

Response Surface Methodology for the Optimized and Validated Investigation of Ropivacaine in Bulk and Tablet Dosage form by RP – HPLC

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Abstract: This study explains the response surface methodology-based novel RP-HPLC method for determining and validating ropivacaine in bulk and tablet dosage form. The effect of three critical, independent variables, the volume of ACN (acetonitrile) in the mobile phase, flow rate and wavelength on the responses Retention time, tailing factor, and Theoretical plates, were studied using Box- Behnken designs. The design was statistically analyzed using ANOVA, normal plot of residual, Box-Cox plot, perturbation, and 3D response surface plots. The quadratic model was used to predict response values. Chromatographic separation was obtained by ODS C18,5µm column with Isocratic elution. The mobile phase used is Acetonitrile (ACN): Methanol: Water (40:30:30) v/v, with 0.1% TEA. The flow rate was 1.5ml/min, and detection was carried out at 270nm. Retention time, R₂, was 6.670mins and 0.9892, respectively. The %RSD was not more than 2.0% in accuracy. The validation of the optimized chromatographic method was carried out as per ICH guidelines. The DOE using Box- Behnken design explained a significant effect of the mobile phase on the responses. The results show that the method is accurate, simple, reproducible, economical, robust, and rapid for routine qualitative analysis.

Keywords: design of experiments (DOE); RP- HPLC; ropivacaine; analysis of variance (ANOVA); UV detector; Box-Behnken design.

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1. Introduction

Ropivacaine is a local anesthetic agent. It is an amino amide local anesthetic indicated to produce local or regional anesthesia or analgesia for surgery, for oral surgery procedures, diagnostic and therapeutic procedures, and obstetrical procedures [1-5]. Ropivacaine blocks the generation and conduction of nerve impulses, presumably by increasing the threshold for electrical excitation in the nerve, slowing the propagation of the nerve impulse, and reducing the rising action potential rate. Specifically, it blocks the sodium channel and decreases the chances of depolarization and consequent action potentials [6]. The progression of anesthesia is connected to the diameter, myelination, and conduction velocity of affected nerve fibers [7,8]. The IUPAC name is (2S)-N-(2,6-dimethylphenyl)-1-propyl piperidine-2-carboxamide.

Figure 1. The molecular formula is $C_{17}H_{26}N_2O$. It has a pKa of 8.07, a molecular weight of 274.4 g/mol, and a solubility of 57.6mg/L in water.

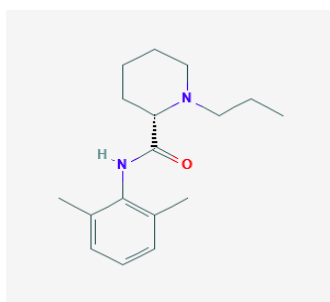


Figure 1. Chemical structure of ropivacaine.

The main goal of the design of experiments is to ensure the quality of the products. QbD ensures the combination of both the process and the product knowledge obtained during development, which can consistently deliver its planned performance [9]. Quality by Design (QbD) is constituted well for manufacturing processes in the pharmaceutical industry and development and manufacture of drug substances as per ICH Q8 [10] and ICH Q11 [11]. Analytical QbD (AQbD) is the terminology used when the QbD concepts are applied to analytical method development [12-17]. Development measurements are based upon analytical target profile (ATP) and critical quality attribute (CQA) to assess the performance characteristics. According to FDA guidance for process validation, three approaches can be applied to method validation [18]. That is method design, method qualification, continued method verification. QbD approach overcomes the disadvantages of traditional development methods such as time delay in studying one parameter variation one time, the number of runs required, and further optimization.

