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Optimization of Chromatographic Conditions with QbD for Method Development and Validation of Bosutinib by HPLC: Applications in Dosage Forms and Rat Plasma Analysis

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Abstract: Aim: Bosutinib (BST) is an anti-cancer medicine that is used to treat a variety of different types of cancer. Using the HPLC method of analysis and the Quality by Design (QbD) strategy, the study aimed to precisely quantify the drug in tablet form and in rat plasma. Methodology: For the developed method's validation, the chromatographic settings were fine-tuned by making use of the Box–Behnken Design (BBD). In the BBD, two dependent variables and three independent variables were selected. Isocratically, samples were eluted, having eluent phase composition of ammonium acetate (CH_3COONH_4) buffer pH 3.0 and acetonitrile (CH_3CN) (60:40% v/v), in Raptor C-18 column at temperature 25 °C with a flow rate of 1 mL/min for 5 min. The wavelength of detection was set at 260 nm. In this study, encorafenib (ENC) was employed as an internal standard. Result: A sharp and resolved peak of BST and ENC at a retention time of 1.92 min and 4.01 min, respectively, was observed by the developed method. The limits of quantification and detection of the newly established method were found to be 1.503 μg/mL^{-1} and 0.496 μg/mL^{-1}. The calibration curve's observed linearity range was between 2 and 20 μg/mL^{-1}, with an r^2 of 0.999. The developed and optimized method was verified in compliance with the ICH guidelines. The results of all validation parameters were within the acceptable range, for example, % RSD of system suitability (0.63–4.46), % RSD of linear regression (1.659), interday and intraday precision % RSD value (1.723–1.892), and (1.762–1.923), respectively, and accuracy (1.476–1.982). Conclusion: The quantity of BST in tablet dosage form and in rat plasma samples was determined using a simple, quick, and robust method that was devised and validated.

Keywords: Bosutinib; Box–Behnken Design (BBD); dosage form; independent variables; method validation; rat plasma; risk assessment

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1. Introduction

Bosutinib (BST) is chemically 4-[(2,4-Dichloro-5-methoxyphenyl)amino]-6-methoxy-7-[3-(4-methyl-1-piperazinyl) propoxy]-3-quinolinecarbonitrile monohydrate (Figure 1) and designated as a BCS Class IV drug because of its reduced solubility and decreased skin penetration [1]. This drug is referred to as a chemotherapeutic agent and is used to treat a range of various types of cancer. Protein kinase inhibitory activity is the main mechanism of action of BST, which is primarily used to treat resistant and intolerant chronic myelogenous leukemia (CML) patients [2]. It is a novel dual SRC/ABL kinase inhibitor in advanced clinical development that is primarily used for the treatment of SRC overexpressing solid tumours and gives a positive result. In oral administration, peak plasma concentration occurs after 4–6 h and is very extensively distributed into the tissue [3]. The absorption of

the drug can be improved when taken with food. The metabolic site of this drug is the liver, and the liver enzyme cytochrome P450 3A4 metabolizes the drug. The drug was reported to have 94% plasma protein binding. It is considered as a drug with a narrow therapeutic index, as BST therapy cannot be continued without interruption due to various adverse effects, especially diarrhoea and liver dysfunction [4].

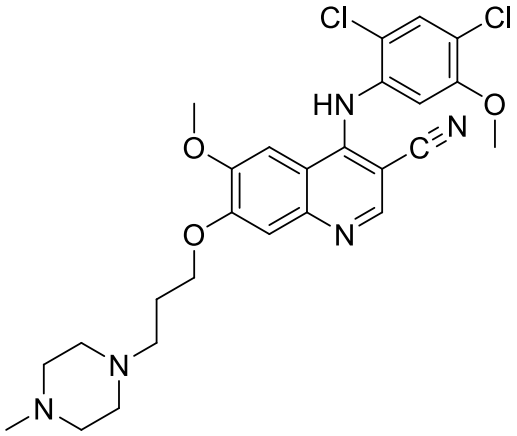


Figure 1. Structure of Bosutinib (BST).

The most non-polar molecules, as well as ionized and non-ionic chemicals, are separated and quantified using the liquid chromatography technique. When it comes to organic compound analysis, high-performance liquid chromatography (HPLC) is by far the most widely utilized technology [5,6]. Dziadosz et al. presented an HPLC method using a diode array detector for the separation of seven out of seven kinase inhibitors, including vatalanib, bosutinib, canertinib, tandutinib, pazopanib and dasatinib, using erlotinib as the internal standard. The method was claimed to be highly sensitive comparable to those of the LC/MS/MS methods, but studies showed a recovery of 73–90% of all analytes except pazopanib, which showed 50% recovery only [7]. Sumimoto et al., reported a method using a UV detector with good sensitivity for the determination of bosutinib in human plasma but recovery was only 85.62%. Furthermore, the method uses Oasis HLB cartridge for solid-phase extraction for human plasma samples. In patients with chronic myeloid leukemia, this study confirmed the validity of this technique for therapeutic drug monitoring of bosutinib [8]. A study conducted by Mita et al. that correlated the adverse effects of bosutinib treatment with its plasma concentration in patients with chronic myeloid leukaemia, used either conventional doses or dose-escalation regimens. The dose-escalation regimen was better suited to avoid treatment interruption. This study emphasized that the therapeutic drug monitoring in everyday practice may allow the daily dose of bosutinib to be changed based on the target concentration to prevent side effects, highlighting the significance of a good and reliable method for the determination of bosutinib [9]. Wang et.al., devised a UPLC-MS/MS method to determine bosutinib in rat plasma and tissue using diazepam as the internal standard. They used acetonitrile as the protein precipitating agent for plasma sample analysis. The study further claimed that the method of analysis can be applied effectively after the oral administration of bosutinib in animals for pharmacokinetic and tissue-distribution studies [10]. Xu et.al., conducted the first pharmacokinetic studies of bosutinib in rat plasma using positive-ion electrospray tandem mass spectrometry with a multiple-reactions monitoring (MRM) technique which lay the groundwork for the drug's future research and use. The reported UPLC-MS/MS method uses pirfenidone as the internal standard and showed good sensitivity and recovery (81.22%) [11]. Another simple, precise, economical, and accurate RP-HPLC method was developed by Jhadav el. al. The mobile phase composition was methanol and sodium phosphate buffer 10 mm pH 6.5 in a ratio of 85:15 v/v. Bosutinib's retention time was observed at 7.43 min. The developed method was validated in accordance with regulatory criteria. The method was applied for analysis of bosutinib in tablet dosage form; however, it had a longer run time and

no internal standard was used [12]. Another study used a C18 column and 1.0 percent triethylamine (v/v) in water (pH 7.0, balanced by ortho-phosphoric acid) as a mobile phase in an RP-HPLC analytical method utilizing a quality--by-design (QbD) strategy [13]. The MS detector approach has its own limits due to its cost, whereas methods based on cheap UV detectors require the solid-phase extraction process with cartridges, which increases the price of sample processing. As a result, the current investigation was performed to reduce the cost of BST analysis by employing simple UV detectors and extracting samples with acetonitrile as a protein aggregating agent.

