A validated reverse phase stability-indicating HPLC method for bortezomib in the presence of degradation products and its process-related impurities

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Abstract

Degradation pathway of bortezomib was established as per ICH recommendations in terms of validated and stability indicating reverse phase liquid chromatographic method. Bortezomib was subjected to stress using conditions of acid, base, oxidation, thermal and photolysis. Significant degradation was observed in acid and base stress conditions. Ten impurities were studied and the major degradant was hydroxyamide impurity. The stress samples were assayed against a qualified reference standard and the mass balance is found close to 98.2%. Efficient chromatographic separation was achieved on a Zorbax Extend C18(100 x 4.6 mm, 1.8 µm) stationary phase with simple mobile phase combination In the developed LC method, the resolution between bortezomib and ten potential impurities such as Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G, bortezomib isomer, hydroxyamide and bortezomib ester) was found to be greater than 2.0. Regression analysis showed r value (correlation coefficient) of greater than 0.999 for bortezomib and ten potential impurities. This method was capable of detecting the impurities of bortezomib at a level of 0.02% with respect to test concentration of 2.0 mg/mL. The developed rapid LC method was validated with respect to specificity, linearity, range, accuracy, precision and robustness for impurities.

Keywords: Bortezomib, Analytical Method development, Analytical Method validation and High Performance Liquid Chromatography.

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Introduction

Bortezomib, is chemically (R)-3-methyl-1-((S)-3-phenyl-2- (pyrazine-2-carboxamido) propanamido butyl boronic acid, belongs to new class of drugs, contains a boronic acid moiety. It is effective against different types of tumours, but mainly used for the treatment of multiple myeloma[1], which accounts for 10% of all blood system malignancies[1]. Bortezomib is one of the first therapeutic proteasome inhibitor evaluated in humans by administering into intravenous bolus[4-5]. It is a peptidomimetic compound consist of modified leucine-phenylalanine dipeptide with boronic acid at the C-terminal region. It is able to interact with proteasome, an intracellular apparatus, which breaks down damaged or unfolded proteins and inhibiting[2]. The photolytic action a few chromatographic methods bortezomib is reported to quantified by including SPE–LC-MS/MS, human urine and a LC–tandem mass spectrometric assay in human plasma,[8] RP-HPLC method for analysis of bortezomib in a pharmaceutical dosage forms and Ultra-Fast LC[6-7], method in Pharmaceutical dosage form samples in the presence of degradation products and potential impurities. The present study is aim to develop a single stability-indicating LC method[3], for the determination of bortezomib and its related impurities. The developed LC method is validated with respect to specificity, LOD, LOQ, linearity, precision, accuracy and robustness.The present study also aimed to establish degradation pathway of bortezomib through stress studies under a variety of ICH recommended test conditions.

Experimental

Chemicals: The bortezomib and its related impurities such as Imp-A, B, C, D, E, F and G, bortezomib isomer, hydroxyamide and bortezomib ester used in the present study were of > 99% purity and obtained from Terra Scientific Pvt. Ltd, Hyderabad and India (Fig. 1). Acetonitrile (ACN), tetrahydrofuran, formic acid, dimethyldichlorsilane and Ammonium formate were HPLC grade and purchased from Merck (Darmstadt, Germany). All other chemicals used in the present were analytical grade and obtained from commercial source.

Equipment's: The LC system was used for the method development, forced degradation studies and method validation. The system consists of HPLC 2695 model binary pump equipped with an auto sampler and a photo Diode array detector (Waters, USA). The output signal was monitored and processed using Empower software (Pro version 2) view sonic computer. Photo stability studies were carried out using photostability chamber (New tronic life care, India). Thermal stability studies were carried out using dry hot air oven (Newtronic life care, India.

Chromatographic conditions: The chromatographic Zorbax Extend C18column with(100 mm long x 4.6 mm diameter, 1.8 µm particle size), was used with a mobile phase A containing 85% (10mM) ammonium formate, 15% tetrahydrofuran and 1% acetonitrile and mobile phase B containing of 11% water, 14% tetrahydrofuran and 75% acetonitrile. The flow rate was fixed to 0.9 mL/min, column temperature was maintained at 35°C, detection was set at 271 nm. The injection volume was 10µL, the acetonitrile (0.1%) in water was used as diluent, sampler cooling temperature was set at 10°C and run time was 35 min. The gradient program was fixed as shown in the Table 1.

