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\textsubscript{17}H\textsubscript{26}N\textsubscript{2}O. It has a pKa of 8.07, a molecular weight of 274.4 g/mol, and a solubility of 57.6 mg/L in water.

![Chemical structure of ropivacaine](https://doi.org/10.33263/BRIAC131.022)

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\textsubscript{1}, %v/v), Flow rate (X\textsubscript{2}, ml/min), and Wavelength (X\textsubscript{3}, nm). Retention time (RT) (R\textsubscript{1}, min), tailing factor (R\textsubscript{2}), and theoretical plate count (TP) (R\textsubscript{3}, N) were considered as co-variates 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(X1) | Flow rate (X2) | Wave length (X3) | Rt (R1) | Tailing factor (R2) | Theoretical plate (R3) | 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 (R₁)    | 2.70| 9  | 0.2999 | 34.52  | 0.0158  | 34.52         | 0.0019        |
| Tailing factor (R₂)    | 0.3218 | 12 | 0.0268 | 838.06 | 0.0250  | 838.06        | 0.0270        |
| Theoretical plates (R₃)| 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:
Retention time = +6.55 + 0.3436X_1 +0.1256X_2 – 0.0353X_3 +0.2986X_1X_2 +0.1768X_1 X_3 – 0.1046X_2 X_3 – 0.3240X_1^2+0.0838X_2^2+0.0845X_3^2

Tailing factor = +2.85 + 0.7371X_1 + 0.3662X_2 + 0.0835X_3 +0.2145X_1 X_2 +0.33X_1 X_3+0.1363X_2 X_3 + 0.6989X_1^2 - 0.2787X_2^2 – 0.9704X_3^2

Theoretical plates = +25877.50 +1932.41X_1 +532.30X_2 + 1649.59X_3 +1011.38X_1 X_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.

![Desirability chart](https://biointerfaceresearch.com/)

**Figure 5.** Desirability chart.

![Box-Behnken design](https://biointerfaceresearch.com/)

**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
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  | X₁     | 36  | 38  | 40  | 42  | 44  |
| Flow rate     | X₂     | 1.0 | 1.1 | 1.5 | 1.6 | 1.7 |
| Wavelength    | X₃     | 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 $R^2$, 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, $R^2$ 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 5μm |
|-------------------------|------------------------------------------------------|
| Flow rate               | 1.5ml                                                |
| Column temperature      | Room temperature                                     |
| Wavelength              | 270nm                                                |
| Injection volume        | 20µL                                                 |
| Retention time          | 6.670minutes                                         |
| Run time                | 10minutes                                            |
| Mobile phase            | Acetonitrile: methanol: water (40:30:30), 0.1%TEA    |

Figure 7. Chromatogram of the blank.
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.

| S. No | Parameter                | Results obtained (Ropivacaine) | Acceptance criteria                  |
|-------|--------------------------|---------------------------------|--------------------------------------|
| 1     | System suitability       | %RSD 1.205                      | %RSD NMT 2.0%                        |
| 2     | Linearity                | Correlation coefficient 0.9982  | R²=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 NMT 2.0%                        |
|       | System precision         | 0.34                            |                                      |
|       | Method precision         | 0.88                            |                                      |
|       | Intermediate precision   | 1.35                            |                                      |
|       | (Ruggedness)             |                                 |                                      |
| 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. R² 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.
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 |
|------|--------------------|-------------|
| 1    | 1                  | 389055      | 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    |                  |
| 2     | Sample 1    | 6.671          | 382635    |                  |
| 3     | Sample 2    | 6.670          | 383123    | 98.2%            |

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.

Funding

This research received no external funding.