The study of drugs and their metabolites has relied heavily on the liquid–liquid extraction (LLE) process. In HPLC analysis, clean samples are essential to minimize the ion suppression and matrix effects. In controlled bioanalysis, liquid–liquid extraction (LLE) is a popular technique for sample preparation. LLE is thought to be inexpensive and can produce clean extracts with high analyte recoveries. To concurrently achieve significant recoveries for metabolites and related molecules, as well as the core analyte, extraction solvents may need to be acidified, basified, or contain low concentrations of more polar solvents. The use of LLE solvents can frequently result in extracts that are unusually saturated with phospholipids (PLs). Residual PLs can accumulate on the analytical column and the LC system in addition to contributing to matrix effects. PL build-up could result in fluctuating analyte signals, the suppression of MS responses, and even instrument downtime. Phospholipid removal (PLR) plates offer high analyte recoveries and clear extracts, just like LLE plates do. In LLE, the idea of "like dissolves like" works effectively. According to how differently the components of the sample combination divide themselves between the two immiscible solvents, by carefully selecting the extraction solvent, the compound of interest can be selectively partitioned into one of two partially miscible phases [14]. In this study, acetonitrile was optimized for protein precipitation in rat plasma samples and extraction in tablet dosage form samples.

In our approach, the separation of bosutinib was optimized in different types of solvents or mixtures of solvents and we considered the one that showed the best results. The same as for the recoveries of analytes in a plasma sample, different extracting or protein precipitating techniques were employed with various types of solvents or in a mixture of solvents. A trial to select the best for the preparation of the sample is required. For extracting bosutinib from plasma samples, we utilized dichloromethane, n-hexane, chloroform, petroleum ether, acetonitrile, ethyl acetate, and other solvents alone or a mixture of various solvents in definite proportions. After the method development, the validation processes were carried out strictly following the ICH Q2 (R1) regulations [15–17].

We strictly followed ICH Q11 for the screening, selection, and optimization of various factors that influence the method development and authentication, and in this analysis, the QbD approach was followed. In the context of analytical method development, QbD is referred to as AQbD and is carried out using scientific methods. Risk assessment, Design of Experiment (DoE), Analytical Target Profile (ATP), and Critical Quality Attributes (CQA) were among the methodologies used [18]. We followed the previous reports in which the different trials of factors helped to optimize the chromatographic condition for the robust and precise method development and validation and its application in the quantitative analysis [19]. A one-factor-at-a-time (OFAT) technique, in which one chromatographic parameter is adjusted in subsequent tests until a suitable resolution between chromatographic peaks is attained, is frequently used to develop HPLC procedures. In the event that there are numerous factors influencing the separation, this considerably raises the number of experiments. Because only one factor's level changes while the others stay the same, the conventional univariate approach is insufficient. The full factorial design, on the other hand, included numerous experiments. Block combinations of the three factorial analyses were first proposed by Box and Behnken (BBD) in 1960. The corresponding decrease in the number of experiments was this analysis' principal benefit. The combinations in which all variables are either at their highest or lowest levels are not included in BBD. Such testing of the values can produce disappointing outcomes. However,

when we want to know the reaction at the extreme values of independent variables, this strategy is likewise not recommended [20].

Herein, we report an HPLC method using a UV spectrophotometer detector with a QbD approach. As discussed above, most of the earlier reported methods use MS detectors which are comparatively more expensive, especially for plasma analysis. Although some methods use UV-detectors, these are for tablet dosage form only. Furthermore, for plasma analysis, solid–liquid extraction techniques were used by earlier reported methods which require costly cartridge/columns for the removal of the complex matrix of the sample prior to its injection into HPLC. The method reported here uses acetonitrile which precipitates the proteins (in plasma samples) almost completely (98–102% recovery), and subsequent sample preparation, such as centrifugation, drying (under nitrogen), and making the desired concentration, the analysis of plasma samples, can be performed more cost-effectively. Similarly, the acetonitrile completely dissolves both bosutinib and encorafenib in tablet samples and on filtration the samples can be directly injected into HPLC. The method also takes care of extraction losses during sample preparations by using encorafenib as the internal standard. The application of the QbD approach with HPLC has a further advantage, as fewer numbers of experiments are required to obtain more reliable results, thus saving the time and resouces during the method development stage. In addition, to the best of our knowledge, no HPLC-UV method with the QbD approach has been reported until now. Therefore, a simple, accurate, and fast HPLC method was developed based on the principle of QbD. In the past, QbD approaches were used to refine chromatographic settings using central composite design (CCD); however, here, we used the Box–Behnken Design (BBD).

2. Experimental Work

2.1. Materials

Sigma-Aldrich (St. Louis, MO, USA) supplied the reference standard for bosutinib and other drugs. The tablet dosage form of bosutinib was prepared in-house using commonly available tablet excipients. Solvents and buffers, distilled water (in-house), organic/inorganic solvents, and buffers (phosphate, acetate, and formate) were acquired from Sigma-Aldrich. The rat plasma was procured form Sigma-Aldrich.

2.2. Instruments/Apparatus

A Waters HPLC system (Waters Breeze 1525, The Netherlands, and Germany) fitted with an auto-sampler, a binary pump, and UV-Visible detector was used for HPLC analysis. Separation was achieved in an analytical Raptor C-18 reverse phase column having dimensions of 100 m $\times$ 4.6 mm $\times$ 5 µm internal diameter and fitted with a raptor guard column. Mettler Toledo analytical balances (Kern & Sohn GmbH, Balingen, Germany) and micropipettes (Eppendorf, Framingham, MA, USA) were used.

2.3. Methods

2.3.1. Risk Assessment Studies

The goal of risk evaluations is to investigate the impact of different conditions on the target method's quality profile (TMQP) [20]. With the help of critical analytical attributes (CAAs), it is easier to interpret the relationships among the critical method parameters of the TMQP before risk-assessment studies. The data from risk-assessment studies help to examine the cause of the problems and fix the the reasons for imperfections, variations, defects, or failures. An additional role of risk assessment studies is to collect information about the individual's risk factor, which can be further segregated into high, medium, and low risk. In this study, a total of seven factors were considered for screening. Among these, three factors were selected based on the high-, medium-, and low-risk scores for systemic optimization [21–23].

2.3.2. Optimization

With the help of QbD, the appropriate trials were constructed. The trials differed from one another due to the interaction impact of factors on each other over the dependent variables. The response surface morphology of the BBD study was planned with three independent components and two dependent response factors, and the Design Expert 13.0.3.0 programme was used for the research setup. Tailing factor (percent) and retention time (minute) were evaluated as dependent variables after the optimization of independent variables, such as mobile phase component flow rate (mL/min), acetonitrile (percent v/v), and wavelength (nm), and their individual and combined effects. To obtain a chromatographically optimized condition, a total of seventeen runs were performed [24,25].

2.3.3. Method Development

Separation and identification of the drug BST were carried out in a Raptor C-18 column at a temperature of 25 °C with a mobile phase flow rate of 1 mL/ min in a 5 min run time after the chromatographic conditions were customized. An injection volume of 20 µL was employed with a mixture of the mobile composition phase of ammonium acetate buffer pH 3.0 (A) and acetonitrile (B) (60:40 percent v/v) in isocratic conditions. The detection of elutes was performed at 260 nm with an internal standard Encorafenib (ENC).