The study performed is based upon Box-Behnken Design which plays a major role in investigating the effect of independent variables on specific responses. This experiment design consists of points lying at the midpoint of each edge and the replicated center point of the multi-dimensional cube [19-23]. The experimental design and statistical data analysis were obtained by Design-Expert software (version 12.0.3.1) using the Box-Behnken Design (BBD). It requires fewer runs than a central composite design in cases of three or four variables to assess the method's robustness. The independent variables selected were the ACN concentration in the mobile phase (X_1 , %v/v), Flow rate (X_2 , ml/min), and Wavelength (X_3 , nm). Retention time (RT) (R_1 , min), tailing factor (R_2), and theoretical plate count (TP) (R_3 , N) were considered as co-variables or dependent variables. A 3-factor, 3-level Box Behnken design constructed 15 experimental runs. The significance of the design was rendered by the evaluation of statistical parameters, that is ANOVA method and the Good fit evaluation. The expansion of the method parameters was done based on the response surface method [24-29].

The chromatographic method is developed based on the design of the experiment's approach as per ICH guidelines. HPLC helps separate samples and allows better qualitative and quantitative analysis [30-33].

On the literature survey, it was noticed that there is no economical method available for the estimation and validation of ropivacaine in bulk and solid dosage form [34-41]. No risk assessment parameters were reported in the earlier methods. So, an attempt was made to develop a simple, accurate, precise, and robust RP-HPLC method for ropivacaine in bulk and a solid dosage form using QbD approach.

2. Materials and Methods

2.1. Chemicals.

Analytically pure Ropivacaine raw material was obtained from APP pharmaceuticals as a gift sample. HPLC grade (acetonitrile, methanol, water) were purchased from Loba Chemie chemical and reagents Pvt. Ltd.

2.2. Instruments.

The analysis of ropivacaine was carried out using Shimadzu Prominence HPLC System with lab solutions software and using a C₁₈ (250 X 4.6 mm, 5 µm) column. The system consists of UV detector. An Enertech Ultrasonic Cleaner was used for degassing the mobile phase. In addition, Shimadzu electronic weighing balance was used in this study.

To perform ropivacaine's response surface methodology analysis, design expert software version 12.0.3.1 was used.

2.3. Chromatographic conditions.

Chromatographic separation is achieved by C₁₈ column (250 X 4.6 mm, 5 µm) with UV detector and detected at 270nm. The mobile phase was constructed using acetonitrile, water, and methanol in the ratio of 40:30:30 (V/V) respectively, 0.1% TEA (Triethanolamine) was used. The mobile phase was passed through C₁₈ column at a flow rate of 1.5ml/min.

2.4. Quantitative estimation.

2.4.1. Preparation of standard stock solution.

Ropivacaine API 50mg was accurately weighed and transferred into a 50ml volumetric flask. The sample was solvated in a few ml of methanol and sonicated to degas. Volume was made up with the diluent to get 1000µg/ml concentration. This solution is used as a standard stock solution.

2.4.2. Preparation of working standard solution.

Further from the standard stock solution, 2ml was pipetted out into a 20ml volumetric flask and made the volume diluent to obtain the final concentration of 100µg/ml. This is used as a standard working solution.

2.4.3. Preparation of sample stock solution.

Ropivacaine sample (injection) 1ml was taken and transferred into a 20ml volumetric flask. Diluted with a few ml of methanol and sonicated it until it was completely dissolved. Volume was made up with the diluent to obtain the concentration of 1µg/ml. This solution is used as a sample stock solution.

The sample solution was injected twice, and the average area obtained was calculated and reported.

2.5. Design of experiments.

The method is performed using Design experts 12.0.3 software. A 2 level 3 factorial Box-Behnken design was established. The 15 experiments given by the software were performed and reported in Table 1. The significance of the design was determined by the evaluation of statistical parameters like the ANOVA method and good fit evaluation. The three independent variables used to investigate the responses are a percentage of ACN in the mobile phase, Flow rate, wavelength with Retention time, Theoretical plates, and tailing factor as responses. The optimized responses after 15 experimental runs were fed back into DOE software.

