ORIGINAL ARTICLE

Development and Validation of Boceprevir using Stability Indicating Reversed-Phase High-Performance Liquid Chromatography Method with Diode Array Detector

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Abstract

Background: Nowadays, demands for anti-viral drugs have significantly increased in recent years because of global susceptibility to viral infections, notably the COVID-19 pandemic and now emergence of human metapneumovirus. Ensuring quality standards and validation of these drugs is essential in healthcare. Boceprevir (BCP) is an antiviral drug classified as a protease inhibitor, primarily used to treat chronic hepatitis C virus infection, with a particular focus on genotype 1. **Objective:** The primary objective is to provide a reversed-phase high-performance liquid chromatography (RP-HPLC) method for the analysis of BCP that is simple, reliable, accurate, and rapid, and to validate it as per ICH guidelines with stability-indicating studies. **Materials and Methods:** The chromatographic analysis was performed using Ainertsil ODS-3V, C18 (250 mm × 4.6 mm, 5 µm) by GL Sciences. The mobile phase comprised acetonitrile: methanol and buffer (0.05 M potassium dihydrogen phosphate) in a 50:40:10 (v/v/v) ratio, with an adjusted pH of 3.5 using orthophosphoric acid. The flow rate was maintained at 0.25 mL/min, with column temperature set at 25°C. Detection was conducted at 206 nm, and the injection volume was 20 µL. **Results:** The BCP exhibited a retention time of 3.69 min. The method demonstrated outstanding linearity within the concentration range of 10–50 µg/mL, with a correlation coefficient (r^2) value of 0.9998.Validation parameters were evaluated following ICH Q2 guidelines, including limit of detection, limit of quantification, accuracy, precision, robustness, and ruggedness. The % relative standard deviation for all parameters was <2%. The stability-indicating method revealed small degradation products under forced degradation conditions, confirming the method's specificity and selectivity. **Conclusion:** The developed RP-HPLC method demonstrated a high theoretical plate count, good resolution, and a symmetric peak, ensuring its accuracy, linearity, specificity, selectivity, and robustness. The method is suitable for detecting impurities and assessing degradation products during the forced degradation study. The validated method is suitable for repetitive pharmaceutical quality control examination and safety assessment of BCP.

Key words: Boceprevir, force degradation study, method development, method validation

INTRODUCTION

The primary objective of the stability-indicating study is to generate data on stress testing parameters to assess the integrity of the drug and its dosage form.[1] This study is essential for ensuring stability, considering climate variations across different regions. Chromatography, an economical and precise technique for analyzing the stability of various drugs, was employed in this research work using reversed-phase high-performance liquid chromatography (RP-HPLC) analytical methods.[2]

Boceprevir (BCP), chemically designated as [(1R,2S,5S)-N-(4-amino-1-cyclobutyl-3,4-dioxobutan-2-yl)-3-[(2S)-2-(tert-butylcarbamoylamino)-3,3-dimethylbutanoyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexane-2-carboxamide], has a molecular

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formula of $C_{27}H_{45}N_5O_5$ and a molecular weight of 519.7 daltons. It is a newly developed direct-acting antiviral drug and NS3 protease inhibitor. The United States Food and Drug Administration approved BCPon13 May 2011, to treat chronic hepatitis C virus (HCV) genotype 1 infection. It is indicated for use in combination with ribavirin and peginterferon [Figure 1].[3,4]

BCP functions as a covalent, reversible HCV NS3 serine protease inhibitor, binding to its active site through a ketoamide functional group. By inhibiting the NS3 protease, it suppresses RNA replication and ultimately reduces the production of virions.[3]

According to the literature survey, there was one method available for method development by HPLC.[2,5] In contrast, other methods developed with HPLC with stability stability-indicating methods, but the sources of those articles are unreliable.[6] One article is available with ultraviolet (UV)-spectroscopy and one with Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS).[7,8] The study aimed to develop a sensitive and stability-indicating method for the assessment of BCP for standard research using a photodiode array detector as per ICH Q2 guidelines.[9]

MATERIALS AND METHODS

Materials and chemicals

BCP (Reference standard procured from Sigma Aldrich, USA), Methanol and Acetonitrile (HPLC grade, Merck Ltd), Milli-Q water (Ultrapure type 1), and O-phosphoric acid (SD Fine Chem Ltd.). Other than these, reagents and chemicals are used with high quality and purity.