2.3.4. Stock, Standard, and Quality-Control Sample

1 mg/mL strength stock solution of the drug (BST) was prepared in methanol and stored at 4 °C. A final dilution of 100 µg/mL was prepared by mixing 1 mL of (1000 µg/mL) solution with 9 mL of mobile phase solvent (A: B 60:40 v/v) for the preparation of the calibration curve in a concentration range of 2–20 µg/mL (2, 4, 8, 12, 16 and 20 µg/mL). Similarly, the internal standard solution of ENC (100 µg/mL) was prepared and added to calibration standard solutions to obtain the final concentration of 10 µg/mL of ENC in each. Three levels of quality assurance samples were prepared: (a) HQC (High quality control) (20 µg/mL), (b) MQC (Medium quality control) (12 µg/mL), and (c) LQC (Low quality control) (2 µg/mL). For subsequent analysis, all of the solutions were kept at 20 °C.

2.3.5. Preparation of Calibration Curve

A series of six dilutions over the range (2–20 µg/mL) of the standard drug (BST), each containing 10 µg/mL of internal standard ENC, were prepared to have an r^2 value of 0.9996 and an average standard deviation value of 0.09766. The limit of detection and the quantification were determined by the formulae 3.3 × SD/Slope and 10 × SD/Slope, respectively.

2.3.6. Preparation of Tablet Formulation Solution

We weighed 5 mg of pure BST (21.0 mg of tablet powder) and dissolved it in 5 mL of methanol (1 mg/mL; 1000 µg/mL). After agitating for 10 min by the use of a sonicator, the solution was passed through a 0.2 µm filter. Three concentration ranges [80–120%] of test concentration (10 µg/mL) were prepared, as shown in Table 1 and the chromatogram in Figure 2 For quantitative analysis, the developed method and the standard calibration curve were employed.

Table 1. Test concentration of tablet formulation.

Concn. (µg/mL)	Standard Stock Solution (mL) (BST) (µg/mL)	Internal Standard (mL) (ENC) (100 µg/mL)	Mobile Phase (mL) (A:B 60:40 v/v)	Total Volume (mL)
8 µg/mL (80%)	0.16	0.4	1.44	2.0
10 µg/mL (100%)	0.20	0.4	1.40	2.0
12 µg/mL (120%)	0.24	0.4	1.36	2.0

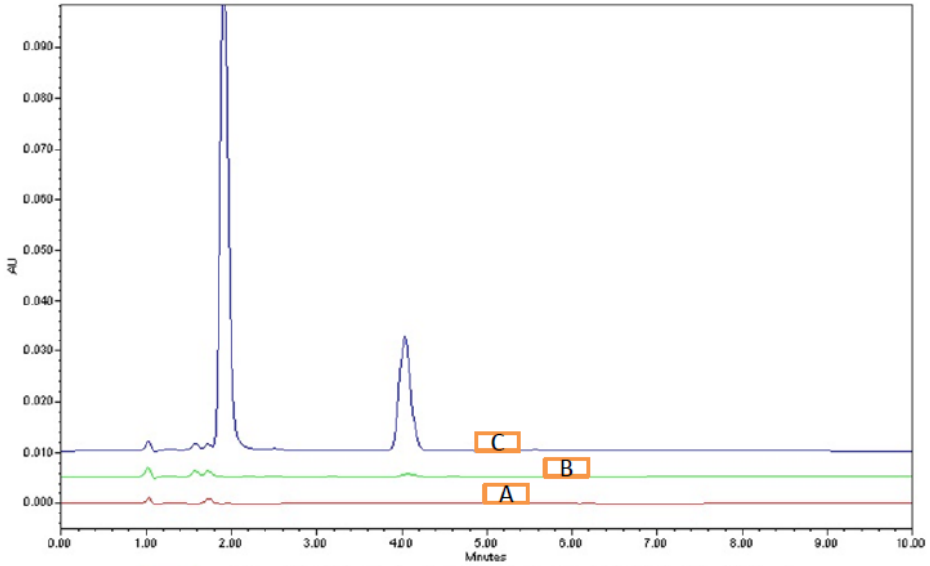


Figure 2. Chromatogram showing blank (A), placebo (B) at test concentration, and tablet sample (C) at test concentration.

2.3.7. Preparation of Rat Plasma Samples

Three ranging concentrations [50–150%] of the sample (10 µg/mL) were prepared. To 150 µL plasma (Sigma-Aldrich), 10, 20 and 30 µL of BST solution (500 µg/mL) was added to make a concentration of 5, 10, and 15 µg/mL, respectively, then vortexed the solution for 10 min. Thereafter, 40 µL of internal standard (ENC) solution in a 500 µg/mL concentration was added to it. After vortexing the entire solution for ten minutes, 750 µL of acetonitrile was added. The resulting mixture was centrifuged at 5000 rpm (2800 RCF or g) at 40 °C for 10 min and acetonitrile layer carefully separated. The solution was then dried under nitrogen at 40 °C and reduced to a final volume of 1 mL with the mobile phase. The solution was filtered through a 0.2 µm filter, degassed for 1 min, and injected into HPLC for analysis. The chromatogram is shown in Figure 3.

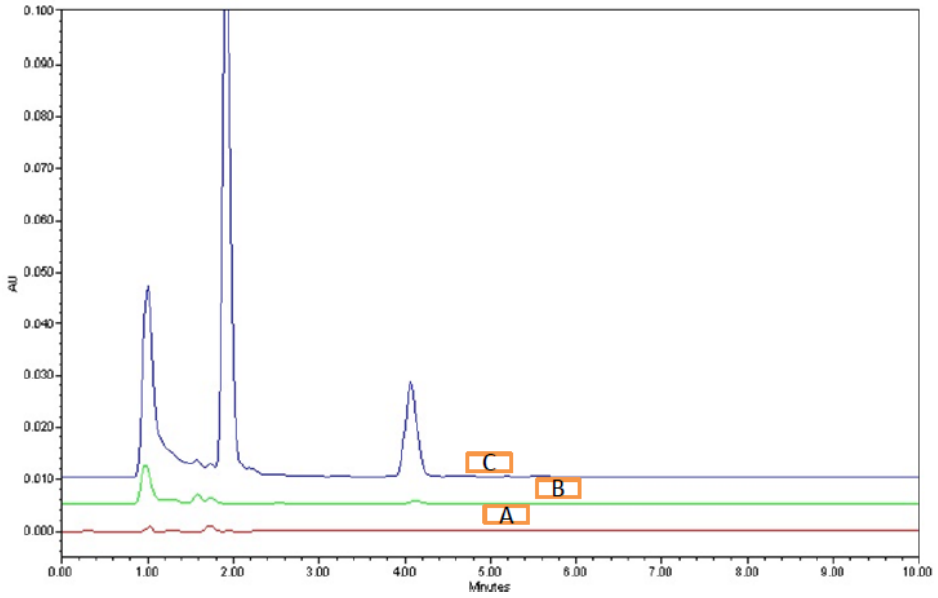


Figure 3. Chromatogram showing blank (A), plasma blank (B) at test concentration, and (C) plasma sample at test concentration.

2.4. Method Validation

All validation parameters, including system appropriateness, linearity, LOD, LOQ, accuracy, precision, robustness, and solution stability, were determined in accordance with ICH Q2 (R1) criteria [16,26].