2.6. Method validation parameters.

2.6.1. Linearity.

Linearity was evaluated over the range of 50-150 µg/ml. The peak area was calculated for each injection, and the correlation coefficient value was determined by plotting the linearity graph with area in the y-axis and concentration in the x-axis.

2.6.2. Accuracy.

The % recovery studies were carried out by spiking the sample concentrations of 50%,100%,150% of standard solution were prepared in triplicate levels, and peak area was noted. The %RSD for recovery of triplicate samples at 50% to 150% levels should be within limits.

2.6.3. Precision.

The method's precision is determined by obtaining a sufficient number of samples, and relative standard deviation was calculated. The working sample solution was injected six times, and the area was measured for all six injections in HPLC. The %RSD for the area for six replicate injections should be within the specified limits.

2.6.4. System suitability.

The system suitability parameters were checked with six replicate injections, and the parameters, including relative standard deviation between standard injections, column efficiency, and tailing factor, were obtained.

2.6.5. Robustness.

The standard working solution was prepared and injected with specified conditions. The same standard solution was re-injected by changing one parameter and keeping other parameters constant.

2.6.6. Specificity.

A placebo was prepared, which contained all the materials except ropivacaine in the same proportion as present in the formulation. A sample solution is injected into the system. The chromatograms obtained were recorded.

2.6.7. QbD approach.

In the present study, three independent variables, percentage of acetonitrile in mobile phase composition (X₁), Flow rate (X₂), and wavelength (X₃), were selected based on the knowledge gained during the trial runs and from previous optimization studies. The experiments were carried out based on the experimental domain, and the qualitative responses studied were the retention factor (R₁), tailing factor (R₂), and theoretical plates (R₃). The responses were observed and listed in Table 1. The design suggested a classical second-degree model with an experimental quadratic domain. This model has the highest least squares regression value for all responses compared to other models. The lack of fit test, indicating an insignificant lack of fit value corresponding with higher p-Value than the model F-Value was used to examine the model. The response surfaces and perturbation plots showed the correlation of the effect of the factors on the covariants. The evaluation of the model was performed by studying the effect of individual factors on the responses in the form of contour plots.

3. Results and Discussion

3.1. Model fitting.

Three independent factors (Percentage of ACN in mobile phase, Flow rate, Wavelength) were taken based on the observation during the trials and from various optimization studies. The experiment was carried out based on the qualitative responses of the co-factors (Retention time, tailing factor, theoretical plates), and observed responses were noted and summarized in Table 1.

Table 1. Experimental Design for Independent variables and co-variants, experimental and predicted values.

S.NO	% of ACN(X ₁)	Flow rate (X ₂)	Wave length (X ₃)	Rt (R ₁)	Tailing factor(R ₂)	Theoretical plate (R ₃)	Desirability
1	40.000	1.500	270.000	6.754	1.532	25919.000	1.000
2	38.000	1.200	269.000	6.661	3.344	24764.000	1.000
3	42.000	1.000	271.000	6.234	1.264	26752.000	1.000
4	36.000	1.700	270.000	6.433	2.421	24562.000	1.000
5	42.000	1.600	268.000	6.771	1.132	25678.000	1.000
6	40.000	1.125	271.230	6.855	1.876	25679.148	1.000
7	36.000	1.481	269.389	6.277	3.321	25689.526	1.000
8	44.000	1.535	272.287	6.622	3.379	26783.050	1.000
9	38.000	1.322	271.433	6.336	1.390	28728.390	1.000
10	42.000	1.196	271.239	6.333	2.492	25794.613	1.000
11	44.000	1.078	274.736	6.207	2.120	25779.916	1.000
12	36.000	1.101	269.903	6.673	1.659	26439.541	1.000
13	38.000	1.459	269.361	6.827	3.130	24562.072	1.000
14	44.000	1.321	270.731	6.551	1.673	25569.277	1.000
15	42.000	1.137	270.319	6.543	1.149	25556.601	1.000

Among various models' the quadratic model was selected and examined using the lack fit-test. The procedure was evaluated for the effect of individual factors and their covariant responses Table 2 (ANOVA analysis).

