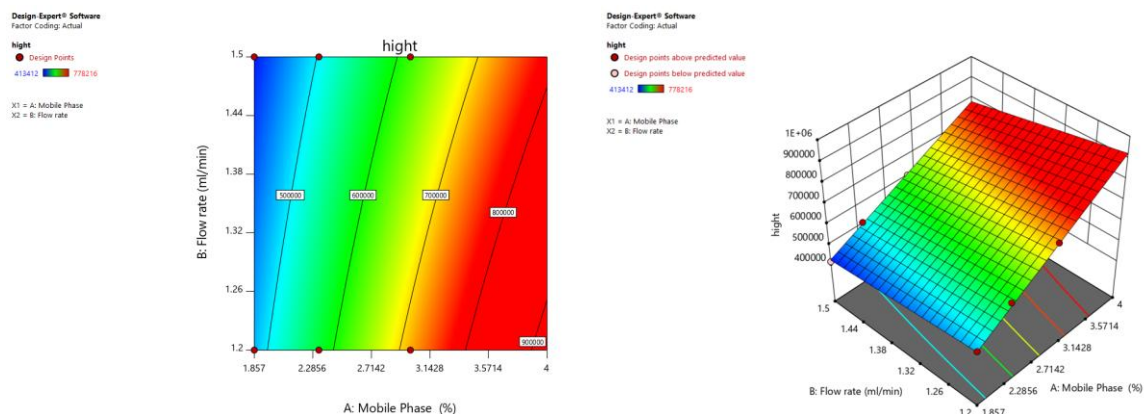


Fig. 9 Contour plot (left) and 3D response surface (right) showing the effect of the critical method parameters on peak height

Design space and optimisation

Fig. 10 displays the overlay plot (design space) generated by applying all the predefined acceptance criteria for all three critical analytical responses: tailing factor < 1.3, theoretical plate count > 12,000, and peak height > 550,000. The shaded area in the overlay plot is the analytical design space, where all the critical quality attributes (CQAs) are met simultaneously, ensuring a strong and reliable chromatographic performance. The best chromatographic conditions predicted by the statistical model were: mobile phase, a mixture of 75% acetonitrile and 25% water (Trial 2) and flow rate, 1.5 mL/min (Trial 2). This operating point is well within the design space set, and was therefore chosen as the final analytical point. The optimised chromatographic conditions adopted for the final method are summarised in Table 9, and the response values recorded at this operating point are presented in Table 10. The chromatogram of regorafenib obtained under the optimised conditions is shown in Fig. 11. The method under these optimized conditions gave the optimized balance of acceptable retention time and excellent peak symmetry as well as high column efficiency and strong detector response confirming the robustness and suitability of the developed AQbD-based RP-HPLC method for routine quantitative analysis of regorafenib.

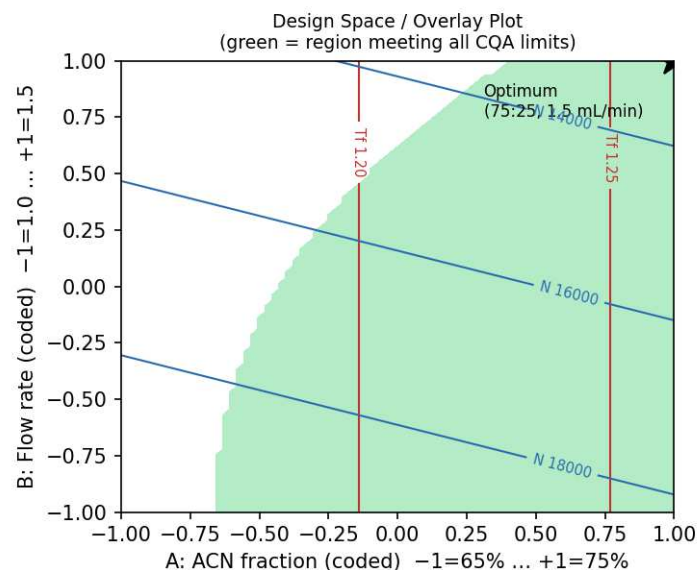


Fig. 10 Design space / overlay plot; the shaded region satisfies all critical-quality-attribute limits, with the optimum marked (★)

