Development and Validation of a Ultra Flow Liquid Chromatography Method for the Assay of Boceprevir Using a Quality-by-Design Approach

Manish Majumder¹, Ramesh B*¹, Minaketan Tripathy²

¹Department of Pharmaceutical Chemistry and Analysis, Sri Adichunchanagiri College of Pharmacy, Adichunchanagiri University (ACU), B G Nagara, Karnataka, India
²Centre for Molecular Pharmaceutics and Advanced Therapeutics (CMPAT), Sri Adichunchanagiri College of Pharmacy, Adichunchanagiri University (ACU), B G Nagara, Karnataka, India

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ABSTRACT

Quality by design guided. The assay method of Boceprevir is developed in accordance with ICH Q8(R2) guideline with due validation. In this process, the Target analytical profile (TAP) of the drug was set and critical method parameters (CMP) were investigated by systematic risk assessment experimentation to control critical Quality Attributes (CQA). In this, A Cause Effect Risk Assessment Matrix with Control-Noise-Experiment (CNX) is used for identifying the high-risk variables i.e Percentage of Organic Modifier (% methanol), pH of the Buffer and flow rate of the mobile phase. The surface response methodology was applied to optimize the critical method parameters (CMP) as well as Critical Quality Attributes (CQA) to find out the Design space of the method. The Optimum assay method condition was mobile phase Acetate Buffer (50mM) pH 5.4: Methanol (11:89), Flow rate: 0.9 ml/min, Lambda Max: 207. The separation was achieved in the Eclip Plus C-18 column (250 × 4.6 mm, 5μm) at ambient temperature. The retention time of Boceprevir was found to be 4.2 min. The method evaluation was performed according to the (Q2R1) ICH guideline.

INTRODUCTION

Hepatitis C virus (HCV) is a seriously increasing health problem globally. Each year, 500000—700000 people died of HCV infected liver disease (Lavanchy, 1999). Boceprevir is an HCV protease inhibitor used for the treatment of chronic hepatitis C (Ascione, 2012). Traditional Method Development by RP-UFLC is a time-dependent process because of the fact one-factor-at-a-time (OFAT) system is utilized for finalizing the method performance. But in today’s competitive scenario, the need of Pharmaceutical Industry is to develop a fast, economic and robust method for estimation of Boceprevir. This type of method can be developed and optimized using the Quality by Design Approach (QbD) (Mallik et al., 2015).

Analytical QbD begins with Analytical Target Profile (ATP) describing the aim of the method, subsequently followed by the Quality Target Method Profile (QTMP) identifying the potential method variables using Initial Risk factor analysis. Analytical Quality by Design (AQbD) includes the Design of Experiments (DOE) to study instantly a high number of investigational dynamics in a limited number of trials. The final target of the QbD approach is to concurrently optimize the separation and method.
robustness that is established through the construction of a Design Space (DS) (Vogt and Kord, 2011; S, 2009; Ferety et al., 2018).

Till date, to our knowledge, few analytical methods are available in literature viz., Anuradha et al., using Uv-Visible Spectrophotometer (Anuradha and Nizami, 2018). Liquid Chromatography(LC) being reported by Ganji et al., Damle M et al., and Chandramowli et al., (Damle and Salunke, 2016; Chandramowli et al., 2018). Two Boceprevir estimation method in Human Plasma by LCMS are also reported (Aouri et al., 2013; Chandramowli and Rajkamal, 2016). However, these reported methods possess such kinds of limitations like method developed by the traditional approach, lack of deep understanding of critical method parameters etc.

Hence, deep monitoring and a detailed understanding of the key variables are expected to establish a robust analytical method. In the current study, it is envisaged to develop a sensitive and economical UFLC method for estimation of Boceprevir using QbD with due validation. The study is planned to be carried out in three phases, (i) the initial Risk factor identification by Control-Noise-Experimentation (CNX) approach, (ii) Optimization of the chromatographic condition by Central Composite Design (CCD) to find out DS and Method Operable Design Region (MODR), and (iii) Validation of the method according to ICH guideline.