Table 2. Statistical parameters by ANOVA analysis for the response.

Parameter	SS	Df	MS	F value	P value	Model F value	Model P value
Retention time (R ₁)	2.70	9	0.2999	34.52	0.0158	34.52	0.0019
Tailing factor (R ₂)	0.3218	12	0.0268	838.06	0.0250	838.06	0.0270
Theoretical plates (R ₃)	1.949	3	6.496	4495.40	0.2195	4495.40	0.0110

The classical polynomial quadratic equation for all the individual responses in terms of coded factors are shown as follows:

$$\text{Retention time} = +6.55 + 0.3436X_1 + 0.1256X_2 - 0.0353X_3 + 0.2986X_1X_2 + 0.1768X_1X_3 - 0.1046X_2X_3 - 0.3240X_1^2 + 0.0838X_2^2 + 0.0845X_3^2$$

$$\text{Tailing factor} = +2.85 + 0.7371X_1 + 0.3662X_2 + 0.0835X_3 + 0.2145X_1X_2 + 0.33X_1X_3 + 0.1363X_2X_3 + 0.6989X_1^2 - 0.2787X_2^2 - 0.9704X_3^2$$

$$\text{Theoretical plates} = +25877.50 + 1932.41X_1 + 532.30X_2 + 1649.59X_3 + 1011.38X_1X_2 - 1769.76X_1X_3 - 433.50X_2X_3 + 412.86X_1^2 - 536.72X_2^2 - 949.11X_3^2$$

The independent variables X_1 , X_2 , and X_3 provide the average response of varying factors from low to high. When two factors are modified, the interaction variables X_1X_2 , X_2X_3 , and X_3X_1 illustrate how the outcome changes. The polynomial quadratic was selected to explain the model.

The variables X_1 , X_2 , and X_3 and quadratic terms with positive signs indicate synergistic effect, while a negative sign indicates antagonistic effect in the polynomial equation.

When all the variants were compared, it was observed that X_1 (0.3436, 0.7372, 1.1932.41) coefficient value was found to be the highest varied, and hence mobile phase was considered a major contributing factor on R_1 , R_2 , and R_3 .

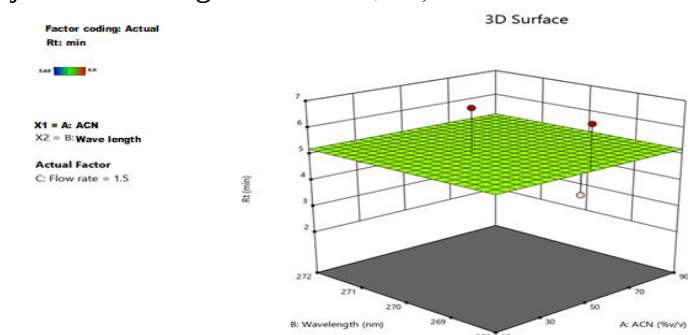


Figure 2. 3D- Response of retention time (RT) (R1).

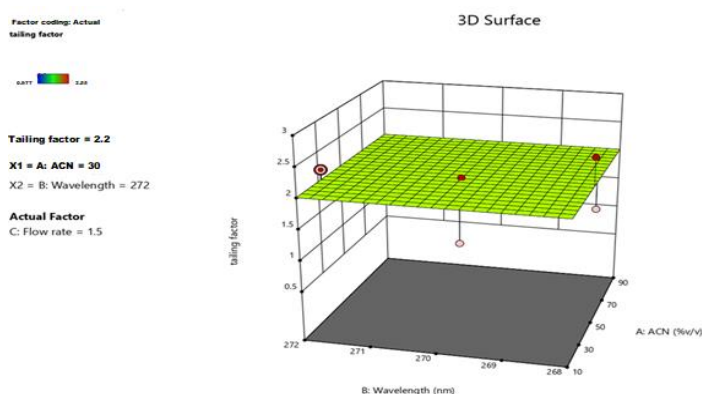


Figure 3. 3D- Response of tailing factor (R2).