2.4.1. System Suitability Test

System suitability tests were checked for equipment's performance analysis following USP 24/NF 19 to obtain reproducible results. The system chromatographic reproducibility was checked before analysing the sample batch. The consistency of individual results for six batches (n = 6) of samples was checked and the percentage-relative standard deviation (%RSD) was reported using three factors: tailing factor, retention times, and theoretical plate [27].

2.4.2. Linearity

Linearity of the calibration curve was performed (n = 6) on the sample of the standard drug BST of six dilutions in the range of 2–20 µg/mL. It was ensured by the detector's response of individual concentrations, and determined the r^2 value using the regression equation. A calibration curve was plotted between the average peak ratio and concentration of a sample of the working standard solution with internal standard ENC (10 µg/mL) [28].

2.4.3. Precision and Accuracy

The precision was determined in terms of interday and intraday on three quality-control samples; LQC (2 µg/mL), MQC (12 µg/mL), and HQC (20 µg/mL) of BST with %RSD of less than 2% of each concentration level.

For the accuracy test, the traditional addition approach (percent recovery) was employed. The percent recovery and percent RSD for each concentration were determined using the pre-estimated sample solution (8 g/mL) loaded with an additional 0, 50, 100, and 150 percent of the standard BST solution [29,30].

2.4.4. Solution Stability

The solution stability was determined at MQC level (12 µg/mL) at 25 °C for 14 days and 2–8 °C for 30 days. The % recovery and % RSD of the experiment were calculated [31].

2.4.5. Robustness Study

A robustness study was carried out to assess the impact of a slight but deliberate alteration in the chromatographic conditions. The value of retention time, tailing factor, and plate count by changing the ammonium acetate buffer pH, flow rate, and detector wavelength were measured [32].

3. Results and Discussion

3.1. Risk Assessment Studies

The findings revealed that risk assessment was used to determine the effects of each element on the chromatographic condition. After screening, three factors, i.e., retention time, peak area, and tailing factor (TF) were selected for the efficient method shown in Table 2.

Table 2. Bosutinib method development risk evaluation.

	Bosutinib Chromatographic Technique Features						
CAAs	**Eluent System Components**	**Wavelength of the Sensor**	**Column Temperature**	**Sample Volume**	**Flow Speed**	**Flow Pattern**	**Column Sizes**
Retention time	+1	0	0	0	+1	0	0
Peak Area	+1	0	−1	0	+1	−1	0
Tailing Factor	+1	−1	−1	0	0	0	0

3.2. Optimization

With experimental design optimisation, it is possible to develop a mathematical model that illustrates how each element affects the outcome of the experiment as well as to identify the factors' ideal values. The process of figuring out the ideal values for the significant elements discovered during screening is known as optimisation. The selection of conditions that yield the appropriate combination of chromatographic parameters presents a challenge during the chromatographic optimisation processes. Multiple-answer variables must be simultaneously optimised in order to solve this problem.

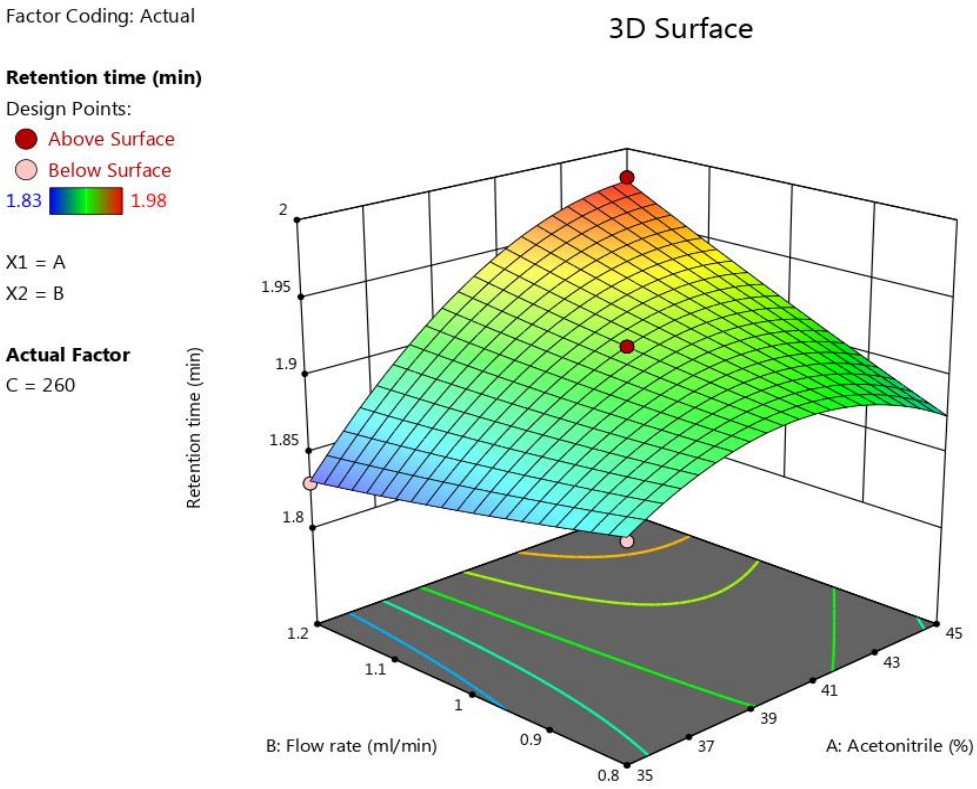
Retention time: Using the three independent variables, the retention time was optimized. The BBD surface response was robust as five trials results showed analysis of variance for the Quadratic model (trial 13–17). The results revealed that the *p*-values < 0.0500, suggesting that the analytical model was significant. The S/N was calculated with sufficient precision. A ratio (S/N) larger than 4 was advantageous. A signal-to-noise ratio of 56.376 indicated a sufficient signal. This paradigm might be useful for navigating the design space Table 3, Figure 4a,b.

Table 3. Independent variables based on the BBD and their impact on dependent variables.