Instrumentation

RP-HPLC system utilized a Shimadzu LC-2030 Plus equipped with UV-visible SPD 20A detector. The data acquisition and processing were performed using Windows XP-based LC solution software. The chromatographic separations were performed on a RP Inertsil ODS-3V, C18 (250 mm × 4.6 mm, 5 µm) by GL Sciences column.

Chromatographic conditions

The mobile phase comprised acetonitrile: Methanol and buffer (0.05 M potassium dihydrogen phosphate [KH_2PO_4]) in a 50:40:10 (v/v/v) ratio, with an adjusted pH 3.5using Orthophosphoric acid, and carried at a flow rate of 0.25 mL/min. The column temperature was kept 25°C, and the injection volume was fixed 20 µL. The drug exhibited maximum absorbance at 206 nm. Hence, 206 nm was chosen as the wavelength for detection. The UV spectrum is represented in Figure 2.

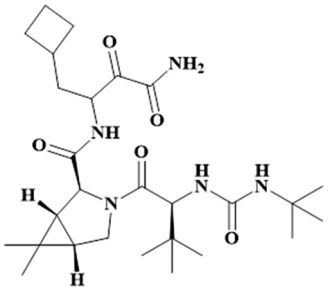

Figure 1: Chemical figure of boceprevir

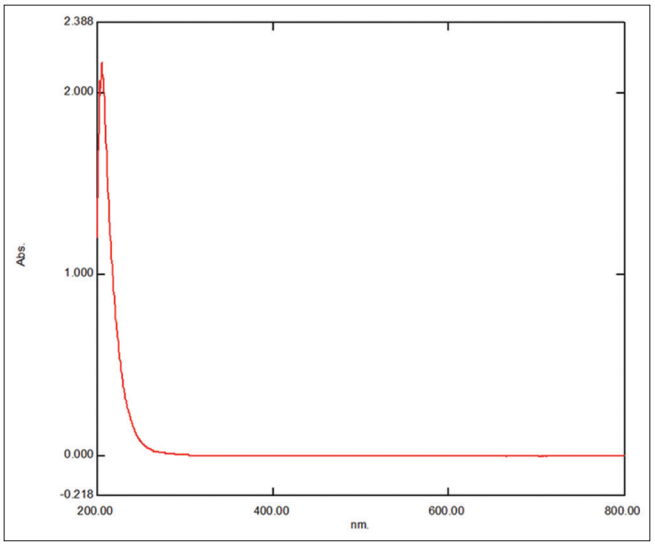

Figure 2: Ultraviolet spectrum of boceprevir

Preparation of standard solution and calibration curve solutions

A precisely weighed 10 mg BCP was transferred into a 10 mL volumetric flask and made up to the volume with mobile phase. The solution was vortexed for 10 min to ensure complete dissolution. This solution was designated as the stock solution. From this stock, 2 mL was pipetted into a 10 mL volumetric flask and diluted to volume with the diluent, yielding a working standard solution of 200 µg/mL. Five different diluents were prepared from the working standard to construct the linearity curve, covering a concentration range of 10–50 µg/mL.

Analytical method validation[10]

The primary goal of method development and validation is to confirm that the proposed analytical method is suitable for its intended purpose, as outlined in the ICH Q2(R1) guidelines. The validation process involves evaluating critical parameters such as linearity, precision, accuracy, robustness, LOD and LOQ, along with performing stability-indicating studies.

System suitability

For the optimization of the proposed method, a system suitability test was conducted. This test is crucial in evaluating the resolution and reproducibility of the method, ensuring its adequacy for analysis. Key parameters assessed during the system suitability test include the tailing factor and column efficiency. The acceptance criteria require a minimum of 2000 theoretical plates and a tailing factor not exceeding 2.0. System suitability was determined through six replicate analyses of the drug at a 30 µg/mL concentration.

Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined using the peak area obtained from the linearity calibration curve. Formulas used for the estimation are, LOD is described by the lowest concentration level emerging in a peak area, while LOQ is defined as the lowest quantification concentration estimated by the peak area response. Formulas for determining LOD and LOQ are LOD = 3.3(SD/Slope) and LOQ = 10(SD/Slope), where SD denotes the standard deviation of the Y-intercept from the regression line.

