Research Article

DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING HPLC METHOD FOR DETERMINATION OF BOCEPREVIR

Mrinalini C. Damle *, Sunny R. Salunke

AISSMS College of Pharmacy, Kennedy Road, Near RTO, Pune, Maharashtra, India

*Corresponding Author Email: mcdamle@rediffmail.com

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ABSTRACT

A simple and rapid stability indicating HPLC method for Boceprevir was successfully developed and was validated. This method is based on HPLC separation followed by UV detection at 215 nm. HPLC method was developed on a symmetry Thermo C₁₈ (250 x 4.6 mm i.d, 5 µm) column (sr no. 0860953Q) with a mobile phase consisting of methanol : 10mM di-sodium hydrogen phosphate buffer(pH 2.5):triethylamine 90:10:0.05 % v/v/v, pumped at 1.0 ml min⁻¹ flow rate. The pH of buffer was adjusted to 2.5 with ortho phosphoric acid. The column was maintained at ambient temperature. The eluted compound was detected by using PDA detector. Boceprevir eluted at 3.33± 0.2 min. Stress degradation study shows that sample degraded with acid and base hydrolysis, under oxidation, thermal and photolytic stress conditions. The method was validated in accordance with requirement of ICH guidelines.

Keywords: Boceprevir, HPLC, Forced degradation, Validation.

INTRODUCTION

Boceprevir is a direct acting protease inhibitor for the treatment of hepatitis C¹. Chemical name of boceprevir is 3-[[[(1R,2S,5S) – 3-[(2S) – 2-[(tert-butylcarbamoyl)amino] – 3,3 – dimethylbutanoyl] – 6,6-dimethyl-3-azabicyclo[3.1.0]hexan – 2 – yl]formamido] – 4 – cyclobutyl – 2 – oxobutanamide.¹ Chemical structure of Boceprevir was shown in Figure 1. Boceprevir contains 5 chiral centres, four of them have a fixed stereochemical configuration controlled during the synthesis and the last one is obtained as a mixture of 2 configurations R and S. Thus, Boceprevir is manufactured as an equal mixture of two diastereoisomers in an approximate amount of 1:1.² A thorough literature search indicate that there are some methods reports for estimation of boceprevir by HPLC³ and UPLC-MS/MS^{4,5} methods. There is no Stability-Indicating HPLC Method (SIM) have been reported for Boceprevir. The objective of current work was to develop Stability-Indicating HPLC Method (SIM) as per ICH Q1A (R2) guidelines. It was aimed to establish inherent stability of the Boceprevir through stress studies under a variety of stress conditions and to develop a validated Stability-Indicating Assay method.

MATERIALS AND METHODS

Chemicals and reagents

Working standard of Boceprevir was kindly supplied by Mylan Laboratories Ltd. (Hyderabad) India. Methanol (HPLC grade), sodium hydrogen phosphate, orthophosphoric acid, triethylamine were purchased from Merck specialties Pvt. Ltd. (Mumbai, India).

Instruments

Quantitative HPLC was performed using isocratic high performance liquid chromatography (Jasco HPLC system) with a LC-PU 2080 Plus pump, manual injector with loop volume of 20µl (Rheodyne), programmable MD 2010 PDA detector and HiQsil C₁₈ (150 x 4.6 mm i.d, 5 µm). The HPLC system was equipped with “Borwin- PDA software (version1.5). An electronic balance (Shimadzu AY-120), UV-Visible (Jasco model V-550) spectrophotometer, Elga Lab water (PURELAB UHQ-II) water purification system were used in this study.

Optimized chromatographic conditions

The mobile phase consisting of Methanol: 10mM sodium dihydrogen phosphate buffer (pH 2.5): triethylamine in the ratio of (90:10:0.5) % v/v/v. It was then filtered through 0.45 µm membrane filter paper using vacuum filtration assembly and then sonicated on ultrasonic water bath for 15 min. The flow rate of mobile phase was maintained at 1ml/min and the column and the HPLC systems were kept in ambient temperature.

Preparation of standard Stock Solution

Standard stock solution of Boceprevir was prepared by dissolving 10 mg of drug in 10 ml of methanol to get concentration of 1000 µg/ml. Further dilution was made in methanol to get final concentration range of Boceprevir 50,100,150,200,250 µg/ml.

Preparation of Buffer

Preparation of buffer procedure in IP Dissolve 90 mg of di sodium hydrogen phosphate in 100 ml of HPLC grade water and adjust the pH 2.5 with 10% orthophosphoric acid.

Preparation of 1 N Hydrochloric acid solution

Hydrochloric acid (1N) was prepared by diluting 0.85 ml of concentrated hydrochloric acid solution with methanol up to 10 ml.

