DEVELOPMENT AND VALIDATION OF AN LC-MS-MS METHOD FOR CABOZANTINIB IN HUMAN PLASMA USING NAPROXEN AS AN INTERNAL STANDARD USING QUALITY BY DESIGN (QBD)

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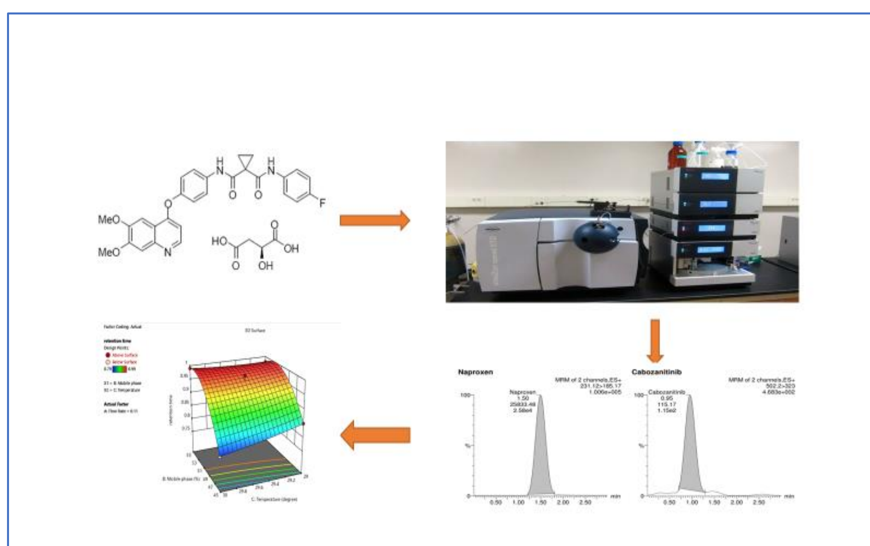
GRAPHICAL ABSTRACT



ABSTRACT

The current studies describe the development and validation of an LC-MS-MS method for Cabozantinib in human plasma using naproxen as an internal standard using Quality by Design (QbD) (IS). For sample preparation, solid-phase extraction was used and linearity was observed for drug concentrations ranging from 5 to 1000 ng/mL The method was systemically optimized using the Box-Behnken design, with mobile phase flow rate (X1), pH (X2), and mobile phase composition (X3) as method variables and retention time (Rt) (Y1) and peak area (Y2) as responses. Optimization studies revealed a reduction in the variability associated with method variables which improved method robustness. Validation studies of the developed method revealed that Cabozantinib in human plasma has good linearity, accuracy, precision, selectivity, and sensitivity. Stability studies in human plasma using freeze-thaw, bench-top, short-term and long-term cycles and auto sampler stability revealed no change in the drug's percent recovery. In a nutshell, the developed method yielded satisfactory results for Cabozantinib analysis in human plasma.

Keywords: Development and validation; LC-MS-MS method; naproxen as an internal standard; Quality by Design (QbD) (IS); Cabozantinib ; human plasma.

1. INTRODUCTION

Cabozantinib's chemical formula is N-(4-(6,7-dimethoxyquinolin-4-yloxy) phenyl)-N'-(4-fluorophenyl) cyclopropane1,1-dicarboxamide, (2S)-hydroxybutanedioate. It's a white to off-white powder. It has a pKa of 5.9-13.46 [1-5]. Cometriq and Cabometyx are the brand names for Cabozantinib. It is used to treat thyroid medullary carcinoma [6,7], renal cell carcinoma [8, 9], and hepatocellular carcinoma [10, 11]. It is a small molecule that blocks c-Met, VEGFR2, AXL, and RET. Evelix's Inc. was responsible for its discovery and development. Cabozantinib is available in two doses. A capsule formulation has been used since 2012 to treat medullary thyroid cancer, while a tablet formulation has been utilized as a second-line treatment for renal cell carcinoma since 2016. Cabozantinib has not been evaluated in pregnant women. However, it has been shown in rats to be harmful [11]. This medication should be avoided by pregnant women and women who are already pregnant. Cabozantinib has not been shown to cross the placenta [12]. Patients with a history of aberrant cardiac rhythms [13] should be treated with caution, particularly those with a prolonged QT interval. Plasma concentrations approach a peak between two and five hours after taking an oral dose. This medicine has a half-life of around 55 hours.