Linearity

A linearity of BCP was estimated by the dilution of the working standard of the analytical procedure with a minimum of 5 concentrations. Standard dilutions were prepared across a concentration range of 5–50 µg/mL. The linearity of the responses was evaluated using linear regression analysis, with calculations performed using the least squares method.

Precision and accuracy

Precision is a measurement of how closely data values align with each other for several measurements under identical analytical conditions. The precision of the assay was assessed by repeatability consistency with three injections in a single day (intra-day) and triplicate injections per day for three days continuously (inter-day). The accuracy of the proposed method was evaluated by fortifying the standard with a known concentration of BCP at three different levels, each performed in triplicate.

Robustness studies

The robustness of the method was assessed by evaluating its resistance to minor variations in chromatographic conditions. To determine the robustness, deliberate alterations were made to parameters such as column temperature, mobile phase composition, pH of mobile phase, and flow rate.

Stress degradation studies

For the evaluation of stress degradation of the drug BCP, different conditions were applied. Acid, base, and oxidative-induced stress degradation were conducted by taking 5 mL of a 500 µg/mL solution for each condition. These were treated with 1 N methanolic hydrogen chloride (HCl), 0.1 N sodium hydroxide (NaOH), and 3% v/v hydrogen peroxide (H_2O_2), respectively. All these conditions were maintained for 24 h. Thermal and photodegradation were carried out on the solid form of the BCP drug. For thermal degradation studies, the drug was subjected to a temperature of 105°C for 6 h, and for photodegradation, it was exposed to UV radiation for 24 h. Following these stress treatments, the samples were injected into the HPLC system, and the results were compared with those of freshly prepared samples.

RESULTS AND DISCUSSION

Method development and optimization

A stability-indicating analytical method was optimized using RP-HPLC. Initially, the solubility of the drug was tested in different solvents, including methanol, water, and ethanol. Methanol exhibited superior solubility for the drug. Multiple mobile phases were evaluated in both isocratic and gradient modes. When methanol (100%) was used in isocratic mode, followed by acetonitrile (100%), a hump was observed in the chromatogram. To address this, various compositions of acetonitrile, methanol, and buffer, ranging from 80:10:10 v/v/v to 10:80:10 v/v/v in isocratic mode, were tested, but these resulted in significant tailing of the peak. Trials of various mobile phase chromatogram are shown in Figures 3 and 4. After exploring numerous compositions in isocratic modes, a mobile phase consisting of acetonitrile, methanol, and buffer (0.05 M KH_2PO_4) in a50:40:10v/v/vratio produced a peak at approximately 3.6 min [Figure 5]. However, the peak was not sharp, and fronting was observed. To reduce fronting and enhance peak sharpness, different compounds were investigated to adjust pH, including ammonium formate, dipotassium hydrogen orthophosphate, and orthophosphoric acid. Among these, orthophosphoric acid is used to adjust pH to 3.5. The optimized method utilized an inertsil ODS-3V,

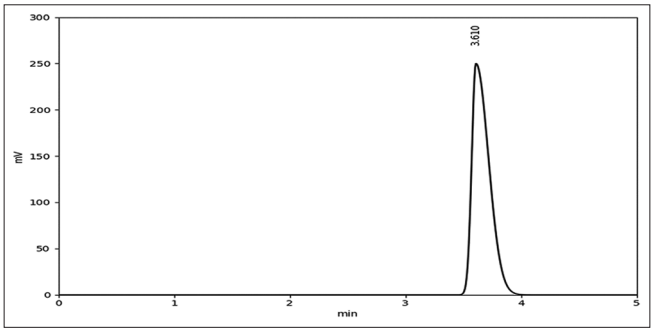

Figure 3: Optimized trial chromatogram using ACN: MeOH: Buffer (60:30:10 v/v/v)

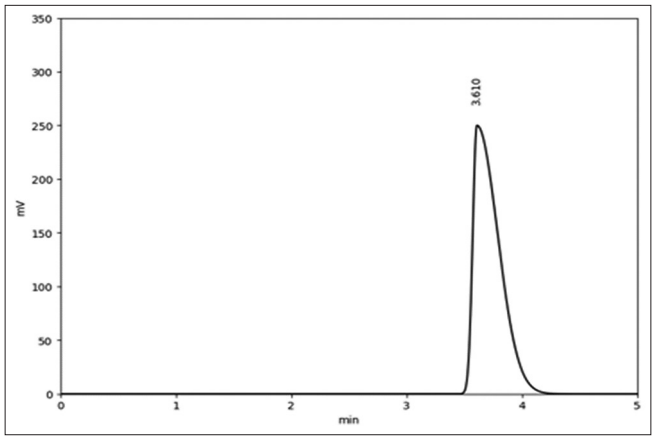

Figure 4: Optimized trial chromatogram using ACN: MeOH: Buffer (70:20:10 v/v/v)