Acknowledgments

We thank the management of CL Baid Metha College of Pharmacy for providing the necessary support throughout the work.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Hershberger, L.; Callis, J.; Christian, G. Liquid chromatography with real-time video fluorometric monitoring of effluents. Anal. Chem 1981, 53, 971-975, https://doi.org/10.1021/ac00230a011.
2. Napoleon, A.A. Pharmaceutical Titrimetric Analysis theory and practical. Kalaimani publishers and distributors: Kanchipuram, India, 2013; pp. 1.1-1.4.
3. Ravi Shankar, S. Text Book of Pharmaceutical Analysis. 4th ed.; RX Publishers: Otacamund, India, 2010; pp. 22-28.
4. Muralidhar Rao, D.; Reddy, S. Instrumental Methods of Analysis. 1st ed.; CBS Publishers & Distributors Pvt Ltd, India, 2013.
5. Wenzel, T.; Douglas A.; Skoog.; Donald M.; West, F.; James Holler.; Stanley R. Crouch: Fundamentals of analytical chemistry, 9th ed.; international ed. Anal. Bioanal. Chem 2013, 405, 7903-7904, https://doi.org/10.1007/s00216-013-7242-1.
6. Bower, N.W.; Skoog, D.A.; Holler, F.J.; Crouch, S.R. Principles of Instrumental Analysis. J. Chem. Educ 1992, 69, A224.
7. Guthrie, R.; Pavia, D.; Lampman, G.M.; Kriz, G.S.Jr. Introduction to Spectroscopy. J. Chem. Edu. 1979, 56, A323.
8. Nascimento Vieira, A.; Franz-Montan, M.; Cabeça, L.; de Paula, E. Anaesthetic benefits of a ternary drug delivery system (Ropivacaine-in-Cyclodextrin-in-Liposomes): in-vitro and in-vivo evaluation. J. Pharm. Pharmacol 2020, 72, 396-408, https://doi.org/10.1111/jphp.13211.
9. Gurdeep, R.; Chatwal, S.; Anand, K. Instrumental Methods of Chemical Analysis. 5th ed.; Himalaya Publishing house, 2016; pp. 21-67.
10. Singh, J. International conference on harmonization of technical requirements for registration of pharmaceuticals for human use. J. Pharmaco. Pharmacother 2015, 6, 185-187, https://dx.doi.org/10.4103/2F0976-500X.162004.

11. Guideline, ICH Harmonised Tripartite. Development and manufacture of drug substances (chemical entities and biotechnological/biological entities) Q11. London: European medicines agency. 2011.

12. Azhakesan, A.; Kuppusamy, S. QbD Based Development and Validation of Novel Stability Indicating Method for the Assay and Dissolution of Garenxocin in Garenxocin Tablets. J. AOAC Int 2021, https://doi.org/10.1093/jaoacint/qsa157.

13. Prajapati, P.B.; Jayswal, K.V.; Shah, S.A. Estimation of Multiple Fixed-Dose Combination Products of Ramipril and Aspirin by GERV-Chromatography Using DoE and Risk-Based Enhanced Analytical Quality by Design Approach. Journal of AOAC International 2021, 104, 1726-1741, https://doi.org/10.1093/jaoacint/qsa073.

14. Prajapati, P.B.; Jayswal, K.; Shah, S.A. Application of quality risk assessment and DoE-based enhanced analytical quality by design approach to development of chromatography method for estimation of combined pharmaceutical dosage form of five drugs. J. Chromatogr. Sci 2021, 59, 714-729, https://doi.org/10.1093/chromsci/bmaa118.

15. Khalil, A.; Kashif, M. Development of UV–visible spectrophotometric methods for the quantitative and in silico studies for cilazapril optimized by response surface methodology. Drug Dev. Ind. Pharm. 2021, 47, 1-12, https://doi.org/10.1080/03639045.2021.1957918.

16. Prajapati, P.; Naik, T.; Tailor, P.; Shah, S. Screening Design and Response Surface Methodology for the Simultaneous Estimation of Carvedilol and Ibabradine HCI by HPTLC Method. J. Chromatogr. Sci 2021, https://doi.org/10.1093/jaoacint/bma130.