Table 1: Gradient Program

Time in Minutes	Mobile phase A (%)	Mobile phase B (%)
0.00	98	2
10.00	61.0	39.0
23.00	30.0	70
24.00	0.0	100.0
25.00	0.0	100.0
26.00	98	2
35.00	98	2

Table 2 Preparation of bortezomib Impurities mixture

S. No.	Name of the impurity	Nature of Impurity	Concentration in PPM
1	Impurity A	degradation product	2.5
2	Impurity B	degradation product	2.5
3	Impurity C	degradation product	2.5
4	Bortezomib isomer	Isomer	2.5
5	Hydroxyamide	degradation product	2.5
6	Impurity D	degradation product	2.5
7	Impurity E	degradation product	2.5
8	Impurity F	degradation product	2.5
9	Impurity G	degradation product	2.5
10	Bortezomib ester	Intermediate	2.5

Table 3: Degradation condition of stress studies

S. No.	Stress agent	Concentrations of solution (PPM)	Temperature (°C)	Time (h)
1	1 mL Con. HCL	650	35	1
2	0.5 mL of 24% NH4OH	650	35	1
3	100uL of 3% H2O2	650	35	1
4	100 W Hg-lamp (~ 1m distance)	650	35	1
5	Heating in normal atmosphere	650	60	24
6	100 W Hg-lamp (~ 1m distance)	1000	35	72
7	Heating in normal atmosphere	1000	100	5

Preparation of standard solutions and sample solutions: The resolution test bortezomib standard solution (mg/ml) was prepared by dissolving bortezomib standard in ACN using ultrasonic bath. The sensitivity test solution was prepared bydiluting7.5 µL of resolution test solution to 25mL with acetonitrile. Unless otherwise stated freshly prepared solution was used. The commercially obtained bortezomib sample was reconstituted with 10 mL water and diluted with acetonitrile to get final concentration of 1 mg/mL. The solution was then filtered through a 0.45µm nylon disk membrane filter.

Stress studies/ Specificity: The stress studies/specificity was used to evaluate the ability of the method to resolve possible substance, impurities and degradation products of bortezomib and other impurities. The impurity samples and degradation products were spiked at a concentration of 2.5 ppm in the bortezomib sample. Forced degradation studies were also carry out on bortezomib to infer stability-indicating property and specificity of the validating method. The stress conditions used for the degradation study are light(ICH Q1B), heat (105°C), acid hydrolysis (0.2 M HCl), base hydrolysis (0.2 M NaOH) and oxidation (3% Hydrogen peroxide). The samples were exposed for 24 h, heat and light studies whereas samples were treated for 2 h for acid and base hydrolysis and also for oxidation. The peak purity of the bortezomib stressed samples were also validated using a Waters photo diode array detector (PDA).The

purity angle was set within the purity threshold limit for all of the stressed samples and contents of impurities were calculated for the stress samples against a qualified reference standard. The mass balance[9-11], (% assay + % of impurities + % of degradation products) was calculated for all of the samples.

Method validation

Precision: The method was validated as per ICH recommendations. The system precision was investigated by injecting six individual preparations (2.5µg/ml) of bortezomib spiked with 0.03% each of impurity A, B, C, D, E, F and G, bortezomib isomer, hydroxyamide and bortezomib ester. Then, % RSD of the areas of impurity C, bortezomib isomer and hydroxyamide was calculated. The intermediate precision of the method was confirmed by different analysts and instruments. The precision of the method was evaluated by the analysis out six independent analysis of a test samples of bortezomib against a qualified reference standard. The % RSD of six independent test values were calculated.

Limit of detection (LOD) and limit of quantification (LOQ): The LOD and LOQ for impurity A, B, C, D, E, F and G, bortezomib isomer, hydroxyamide, bortezomib and bortezomib ester were evaluated at a of 3:1 and 10:1, signal-to noise ratio by injecting a series of dilute solutions with known concentrations. The precision study was also performed at the LOQ level by injecting six individual samples and calculated the %RSD of the areas.

Linearity: Linearity test solutions were prepared from a stock solution at six concentration levels from 50 to 150% of the analyte at concentration for the related substance method. The peak area versus concentration details analyzed with least-squares linear regression. The Linearity test solutions were prepared by diluting the impurity stock solution to the required concentrations for the related substance method. The solutions were diluted at six different concentration levels from the LOQ to 150%. The slope and y-intercept of the calibration curve were observed. The peak area versus concentration data was analyzed using least squares linear regression. The linearity test solutions were prepared by diluting the impurity stock solution to the required concentrations for the related substance method. The slope and y-intercept of the calibration curve were reported.

Accuracy: The accuracy of the related substance method was evaluated in triplicate at three concentration levels, 50, 100 and 150% and the percent recovery was also calculated. The impurities A, B, C, D, E, F and G, Bortezomib isomer, hydroxyamide, bortezomib ester and bortezomib were spiked into bortizomib and recovery experiments were performed to determine the accuracy of the related substance method for quantification of impurities. The study was carried out in triplicate at 0.2, 0.4 and 0.6% of the analyte concentration (2.0 >g/ml). The percent of recovery for Impurities A, B, C, D, E, F and G, Bortezomib isomer, hydroxyamide, bortezomib ester and bortezomib were calculated.

Robustness: The robustness of the developed method of evaluated at different experimental conditions such as flow rate, pH and column temperature. The flow rate of the mobile phase was maintained 0.9 ml/min. To study the effect of the flow rate on the resolution, the flow rate was changed by 0.1 units from 0.8 and 1.0 ml/min. The effect of pH on the resolution of the impurities was studied by varying the pH by ± 0.2 units from buffer pH 2.8 and 3.2. The effect of the column temperature on the resolution was studied from 30-40°C. In all these varied conditions, the components of the mobile phase was remain constant during this study.