[Insert Table 9 here]

Table 9 Optimised chromatographic conditions

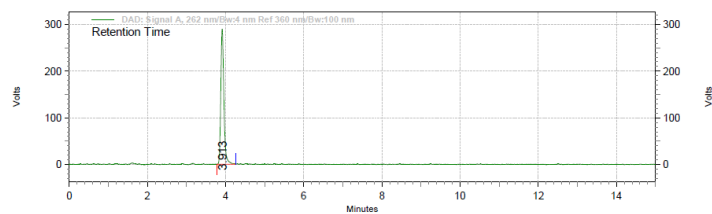
Parameter	Optimised condition
Mobile phase	Acetonitrile:water (75:25 v/v)
Column	C18 (250 × 4.6 mm i.d., 5 µm)
Flow rate	1.5 mL/min
Column temperature	25 °C
Injection volume	10 µL
Run time	10 min
Detection wavelength	262 nm

[Insert Table 10 here]

Table 10 Response values at the optimised condition (Trial 2)

Trial	Mobile-phase ratio	Flow rate (mL/min)	Retention time (min)	Tailing factor	Theoretical plates
2	75:25	1.5	3.9	1.25	12,760

[Insert Fig. 11 here]



DAD:
Signal A,
262
nm/Bw:4
nm Ref
360
nm/Bw:10
0 nm

Results

Retention Time	Area	Area %	Height	Height %	Asymmetry	Theoretical plates (USP)	Asymmetry (10%)
3.913	3321503	100.00	607785	100.00	1.40854	12760	1.25408
Totals	3321503	100.00	607785	100.00			

Fig. 11 Chromatogram of regorafenib under the optimised conditions ($t_R \approx 3.9$ min)

METHOD VALIDATION

The developed RP-HPLC method was successfully validated as per the recommendations of ICH Q2(R2). Validation showed that the method met all the acceptance criteria set for the performance characteristics evaluated such as Linearity, Analytical Range, Accuracy, Precision, Limit of Detection (LOD), Limit of Quantification (LOQ), Robustness and System Suitability. The validation results obtained were satisfactory and established the reliability, accuracy, precision and suitability of the proposed analytical procedure for routine quantitative estimation of regorafenib in pharmaceutical dosage forms [22,23].

1. Linearity and range

Linearity is an indicator of the property of an analytical method to yield test results which are directly proportional to the concentration of the analyte over a stated range of working [23]. The developed RP-HPLC method was validated with the linearity of the method between 20–70 µg/mL at six different levels of concentrations. The chromatographic peak areas were plotted against concentrations of regorafenib (Table 11 and Fig. 12) to obtain a calibration curve. The regression equation $y = 71061.8x - 42667.1$ and the coefficient of determination (R^2) of 0.9997, showed excellent linearity over the range investigated, and the analytical curve was well defined, with a good linear relationship between the concentration of the analyte and the detector response.

[Insert Table 11 here]

Table 11 Calibration data for regorafenib

Sr. no.	Concentration (µg/mL)	Peak area
1	20	1,408,659
2	30	2,078,638
3	40	2,760,512
4	50	3,506,682
5	60	4,238,142
6	70	4,938,048
Regression: $y = 71061.8x - 42667.1$		$R^2 = 0.9997$

[Insert Fig. 12 here]