The development of bio-analytical methods based on the holistic principles of Quality by Design (QbD) has gained immense popularity for enhanced understanding of the high degree of variability associated with the LC-MS/MS method development and attaining optimal chromatographic separation. As per the QbD approach, the Design of Experiments (DoE) is considered a vital tool that helps in systematically executing the chromatographic method development. DoE helps identify the "vital few" methods variables critically influencing the method performance and optimizes them with minimal investment of time, efforts, and cost t (14, 15). Literature reports, in this regard, are testimony to the higher fruition of the QbD approach for efficient development of the liquid chromatographic methods with greater flexibility and enhanced method performance (16–19).Thus attempts, therefore, were made for the development of a simple, fast, sensitive, and accurate LC-MS-MS method of Cabozantinib followed by optimization of the selected method variables using Box-Behnken design (BBD) for practical, cost-effective, and efficient chromatographic separation. The developed method was extensively evaluated as per the ICHguideline, followed by evaluating the drug stability in rat plasma

2. Materials and Methods

2.1 Reagents and chemicals

A gift of Cabozantinib and naproxen (internal standard) was provided by Rk Laboratories. The HPLC-quality acetonitrile and formic acid were from the Mumbai lab chemistry manufacturer. Ebelly services Hyderabad, Telangana, acquired rat plasma.

2.2. LC-MS/MS instrumentation

The UPLC Waters Acquity was employed. MRM was used to analyze the Quattro premier xe. Data were processed using mass link software. The detection was performed in positive ion mode (ESI) with a resolution of one unit using a channel electro multiplier (CEM). To isolate carbozantinib, detected the MH+ precursor ion (m/z 502.13) and identified a fragment with an

m/z of 323.07 as the product ion. Internal standard monitoring was carried out using a product with a mass of 185.17 and a precursor ion with a mass of 231.24. The source temperature was set to 120 degrees Celsius; the desolvation temperature was 4000C; the capillary voltage was set to three; the desolvation gas flow was set to 850 liters per hour; the RF lens was set to zero; the extractor was set to three; the collision gas flow was set to one hundred liters per hour; the cone (v) was set to thirty, and the collision energy was set to twenty-seven.

2.3 Chromatography and Mass spectroscopy conditions

The samples' separation was accomplished using an Agilent Zorbax XDB column (2.1× 50mm,3.5). The column's temperature was maintained at 30oC. The mobile phase was made up of acetonitrile and formic acid in a 50:50 (v/v) ratio.

2.4 Sample preparation for standard and Quality control

Cabozantinib standard stock solution (100µg/mL) and internal standard (100µg/mL) were prepared acetonitrile. The internal spiking solutions were designed in 50%Acetonitrile from the internal stock solution to retain the IS spike solution at 2-8 ° C.. It was placed in a refrigerator. Sterile pharmaceutical-grade rat plasma was used to standardize the Cabozantinib concentrations, which were 5, 10, 25, 85, 400, 500, 800, and 1000 ng/ml. The solution was reconstituted by using (50 percent acetonitrile in 0.2 percent formic acid).

2.5 Sample Preparation

Cabozantinib and its internal standard were extracted from rat plasma using a protein precipitation extraction technique|; 20µL of a -spiking solution is added to 180µL of rat plasma.Then the final volume is up to 200µL.From 200µL, take 100µL, then the solution is called allocation volume. This solution is vortexed for 3 minutes, and add 400 µL of acetonitrile for precipitation and close cap and again vortex the solution for 5 minutes. Then the samples are centrifuged for about 10 minutes at 4000rpm. The supernatant layer was transferred to a labeled container into an autosampler.