C18 (250 mm × 4.6 mm, 5 µm). The flow rate was varied from 0.1 mL/min to 0.8 mL/min to achieve a sharp peak and shorter elution time, with an optimal flow rate determined to be 0.25 mL/min. At this flow rate, the column pressure was recorded at 140 bar, well within the maximum column pressure limit of 400 bar. Total runtime was 5 min. Detection was performed at 206 nm, with the column maintained at room temperature. This method is cost-effective and suitable for long-term routine quality control analysis. Developed parameters are concise in Table 1.

System suitability

The % relative standard deviation (% RSD) of a drug's retention time and peak was found to be within the acceptable limit, as shown in Table 2 and the chromatogram of standard BCP Figure 5. The column's efficiency, expressed in the number of theoretical plates and USP tailing factor, was determined to be 3404 and 1.98 ± 0.05, respectively.

LOD and LOQ

LOD and LOQ were estimated based on calibration curves with the help of a formula. The developed method is sensitive, which could be identified by the data obtained from the system, as illustrate in Table 3.

Linearity

A linear relationship was observed for BCP concentration ranging from 10 to 50 µg/mL. The peak area and corresponding concentrations were analyzed using least squares regression to determine the regression equation. The peak areas of BCP were linear with respect to concentration. The linearity was confirmed through triplicate analysis. The results are summarized in Table 4, and the linearity curve is depicted in Figure 6.

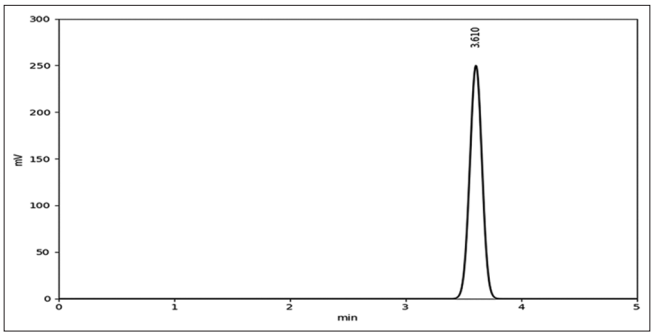

Figure 5: Final chromatogram using ACN: MeOH: Buffer (50:40:10 v/v/v)

Table 1: Developed separation method for Boceprevir

Parameters	Conditions
Stationary phase	Inertsil ODS-3V, C18 (250 mm×4.6 mm, 5 µm)
Mobile phase	acetonitrile, methanol and buffer (50:40:10 v/v/v) (pH 3.5 O-phosphoric acid)
Retention time (min)	3.6
Detection wavelength (nm)	206
Flow rate (mL/min)	0.25
Run time (min)	5
Injection volume (µL)	20

Table 2: System suitability test for boceprevir

S. No.	Retention time	Peak area	Theoretical plates	Tailing factor
1.	3.6	182,304	3440	1.75
2.	3.5	183,105	3464	1.84
3.	3.6	179,657	3428	1.82
4.	3.6	185,998	3379	1.78
5.	3.6	184,342	3426	1.76
6.	3.7	185,213	3289	1.81
Mean	3.6	183,436	3404	1.98
Standard deviation	0.049	2289	62.93	0.03
% CV	1.36	1.24	1.84	1.98

Accuracy and precision

The accuracy and precision of the quality control standards were assessed, with accuracy ranging from 99.98% to 102.34%. The precision data showed that both inter-day and intra-day variations were within 2% RSD. The data for accuracy and precision are provided in Table 5a and b.

