

**DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING
HPTLC METHOD FOR DETERMINATION OF BOCEPREVIR****Mrinalini C. Damle* and Sunny R. Salunke**

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Corresponding Author*Dr. Mrinalini C. Damle**AISSMS College of
Pharmacy, Kennedy Road,
Near RTO, Pune-411001.**ABSTRACT**

A simple, accurate, precise and rapid validated stability indicating HPTLC method of Boceprevir was successfully developed. This method is based on HPTLC separation followed by UV detection at 215 nm. The separation was carried out on merck TLC aluminium sheets precoated with silica gel 60F₂₅₄ using Ethyl Acetate:Toluene (6:4, v/v) as a mobile phase. Boceprevir gave well defined and sharp peak at R_f 0.42 ± 0.02. Calibration curve was linear in range 2000-10000 ng/band. Stress degradation study shows that sample degraded with acid and base hydrolysis, under oxidation, thermal and photolytic stress conditions. The peak purity parameter ensured noninterference

by product of degradation. This method can be applied to determination of stability of Boceprevir. The suitability of this HPTLC method for quantitative determination of Boceprevir was proved by validation in accordance with requirements of ICH guidelines.

KEYWORDS: Boceprevir, HPTLC, Forced degradation, Validation.**INTRODUCTION**

Boceprevir is 3-{[(1R,2S,5S)-3-[(2S)-2-[(tert-butylcarbonyl)amino]-3,3-dimethylbutanoyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexan-2-yl]formamido}-4-cyclobutyl-2-oxobutanamide.

Boceprevir is a direct acting protease inhibitor for the treatment of hepatitis C^[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]

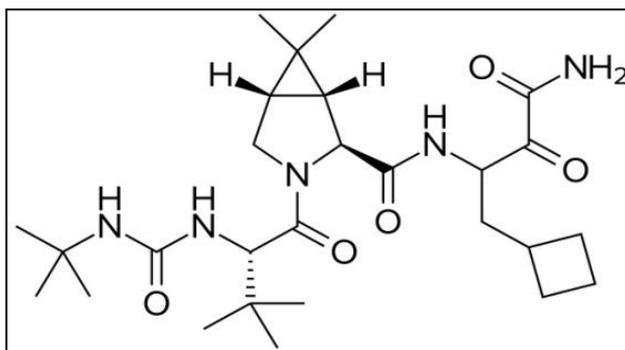


Fig 1: Chemical structure of Boceprevir

A thorough literature search indicate that there are some methods reports for estimation of boceprevir by HPLC^[3] and UPLC-MS/MS^[4,5] methods. There is no Stability-Indicating HPTLC Method (SIM) have been reported for Boceprevir. Hence, considering inherent advantage of HPTLC over HPLC, the objective of current work was to develop Stability-Indicating HPTLC 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. Ethyl Acetate, Toluene and methanol (HPLC grade) were purchased from Merck specialties Pvt. Ltd. (Mumbai, India).

Instrumentation and chromatographic conditions

Precise analytical weighing balance (Shimadzu AY120) was used for weighing. Chromatographic separation of drug was performed using aluminium plate precoated with silica gel 60 F₂₅₄ (10 × 10) with 250 μm thickness (E. MERCK, Darmstadt, Germany) using a CAMAG Linomat 5 sample applicator (Switzerland). Samples were applied on the plate as a band with 6 mm width using Camag 100 μL sample syringe (Hamilton, Switzerland). Thermal degradation study was carried out in hot air oven (Make - Kumar lab).

Linear ascending development was carried out in 10 x 10 cm twin trough glass chamber (CAMAG, Muttensz, Switzerland) by using Ethyl Acetate: Toluene (6:4, v/v) as mobile. The optimized chamber saturation time for mobile phase was 15 min. The length of chromatogram run was 9 cm and development time was approximately 15 min. TLC plates

were dried in a current of air. Densitometric scanning was performed on CAMAG thin layer chromatography scanner at 215 nm operated by WINCATS software version 1.4.2. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 200 to 400 nm.