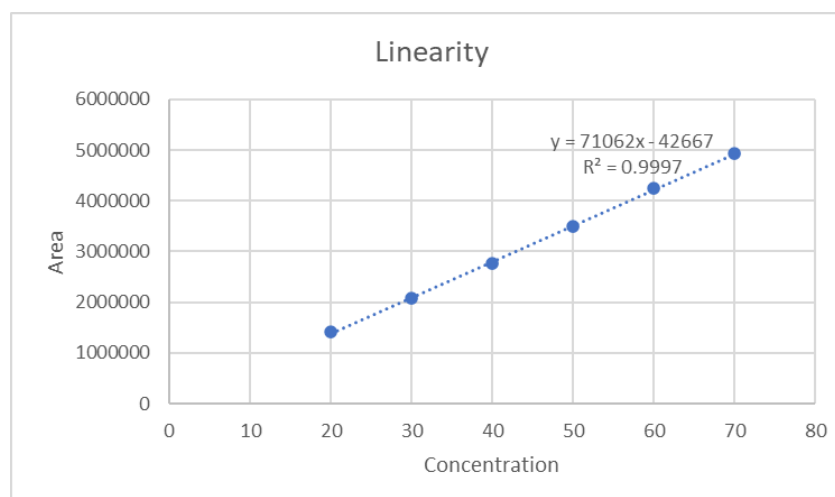


Fig. 12 Calibration curve of regorafenib (20–70 µg/mL)

Interpretation: The built calibration model showed excellent linearity with the coefficient of determination (R²) of 0.9997, indicating a good proportional relationship between detector response and concentration of regorafenib in the investigated analytical range. The correlation coefficient obtained met with the criteria defined in the ICH Q2(R2) guideline for linearity, and showed good calibration results. Additionally, the validated linear range covered the routine assay concentration of 50 µg/mL which was well within the validated calibration interval allowing the quantitative estimation of the level with sufficient accuracy, precision and reliability during routine quality control analysis.

2. Accuracy

The accuracy can be defined as the proximity of the value found in the experiment to the true value or the accepted reference value of the analyte. The accuracy of the developed RP-HPLC method was determined by the standard addition (recovery) method at three concentration levels, 80%, 100% and 120% of nominal assay concentration. The percentage recoveries for each level were determined for evaluation of trueness of the method (Table 12). The results of the recovery showed that the proposed analytical method was capable of accurately quantifying regorafenib in presence of pharmaceutical excipients thus making it suitable for routine assay applications.

[Insert Table 12 here]

Table 12 Recovery study of regorafenib

Sr. no.	Level (spiked)	Amount found (µg/mL)	Peak area	Mean area	% Recovery
1	80%	39.23	2,745,211	2,781,324	98.07
2	80%	39.79	2,785,200		99.48
3	80%	40.19	2,813,562		100.48
4	100%	50.15	3,521,623	3,519,145	100.31
5	100%	50.00	3,510,814		100.01
6	100%	50.20	3,525,000		100.40
7	120%	60.23	4,237,884	4,224,171	100.39
8	120%	59.99	4,201,630		99.99
9	120%	60.16	4,233,000		100.28

Interpretation: The analytical accuracy of the recovery study was good for all three concentrations. The mean percentage recoveries obtained at 80%, 100%, and 120% spiking levels were 99.34%, 100.24%, and 100.22%, respectively, with an overall recovery range of 98.07–100.48%. All the recovery values were within the acceptance range of 98–102% as recommended in the ICH Q2(R2) guideline and hence the developed analytical method was found to be highly accurate and true [27]. These results suggest that the formulation excipients, and other components of the sample matrix, did not significantly affect the quantification of regorafenib. Furthermore, the recovery results were not significantly different from or were slightly better compared with the previously reported one factor at a time (OFAT) RP-HPLC methods of regorafenib, which further confirmed the reliability and practical application of the proposed AQbD-based RP-HPLC method for routine pharmaceutical quality control [14,16,18].

3. Precision

Precision is the closeness of agreement between a series of independent test results obtained from repeated tests on a homogeneous sample under the same analytical conditions [23]. It is representative of the repeatability of the analytical procedure when measurements are repeated with the same analyst using the same apparatus over a short period of time and the reproducibility of the analytical procedure when measurements are made by different analysts, using the same apparatus over a long period of time, or by different laboratories, using different apparatus over a long period of time (intermediate precision). In general, an analytical method is acceptable if the %RSD of the results is within the acceptance limit, usually $\leq 2.0\%$, which signifies that the analytical method is consistent and reliable.