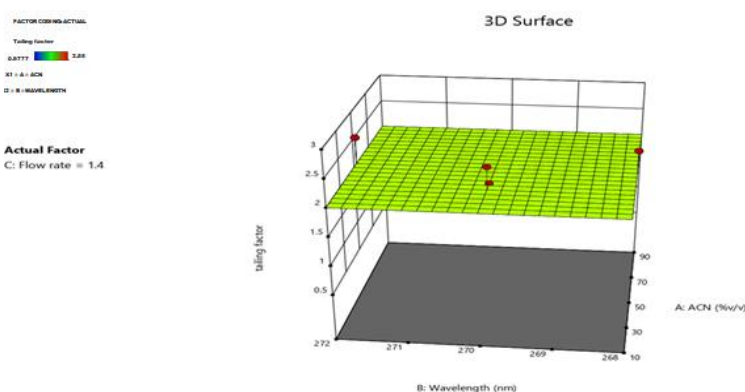


Figure 4. 3D- Response of theoretical plate (R3).

The 3D surface response and their contour plots of the quadratic model given by the software provide the interactive relationship of two factors on the response by keeping the third factor constant. The 3D surface plots of the interaction effect are shown in Figures 2-6.

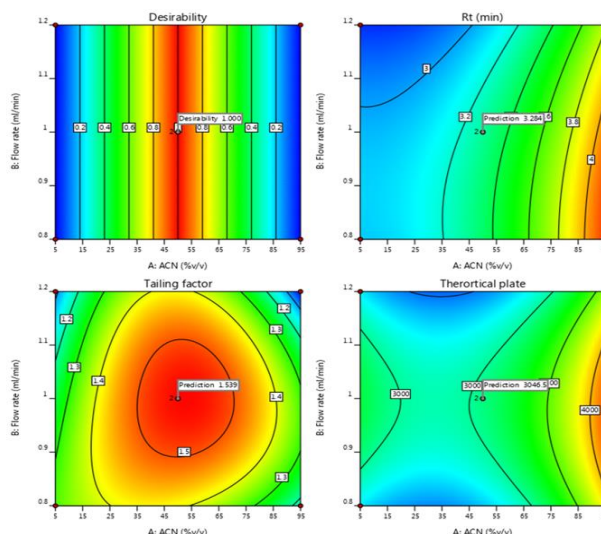


Figure 5. Desirability chart.