Preparation of 0.1 N Sodium hydroxide solution

Sodium hydroxide powder (40mg) was dissolved in methanol in 10 ml volumetric flask the volume was made up with methanol to get 0.1N sodium hydroxide solution.

Preparation of 3 % v/v Hydrogen peroxide solution

Hydrogen peroxide (3 % v/v) was prepared by appropriately diluting 1 ml of 30 % v/v hydrogen peroxide to 10 ml with methanol.

Selection of Detection Wavelength

The UV spectrum of Boceprevir (100µg/ml) solution was obtained over the range of 200-400 nm. It was observed that drug showed considerable absorbance at 215 nm. So, wavelength 215 nm was selected as the wavelength for detection was shown in Figure 2.

Table 1: System suitability parameter

Name	RT (min)	Conc. (µg/ml)	Area	Plates	Asymmetry
Boceprevir	3.33	150	3150023	2650.4	1.36

Table 2: Data of forced degradation studies of Boceprevir

Sr. No	Stress conditions/ duration	% Recovery	Rt of degradation products (min.)	Peak purity	
				Peak front	Peak tail
1.	Acidic hydrolysis / 1 N HCl reflux for 15 min.	72.81	2.7	994.1	996.3
2.	Alkaline hydrolysis /0.1 N NaOH/ kept at room temperature for 1 Hr.	73.41	2.9, 3.7	992.4	994.8
3.	Oxidative /3 % v/v H ₂ O ₂ / kept at room temperature for 3 Hours	86.03	-	996.8	996.5
4.	Neutral hydrolysis /H ₂ O reflux for 1 hr	88.03	-	994.5	995.8
5.	Dry heat/ 80°C/ 12 hours	90.39	-	992.9	995.3
6.	UV light (200 Watt hours/Square meter)	53.48	-	995.7	997.1
7.	Fluorescence light (1.2 million lux hours).	77.18	-	993.6	995.2

Table 3: Specificity

Drug	Purity tail	Purity front
Boceprevir	993.712	996.17

Table 4: Assay of Spiked blend powder

Sr. No.	Peak area of Boceprevir	Amount Recovered (µg/ml)	% assay
1	3122101	153.00	102.00
2	3086274	151.14	100.76
3	3150130	154.46	102.97
4	3052443	149.39	99.59
5	3020148	147.71	98.47
6	3100341	151.87	101.25
Mean	3088572.83	151.26	100.84
SD	46998.97	2.43	1.62
%RSD	1.52170	1.61	1.61

Table 5: Recovery studies of Boceprevir

Drug	Amount taken (µg/ml)	Amount added (µg/ml)	Total Area found	% Recovery	% RSD
Boceprevir	100	80	3697489.33	101.59	1.56
	100	100	3955648.00	98.13	1.36
	100	120	4418772.66	100.13	0.73

*Average of three determinations

Table 6: Intra-day precision study of Boceprevir

Concentration (µg/ml)	Area	Mean Area	SD	% RSD
100	2113417	2103596	9379.61	0.44
	2113417			
	2113417			
150	3140487	3124115	25520.14	0.81
	3094710			
	3137148			
200	3943482	3932470	36765.40	0.93
	3891457			
	3962471			

Table 7: Inter-day precision of Boceprevir

Concentration (µg/ml)	Area	Mean Area	SD	% RSD
100	2134716	2110094.00	32104.98	1.52
	2073782			
	2121784			
150	3064711	3095309.33	26750.70	0.86
	3106947			
	3114270			
200	3968120	3934867.33	39403.62	1.00
	3891346			
	3945136			

Table 8: Robustness study

Drug	% RSD Found For Robustness Study(peak area)					
	Flow Rate (1 ml/min)		pH. of Buffer (pH 2.5)		Mobile phase ratio (90:10)	
	0.9	1.1	2.4	2.6	89:91:0.5	91:09:0.5
Boceprevir	0.61	0.58	0.34	0.73	0.99	1.06

Table 9: Summary of validation parameter

Validation parameters	Boceprevir
Linearity Equation	$y = 19270x + 173614$
(r ²)	$r^2 = 0.9977$
Range	50-250 µg/ml
Precision (% RSD)	
Interday	1.00
Intraday	0.93
Accuracy	% Recovery
80%	101.59
100%	98.13
120%	100.13
Limit of Detection	3.49 µg/ml
Limit of Quantitation	10.59 µg/ml
Specificity	Specific
Robustness	Robust

Figure 1: Chemical structure of Boceprevir

Figure2: UV Spectrum of Boceprevir (100µg/ml)

Figure 3: Chromatogram of standard solution of Boceprevir(RT = 3.33 ± 0.2 min)

Figure 4: Chromatogram obtained from sample subjected to acid degradation with degradation product RT 2.7 min (150µg/ml)

(A) (B)

Figure 5: A) UV spectrum of Boceprevir at RT- 3.33 min., B) UV spectrum of Degradation product at RT- 2.7

Figure 6: Chromatogram obtained from sample subjected to Base degradation for overnight with degradation product RT 2.9 and 3.7 min (150µg/ml)

(A) (B) (C)

Figure 7: A) UV spectrum of Boceprevir at RT- 3.33 min., B) UV spectrum of Degradation product at RT- 2.9 min., C) UV spectrum of Degradation product at RT- 3.7 min.