2.6 Method validation

2.6.1. Selectivity and Specificity

Three independent batches of plasma were collected, processed, and analyzed using a conventional technique in a controlled environment. No evidence of interference was seen for the Cabozantinib and Naproxen retention time intervals. During the retention time, the peak area of Cabozantinib should not exceed 20% of the mean peak area of the LOQ for Cabozantinib. If the Naproxen peak area exceeds 5% of the mean of the Naproxen LOQ for the relevant retention period, the sample should be discarded.

2.6.2 Matrix factor

Consider a matrix factor to obtain the variance for the matrix effect. Three distinct concentrations of quality standards (LQC, HQC, and MQC) were injected into three separate biological matrix samples in a total of nine injections. It should be 15%. %CV

2.6.3 Linearity

An analyte concentration ranging from 5-1000 ng/mL was employed to develop the analytical curves for Cabozantinib in rat plasma. To produce the calibration curve, we use a calibration weight of 0.99 /conc2. Studies have found that the concentration of naproxen peaks is inversely related to that of Cabozantinib. A correlation coefficient of 0.989655 was observed when a linear regression model was used

2.6.4. Accuracy and precision

Repeating measurements on a single homogeneous volume of the biological matrix defines the closeness of the results for a specific analyte. Precision is functional when less than five measurements are required to determine the concentration. Rather than just adjusting the concentration to the projected value, select three significantly more or less than the predicted value.

When samples are known to contain the analyte at a specified concentration, an assessment of the analysis's Accuracy can be made. To assure Accuracy, each concentration should be tested at least five times. Each level within the typical range must have a minimum of three additional levels.

2.6.5 The Stability studies

Benchtop stability was done at 12 hours under control dosage levels. Stability tests were performed on cabozantinib's long-term plasma stability at -700C, as well as the stock solution's stability for 24 hours at room temperature. The Nominal concentration of stability samples should be within ≤15% and ±15%.

2.6.6 Lower limit of Quantification

Blank and spiked lower limit of quantification samples obtained from the same plasma were monitored and observed for the response peak area. Naproxen and Cabozantinib LLOQ samples should have a blank area of about 20 percent of the mean peak area. To achieve the required precision and average Accuracy, the LLOQ blank concentration must be ≤20 % and ±20% respectively.

Table-1 Summary of Design matrix as per BBD

Run	Factor-1 Flow rate ml/min	Factor-2 Mobile phase composition	Factor-3 temperature	Response -1 Retention time minutes	Response-2 peak area Cm^2
1	0.12	50	29	0.98	145.112
2	0.12	50	30	0.95	144.581
3	0.1	55	29.5	0.98	148.521
4	0.11	50	29.5	0.94	143.581
5	0.11	45	29	0.85	140.28
6	0.11	50	29.5	0.99	146.581
7	0.11	50	29.5	0.99	146.581
8	0.11	50	29.5	0.99	146.581
9	0.11	45	30	0.79	139.51
10	0.12	45	29.5	0.79	138.51
11	0.12	55	29.5	0.97	148.521

12	0.11	55	30	0.99	150.211
13	0.1	45	29.5	0.84	138.51
14	0.11	55	29	0.98	149.521
15	0.11	50	26.5	0.94	143.581
16	0.1	50	30	0.97	144.581
17	0.1	50	29	0.98	145.112

3. Results

3.1 Method Development

Due to the selectivity, sensitivity, and reproducibility of LC-MS/MS, it is widely used in clinical pharmacokinetics. The scope of this study was to develop and promote a simple, efficient, and selective test for determining Cabozantinib levels in plasma samples. Simple plasma sample processing procedures are employed to separate Cabozantinib and naproxen from plasma samples. The methodologies used were gradually adjusted during these trials until a resolution and signal suitable for therapeutic application were reached. MS optimization was performed utilizing a cabozantinib and naproxen (ESI) solution that was subsequently injected into the mass spectrometer's source.