Robustness study

Robustness was evaluated to determine the method's ability to remain unaffected by deliberate minor variations

Table 3: Sensitivity

S. No.	Linearity equation	Determination coefficient (R^2)	Linearity range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
1.	Y=8717.4X−6414	0.998	10–50	2.697	8.174

LOD: Limit of detection, LOQ: Limit of quantification

Table 4: Linearity data of boceprevir

S. No.	Concentration (µg/mL)	Peak area
1.	10	85120
2.	20	159780
3.	30	255310
4.	40	348900
5.	50	426430

in chromatographic conditions. To evaluate robustness, minor alterations were made to the experimental conditions. Specifically, variations in flow rate (±0.1 mL/min), detection wavelength (206 nm ± 2 nm), and column temperature (25°C ± 5°C) were investigated, as presented in Table 6. The results showed no significant variation when these parameters were changed.

Force degradation studies[11]

The forced degradation study was conducted following ICH guidelines Q1B and Q1A (R2),[12] Samples were subjected to various stress conditions, including acidic, basic, oxidative, thermal and photolytic, for a duration of 24 h. After degradation, the degradant product was injected into HPLC, and the results obtained were compared with the control sample. For every degradation study, two samples were prepared: a blank and a working standard. The blank sample was exposed to stress environments in the same manner as the working standard solution. Photolytic and dry heat degradation studies were performed in the solid state. The degradation study data are presented in Table 7.

Acidic degradation

Acid hydrolysis was performed by taking a 5 mL working standard solution of 500 µg/mL concentration in a volumetric flask, then 5 mL of 0.1 N methanolic HCL and 40 mL methanol were added to the volumetric flask and mixed well. After mixing, the solution was refluxed for 15 min. The refluxing mixture was neutralized with the help of 5 mL of NaOH solution. A 30 µg/mL solution was injected into the system. Results showed that 9.8% of BCP was degraded, as shown in Figure 7.

Base degradation

Base degradation of the BCP standard solution was conducted by mixing 5 mL of 500 µg/mL BCP solution with 5 mL of 0.1 N NaOH solution in a volumetric flask. The volume was adjusted to 50 mL using methanol and

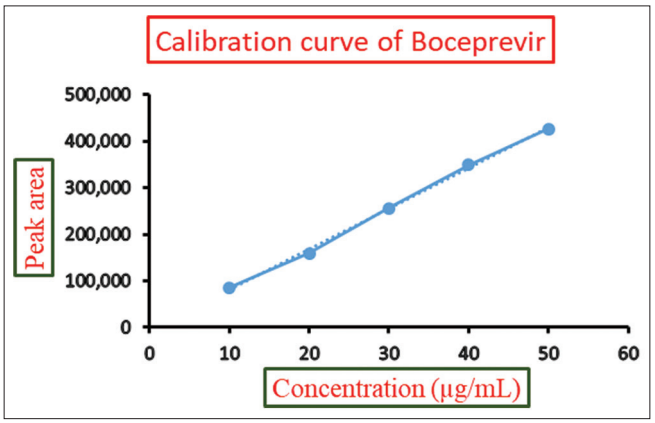

Figure 6: Linearity curve of boceprevir

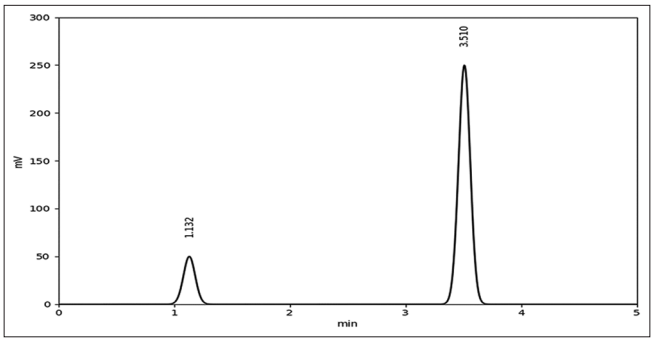

Figure 7: Chromatogram of acid degradation with degradation product at R_f1.132

allowed to stand at room temperature for 1 h. Following the degradation period, the mixture was neutralized with 0.1 N HCL. A final concentration of 30 µg/mL was injected into the chromatographic system that shows a degradation of 2.5%. The corresponding chromatogram is presented in Figure 8.

Oxidative degradation

Oxidative degradation of BCP was estimated by adding 5 mL of 3% v/v H_2O_2, to a volumetric flask holding 5 mL of a 500 µg/mL BCP standard solution. Volume made up of a volumetric flask by adding methanol up to 50 mL and kept the solution for 3 h. After the degradation period, a final concentration of 30 µg/mL was injected into the chromatographic system, showing the degradation of 4.2% The corresponding chromatogram is presented in the Figure 9.