Solution stability and Mobile phase stability: The stability of bortezomib solution in the proposed method was carried out by placing the both sample and reference standard solutions in a tightly capped volumetric flasks at room temperature for 6 h. The same sample solutions were assayed for in a 6 h interval during the study period. The mobile phase stability was evaluated by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions for 6 h intervals. The prepared mobile phase remained constant during the study period. The % RSD of the bortezomib impurities was calculated for the mobile phase as well as solution stability experiments. The amount of bortezomib and impurity A, B, C, D, E, F and G, bortezomib isomer, hydroxyamide and bortezomib ester was determined up to the study period at 6 h intervals. The stability of mobile phase was evaluated for48 h by injecting the freshly prepared sample solutions at every 6 h interval. The content of bortezomib and impurity A, B, C, D, E, F and G, bortezomib isomer, hydroxyamide and bortezomib ester was determined in the test solutions. The prepared mobile phase remained same during the study period.

Results and Discussion

Method development and optimization: The present study aimed to separate bortezomib and its impurities from the analyte peak during stress studies. As a preliminary study using chromatographic method Impurities and degradation products were co-eluted by using Inertsil ODS (250 mm x 4.6 mm x 4.5µm) with different mobile phases such as phosphate, sulphate and acetate buffers (pH 4.5) in a pump A and organic modifiers including acetonitrile and methanol in a pump B. This leads to merging of peak shapes. Then resolution between the impurities and bortezomib was improved using Zorbax Extend C18, 100 x 4.6 mm, 1.8 µm as a stationary phase with the reduced concentration of acetonitrile in mobile phase. The results show that there is no resolution between the impurities and analyte was data not shown. To further optimize the resolution and retention time of impurities, trails were

carried out with different mobile phase ratios using buffer and acetonitrile. The results indicate that the isocratic trials were not successful in achieving a favorable resolution between the impurity and bortezomib peaks and the elution of the process impurities. Therefore, a gradient method was selected using buffer and acetonitrile as mobile phase A and B. Different gradient programs were investigated and satisfactory results were obtained with a gradient program of 0.00/100, 10/61, 23/29, 24/0, 25/0, 26/100 and 31/100 (time (min) / %B) with a stationery phase Zorbax Extend C18, 100 x 4.6 mm, 1.8µm diameter at a flow rate of 0.9 ml/min. The results indicating that column temperature was found to be 30°C and the detection wavelength was maximum at 271 nm with an injection volume injection volume of 10 µL for successful resolution (Fig. 2 (b))

Method validation

Precision: Then the method was validated in terms of precision, linearity, accuracy, robustness, stability and forced degradation studies as per ICH guidelines[12-16]. The % RSD of bortezomib retention and area was set at ≤ 5% (Table 4). The system precision average of bortezomib retention time was observed at 8.8 min and % RSD was 0.11. However, the system precision average of bortezomib area was noticed at absorbance of 4762034 and % RSD was 0.87. The system precision retention time of the impurities in terms of % RSD was 0.27, 0.63, 0.10 and 0.87 for impurity C, bortezomib isomer, hydroxyamide and bortezomib, respectively (Table 5). The method precision study was carried out within 10.0 % RSD. The results show that % RSD of impurity C was 6.28%, bortezomib isomer was 2.66% and hydroxyamide was 9.34% (Table 6). Batch analysis of bortezomib samples were carried (Table 7) and the results were expressed in % w/w. The results demonstrate that % w/w of batch 1, 2 and 3 was 0.0260, 0.0243 and 0.0252, respectively for Impurity C; 0.0621, 0.0600 and 0.0590 respectively for bortezomib isomer; 0.0413, 0.0351 and 0.0406 respectively for hydroxyamide and 99.8, 99.8 and 99.8 respectively for bortezomib.

Table 4: System precision of bortezomb

S. No.	Bortezomib Retention Time	Bortezomib areas
1	8.77	4775402
2	8.78	4680395
3	8.78	4770777
4	8.78	4766702
5	8.79	4799746
6	8.78	4779182
Average	8.8	4762034
STDEV	0.01	41612.76
%RSD	0.11	0.87

Table 5: System precision retention times

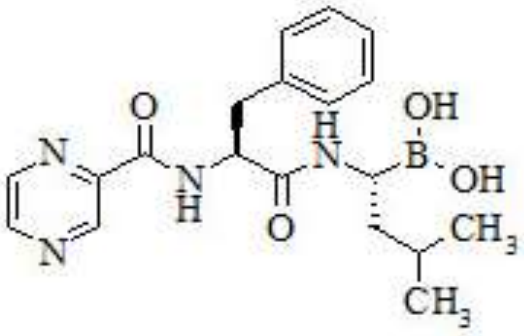
Number of injections	Retention Time		
	Retention of Imp. C (min)	Retention of BTZ isomer (min)	Retention of hydroxyamide (min)
1	7.43	9.5	9.85
2	7.41	9.52	9.86
3	7.44	9.6	9.87
4	7.45	9.64	9.86
5	7.43	9.61	9.87
6	7.41	9.62	9.87
Average	7.4	9.6	9.9
STDEV	0.02	0.06	0.01
% RSD	0.27	0.63	0.1