3.1 Repeatability

Six replicate injections of a 50 µg/mL standard were analysed (Table 13).

[Insert Table 13 here]

Table 13 Repeatability (n = 6)

Injection	Peak area
Replicate 1	3,506,682
Replicate 2	3,498,200
Replicate 3	3,512,400
Replicate 4	3,501,200
Replicate 5	3,517,800
Replicate 6	3,499,600
Mean $\pm$ SD	3,505,980 $\pm$ 7,817
%RSD	0.22%

3.2 Intra-day precision

A 50 µg/mL solution was analysed three times on the same day (Table 14).

[Insert Table 14 here]

Table 14 Intra-day precision

Sr. no.	Concentration	1st determination	2nd determination	3rd determination
1	50 µg/mL	3,502,100	3,507,563	3,563,561
2	50 µg/mL	3,510,500	3,506,291	3,573,216
3	50 µg/mL	3,499,800	3,552,165	3,561,032
Mean		3,504,133	3,522,006	3,565,936
SD		5,633	26,070	6,430
%RSD		0.16%	0.74%	0.18%

3.3 Inter-day precision

A 50 µg/mL solution was analysed on three separate days (Table 15).

[Insert Table 15 here]

Table 15 Inter-day precision

Sr. no.	Day	Peak area*	SD	%RSD
1	I	3,505,867	4,501	0.13%

Sr. no.	Day	Peak area*	SD	%RSD
2	II	3,505,100		
3	III	3,513,251		

*Mean of three determinations.

Interpretation: The precision study showed good reproducibility of the developed RP-HPLC method. The %RSD for repeatability (0.22%), intra-day precision (0.16–0.74%) and inter-day precision (0.13%) were all well below the ICH Q2(R2) acceptance limit of 2.0%, demonstrating the high precision of the analytical procedure. The consistently low %RSD values show that the replicate injections gave a low variation and that good agreement was obtained between analyses performed on different occasions. The results obtained indicate that the proposed method can give reliable and reproducible quantitative results in repeatability and intermediate precision conditions. In addition, the precision observed in this method was equivalent to or better than that reported in the previous RP-HPLC method to determine regorafenib in the literature, which indicated the robustness and suitability of the proposed AQbD based analytical method for routine quality control analysis.

4. Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were computed on the basis of standard deviation of response and slope of calibration curve ($LOD = 3.3/S$; $LOQ = 10/S$) [23] (Table 16).

[Insert Table 16 here]

Table 16 LOD and LOQ of regorafenib

Sr. no.	Parameter	Result
1	LOD	0.25 µg/mL
2	LOQ	0.75 µg/mL

Interpretation: The method is suitable to assay the bulk drug and its dosage form as the low value of LOD and LOQ indicate that the method can be used to analyze the concentration of regorafenib even below 20 µg/mL range of the calibration curve.

5. Robustness

To check the robustness of the analytical method, the detection wavelength was varied by 1 nm from the optimum value (262 nm) and the peak areas of the sample solution at 261 nm and 263 nm were determined (Table 17).

[Insert Table 17 here]

Table 17 Robustness data of regorafenib

Parameter	Area	Mean area	SD	%RSD
Wavelength 261 nm	3,499,500	3,511,000	11,500	0.33%
	3,511,000			
	3,522,500			
Wavelength 263 nm	3,486,800	3,500,500	13,700	0.39%
	3,500,500			

Parameter	Area	Mean area	SD	%RSD
	3,514,200			

Interpretation: 1 nm incremental shifts in wavelength provided %RSD of 0.39% which is less than 2% limits. Therefore, deliberate shifts of the operation parameters do not have a significant impact on the results which demonstrate method robustness, due to operation within the AQbD design space.

6. System-suitability parameters

System-suitability testing was conducted to ensure the chromatographic system was within the validated parameters (Table 18).