17. Ranjan Jena, B.; SN Koteswara Rao, G.; Reddy Alavala, R.; Kumar Desu, P.; Chakravarthi, G.; Swain, S.; Prasad Pradhan, D.; Niharika, P. Response Surface Methodology Driven Systematic Development of a Stability-indicating RP-UPLC Method for the Quantification of Aliskiren: A Renin Inhibitor. J. Pharm. Res. Int 2021, https://doi.org/10.9734/jipri/2021/v33i2B33617.

18. Steehler, J.; Rouessac, F.; Rouessac, A. Chemical Analysis: Modern Instrumentation Methods and Techniques. J. Chem. Educ. 2008, 85, https://doi.org/10.1021/ed085p373.

19. Ettre, L.S.; Sakodynskii, K.I. MS Tswett and the discovery of chromatography I: Early work (1899–1903). Chromatographia 1993, 35, 223-231, https://doi.org/10.1007/BF02269707.

20. Skoog, D.A.; Leary, J.J. Principles of instrumental analysis. J. Chem Educ 1992, A224.

21. Mohan, T.; Jogia, H.; Mukkanti, K. Novel Stability-Indicating UHPLC Method Development and Validation for the Quantification of Perindopril, Amlodipine and Their Impurities in Pharmaceutical Formulations: Application of QbD Approach. Chromatographia 2020, 83, 1197-1220, https://doi.org/10.1007/s10337-020-03936-6.

22. Pawar, A.; Pandita, N. Statistically Designed, Targeted Profile UPLC Method Development for Assay and Purity of Haloperidol in Haloperidol Drug Substance and Haloperidol 1 mg Tablets. Chromatographia 2020, 83, 725-737, https://doi.org/10.1007/s10337-020-03889-w.

23. Ferreira, S.L.Costa.; Bruns, R.E.; Ferreira, H.S.; Matos, G.D.; David, J.M.; Brandão, G.C.; da Silva, E.G.Paranhos.; Portugal, L.A.; Dos Reis, P.S.; Souza, A.S. Box-Behnken design: An alternative for the optimization of analytical methods. Anal. Chim. Acta 2020, 597, 179-186, https://doi.org/10.1016/j.aca.2007.07.011.

24. Ameeduzzafar; El-Bagory, I.; Alruwaili, N.; Imam, S.; Alomar, F.; Elkomy, M.; Ahmad, N.; Elmowafy, M. Quality by design (QbD) based development and validation of bioanalytical RP-HPLC method for dapagliflozin: Forced degradation and preclinical pharmacokinetic study. J. Liq. Chromatogr. R 2020, 43, 53-65, https://doi.org/10.1080/10826076.2019.1667820.

25. Patel, K.; Dedania, Z.; Dedania, R.; Patel, U. QbD approach to HPLC method development and validation of ceftriaxone sodium. Future J. Pharm. Sci. 2021, 7, https://doi.org/10.1186/s43094-021-00286-4.

26. Izma, H.; Martono, S.; Lukitaningsih, E. The Optimization Of Rp-Hplc Condition Using Response Surface Methodology Box-Behnken Design For Simultaneous Determination Of Metformin Hcl And Glimipride In Spiked Plasma. Int. J. A Pharm 2019, 12, 24-35, https://doi.org/10.22159/ijap.2020v12i2.36052.

27. Yabré, M.; Ferey, L.; Sakira, A.; Bonmatin, C.; Fauré, C.; Somé, T.; Gaudin, K. Green Analytical Methods of Antimalarial Artemether-Lumefantrine Analysis for Falsification Detection Using a Low-Cost Handled NIR Spectrometer with DD-SIMCA and Drug Quantification by HPLC. Molecules 2020, 25, https://doi.org/10.3390/molecules25133597.

28. Waghmare, S.; Sumithra, M. QbD Based Development and Validation of RP-HPLC Method for Nintedanib Esylate: Application to Bioanalytical and Stability Study in Plasma. Anal. Chem. Lett 2021, 11, 392-408, https://doi.org/10.1080/22297928.2021.1930581.