Figure 8: Chromatogram obtained from base induced degradation product

Figure 9: LCMC spectra of alkali degradation product

Figure 10: Chromatogram obtained from sample subjected to acid degradation

Figure 11: LCMC spectra of acid reflux degradation product

Figure 12: formation of quaternary compound

Figure 13: Chromatogram of linearity of Boceprevir (50-250µg/ml)

Figure 14: Calibration curve for Boceprevir

RESULTS AND DISCUSSION

Selection of mobile phase

The standard solution of Boceprevir (150µg/ml) was injected into the HPLC system and run in different solvent systems. Different mobile phases like acetonitrile and water, methanol and water, acetonitrile and phosphate buffer, methanol and phosphate buffer in varying proportion of mobile phase components, varying conditions of pH were tried in order to obtain the desired system suitability parameters for the Boceprevir. After several trials, methanol, 10 mM disodium hydrogen phosphate pH 2.5 and triethylamine in the ratio of 90:10:0.5 % v/v/v was chosen as the mobile phase, which gave good resolution and acceptable peak parameters.

Chromatogram and system suitability parameter of drug

The column was equilibrated with the mobile phase (indicated by constant back pressure at desired flow rate). Working standard solution of drug 150µg/ml was injected into the system shown in Figure 3. The retention time for the drug was found to be 3.33 ± 0.2 min. System suitability parameters of Boceprevir are summarized in Table 1.

Stress degradation studies of bulk drug

Stress degradation studies were carried under condition of acid, base, neutral hydrolysis, oxidation, dry heat and photolysis as per ICH Q1A (R2) and Q1B. For each study, two samples were prepared: the blank subjected to stress in the same manner as the drug solution and working standard solution of Boceprevir was subjected to stress condition. Dry heat and photolytic degradation were carried out in solid state.⁶⁻⁷

Acid hydrolysis

5 ml working standard solution of Boceprevir (10000 µg/ml) was mixed with 5 ml of 1N methanolic hydrochloric acid (HCl) and 40 ml of methanol. Solution was refluxed for 15 min. The solution was neutralized with 5 ml of Sodium Hydroxide (NaOH) solution. Final solution (150µg/ml) was injected. After acid hydrolysis, 72.81 % Boceprevir was recovered with the product of degradation at RT 2.7 min was shown in Figure 4 and 5.

Base hydrolysis

Initially trials were conducted using 1 N NaOH by keeping the sample solution for 24 hours. It was observed that drug gets degraded with two peak of degradation products RT 2.9 and 3.7 min (150µg/ml) was shown in Figure 6 and 7. Base induced degradation was performed by adding 1 ml of 0.1N sodium hydroxide (NaOH) to volumetric flask containing 1 ml of Boceprevir standard solution (10000 µg/ml). The volume was made up to 10 ml with methanol kept at room temperature for 1 Hour. The solution was neutralized with 0.1 N of Hydrochloric acid (HCl) solution. Final solution (150µg/ml) was injected. After alkaline hydrolysis, 73.41 % Boceprevir was recovered with no peak of degradation.

Neutral Hydrolysis

5 ml working standard solution of Boceprevir (10000 µg/ml) was mixed with 5 ml water and 40 ml of methanol. Solution was refluxed for 15 min. Final solution (150µg/ml) was injected. After neutral hydrolysis, 88.03 % Boceprevir was recovered with no peak of degradation.

Oxidative Hydrolysis

Oxidative degradation was performed by adding 1ml of Hydrogen peroxide (H₂O₂, 3% v/v) to volumetric flask containing 1 ml of Boceprevir standard solution (10000 µg/ml). The volume was made up to 10 ml with methanol & kept at room temperature for 3 Hour.

After oxidative hydrolysis, 86.03% Boceprevir was recovered with no peak of degradation.

Degradation under dry heat

Dry heat study was performed by exposing Boceprevir in oven (80°C) for a period of 12 hours. A sample was withdrawn after 12 hours, weighed and dissolved in methanol to get solution of 1000 µg/ml and further dilutions were made with methanol to get final concentration (150 µg/ml)

After the dry heat degradation, 90.39 % Boceprevir was recovered with no peaks of degradation.