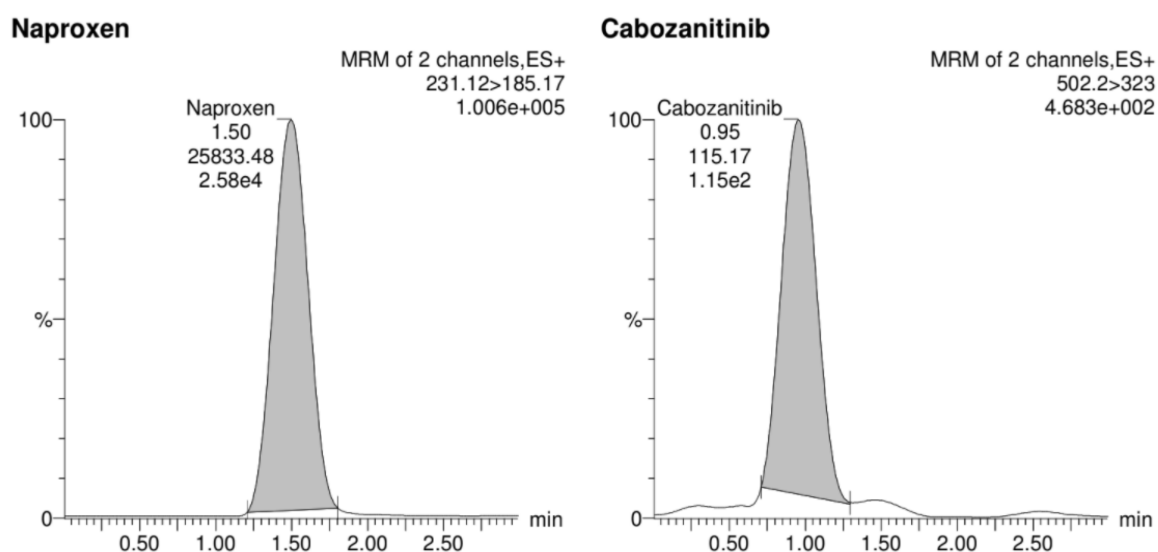


Figure 1. Chromatogram of Cabozantinib and chromatogram of internal standard (i.e., Naproxen

3.2 Method

3.2.1. Selectivity and specificity

Using the MRM function, naproxen and Cabozantinib were analyzed highly. Therefore, no interfering substances were identified. Tests were performed on three lots of rat plasma to determine its specificity. 0.95ng/mL Cabozantinib and 1.50ng/mL naproxen were added to the plasma to develop chromatograms.

3.2.2. Matrix Factor

The overall precision of the matrix factor was 12 ng/mL at the low concentration of Cabozantinib, whereas it was 531 ng/mL at the high concentration.