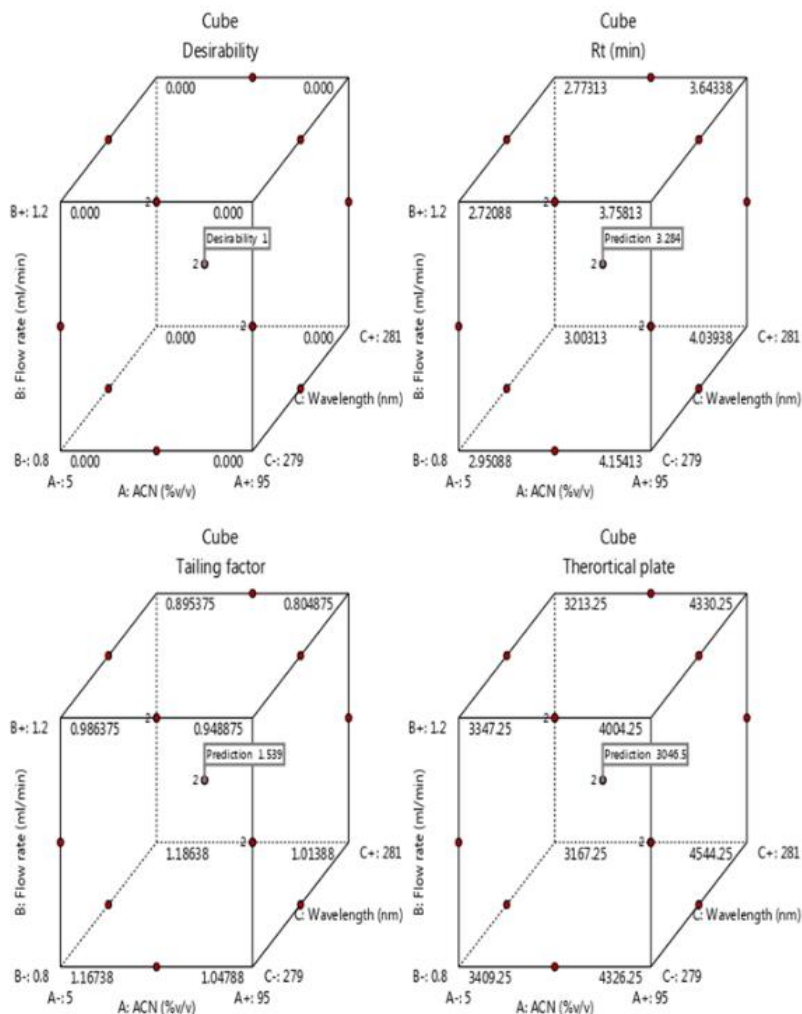


Figure 6. Box-Behnken design.

The predicted R-square value indicates how well the model could predict future data. The adjusted and predicted R-squared concludes that the applied statistical model effectively predicts the response. The high F-values imply the models are significant, and there is only a

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0.01% chance that F-values could occur due to noise. P-values less than 0.0500 indicate model terms are significant. If the value is greater than 0.100, that indicates the model terms are not significant.

3.2. Identification for optimum method condition.

It is obtained by setting the maximum and minimum values for the independent variables and entering their response variables as summarized in Table 3. The numerical optimization suggests the desired method solution. That method is considered to be an optimized method. The desired method parameters were found to be flow rate 1.5ml/min, the wavelength at 270nm with ACN concentration of 40% at the desirability of 1.000. The graphical optimization of desirability is shown in Figure 5.

Table 3. Independent variables and their corresponding levels.

Variable	Symbol	- α	-1	0	+1	+ α
Mobile phase	X ₁	36	38	40	42	44
Flow rate	X ₂	1.0	1.1	1.5	1.6	1.7
Wavelength	X ₃	268	269	272	272	274

3.3. Final optimized chromatographic conditions.

Separation was achieved with isocratic elution using a C₁₈ column, utilizing a mobile phase of acetonitrile: methanol (40:60) at a 1.5 ml/min flow rate with UV detection at 270 nm and retention time of ropivacaine was 6.670 min. From the desirability chart, the desirability value was found to be 1, and it is considered a suitable, good method and fit for analysis. The 3D surface shows that R², P-value, F-value are within limits, showing that the method is significant. Hence, we obtained the optimized method by Box-Behnken design and optimized the chromatographic conditions, which were further validated as per ICH guidelines. From the ANOVA results for the co-variates such as retention time, tailing factor, and theoretical plates, R² was found to be 0.9873, 0.9999, and 0.7398, respectively, and adequate precision was found to be 17.1327, 95.0270, 3.6343, respectively. The optimized chromatographic conditions are presented in Table 4, and the chromatograms are summarized in Figures 7–9.

Table 4. Optimized chromatographic conditions.