Table 6: Method precision Results of Impurity C, bortezomib Isomer and hydroxyamide

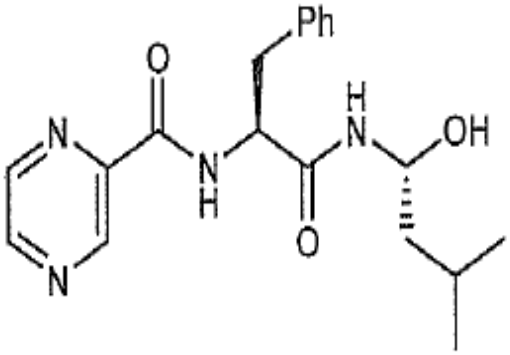
Sample No.	% of Impurity		
	% of Impurity C	% of BTZ isomer	% of hydroxyamide
1	0.025	0.0621	0.0413
2	0.0243	0.0602	0.0394
3	0.022	0.0600	0.0351
4	0.0247	0.0615	0.0359
5	0.0219	0.0578	0.0452
6	0.0252	0.059	0.0406
Average	0.0239	0.0601	0.0396
STDEV	0.0015	0.0016	0.0037
% RSD	6.28	2.66	9.34

Table 7: Batch results of bortezomib

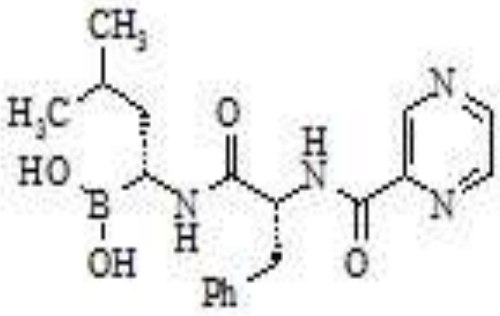
B. No.	Batch Analysis			
	Imp. C	BTZ isomer	hydroxyamide	Bortezomib
BTZ-001	0.026	0.0621	0.0413	99.8
BTZ-002	0.0243	0.06	0.0351	99.8
BTZ-003	0.0252	0.059	0.0406	99.8



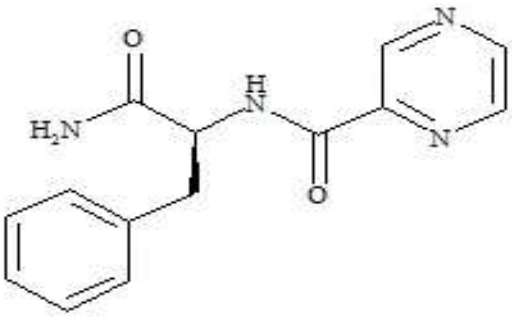
a: Bortezomib
Molecular weight 384.2



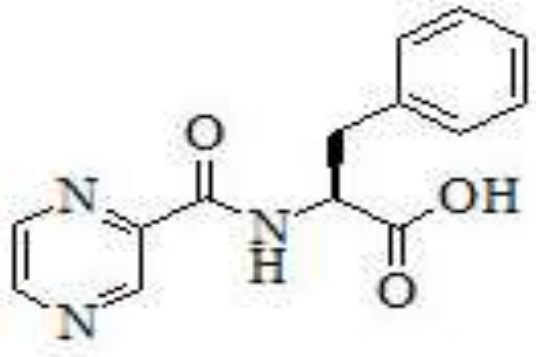
c: Hydroxyamide
Molecular weight 270.29



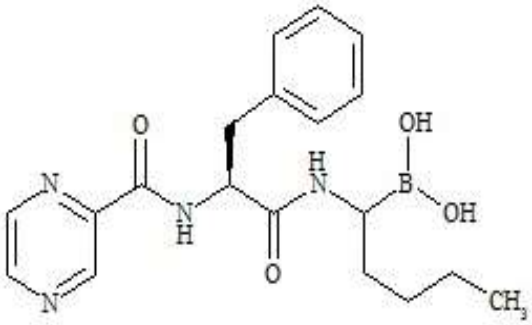
b: Bortezomib isomer
Molecular weight 384.2