Preparation of Stock Solution

Standard stock solution of Boceprevir was prepared by dissolving 100 mg of drug in 10 ml of methanol to get concentration of 10000 $\mu\text{g/ml}$. from which 1 ml was further diluted to 10 ml with methanol to get concentration of solution 1000 $\mu\text{g/ml}$

Selection of Detection Wavelength

The UV spectrum of Boceprevir (100 $\mu\text{g/ml}$) 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.

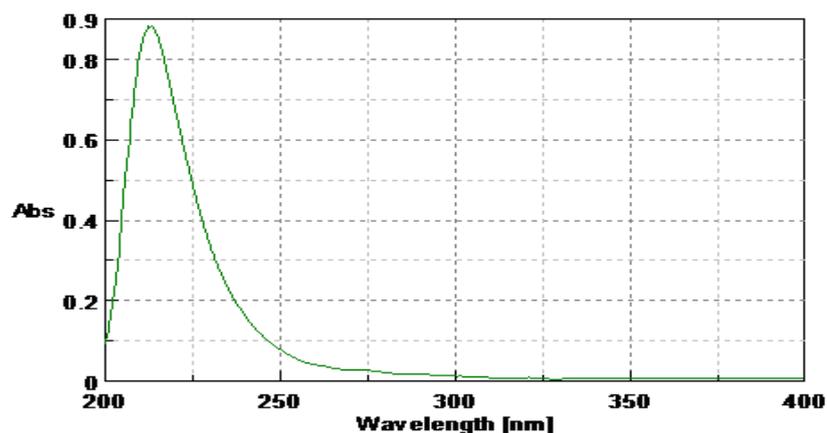


Fig. 2: UV Spectrum of Boceprevir (100 $\mu\text{g/ml}$)

Stress degradation studies of bulk drug

The forced degradation studies were carried out on bulk drug substance in order to prove the stability-indicating property and selectivity of the developed method. The degradation was carried out under acid, base and neutral hydrolytic, oxidative, thermolytic and photolytic conditions.^[6,7]

Acid treatment

5 ml working standard solution of Boceprevir (10000 $\mu\text{g/ml}$) was mixed with 5 ml of 1N methanolic hydrochloric acid (HCl) and 40 ml of methanol. Solution was refluxed for 15 min.

The 6 µl of resulting solution was applied on TLC plate and developed under optimized chromatographic condition.

Alkali treatment

1 ml working standard solution of Boceprevir (10000 µg/ml) was mixed with 1 ml of 0.1 N methanolic sodium hydroxide (NaOH) and 8 ml of methanol. Solution was kept at room temperature for 1 hour. The 6 µl of resulting solution was applied on TLC plate and developed under optimized chromatographic condition.

Neutral Hydrolysis

5 ml working standard solution of Boceprevir (10000µg/ml) was mixed with 5 ml of water and 40 ml of methanol. Solution was refluxed for 1 hour. The 6 µl of resulting solution was applied on TLC plate and developed under optimized chromatographic condition.

Oxidative degradation

1 ml working standard solution of Boceprevir (10000µg/ml) was mixed with 1 ml of 3% v/v methanolic Hydrogen peroxide (H₂O₂) and 8 ml of methanol. Solution was kept at room temperature for 1 hour. The 6 µl of resulting solution was applied on TLC plate and developed under optimized chromatographic condition.

Degradation under dry heat

Dry heat study was performed by keeping drug in oven at 80°C for period of 12 hours. A sample was withdrawn at appropriate times, weighed and dissolved in methanol to get solution of 1000 µg/ml. 6 µl of the resulting solution was applied to HPTLC.

Degradation under Photolytic conditions

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. 6 µl of the resulting solution was applied to HPTLC.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

The primary objective in developing this stability indicating HPTLC method is to achieve the resolution of Boceprevir and its degradation products. Representative densitogram of standard solution of Boceprevir is shown in figure: 3