Thermal degradation

A thermal degradation study of BCP was conducted by exposing the drug to 105°C in an oven for 12 h.

Table 5a: Accuracy data of BCP

S. No.	% Spike level	Amount of BCP present(µg/mL)	Amount of BCP added (µg/mL)	Theoretical Value (µg/mL)	Observed value (µg/mL)	Recovery (%)	Average recovery (%)
1.	80%	2231.25	750	2981.25	2985.15	106.52	100.35
2.	80%	2132.10	750	2882.10	2896.8	105.96	
3.	80%	2194.35	750	2944.35	2933.7	102.58	
1.	100%	2231.25	1500	3731.25	3782.25	103.40	102.34
2	100%	2132.10	1500	3632.10	3652.65	101.37	
3.	100%	2194.35	1500	3694.35	3728.40	102.27	
1.	120%	2231.25	2250	4481.25	4476.90	99.80	99.98
2.	120%	2132.10	2250	4382.10	4387.65	106.91	
3.	120%	2147.55	2250	4397.55	4501.80	104.63	

BCP: Boceprevir

Table 5b: Precision data of boceprevir

S. No.	Conc. (µg/mL)	Intra-day measured area (µg/mL) (*n*=3)	% RSD	Inter-day measured area (µg/mL) (*n*=3)	% RSD
1.		162560		159780	
2.	20	157596	1.86	156684	1.54
3.		163025		161541	
1.		260142		253321	
2.	30	252724	1.56	258120	1.95
3.		259201		248236	
1.		347205		348925	
2.	40	339521	1.23	337675	1.83
3.		340258		338539	

RSD: Relative standard deviation

Table 6: Robustness data of boceprevir

Parameter	% RSD	Tailing factor	Theoretical plate count
Change in flow rate (±0.1mL/min)			
0.15 mL	1.7	1.4	3025
0.35 mL	1.5	1.1	2650
Change in column temperature (25°C±5°C)			
20°C	1.8	1.5	2506
30°C	1.6	1.8	2628
Change in wavelength (206 nm±2 nm)			
204 nm	1.4	1.3	3828
208 nm	1.7	1.5	4200

RSD: Relative standard deviation

After 12 h drug was withdrawn and a prepared solution of 500 µg/mL concentration in methanol. Subsequent dilutions were performed to achieve a final concentration of 30 µg/mL. The final concentration was injected into the system, and degradation was found to be 5.3%. Chromatogram is shown in Figure 10.

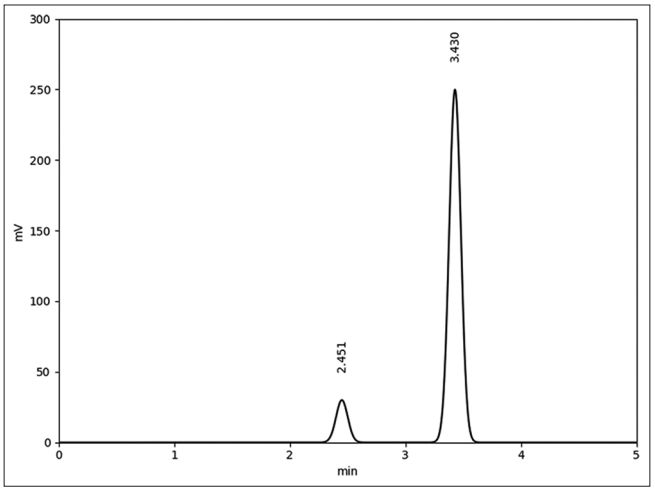

Figure 8: Chromatogram of base degradation with degradation product at R_f2.451

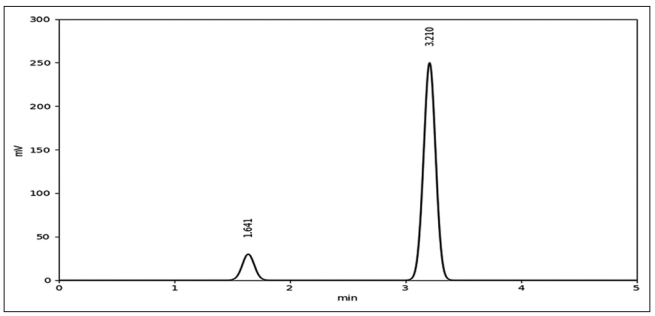

Figure 9: Chromatogram of oxidative degradation with degradation product at R_f1.641