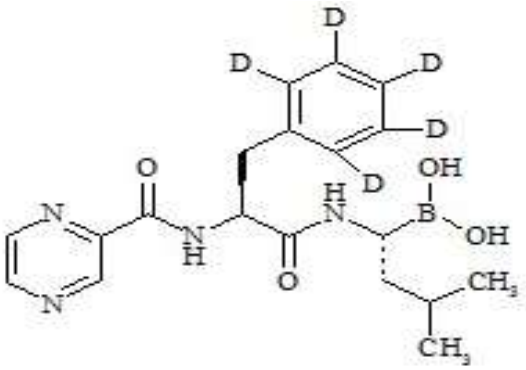
d: Impurity A
Molecular weight 270.29



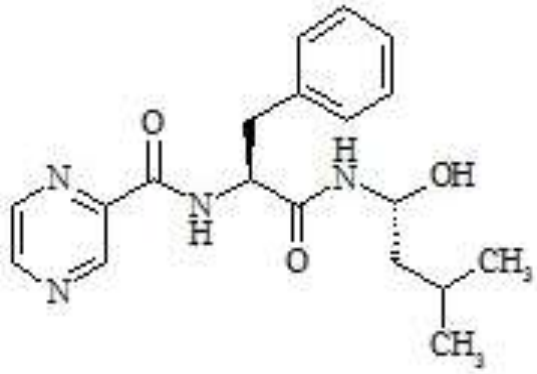
e: Impurity B
Molecular weight: 271.28



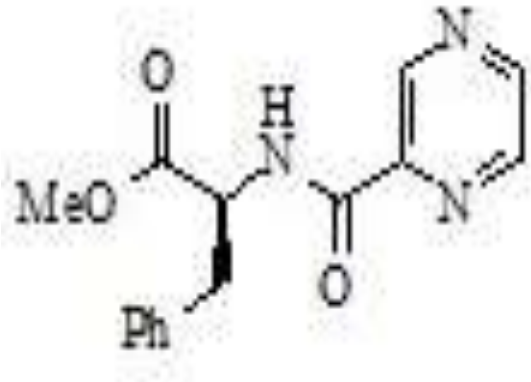
f: Impurity C
Molecular weight: 384.25



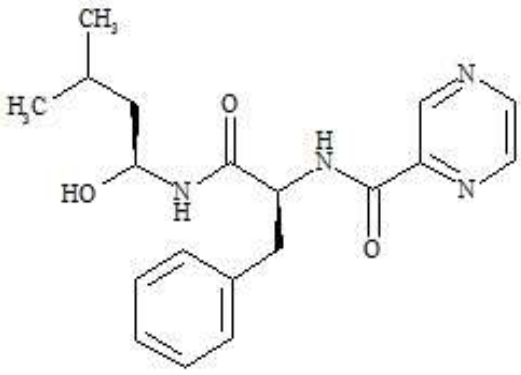
g: Impurity D
Molecular weight: 389.28



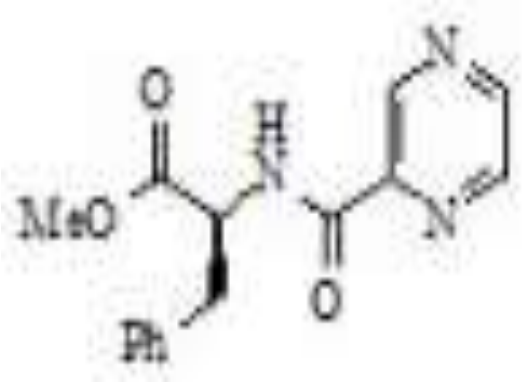
h: Impurity E
Molecular weight: 356.43



i: Impurity F
Molecular weight: 285.31



j: Impurity G
Molecular weight: 356.43



k: Bortezomib ester
Molecular weight: 271.23

Fig. 1: Chemical structure of Bortezomib and its impurities

Linearity: The linear calibration plot of the method was obtained in the tested calibration range of 50-150% level and the correlation coefficient obtained was > 0.999. These results indicating significant correlation between the peak areas and analyte concentration. The linear calibration plot for the related substance was determined in the calibration range for Bortezomib and Impurities was 150% with respective to LOQ, a correlation coefficient of greater than 0.999 was obtained. In this range the linearity was checked for the related substance at same concentration range for three consecutive days. The %RSD values of the slope and y-intercept of the calibration curves were achieved within 10%. These results showed significant correlation between the peak areas and concentrations of bortezomib and impurities. The residuals were within ± 10% scattered with respect to 100% concentration response. The sensitivities were scattered within ± 10% with respect to 100% concentration sensitivity (Table 9).

Table 8: LOQ values of bortezomib Impurities

S. No.	Concentration of LOQ Solution in % (With respect to sample Concentration)	Signal to noise ratio
1	Impurity A	10.5
2	Impurity B	9.8
3	Impurity C	11.5
4	Bortezomib isomer	10.6
5	Hydroxyamide	12.3
6	Impurity D	11.5
7	Impurity E	10.5
8	Impurity F	11.6
9	Impurity G	10.5
10	Bortezomib ester	10.2
11	Bortezomib	10.5

Table 9 (A): Regression and precision data of bortezomib related impurities

Name of the Impurity	Impurity A	Impurity B	Impurity C	Bortezomib isomer	Hydroxyamide
LOD %	0.005	0.006	0.005	0.005	0.006
LOQ %	0.015	0.018	0.015	0.015	0.018
Slope (m)	11325	11562	10234	11569	12365
Intercept (C)	-32.4	-25.4	25.41	24.56	-35.62
Correlation coefficient	0.9995	0.9996	0.9998	0.9997	0.9997
Precision (%RSD)	2.6	3.2	4.5	4.2	3.5