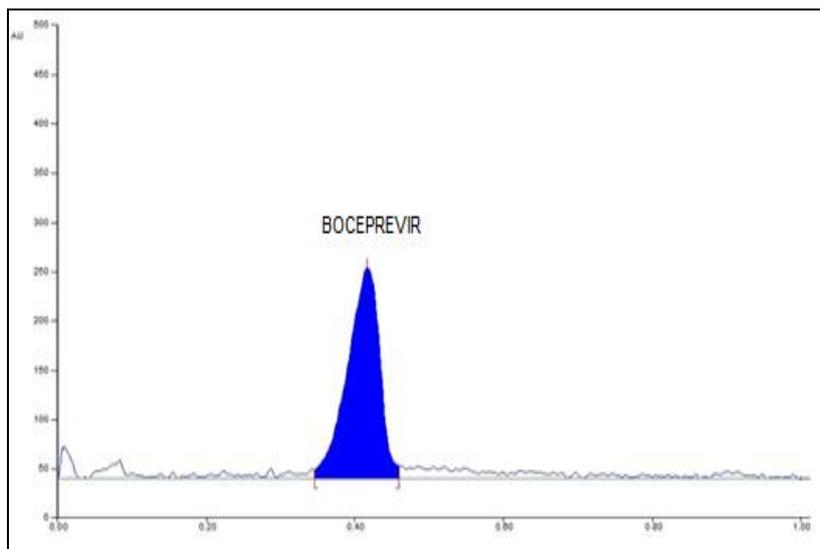


Fig. 3: Representative densitogram of standard solution of Boceprevir (6000ng/band, $R_f = 0.42 \pm 0.02$)

Results of forced degradation studies

Forced degradation study showed that the method is highly specific and there was no interference of degradation products observed at retention factor of drug.

Acid treatment

In Acid hydrolysis condition, 28.90 % degradation of Boceprevir was observed with the product of degradation at $R_f = 0.04 \pm 0.02$. The representative densitogram obtained from acid treatment after refluxed for 15 min. figure 4 respectively.

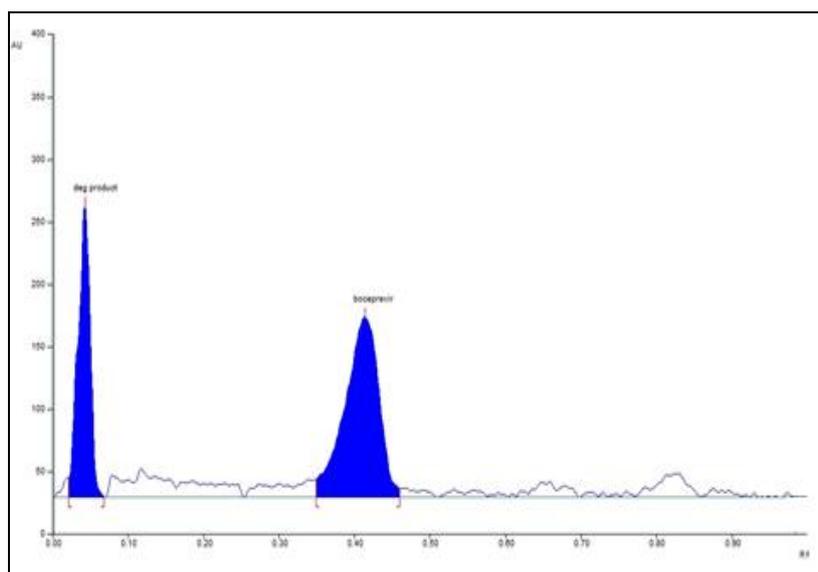


Fig. 4: Densitogram obtained from sample subjected to acid degradation with degradation product at $R_f 0.04$