Column	Shimadzu HPLC system on ODS C18 (4.6 X 250mm) and 5 μ m
Flow rate	1.5ml
Column temperature	Room temperature
Wavelength	270nm
Injection volume	20 μ L
Retention time	6.670minutes
Run time	10minutes
Mobile phase	Acetonitrile: methanol: water (40:30:30), 0.1%TEA

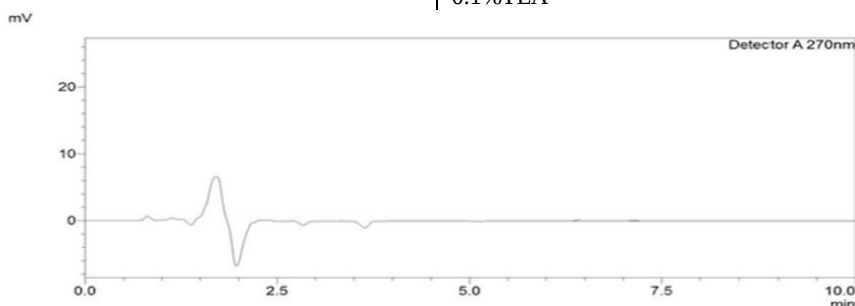


Figure 7. Chromatogram of the blank.

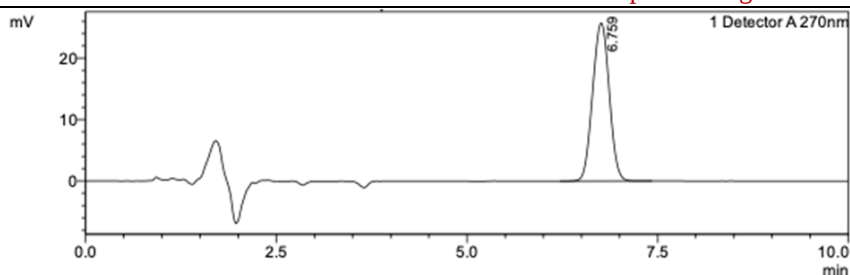


Figure 8. Chromatogram of standard Ropivacaine.

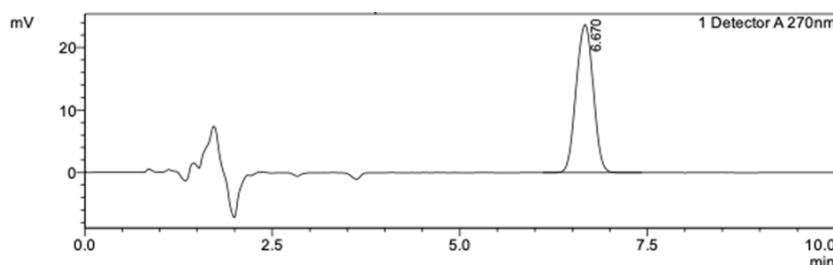


Figure 9. Chromatogram of the sample.

3.4. Validation of optimized method.

3.4.1. Specificity.

Specificity studies show no interfering peak at a retention time of the analyte peak, shown in Figures 7-9.

The developed method was validated, and the results obtained were found to be within limits, as shown in Table 5. The %RSD is 1.205, Rt was 6.670mins, the peak area was 383123, and theoretical plates were found to be 25919.

Table 5. Validation parameters.

S. No	Parameter	Results obtained (Ropivacaine)	Acceptance criteria
1	System suitability	%RSD 1.205	%RSD NMT 2.0%
2	Linearity	Correlation coefficient 0.9892	$R^2=1$
3	Accuracy	%Recovery 90.88%	90% - 110%
4	Range	%RSD 50%= 1.07, 150%= 0.60	%RSD NMT 2.0% for both concentrations
4	Precision	%RSD	%RSD NMT 2.0%
	System precision	0.34	
	Method precision	0.88	
	Intermediate precision (Ruggedness)	1.35	
5	Robustness	No deliberate change in RT with slight changes in flow rate and wavelength.	%RSD NMT 2.0%
6	Specificity	No interference of blank and placebo peaks with the main peak.	-

3.4.2. Linearity.

Linearity of standard solutions was injected, chromatograms were recorded. Linearity of concentration and peak area was plotted. R^2 was found to be 0.9981. The calibration curve shows that the method is linear, and a greater correlation exists between concentration and peak area, shown in Figure 10.