Table 9(B): Regression and precision data

Name of the Impurity	Impurity D	Impurity E	Impurity F	Impurity G	Bortezomib ester	Bortezomib
LOD %	0.004	0.005	0.006	0.006	0.004	0.005
LOQ %	0.012	0.015	0.018	0.018	0.012	0.015
Slope (m)	11563	11563	11456	12365	10256	12563
Intercept (C)	25.63	-45.62	-12.56	15.63	14.25	13.56
Correlation coefficient	0.9998	0.9991	0.9993	0.9994	0.9996	0.9995
Precision (%RSD)	2.6	2.1	4.1	3.6	2.5	2.1

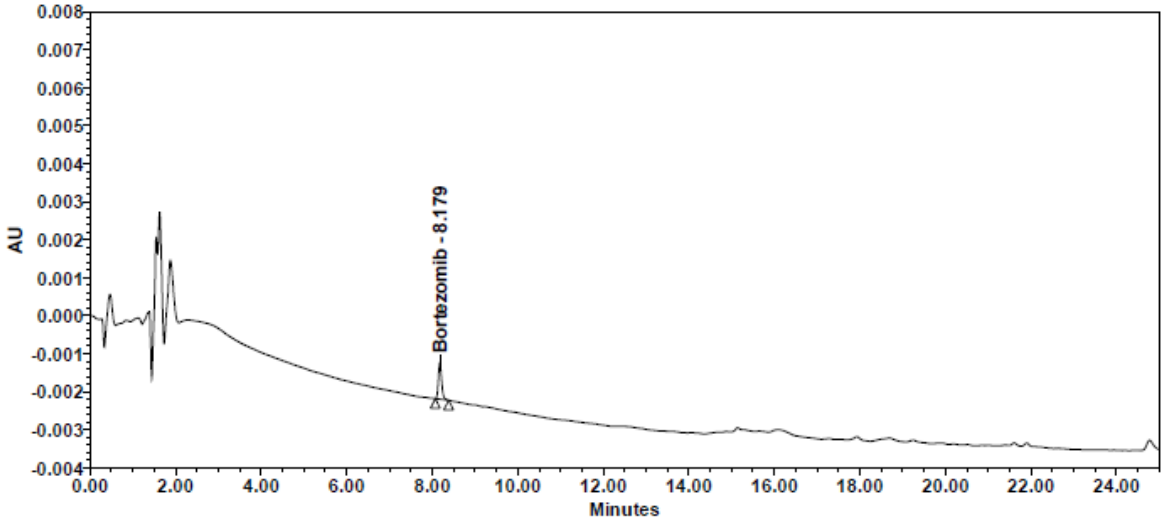
Linearity range was LOQ-150% w.r.t 0.7 mg/ml bortezomib for impurities; Linearity range was 50-150% of bortezomib. Six determinations using LOQ solutions for impurities and bortezomib.

Accuracy: The percent recovery of bortezomib impurities in the drug substances was ranged from 98 to 105, 96 to 101, 97 to 101, 97 to 101and 97 to 102 for impurity C, bortizomib isomer, hydroxyamide, bortizomib ester and bortizomib, respectively. The HPLC chromatograms of spiked samples at the 0.15% level of all impurities in the bortezomib drug substance sample were shown in Fig. 2. These results indicate the accuracy of method.

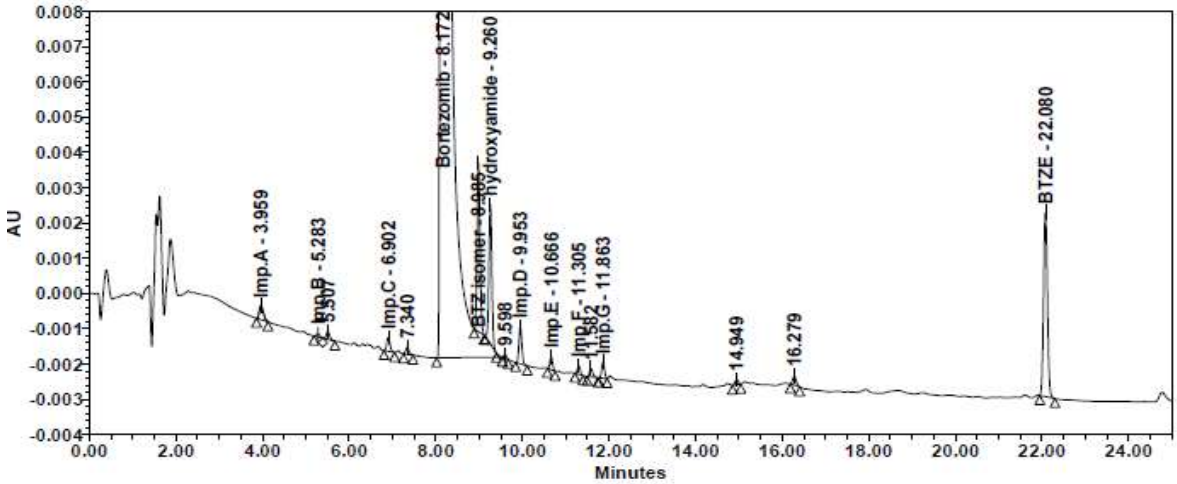
Robustness: At deliberately modified chromatographic conditions such as flow rate, pH, solvent ratio and column temperature etc., the resolution between the closely eluting impurities, bortezomib and Impurity A,B,C,D, E, F and G, bortezomib isomer, hydroxyamide and bortezomib ester, resolution achieved was greater than 2.0. The variability of bortezomib and the impurities area response was within ±2% and within ±3%, respectively. The results emphasizing the robustness of the method.