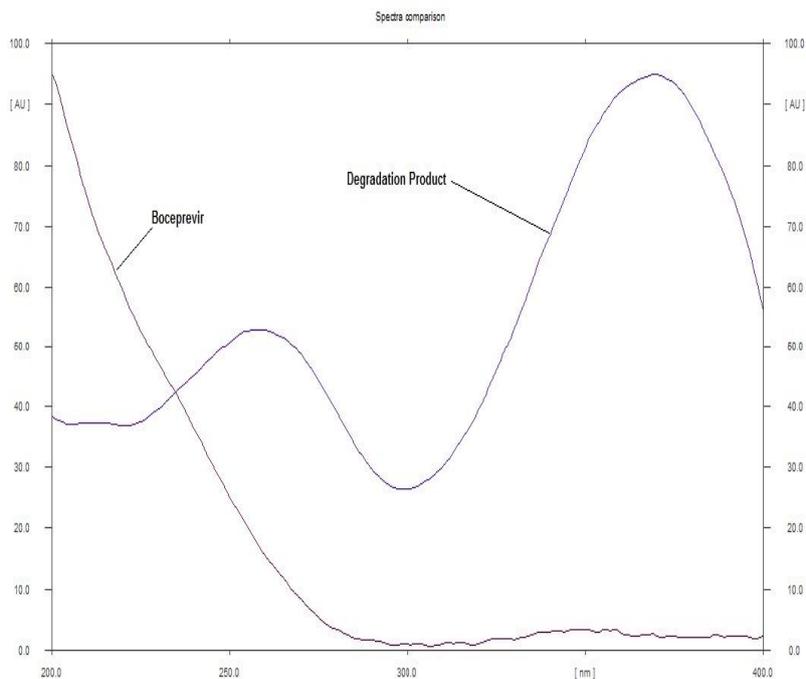


Fig. 5: Overlay of UV spectra of boceprevir drug and degradation product

Alkali treatment

In Acid hydrolysis condition, 28.90 % degradation of Boceprevir was observed with the product of degradation at $R_f = 0.25, 0.41$ and 0.59 ± 0.02 . The representative densitogram obtained from alkali treatment after 12 hours. figure 5 respectively.

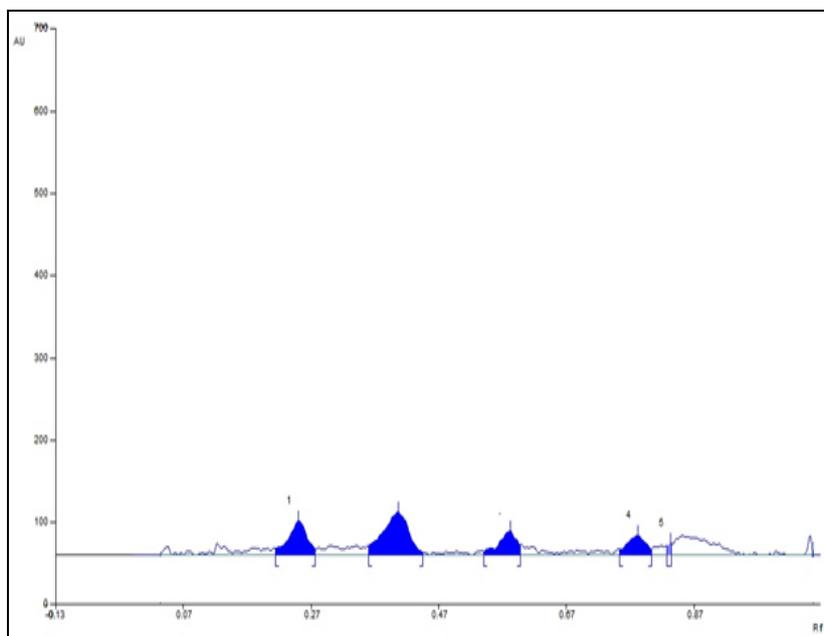


Fig. 6: Densitogram obtained from sample subjected to alkali degradation with degradation product at $R_f 0.25, 0.59, 0.78$

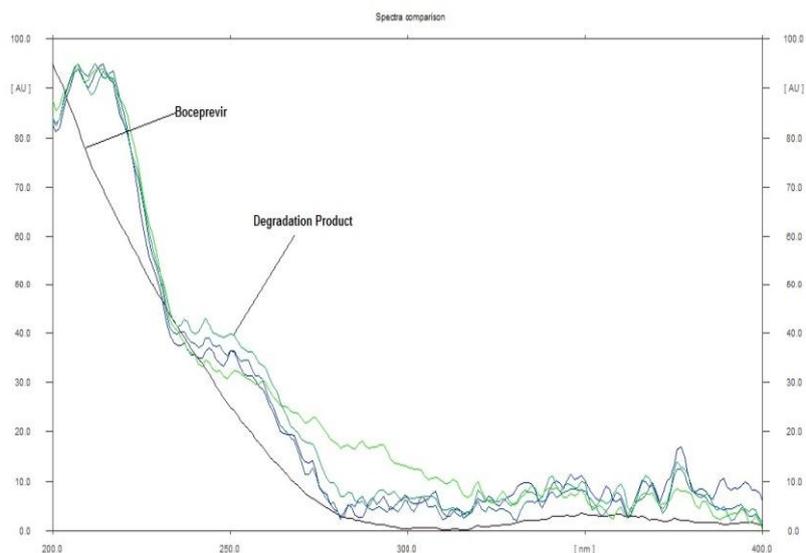


Fig. 7: Overlay of UV spectra of boceprevir drug and degradation product Oxidative degradation