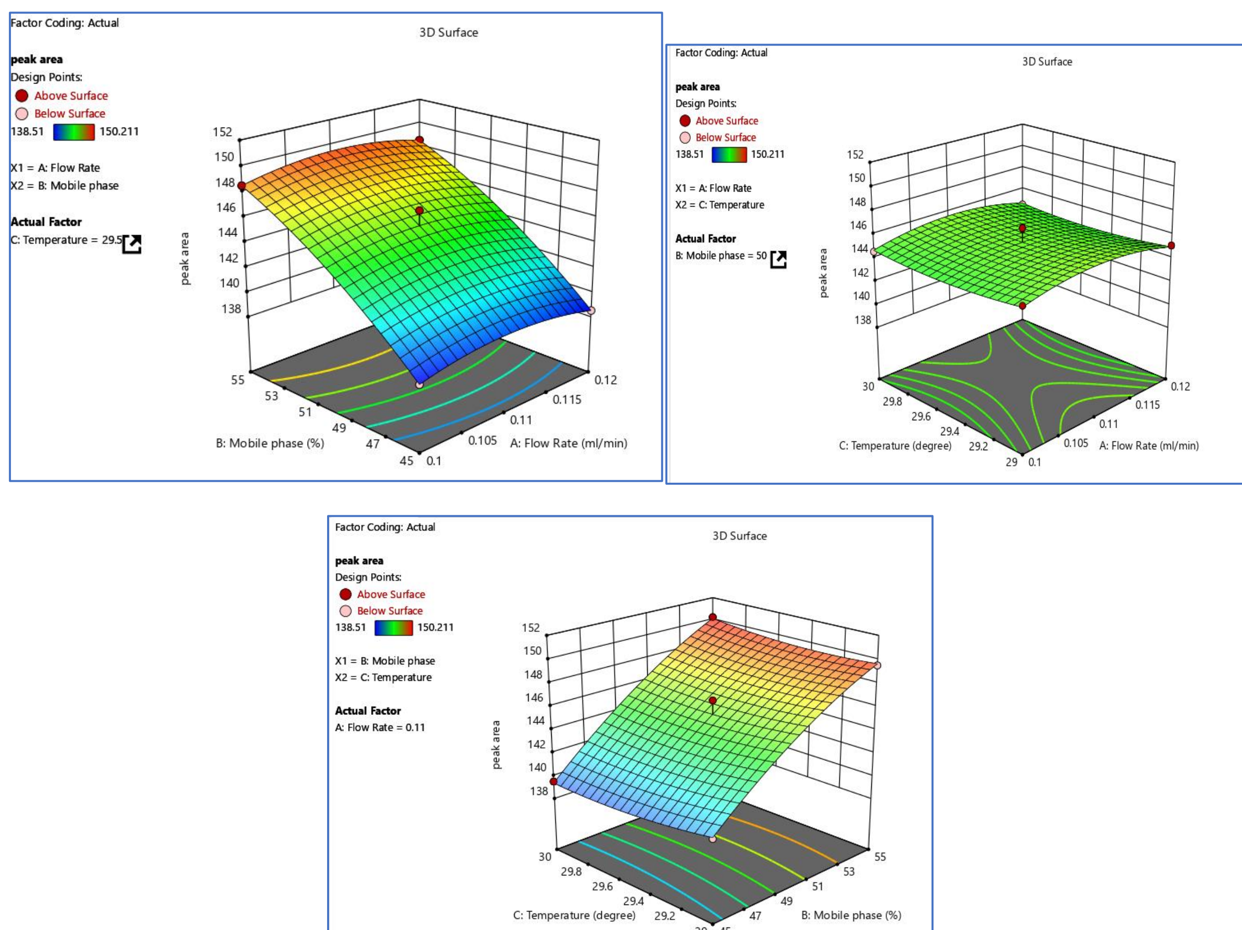


Figure 2. 3D-response surface plots depicting the influence of mobile phase ratio, flow rate, and temperature on the peak area as the response variable

3.2.3 Calibration curve regression

The observed responses for Cabozantinib were subjected to quadratic regression (1/concentration), which showed best-fit results for the coefficient of determination (2) greater than 0.998.

3.2.4Accuracy and precision

Accuracy (% nominal) data for Cabozantinib within the batch and between batches. The data showed that Accuracy was foundbetween 102.4 and 100.8%, while % CV within a batch and betweenbatches was reported as 1.7 and 0.9 %, respectively.

3.2.5. Recovery

It was determined by comparing the peak area of the QC sample before extraction to the peak area after extraction. Cabozantinib recoveries at the low, medium, and high QC levels were 103.46, 100.09and 100.08%, respectively.