Experimental
Chemical and materials
Boceprevir was procured from MNS Laboratories Pvt Ltd. Sodium Acetate Trihydrate (Analytical Grade) was purchased from Ranbaxy laboratories ltd, Mumbai and HPLC grade Methanol was purchased from Thermo Fischer Scientific. Ultra-pure water (HPLC grade) was obtained from a Milli-Q Plus 185 water purification unit.

Instruments
The method development and method validation was performed using one Shimadzu Ultra Flow Liquid Chromatography (UFLC) system with LC-20AD pump and PDA Detector. The posterm analysis was combined and managed using LC Real-time Analysis software. The pH meter (S-3CW microprocessor) was from Anametrics, Bangalore. The weighing Balance ACCULAB Sartorius was used during the experiment.

Initial Chromatographic Condition
The initial chromatographic condition includes mobile phase consisting 20mM Sodium acetate Buffer (pH 5.5): Methanol (10:90% v/v), separation is achieved on Eclip plus C-18 column (250 × 4.6 mm, 5 μm) at 1.0mL/min flow rate with 10 min run time.

Statistics
The obtained results were subjected to Central Composite Design (CCD) using Design-Expert® 11 Software Trial Version.

Preparation of standard and sample solution
Preparation of primary Stock solution (1000μg/ml): 10mg of Boceprevir was dissolved in 10ml of methanol to obtain the strength 1000μg/ml
Preparation of working standard (100 μg/ml): The stock solution was diluted to attain the strength of 100μg/ml solution of Boceprevir.

Initial Risk Assessment
Risk assessments are an integral part of the Analytical QbD process. A cause-effect risk assessment Metrix is utilised for identifying and subsequently ranking the critical method parameters affecting the critical method attributes mostly in conformation to Analytical target profile (ATP) of the Boceprevir (Raman et al., 2015; Beg et al., 2016b). In the present studies, High risk Dependent and Dependent variables are identified and analysed by Control-Noise-Experimentation (CNX) approach (Table 1). Three identified high scored Independent variables viz. percentage of methanol, flow rate and pH of Buffer solution was subjected to Design of Experiment using Surface Response methodology to find and establish a design space of the method.

Experimental Design
In experimental design, the number of experimental runs is constructed to achieve true optimum points. Therefore, The Surface Response Methodology in term of Central Composite Design (CCD) is applied to optimize the effective Independent variables on the proficiency of the method. A quadratic model was constructed between the Critical Method Attributes (Resolution & Asymmetric factor) and Critical Method Parameters (% organic modifier, mobile phase pH & Flow Rate) variables (Box and Wilson, 1951; Hashemi et al., 2010; Sivakumar et al., 2007).

The Main and interactive effects of the variables are studied by the developed model. The Analysis of Variance (ANOVA) is utilized for the significance study of the model’s coefficients. Here polynomial equation is constructed for responses by taking into consideration of coefficient of correlation, %CV and lack of fit as an adopted model fittings (Sivakumar et al., 2007; Rhodadoust and Ghaedi, 2013). The fitness of the model is investigated by the diagno.
Figure 1: Normal probability plot of residuals for Resolution (As)

Figure 2: Normal probability plot of residuals for Asymmetric factor (As)

Figure 3: Plot of residuals vs. predicted values for Resolution (Rs)
### Table 1: Control-Noise-Experimentation (CNX) approach