Oxidative Degradation

Boceprevir when treated with 3% v/v H₂O₂ i.e. oxidative degradation, 15.78 % degradation was observed.

Neutral Hydrolysis

In neutral hydrolysis condition, 9.05 % degradation of Boceprevir was observed.

Dry heat degradation studies:

When the drug substance was exposed to dry heat at 80° C for 12 hrs.10.28% of degradation was observed.

Photodegradation Studies

Boceprevir exhibited 50.0 % of degradation, when exposed to ultraviolet light (200 Watt hours/Sequare meter) and 24.2 % of degradation when exposed to fluorescence light (1.2 million lux hours).

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 micro litter/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.

CHARACTERIZATION OF DEGRADATION PRODUCT BY LCMS**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 fig. 9.

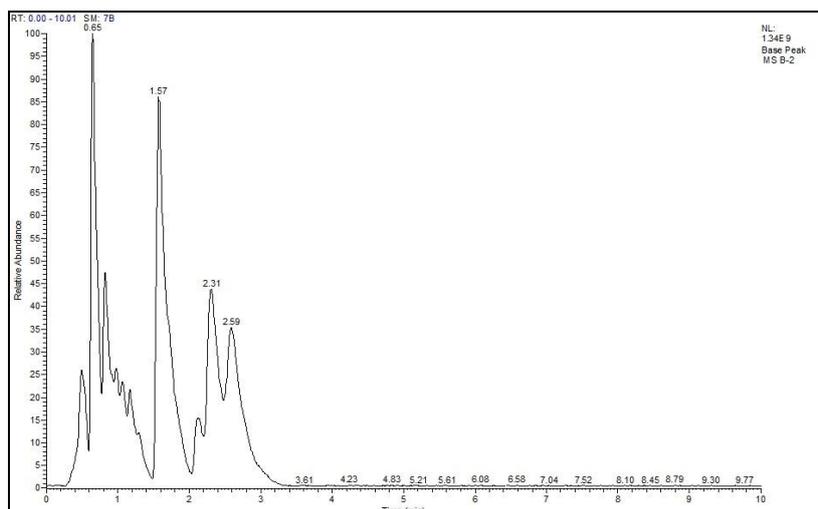


Fig. 8: Chromatogram obtained from base induced degradation product

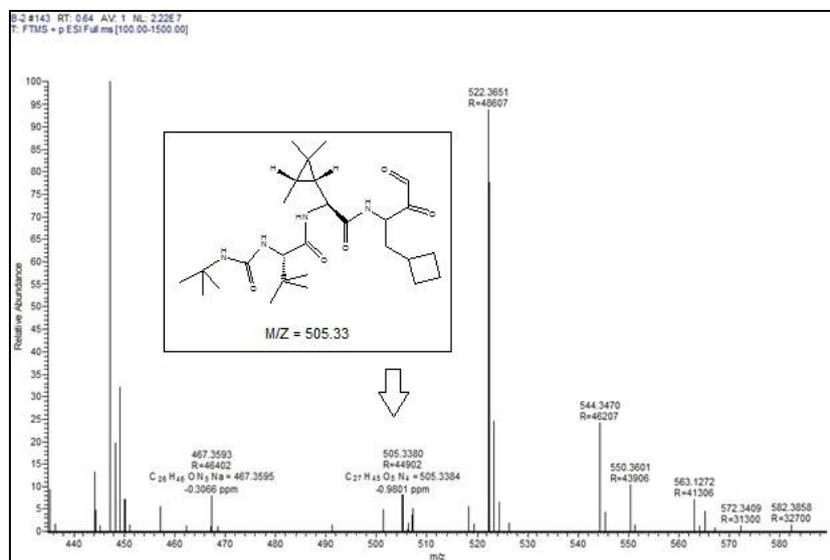


Fig. 9: LCMC spectra of alkali degradation product

Probable Reaction Mechanism**A) Cleavage of ring**

Cleavage of herocyclic 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 fig. 11. And 12.