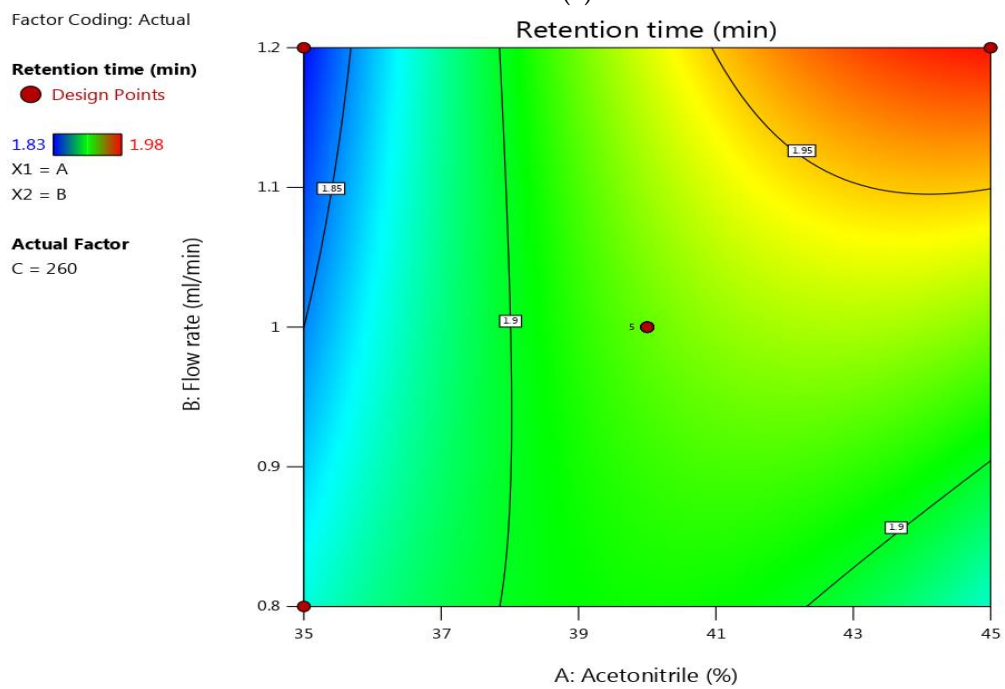
S. No.	Independent Variable			Dependent Variable	
Standard Run	Acetonitrile (%)	Flow Speed (mL/min)	Wavelength (nm)	Retention Time (min)	Tailing Factor
1.	35	0.8	260	1.87	0.88
2.	45	0.8	255	1.89	0.87
3.	35	1.2	260	1.83	0.93
4.	45	1.2	260	1.98	0.88
5.	35	1	250	1.92	0.94
6.	45	1	250	1.93	0.88
7.	35	1	270	1.84	0.9
8.	45	1	270	1.97	0.91
9.	40	0.8	250	1.98	0.86
10.	40	1.2	250	1.95	0.92
11.	40	0.8	270	1.89	0.87
12.	40	1.2	270	1.98	0.88
13 *.	40	1	260	1.92	0.89
14 *.	40	1	260	1.92	0.89
15 *.	40	1	260	1.92	0.89
16 *.	40	1	260	1.92	0.89
17 *.	40	1	260	1.92	0.89

* Optimized trial.

Tailing factor: Symmetry and shape of the peak determined the developed method's accuracy. Here, the tailing factor value was optimized by the BBD surface response with ANOVA for the quadratic model, the results of which are shown in the last five trials (13–17). The *p*-values < 0.0500, as described in the preceding section, demonstrate the sufficient precision measurement of the signal-to-noise ratio (S/N) with a value larger than 4 was desirable. A signal with a S/N ratio of 56.376 was considered sufficient. The design space could be navigated using this paradigm (Table 3, Figure 5a,b).

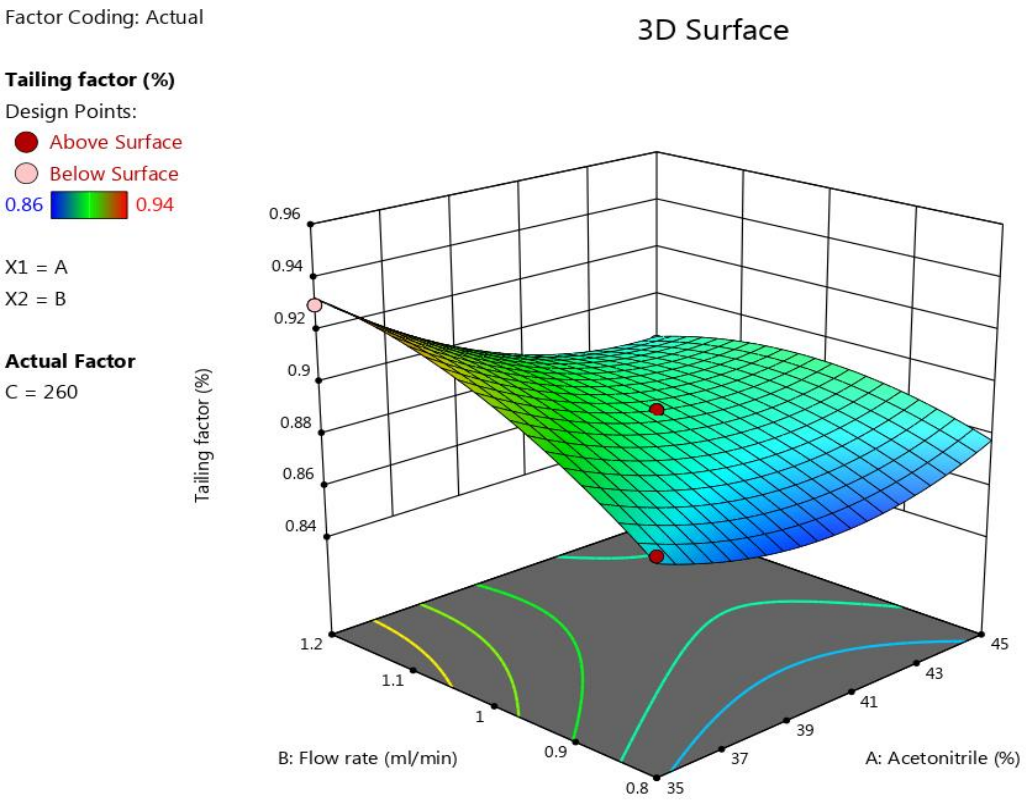


(**a**)

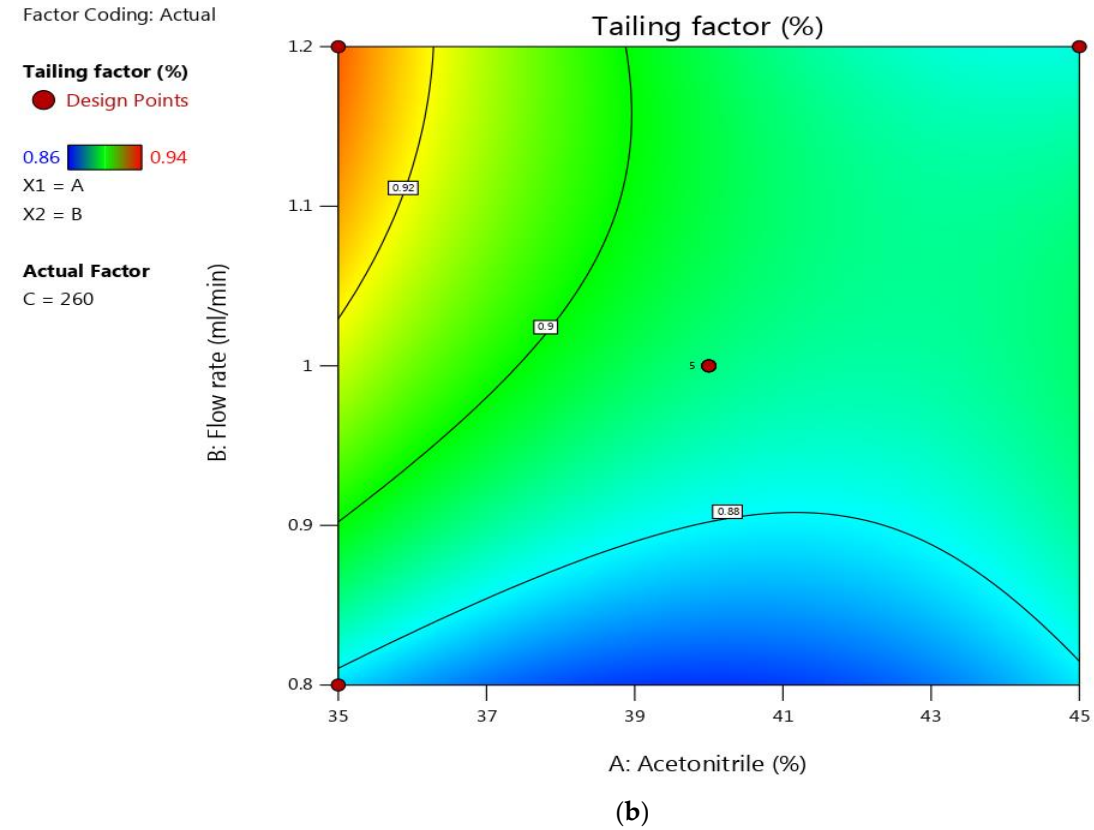


(**b**)

Figure 4. (**a**). Effect of independent factor on retention time 3D sensitive surface. (**b**). Effect of the independent factor on retention time -contour plot.