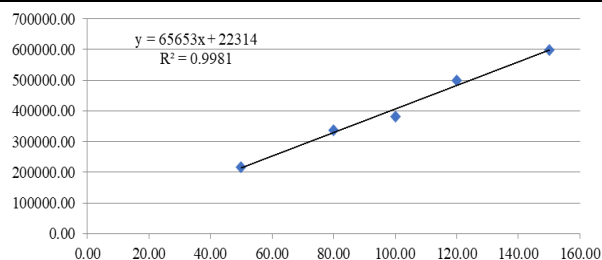


Figure 10. Calibration curve of ropivacaine.

3.4.3. Accuracy.

The mean percentage recovery of 50%, 100%, and 150% accuracy was calculated and observed to be 102.75, 90.88, and 96.53%, respectively. The %RSD was found to be less than 2. It is given in Table 6.

Table 6. Statistical data of accuracy.

Test	Ret time	Peak Response	Amt obt. (mg)	Amount added (mg)	Recovery %	Mean%	RSD
50% Recovery	6.695	206825	0.05	0.05	100.00	102.75	1.89
50% Recovery	6.689	202000	0.05	0.05	104.08		
50% Recovery	6.689	202000	0.05	0.05	104.17		
100% Recovery	6.670	376144	0.10	0.11	90.91	90.88	0.96
100% Recovery	6.661	382635	0.10	0.11	91.74		
100% Recovery	6.661	382635	0.09	0.10	90.00		
150% Recovery	6.667	592962	0.12	0.13	95.24	96.53	1.69
150% Recovery	6.698	597599	0.12	0.13	96.00		
150% Recovery	6.698	597599	0.12	0.12	98.36		

3.4.4. Precision.

The experiment was repeated in terms of system precision, method precision, intermediate precision, and the average were calculated. The %RSD for the six assay determinations was found to be 0.34%, 0.88%, and 1.35%, respectively Table 7a, Table 7b.

Table 7(a). Statistical data for intermediate precision.

S.No	Ropivacaine	
	Retention time	Peak area
1	6.749	401421
2	6.757	394012
3	6.755	390469
4	6.759	398547
5	6.740	390288
6	6.749	388998
Average	6.754	393956
SD	0.008	5040
RSD	0.125	1.28

Table 7(b). Statistical data for intermediate precision.

S.No	Sample weight (mg)	Ropivacaine	
		Peak area	Content
1	1	390858	100.00
2	1	388998	98.00
3	1	389103	96.00
4	1	391113	99.00
5	1	389601	98.00
6	1	387467	98.00
Average		389523	98.17
RSD		0.344	1.35

3.5. Assay.

The percentage purity of ropivacaine in the sample preparation was found to be 98.2%. The quantitative estimation of ropivacaine standard sample was given in Table 8.

Table 8. Quantitative estimation of ropivacaine

S. No	Ropivacaine	Retention Time	Peak area	% Amount present
1	Standard	6.759	389547	98.2%
2	Sample 1	6.671	382635	
3	Sample 2	6.670	383123	

4. Conclusions

The validation study was carried out to determine ropivacaine in bulk, and the solid dosage form shows satisfactory results meeting the acceptance criteria. The method was validated according to ICH guidelines. QbD studies were performed using the Box-Behnken design method, leading to fewer trials.

The results obtained concluded that the given method is simple, precise, accurate, reproducible, and all the parameters were found to be within limits. The method can be applied in routine analysis. QbD approach can be applied to study the stress effects on ropivacaine in the future.

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Conflicts of Interest

The authors declare no conflict of interest.

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