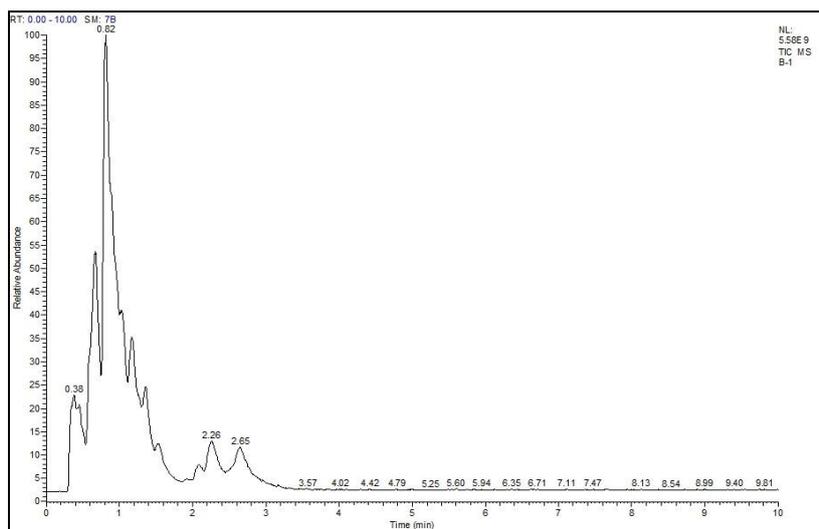


Fig. 10: Chromatogram obtained from sample subjected to acid degradation

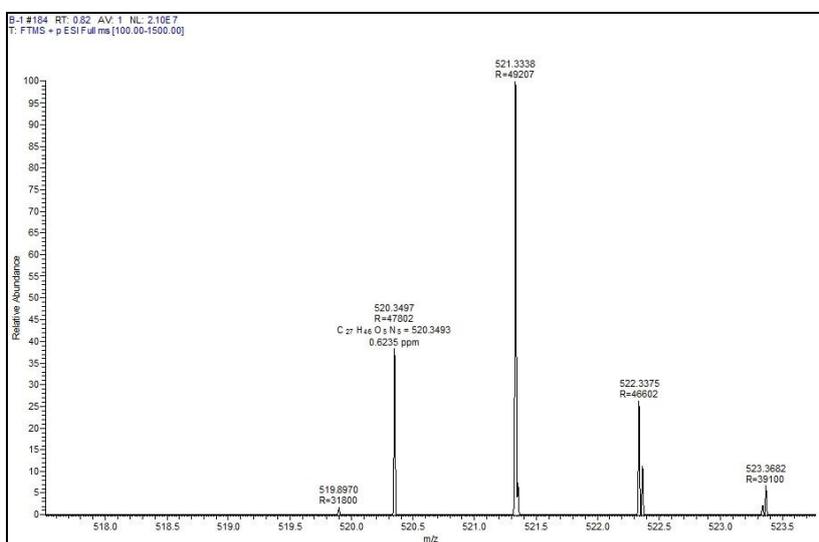


Fig. 11: LCMC spectra of acid reflux degradation product

Probable Reaction Mechanism**A) Tautomerism****B) Formation of quaternary compound**

was shown in fig. 12

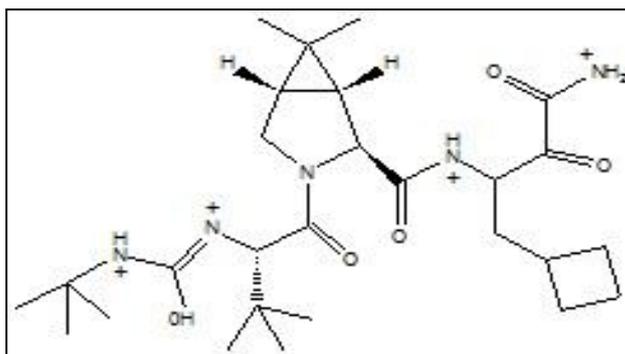


Fig. 12: formation of quaternary compound

The forced degradation studies data is summarized in Table 1.