(**a**)



(**b**)

Figure 5. (**a**). Effect of an independent factor on tailing factor—3-D response surface. (**b**). Effect of an independent factor on tailing factor-contour plot.

The surface sensitivity of BBD shows that the experimental circumstances were matched to the linear and quadratic equations using the multiple regression approach.

3.3. Stock, Standard and Quality Control Sample

The sample was run in the instrument at the optimized condition to check the specificity and retention time of the drug BST and internal standard ENC. The result showed no peak in the blank solution, which confirms the specificity result and retention time of BST (1.92 min) and ENC (4.01 min) (Figure 6a,b).

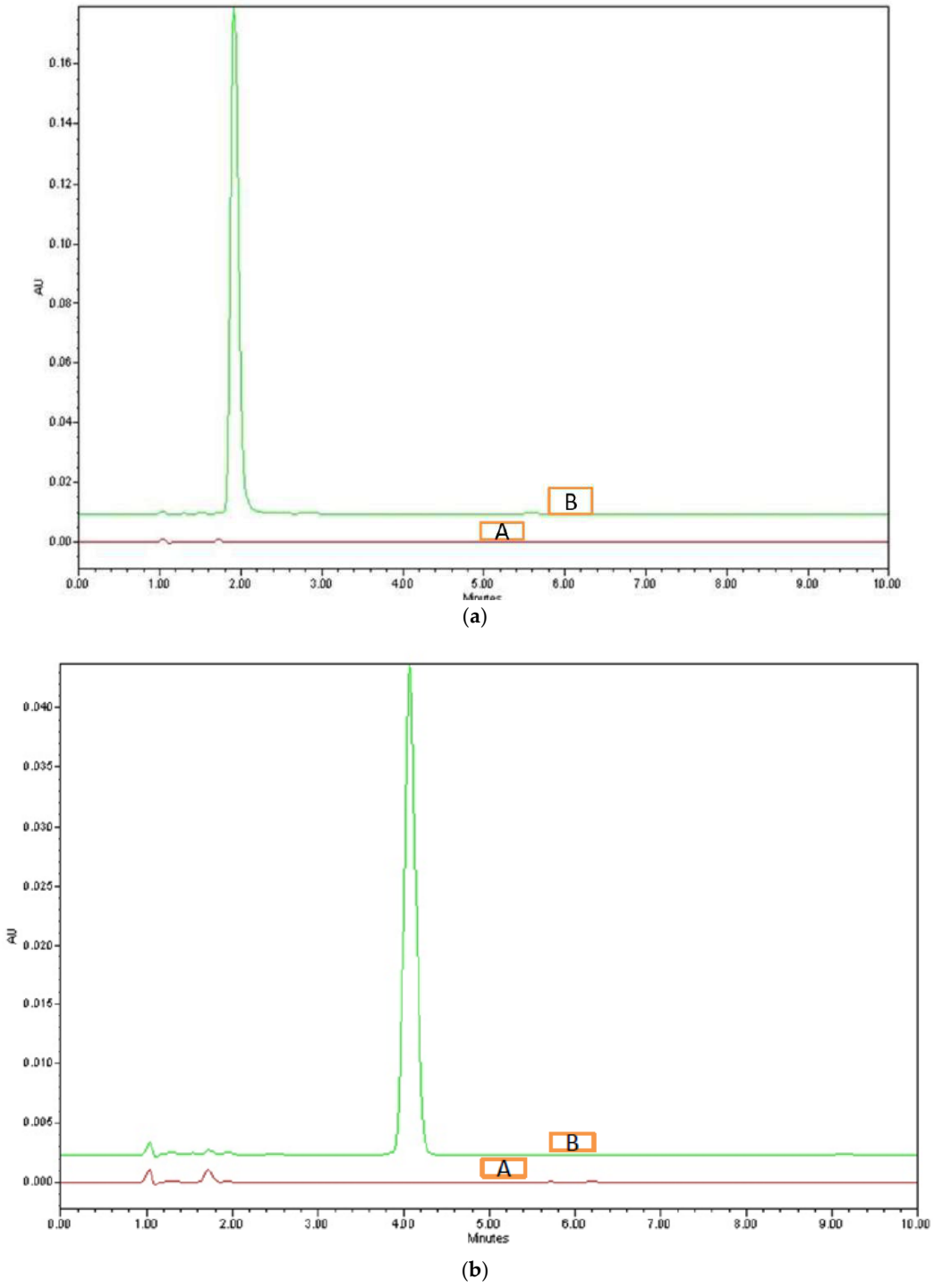


Figure 6. (**a**) Chromatogram of blank (A) and BST (B). (**b**) Chromatogram blank and ENC.

3.4. Method Validation

3.4.1. System Suitability

The analysis of the samples batch showed a reproducible result in factor retention time, tailing factor, and theoretical plate. The value of standard deviation (SD) and percentage of relative standard deviation (% RSD) indicate that the reproducibility of the chromatographic system is good (Table 4, Figure 7). All the values of %RSD below 2% followed the acceptance criteria. Well-resolved peaks of the drug and internal standard were observed.

Table 4. System suitability (n = 6).

	Retention Time (min)		Tailing Factor		Plate Count	
	BST	**ENC**	**BST**	**ENC**	**BST**	**ENC**
1	1.94	4.06	0.9	1.21	2032	4288
2	1.95	3.99	0.88	1.2	2018	4232
3	1.91	3.97	0.9	1.21	2013	4276
4	1.89	4.05	0.91	1.18	2012	4232
5	1.93	3.99	0.87	1.18	2022	4284
6	1.93	4.06	0.91	1.17	2003	4224
Average	1.925	4.02	0.895	1.19167	2016.667	4256
SD	0.0197906	0.03742	0.015	0.01572	9.012337	27.03085
%RSD	0.98	0.92	1.68	1.26	4.46	0.63