| Critical Method Parameter | Critical Method Attributes | Initial Risk assessment Scores | C,N,X | Experimental Strategy |
|---------------------------|----------------------------|-------------------------------|-------|-----------------------|
|                          | Resolution                 | Asymmetric Factor             |       |                       |
| Isocratic Binary Parameter |                           |                               |       |                       |
| Flow Rate                 | 2                          | 2                             | 40    | C                     | Calibrated |
| Stationary Phase          | 5                          | 5                             | 100   | C                     | New Column |
| Particle size             | 2                          | 2                             | 40    | C                     | Optimum    |
| Dimension                 | 2                          | 2                             | 40    | C                     | Standard   |
| Column Temp               | 5                          | 5                             | 100   | N                     | Ambient    |
| Buffer pH                 | 10                         | 10                            | 200   | X                     | DOE        |
| % organic Modifier        | 10                         | 10                            | 200   | X                     | DOE        |
| Solvent Grade             | 5                          | 5                             | 100   | N                     | HPLC grade |
| Injection Vol             | 2                          | 2                             | 40    | C                     | 20μL       |
| Flow Cell                 | 5                          | 5                             | 100   | C                     | 400 °C     |
| temp                      | 5                          | 5                             | 100   | N                     | Standard   |

Note: C-Control, N-Noise and X-Experiment Score Low Risk-1, Medium Risk-5 and High Risk-10 Total Score = (Risk level of First CMA × 10) + (Risk level of Second CMA × 10)

**Figure 4: Plot of residuals vs predicted values for Asymmetric factor (As)**
Figure 5: 3D-response surface plot for interaction study

(a) % of Organic modifier & Flow Rate Vs Resolution, (b) pH & % of Organic modifier Vs Resolution, (c) Flow Rate & pH Vs Resolution, (d) pH & % of Organic modifier Vs Asymmetric factor, (e) flow Rate and % of Organic modifier Vs Asymmetric factor, (f) pH & flow Rate Vs Asymmetric factor.

Figure 6: Design space by using % of organic modifier and buffer's pH

Figure 7: Design space by using flow rate and % of organic modifier
Table 2: Central Composite design: 3 Factors with 2 Responses

| Run | A: % Organic Modifier % concentration | B: pH | C: Flow Rate mL/min | Response 1: Resolution | Response 2: Asymmetric Factor (K) |
|-----|--------------------------------------|-------|---------------------|------------------------|----------------------------------|
| 1   | 90                                   | 6.3409| 1                   | 1.582                  | 0.408                            |
| 2   | 90                                   | 5.5   | 1                   | 1.572                  | 0.403                            |
| 3   | 85                                   | 6     | 1.5                 | 1.922                  | 0.751                            |
| 4   | 90                                   | 5.5   | 1                   | 1.672                  | 0.403                            |
| 5   | 90                                   | 5.5   | 1                   | 1.672                  | 0.403                            |
| 6   | 90                                   | 5.5   | 1.8409              | 1.672                  | 0.403                            |
| 7   | 95                                   | 6     | 1.5                 | 1.5                    | 0.229                            |
| 8   | 90                                   | 5.5   | 1                   | 1.524                  | 0.403                            |
| 9   | 85                                   | 5     | 1.5                 | 1.967                  | 0.733                            |
| 10  | 95                                   | 5     | 1.5                 | 1.507                  | 0.226                            |
| 11  | 90                                   | 5.5   | 1                   | 1.672                  | 0.403                            |
| 12  | 95                                   | 6     | 0.5                 | 1.578                  | 0.234                            |
| 13  | 90                                   | 5.5   | 0.159104            | 1.585                  | 0.393                            |
| 14  | 85                                   | 6     | 0.5                 | 1.564                  | 0.349                            |
| 15  | 90                                   | 4.6591| 1                   | 1.538                  | 0.336                            |
| 16  | 98.409                               | 5.5   | 1                   | 1.501                  | 0.135                            |
| 17  | 81.591                               | 5.5   | 1                   | 1.99                   | 1.099                            |
| 18  | 85                                   | 5     | 0.5                 | 1.76                   | 0.544                            |
| 19  | 95                                   | 5     | 0.5                 | 1.679                  | 0.44                             |
| 20  | 90                                   | 5.5   | 1                   | 1.608                  | 0.403                            |

A: Factor1: % Organic Modifier;  
B: Factor2: pH  
C: Factor3: Flow rate  
R1: Response 1: Resolution  
R2: Response 2: Asymmetric Factor (K)