Table 1: Data of forced degradation studies of Boceprevir

Stress conditions/ duration	% Recovery	% Degradation	Rf values of degraded Products
Acidic hydrolysis / 1 N HCl reflux for 15 min.	71.09	28.90 %	0.04
Alkaline hydrolysis /0.1 N NaOH/ kept at room tempeture for 1 Hr.	75.18	24.82 %	0.25,0.59,78
Oxidative /3 % v/v H ₂ O ₂ / kept at room tempeture for 3 Hr.	84.22	15.78 %	-
Neutral hydrolysis /H ₂ O reflux for 1 hr	90.95	9.05 %	-
Dry heat/ 80°C/ 12 hours	89.72	10.28 %	-
UV light (200 Watt hours/Sequire meter)	50.0	50	-
Fluorescence light (1.2 million lux hours).	75.8	24.2	-

Validation of the method

The method was validated for various parameter in accordance with ICH guidelines.^[8]

1. Specificity

The developed method was specific for analyte. The specificity of the method was ascertained by peak purity profiling studies. The peak purity values were found to be more than 0.9996, indicating the no interference of any other peak of degradation product.

2. 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). Solution was filtered 4 µl volumes was applied on plate

Table 2: Assay of Spiked blend powder

Sr. No.	Peak area Of Boceprevir	Amount Recovered (ng/band)	% assay
1	4985.23	3942.01	98.55
2	5012.1	3968.75	99.21
3	5002.14	3958.84	98.97
4	5100	4056.22	101.4
5	4900	3857.19	96.42
6	5021.11	3977.71	99.44
Mean	5003.43	3960.12	99.003
SD	64.41	64.09	1.602
%RSD	1.2873	1.6185	1.6185

3. Linearity and range

The standard stock solutions of Boceprevir (1000µg/ml) were applied by spotting on TLC plate in range of 2, 4, 6, 8 and 10 µl. Straight-line calibration graphs were obtained $y = 1.0049x + 1023.7$ in the concentration range 2000-10000 ng/band with high correlation coefficient > 0.99 .

4. Accuracy

To check accuracy of the method, recovery studies were carried out by adding standard drug to sample at three different levels 80, 100 and 120 %. Basic concentration of sample was 4000 ng/band from SB. The drug concentrations were calculated from respective linearity equation. The results of the recovery studies indicated that the method is accurate. The results obtained are shown in Table 2.

Table 3: Recovery studies of Boceprevir

Drug	Amount taken (ng/ band)	Amount added (ng/ band)	Total Area found	% Recovery	% RSD
Boceprevir	4000	3200	8239.46	99.72%	1.01
	4000	4000	9072.70	100.11%	0.39
	4000	4800	9947.86	100.91%	0.45

*Average of three determinations

5. Precision

A set of three different concentrations in three replicates of standard solutions of Boceprevir were prepared. All the solutions were analyzed on the same day in order to record any intraday variations in the results. Intra-day variation, as RSD (%), was found to be in the range of 0.35 to 1.23. For Inter day variation study, three different concentrations of the standard solutions in linearity range were analyzed on three consecutive days. Interday variation, as RSD (%) was found to be in the range of 1.24 to 1.48. The lower values of % R.S.D. (< 2) indicated that method was found to be precise.

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

LOD and LOQ were calculated as $3.3 \sigma/S$ and $10 \sigma/S$, 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 157.76 ng/band and 478.07 ng/band respectively.

7. Robustness Studies

Robustness of the method was determined by carrying out the analysis under conditions during which mobile phase composition, chamber saturation time was altered and the effect on the area of drug was noted. Robustness of the method checked after deliberate alterations of the analytical parameters showed that areas of peaks of interest remained unaffected by small changes of the operational parameters (% R.S.D. < 2). The results are given in Table 3.

Table 4: Robustness Data in Terms of Peak Area (% RSD)

Sr. No.	Parameter	(% RSD)
1	Mobile phase saturation time 10 min and 20 min	1.51
2	Mobile phase variation chloroform 9.8 and 10 ml	1.47

*Average of three determinations

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 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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