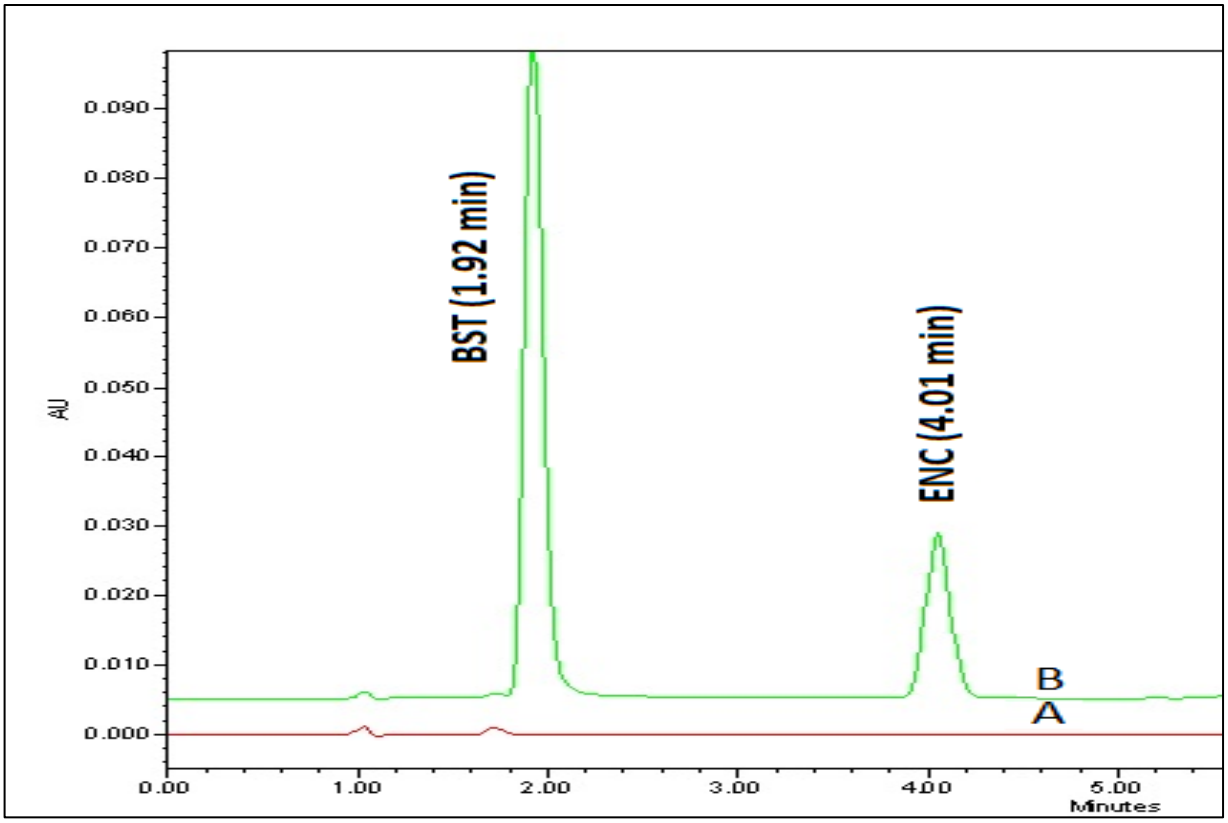


Figure 7. Chromatogram showing blank (A) and retention time of BST and ENC (B).

3.4.2. Linearity

The calibration curve of the standard solution (2–20 µg/mL) was plotted in between average peak ratio and concentration. Results of the linear regression analysis of the

calibration curves (n = 6) of BST are depicted in Table 5 and Figure 8. The r^2 value 0.999 and % RSD 1.659 indicated good linearity, with a limit of detection of 0.496 µg/mL.

Table 5. Linear regression analysis of calibration curves (n = 6) BST.

S. No.	Parameters	Numerical Value
1	Linearity range (µg mL^{-1})	2–20
2	Intercept	0.109
3	Slope	0.561
4	Correlation coefficient (r^2)	0.999
5	SD	0.098
6	%RSD	1.659
7	LOD (µg mL^{-1})	0.496
8	LOQ (µg mL^{-1})	1.503

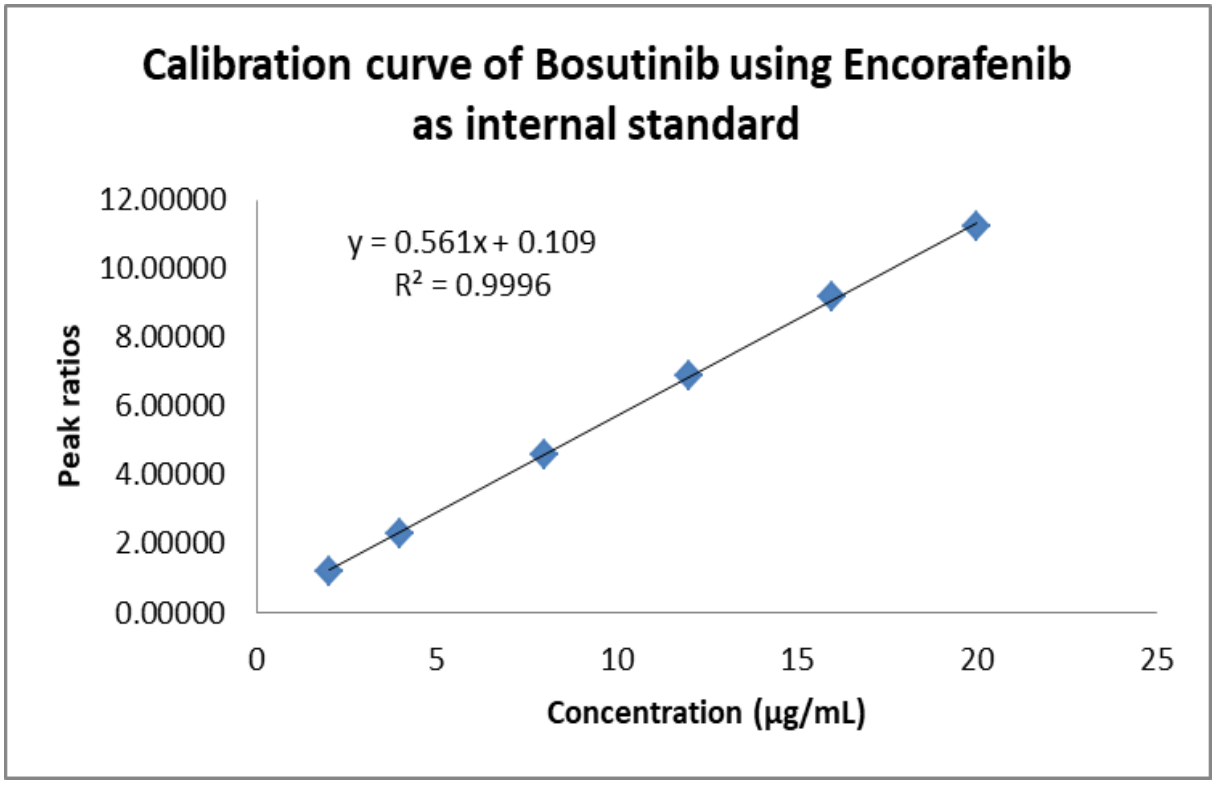


Figure 8. Calibration Curve of Bosutinib (n = 6).

3.4.3. Precision and Accuracy

Inter- and intra-day precision for three quality control levels (LQC, MQC, and HQC) are presented in Table 6, and a chromatogram is shown in Figure 9. The % RSD value of the precision and accuracy was below 2%, which indicated that the developed method is precise and accurate.