Photolytic degradation

Photolytic degradation studies were performed by exposing the BCP drug to UV radiation up to 200 watts/hour and then subjected to fluorescence light lamination at 1.2 M lux hr. The exposed sample was then accurately weighed and dissolved in methanol to prepare a 500 µg/mL solution. Further dilution was carried out to obtain a final concentration of 30 µg/mL,

Table 7: Data on stress degradation of boceprevir

Degradation condition	Main compound RT (min)/ Degradation product RT	Purity Angle	Purity threshold	%Drug degraded
Acid (0.1N HCL)	3.5/1.1	0.223	0.356	9.8%
Base (0.1N NaOH)	3.4/2.4	0.215	0.349	2.5%
Oxidative (3% H_2O_2, 3% v/v)	3.2/1.6	0.247	0.362	4.2%
Thermal (105°C for 6 h)	3.8/4.1	0.198	0.341	5.3%
Photolytic (UV light for 24 h)	3.7/4.2	0.204	0.346	6.1%

HCl: Hydrogen chloride, NaOH: Sodium hydroxide, H_2O_2: Hydrogen peroxide, UV: Ultraviolet, RT: Retention time

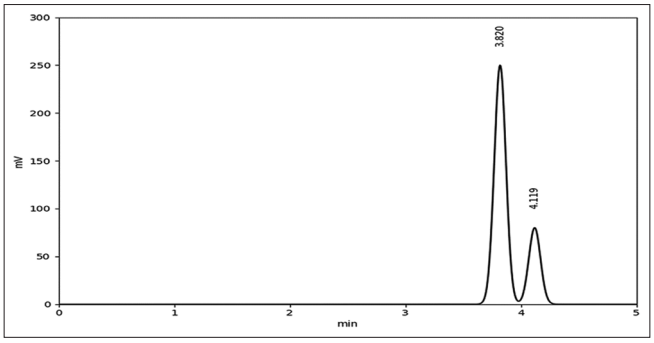

Figure 10: Chromatogram of oxidative degradation with degradation product at R_f4.119

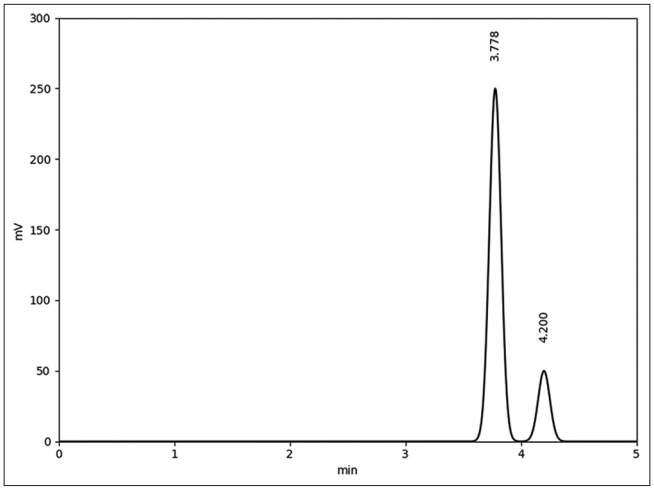

Figure 11: Chromatogram of oxidative degradation with degradation product at R_f4.200

which was injected into the HPLC system. The degradation of BCP samples was found to be 6.1%. The corresponding chromatogram is presented in Figure 11.

CONCLUSION

A stability-indicating RP-HPLC-diode array detector method was developed and validated for the analysis of BCP. Stress degradation studies were conducted under acid, base, oxidative, thermal, and photolytic conditions, and the resulting degradation product exhibited no interference with the analytical peak of BCP. The developed method exhibits high accuracy, with recovery values falling within the acceptable range. Precision was confirmed with RSD value ≤2% ensuring reproducibility. In addition, the method exhibits excellent sensitivity characterized by low LOD and LOQ values. The simplicity of sample preparation, mobile phase composition, and instrumental conditions ensures easy adoption of the developed method in routine laboratory workflows. Collectively, these attributes make the developed method highly suitable for routine quality control and stability assessment of BCP.

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