[Insert Table 18 here]

Table 18 System-suitability parameters

Sr. no.	Parameter	Acceptance limit	Result
1	Theoretical plates (N)	$N > 2000$	12,759
2	Tailing factor (T)	$T \leq 2$	1.2
3	%RSD of peak area	$\leq 2\%$	0.22%

Interpretation: All the chromatographic system suitability acceptance criteria were met. A high theoretical plate count indicated a high column efficiency and effective chromatography separation, and a tailing factor near 1.0 indicated a peak shape without peak distortion, and with symmetrical peak shape. Moreover, the low percentage relative standard deviation (%RSD) of replicate injections also revealed good injection precision and system reproducibility. All these results collectively confirm the optimal working of the chromatographic system and its suitability for reliable routine quantitative analysis of regorafenib.

Overall: With the optimized chromatographic conditions, the developed RP-HPLC method by the AQbD approach showed good chromatographic performance when using the mobile phase of acetonitrile (75:25, v/v) at 1.5 mL/min. The method gave well resolved and symmetrical peaks, tailing factor of 1.25, retention time 3.9 min, and the number of theoretical plates of 12,760, indicating good separation and high performance of the column. The validation of the method, according to ICH Q2(R2) guidelines, showed excellent analytical performance with very good linearity ($R^2 = 0.9997$), accuracy (recovery in the range 98–102%), precision (%RSD $\leq 0.74\%$), robustness (%RSD = 0.39%) and satisfactory analytical sensitivity. In addition to this, the systematic risk assessment and the design of experiments (DoE), statistical optimization, and the establishment of a scientifically justified design space (DS) will add a huge advantage over the traditional one factor at a time (OFAT) approach. Thus, the proposed method is a good analytical procedure which is robust, reliable and transferable for the quantitative estimation of regorafenib in pharmaceutical dosage forms in the routine quality control and regulatory applications.

CONCLUSION

A well-validated analytical quality by design (AQbD) approach based RP-HPLC method has been successfully developed for quantitative estimation of regorafenib in its bulk drug and pharmaceutical dosage forms. To understand and control critical method parameters,

especially the composition and flow rate of the mobile phase, the analytical method was systematically optimized by a 3² full factorial design, risk assessment, analysis of variance (ANOVA), and establishment of an analytical design space. This optimized chromatography condition always gave good separation and high analytical performance. The method was validated according to ICH Q2(R2) guidelines and showed to meet all the validation criteria for linearity, accuracy, precision, robustness, sensitivity and system suitability, making it suitable for routine quality control use.

The AQbD methodology provided a better understanding of the method variability, robustness and method reliability when compared with conventional one factor at a time (OFAT) methodologies, by scientifically justifying the optimization and establishment of a design space for the method. Thus, the proposed method is a robust, repeatable and portable analytical technique for the regular analysis of the regorafenib in quality control laboratories.

Further utility of the proposed method may be extended in future investigations by combining the proposed method with more advanced analytical techniques like UPLC, LC-MS/MS, HPTLC, FTIR for high sensitivity quantification, impurity and structural characterization and rapid analytical screening. Further, the method can be adapted to forced degradation studies, dissolution testing, stability-indicating analysis, and bioanalytical applications with biological matrices (such as plasma), which will expand its use in the pharmaceutical industry and in regulatory studies as well as in therapeutic monitoring.

ETHICAL APPROVAL

Not applicable.

AUTHORS' CONTRIBUTIONS

Pankaj H. Chaudhary conceived and supervised the study and provided scientific guidance throughout the investigation. Shreya P. Bhorkhade performed the experiments, developed and validated the analytical method, acquired the data, carried out the statistical analysis and drafted the manuscript. Ishika D. Pakade assisted with manuscript editing, reviewing and documentation. Abhijeet Welankiwar provided training in HPLC operation and assisted with the HPLC instrumental analysis. Rani B. Navale reviewed the manuscript and provided academic support during completion of the project. Prashant J. Burange contributed to the critical review of the work and provided technical expertise during the study. All authors read, reviewed and approved the final version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.