Table 6. Precision and accuracy (n = 6).

Precision						
	Inter-Day			Intraday		
	Theoretical concentration (µg mL^{-1})	Concentration found	%RSD	Theoretical concentration (µg mL^{-1})	Concentration found	%RSD
LQC	2	1.97 ± 0.037	1.892	2	2.08 ± 0.04	1.923
MQC	12	11.91 ± 0.222	1.874	12	11.96 ± 0.21	1.762
HQC	20	19.89 ± 0.342	1.723	20	19.91 ± 0.362	1.823
Accuracy (% Recovery) (Rat Plasma Samples)						
	Excess drug added to analyte %	Theoretical Content (µg mL^{-1})		Concentration found (Mean ± SD)	% Recovery (Mean ± SD)	%RSD
	0	8		7.86 ± 0.213	98.25 ± 1.847	1.881
	50	12		11.84 ± 0.517	98.66 ± 1.954	1.982
	100	16		16.2 ± 0.673	101.25 ± 1.783	1.763
	150	20		20.08 ± 0.382	100.4 ± 1.482	1.476

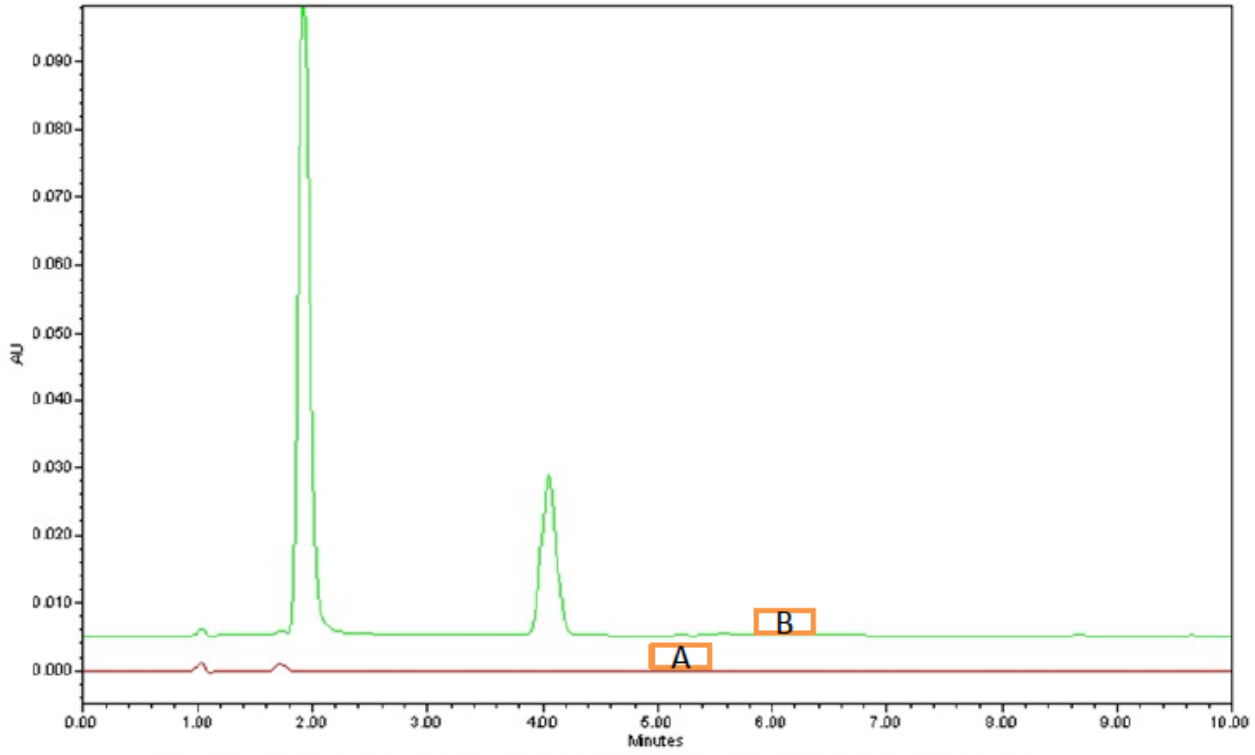


Figure 9. Chromatogram showing Blank (A), BST and ENC at MQC level (B).

3.4.4. Solution Stability

The stability of the drug solution (BST) was checked at a temperature of 25 °C for 14 days and 2–8 °C for 30 days for a concentration level of 12 µg/mL (MQC). The % RSD of was found to be <2%. Results are shown in Table 7.

Table 7. Solution stability (n = 6).

At 25 °C for 14 Days				At 2–8 °C for 30 Days			
A	**B**	**C**	**%RSD**	**A**	**B**	**C**	**%RSD**
12	11.85 ± 0.223	98.75 ± 1.852	1.875	12	11.81 ± 0.186	98.41 ± 1.624	1.651

A = Theoretical concentration (µg mL^{-1}); B = Concentration found (µg mL^{-1}) (Mean ± SD); C = % Recovery (Mean ± SD).

3.4.5. Robustness Study

The tailing factor, the retention time, and the plate count of the BST and internal standard were not significantly affected by changes in the developed method, such as changes in buffer pH, flow rate, and detector wavelength (ENC). Table 8 summarizes the findings.

Table 8. Robustness (n = 6).

	Retention Time (min)		**Tailing Factor**		**Plate Count**	
	BST	**ENC**	**BST**	**ENC**	**BST**	**ENC**
Ammonium acetate buffer pH 3.1 (Mobile phase A)	1.93	4.08	0.85	1.21	2089	4421
Ammonium acetate buffer pH 2.9 (Mobile phase A)	1.91	4.12	0.91	1.16	2098	4212
Flow rate at 0.9 mL min^{-1}	1.90	3.95	0.92	1.22	1988	4098
Flow rate at 1.1 mL min^{-1}	1.95	4.13	0.93	1.18	1997	4235
Detector wavelength at 258 nm	1.93	3.98	0.86	1.23	2095	4312
Detector wavelength at 262 nm	1.94	4.05	0.88	1.17	2084	4308

4. Conclusions

Quality by design is applied to the optimization of chromatographic conditions. The new simple, rapid, accurate, and precise method was developed for HPLC analysis and has been validated according to ICH guidelines. The new method is economical, the mobile phase is easily available, and it employs a simple UV detector and a simple extraction procedure for rat plasma and tablet dosage form samples. The validation of the study results revealed that the developed method could be adapted for the routine analysis of tablet dosage form and rat plasma samples. This HPLC method can be used for drug therapeutic monitoring (TDM) and bioavailability studies.

Pharmaceutical liquid chromatographic separation was improved with the use of QbD. When compared to the traditional optimisation procedure, which involves modifying one variable at a time, the provided approach allows for the performance of fewer trials for each optimisation stage. The introduction of a multi-objective approach to the optimisation process is made possible by the computation of the desirability function since numerous parameters are merged into a single score. In this instance, the global desirability was expressed as the resolution between analyte peaks, chromatographic responses, and chromatographic separation time. By minimising the use of harmful solvents, one of the optimisation aims, the presented approach enables the integration of the green analytical chemistry element. The described methodology is straightforward since it makes use of technologies that are widely available but slightly modified and necessitates a remarkably little number of chromatographic runs.

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