Photo-degradation studies⁸

Photolytic degradation studies were carried out by exposure of drug to UV light up to 200 watt hours /square meter and subsequently to fluorescence light illumination not less than 1.2 million lux hours. Sample was weighed, dissolved in methanol to get concentration of 1000 µg/ml. and further dilutions were made with methanol to get final concentration (150 µg/ml)

After the photo degradation study under UV light 53.48 % and Fluorescence light 77.18 % Boceprevir was recovered with no peak of degradation.

Characterization of degradation product by LCMS

LCMS conditions

For the identification of degradation product during forced degradation study (acid and alkali stress conditions) LC-MS system was used. Column: Thermo scientific-50*2.1 micron size was used as stationary phase. The flow rate was 350 µl/min and the column temperature was maintained at 23°C. Mobile phase contains a mixture of acetonitrile: water (50:50 v/v). The analysis was performed in positive electrospray ionization mode.

Alkali treatment

From LC-MS data the degradation product which was observed in alkali degradation sample shows protonated molecular ion at m/z = 505.33. The possible probable structure of the degradation product (m/z = 505.33) was shown in Figure 8 and 9.

Probable Reaction Mechanism

- A) Cleavage of heterocyclic ring.
- B) alkaline hydrolysis of amide.

Acid treatment

From LC-MS data the degradation product which was observed in acid degradation sample shows protonated molecular ions at m/z = 520.34, 522.33, 521.33. The possible probable structure of the degradation product (m/z = 520.34, 522.33, 521.33.) was shown in Figure 10 and 11

Probable Reaction Mechanism

- A) Tautomerism.
- B) Formation of quaternary compound.

Probable structure was shown in Figure 12

The forced degradation studies data is summarized in Table 2.

Method Validation

The method was validated for various parameter in accordance with ICH guidelines.⁹

Specificity

The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 990, indicating the non-interference of any other peak of degradation product or impurity was shown in Table 3.

Linearity

Linearity was tested for the range of concentrations 50-250 µg/ml. Each sample in five replicates was analyzed and peak areas were recorded. The response factors were plotted against the corresponding concentrations of Boceprevir to obtain the calibration curve. Figure 10 and 11 represents the linearity and calibration curve for Boceprevir respectively.

Range

Boceprevir: The range was found to be 50-250 µg/ml

Assay

Marketed sample was not available in India therefore spiked blend was used. Accurately weighed quantity of Boceprevir was mixed with blank blend (BB) containing starch and lactose. Spiked blend (SB) was assayed and used for accuracy studies. To determine accuracy, 200 mg blank blend and 800 mg drug were mixed properly and finally 1000 mg SB were prepared. Blend equivalent to 10 mg of drug was transferred to 10 mL volumetric flask and was diluted with methanol, sonicated for 10 min and volume made to 10 mL (1000 µg/mL). Further dilution was made in methanol to get final concentration of Boceprevir 150 µg/ml. The results obtained are shown in Table 4.

Accuracy

To check accuracy of the method, recovery studies were carried out by adding standard drug to Blend (Excipients) at three different levels 80%, 100% and 120 %. Basic concentration of sample chosen was 100 µg/ml of standard. These solutions were injected into HPLC system in triplicate to obtain the chromatogram. The drug concentrations were calculated by using linearity equation of Boceprevir. The results obtained are shown in Table 5.

Precision

The precision of the method was demonstrated by intra-day and inter-day studies. In the intra-day studies, 3 replicates of 3 standard solutions (100, 150 and 200 µg/ml) were analyzed in a same day and percentage RSD was calculated (Table 6). For the inter-day variation studies, 9 replicates of 3 standard solutions (100, 150 and 200 µg/ml) were analyzed on 3 consecutive days and percentage RSD was calculated and shown in Table 6 and 7.

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S, respectively; where σ is the standard deviation of the response at lowest concentration in range and S is the slope of the calibration plot. The LOD and LOQ were found to be 3.49 µg/ml and 10.59 µg/ml respectively.

Robustness Studies

Robustness of the method was determined by carrying out the analysis under conditions during which flow rate, Concentration (Strength) of buffer, mobile phase ratio were altered and the effects on the peak area were noted and was shown in Table 8.

Summary of validation parameter

Summary of validation parameter was shown in Table 9.

CONCLUSION

The developed method is stability indicating, since the drug peak was found to be pure as confirmed by peak purity profiling study. This proves that there is no interference of degradation product in analytical peak. The degradation product obtained under hydrolytic conditions are well resolved. The method is specific, accurate, precise, and robust and can be used for routine quality control as well as assessing the stability of Boceprevir.

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