Figure 8: Derringer’s desirability plot for optimizing % of Organic Modifier, pH and Flow Rate of the method.
### Table 3: Summary of Validation

| Assay Parameters | Result | Acceptance Criteria |
|------------------|--------|---------------------|
| **Specificity**  | The retention Time of Boceprevir is observed to be 4.2 min with Peak Purity Index: 0.9999. | The chromatographic peak excipient must not interfere with the targeted analyte peak. |
| **Linearity**    | The standard linearity for Boceprevir was generated from 100 µg/mL to 800 µg/mL. R² was found to be 0.9987 with \( y = 14766x + 255855 \). And Standard error was found to be 118550. P-value was found to be 0.097085. | The correlation coefficient for six concentration levels will be ≥0.997 for the range of 80 to 120% of the target Concentration. |
| **Range**        | 200 µg/mL to 600 µg/mL range was used for the Accuracy and Precision study. %RSD was less than 2. | The acceptable range will be defined as the concentration interval over which linearity and accuracy are obtained per the above criteria, and in addition, that yields a precision of ≤3% RSD. |
| **Accuracy**     | For drug substance: Average %assay at three concentration level (50%, 100% & 150%) for Boceprevir were found to be 100%, 100.75% & 100% respectively. And % RSD were found to be 0.422, 0.209 & 0.177 for three different level. For Accuracy of Drug Product (Boceprevir tablet): Average Recovery (%) and Standard Deviation (SD) Values at Each level (80%, 100%, and 120%) for Boceprevir were found to be 99.42%±0.4233, 98.69%±0.4618 and 99.26±0.1456, respectively. | The Relative Standard Deviation (RSD %) for the individual recovery result at each level, not more than 2.0%. The average recovery at different concentration levels: 98.0-102.0%. |
| **Precision**    | The % RSD for the repeatability study was found to be 0.1197 for Retention Time and 0.7326 for Peak Area of Boceprevir, respectively. Therefore, the precision of the analytical method was found to be within acceptable limits. | The %RSD for repeatability, not more than 2.0 |
| **LOD**          | 29.24 µg/mL | Complies the report |
| **LOQ**          | 87.51 µg/mL | Complies the report |
| **Assay**        | 198.68 mg 99.34% | 98% to 102% |
RESULTS AND DISCUSSION

Design of Experiment

In Central Composite design (CCD) 20 experiments are run in randomized order to reduce the effects of unrestrained variables as shown in Table 2. This design is utilized to optimize the quadratic and interactive effect (Hashemi et al., 2010). The experimental results of the CCD have been fitted with coded expressions for (Resolution) R1 and R2 (Asymmetric factor) as expressed

\[
R1 = +1.62 - 0.1297A - 0.0201B + 0.0338C + 0.0166AB - 0.1019AC + 0.0306BC + 0.0525A^2 - 0.0131B^2 + 0.0111C^2
\]

\[
R2 = 0.4039 - 0.2111A - 0.0299B + 0.0835C + 0.0155AB - 0.1450AC + 0.0340BC + 0.1175A^2 - 0.0045B^2 + 0.0042C^2
\]

Response R1 = Resolution, Response R2 = Asymmetric factor, Factor A = %Organic modifier

Factor B = pH of the Buffer, Factor C = Flow Rate of the mobile Phase

The ANOVA is applied to understand the variable’s effect and interaction using Design expert 11. The P-value for R1 & R2 is observed to be 0.0004 & 0.002, indicating the statistical significance of an effect at a 95% confidence level. The Model F-values of 11.22 for R1 & 11.03 for R2, respectively, implies the model is significant (Sivakumar et al., 2007; Khodadoust and Ghaedi, 2013). The quality of fit of the polynomial model equation is expressed by the coefficient of determination $R^2$ as shown in the result with 0.9909 and 0.9098 for the corresponding values of resolution and asymmetric factor, whereas 0.8287 and 0.8261 represented the same for the adjusted $R^2$ values. The large adjusted $R^2$ values ≥ 0.80 indicate a good relationship between the experimental data and the fitted model (Sivakumar et al., 2007; Khodadoust and Ghaedi, 2013). The adequate precision value is a measure of the “signal (response) to noise (deviation) ratio”. A ratio greater than four is desirable (Sivakumar et al., 2007; Khodadoust and Ghaedi, 2013). In this study, the ratio is found to be 12.3243 for R1 & 12.8682 for R2, indicating the model is significant for the separation process. The value of 3.76 in the case of the parameter of %CV for all models is in agreement with previous literature, hence indicate reasonable reproducibility (Sivakumar et al., 2007; Khodadoust and Ghaedi, 2013).