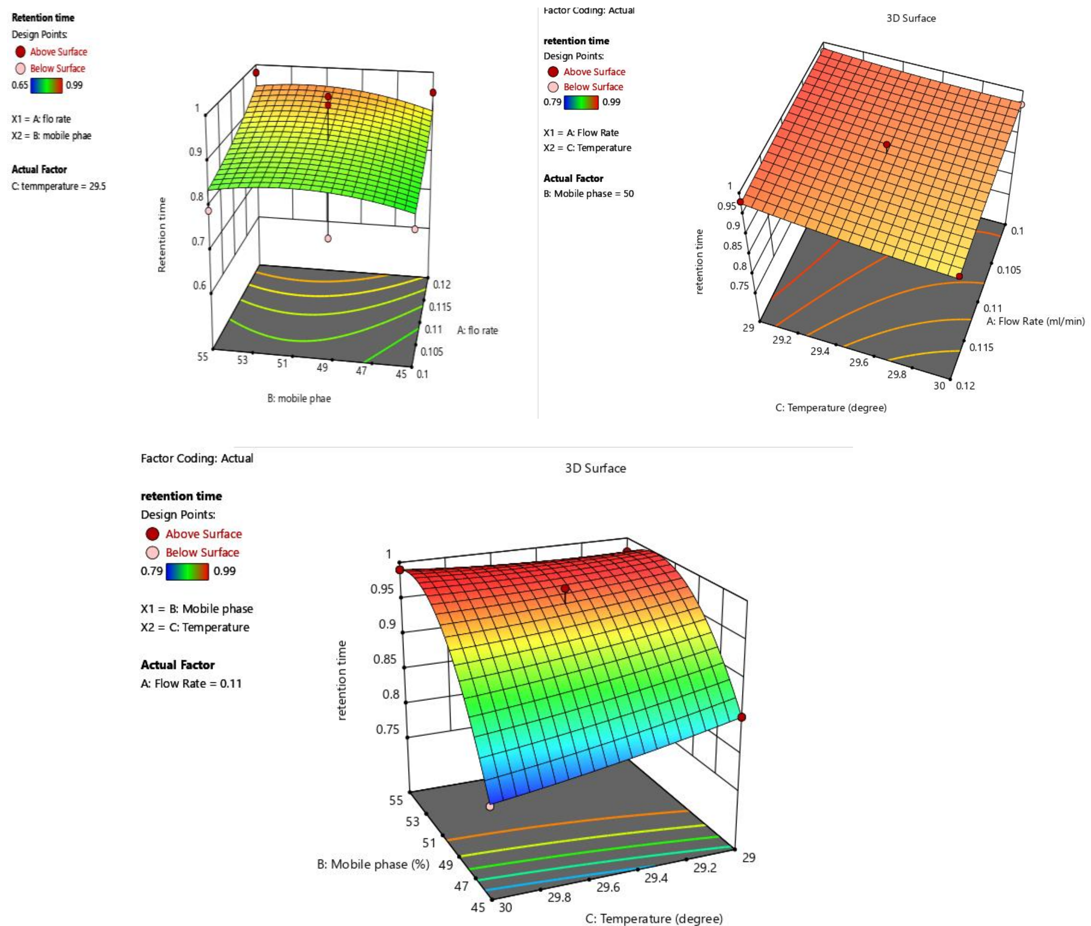


Figure 3. 3D-response surface plots depicting the influence of mobile phase ratio, flow rate, and temperature on the retention time as the response variable

Robustness testing using experimental design

Robustness refers to the capacity to remain resistive to minor and premeditated variations in chromatographic circumstances such as mobile phase composition, flow rate, and column oven temperature. It was used to determine the robustness of three dependent variables, namely flow rate (A) (mL/min), pH (B), and a mobile phase (percent v/v Methanol; Formic acid) (C), as well as column temperature, where their likely effect on two dependent variables (response), namely Rt (min) (Y1) and peak area (cm) (Y2), was investigated. A total of seventeen runs were obtained, and each suggested combination was tested on the system. The method's perfection was determined using the experimental design's outcomes. Among the trial combinations proposed by the chosen design, BBD revealed that a 50:50 mobile phase (Methano1: Formic acid) and 0.120 mL/min flow rate solution produced responses with a peak area of 144.581 and an Rt of 0.95 min, respectively.