29. Balasaheb, D.R.; Vijayalakshmi A. Improvement of Development and Validation of an RP-HPLC Method for The Fluvastatin Sodium Using QbD Approach and Its Application to Forced Degradation Studies. Int. J. Res. Pharm. Sci. 2020, 11, 6938-6948.

30. Ganji, S.; Dhulipala, S.; Nemala, A. Development and validation of RP HPLC method for the estimation of Sofosbuvir and related impurity in bulk and pharmaceutical dosage form. Future J. Pharm. Sci 2021, 7, 1-10, https://doi.org/10.1186/s43094-021-00285-5.
31. Deidda, R.; Orlandini, S.; Hubert, P.; Hubert, C. Risk-based approach for method development in pharmaceutical quality control context: A critical review. *J. Pharm. Biomed* 2018, 161, 110-121, https://doi.org/10.1016/j.jpba.2018.07.050.

32. Kusnul, K.; Sudiyo, M.; Abdul, R. Box–Behnken design-based HPLC optimization for quantitative analysis of chloramphenicol and hydrocortisone acetate in cream. *J. A Pharm. Sci* 2020, 10, 134-139, https://doi.org/10.7324/JAPS.2020.10916.

33. Gurrala, S.; Raj, S.; Subrahmanyam, CSV.; Anumolu, PD. Multivariate optimization of liquid chromatographic conditions for determination of dapagliflozin and saxagliptin, application to an in vitro dissolution and stability studies. *Future J. Pharm. Sci* 2021, 7, 1-11, https://doi.org/10.1186/s43094-021-00229-z.

34. Parr, M.; Schmidt, A. Life cycle management of analytical methods. *J. Pharm. Biomed* 2018, 147, 506-517, https://doi.org/10.1016/j.jpba.2017.06.020.

35. Kovács, B.; Péterfi, O.; Kovács-Deák, B.; Székely-Szentmiklósi, I.; Fülop, I.; Bába, L.; Boda, F. Quality-by-design in pharmaceutical development: From current perspectives to practical applications. *Acta Pharm* 2021, 71, 497-526, https://hrcak.srce.hr/246699.

36. Zhang, Y.; Liu, L.; Zheng, L.; Chen, J.; Huang, L.; Wang, Q.; Shi, K. Comparison of Effectiveness of Ropivacaine Infusion Regimens for Continuous Femoral Nerve Block for Recovery After Total Knee Arthroplasty: A Randomized Double-Blind Trial. *J. Pain Res* 2020, 13, 997–1005, https://dx.doi.org/10.2147%2FJPR.S247158.

37. Huang, L.; Zheng, L.; Wu, B. Effects of ropivacaine concentration on analgesia after ultrasound-guided serratus anterior plane block: a randomized double-blind trial. *J. Pain Res* 2020, 13, 57–64, https://dx.doi.org/10.2147%2FJPR.S229523.

38. Wang, Y.; Xu, M. Comparison of ropivacaine combined with sufentanil for epidural anesthesia and spinal-epidural anesthesia in labor analgesia. *BMC Anesthesiol* 2020, 20, https://doi.org/10.1186/s12871-019-0855-y.

39. Lamy, E.; Fall, F.; Boigne, L.; Gromov, K.; Fabresse, N.; Grassin-Delyle, S. Validation according to European and American regulatory agencies guidelines of an LC-MS/MS method for the quantification of free and total ropivacaine in human plasma. *Clin. Chem. Lab. Med* 2020, 58, 701–708, https://doi.org/10.1515/cclm-2018-1298.

40. Chen, S.; Ma, J.; Wang, X.; Zhou, Q. Simultaneous Determination of Ropivacaine and 3-Hydroxy Ropivacaine in Cerebrospinal Fluid by UPLC-MS/MS. *BioMed Res. Int* 2020, 2020, 1-6, https://doi.org/10.1155/2020/8844866.

41. Kumar, R.; Munipalli, V.K.; Singh, R.M.; Warde, S. Validated RP-HPLC Method for Determination and Quantification of Nintedanib in Pharmaceutical Formulation. *J. Adv. Pharmacol* 2020, 1, 38–47.