The diagnostic plots, (i) a normal probability plot of residuals and (ii) a plot of residuals vs predicted values, are analysed for response R1 and R2. It is observed by closed inspection in Figures 1 and 2 that the distribution of errors is normal, indicating the model fits the data adequately (Khodadoust and Ghaedi, 2013; Olivero et al., 1995; Stalikas et al., 2009). The observation of Figures 3 and 4 tells there is no apparent pattern of residual in the plot of residual versus predicted response due to equal scattering of residuals above and below the x-axis, expressing the suitability of the proposed model. Since the norms of normality and continual discrepancy of the residuals were found to be satisfied, the fitted model for the R1 and R2 may be accepted (Khodadoust and Ghaedi, 2013; Olivero et al., 1995; Stalikas et al., 2009).

Interference study

Response surface analysis has been carried out employing a 2D-response surface plot for identifying the underlying interaction(s) among the studied factors (Hasnain et al., 2016; Jovanović et al., 2015;
The 3D-response surface plot Figure 5 indicates a linear increasing Resolution value with a decrease in the Organic modifier (%) as well as flow rate and a linear declining Resolution value observed with increase in the pH as well decrease in flow rate. And at the highest level of pH and lowest flow rate, Resolution is Maximum.

It is also observed in the 3D-response surface plot that a linear declining Asymmetric factor value increases with Organic modifier (%) and with decreasing pH value. Asymmetric Factor illustrated a linear declining value with an increase in the pH as well decrease in flow rate. At the point of the highest level of pH and lowest flow rate, Asymmetric factor showed minimum.

**Design Space and Desirability Function**

Design Space has been created after treating all data using the modelling software Design Expert-11 Trial Version. Two-dimensional charts are created by taking three factors (% Organic Modifier, pH & Flow rate) and represented in Figures 6 and 7. The Red region of the 2D contour plots depicts the design space, defining the robust region of the method where results are within designated criteria.

Maximising the resolution of symmetrical peak and minimization of asymmetric factors are the main objectives. Derringer’s desirability function (D) is the most suitable technique to optimize the different responses at multiple targets (Hadjmohammadi and Sharifi, 2012). The value of D is zero indicating a desirable range of all responses and subsequently, a value of D is close to one, representing globally optimal (near to the target value) for a combination of different criteria (Beg et al., 2016a). In this study, the value of D is 0.985 obtained from the surface response curve, indicating an excellent mathematical model in Figure 8 (Hadjmohammadi and Sharifi, 2012; Panda, 2015). The coordinates produce the maximum desirability value at % organic modifier 89% v/v, buffer pH 5.4, and a flow rate of 0.9 mL min⁻¹. The represented Chromatogram of Boceprevir is presented in Figure 9.

**Validation**

The method validation is performed according to the (Q2R1) ICH guideline. (26) Summary of Validation is illustrated in Table 3.

**CONCLUSIONS**

In this work, the QbD approach has been successfully implemented for robust method development in the estimation of Boceprevir in its pure form and also in the case of the investigated formulation. Each step of the Analytical QbD process has been studied to find out the Design Space. Response surface plots using CCD illustrated graphically the major effects of independent variables (% Organic modifier, pH and Flow rate) on the separation. Using the QbD approach, a robustness of the method is already available before going for validation. The method has been found to be linear, accurate as well as precise in the range of 200 mg/ml to 600 mg/mL for Boceprevir using Eclip plus C-18 column in 10 minutes runtime.

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**Conflict of Interest**

The authors declare that they have no conflict of interest for this study.

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