Table-2Chromatographic Conditions and Range Investigated during Robustness Testing

Factor	Range	low	High	optimized
A= Flow rate	0.1-0.12	0.1	0.12	0.12
B=Mobile phase composition	45-55	45	55	50
C=Temperature	29-30	29	30	30

To reduce the effects of uncontrolled variables on response variability, tests were conducted in a randomized order. Using a lack-of-fit test, the second-order model with an experimental quadratic domain was chosen for both Y1 (r2 = 0.9559) and Y2 (r2 = 0.9971) when compared to other models indicated by design.

A more significant P-value was observed as compared to the model F-value, indicating negligible lack-of-fit values. This was confirmed and analyzed using analysis of variance (ANOVA) to determine the model's significance for both response variables. The results analysis revealed that both of these responses had statistically significant disparities in their values. The predicted values for all factors, namely flow rate (A), mobile phase composition (B), and column oven temperature (C), are less than satisfactory, with a predicted model F-value of 18.80 indicating that the model is highly significant with a P-value of 0.0001, indicating that there is only a 0.04 percent chance that the model F-value is significant due to noise. The ANOVA findings projected a model F-value of 14.85 for response Y2, indicating that the model is likely to be significant due to the 0.09 percent possibility that the model F-value is considerable due to noise. Additionally, the model recommended that predicted values for both the response and the input be closer to the actual values, indicating that the generated values are more accurate and precise.

The effect of the model's various components on the responses was analyzed using contour plots revealing response surfaces for both Y1 and Y2. This indicated that the influence of both responses is reliant on factors A (flow rate) and B (mobile phase composition percent) but not on factor C (column oven temperature). The model was validated by examining the interaction of several factors on the results. The effect of various factors on the response Y1 (Rt) and Y2 (peak area) was evaluated using a one-factor interaction study, which revealed that the flow rate (A) exhibited a two-factor interaction effect, with the survey showing that both factor A (flow rate) and factor B (mobile phase composition) exhibited multiple interactions on the obtained response, as demonstrated by the contour pl. By contrast, factor C has no effect on either reaction.

DISCUSSIONS

Overall, this study embarks upon the application of QbD principles for developing the LC-MS-MS method of Cabozantinib Systematic method development was carried out by employing BBD for evaluating the method robustness using mobile phase composition, column oven temperature, and flow rate as the method variables, followed by optimization of their effects on the retention time and peak area as the responses The response surface analysis helped in the critical understanding of the method parameters followed by a critical understanding of the relationship between them. Method validation studies revealed a high degree of sensitivity, selectivity, and specificity of the obtained method. Evaluation of the stability in plasma through the stock solution, short-term, long-term, and bench-top stability studies revealed no significant influence of the experimental conditions on the drug stability. The method was highly robust and reliable enough for routine analysis of the drug in bio-analytical samples.

CONCLUSION

The proposed method of Cabozantinib was simple, specific, accurate, and linear and validated in rat plasma over a range of 50–1000 ng/mL The results obtained from the validation of the method were satisfactory and offer a rapid and straightforward sample preparation which can

facilitate the bio-studies of Cabozantinib. This vouched for the routine applicability of the method in the pharmacokinetic analysis of samples. The regulatory requirements for Accuracy, precision, sensitivity, selectivity, stability, and ruggedness were excellent. The applied BBD design for optimization of robustness parameters were highly suitable for validation and able to predict minor changes in the flow rate and mobile phase composition for the responses, i.e., retention time and peak area for the purpose.

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