Solution stability and mobile phase stability: The series of solution stability and mobile phase stability indicate the %RSD of the related substances method of bortezomib and was less than 1%. These results indicate that no significant changes in the content of bortezomib and impurities A, B, C, D, E, F and G, bortezomib isomer, hydroxyamide and Bortezomib ester during the solution stability and mobile phase stability experiments The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the related substance determinations were stable up to72 h. Mobile phase is proved to be stable up to one weekdays.

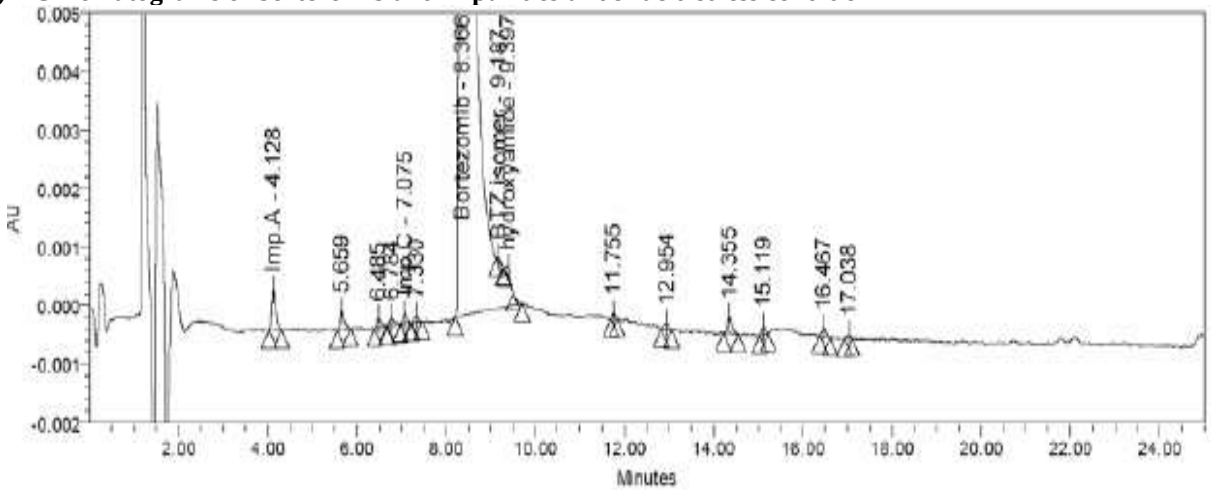
a) Chromatogram of Sensitivity Solution



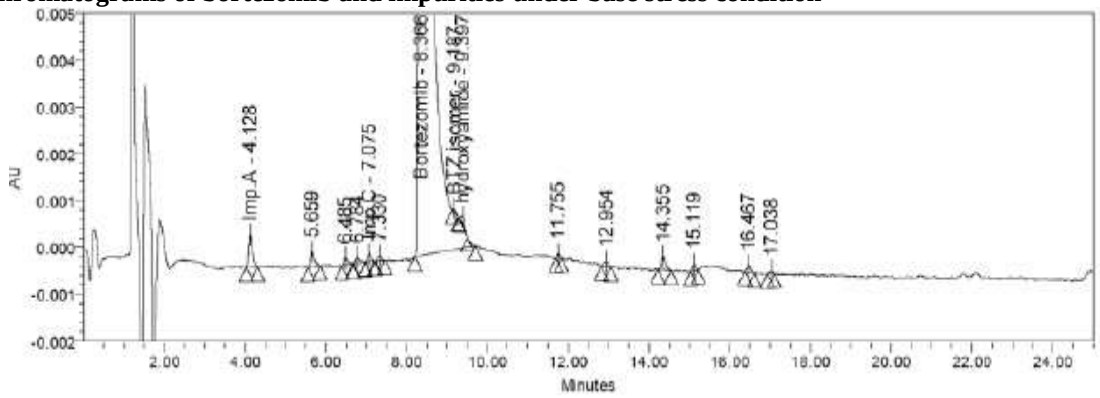
b) Chromatograms of resolution Solution



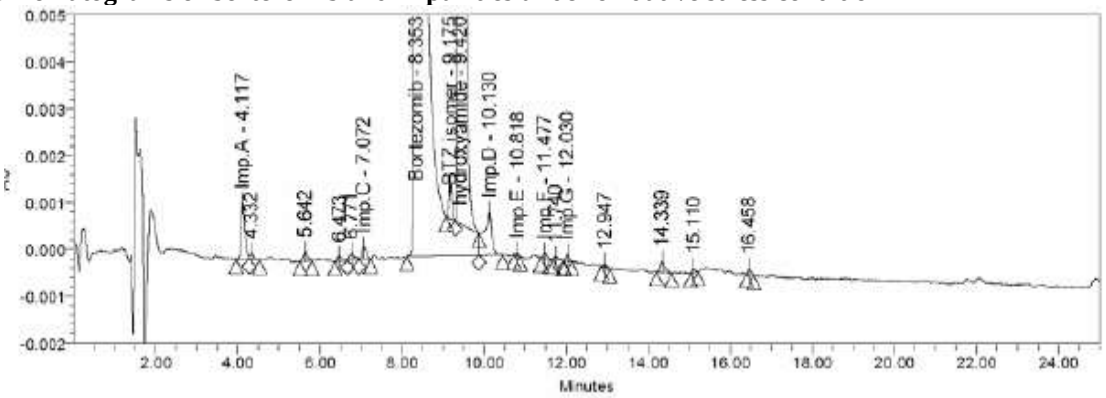
c) Chromatograms of bortezomib and impurities under acid stress condition



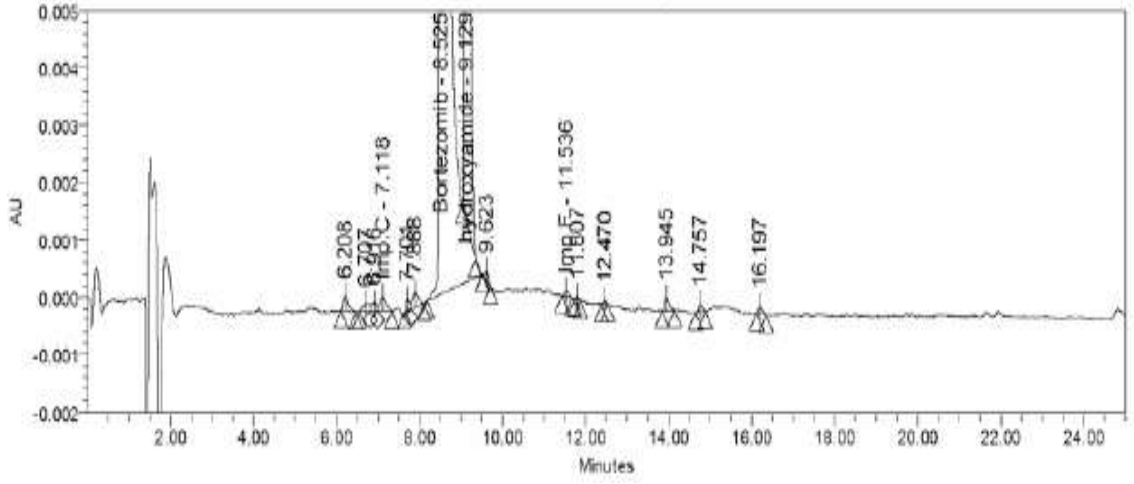
d) Chromatograms of bortezomib and impurities under base stress condition



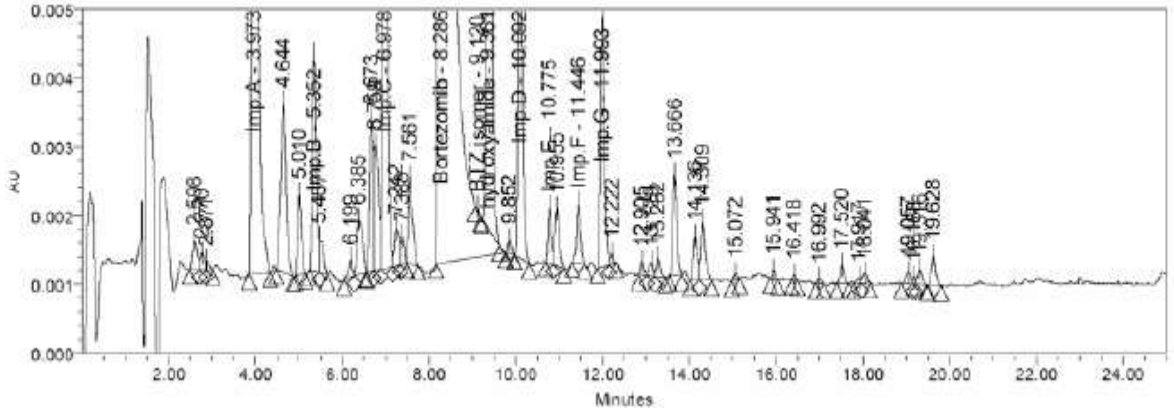
e) Chromatograms of bortezomib and impurities under oxidative stress condition



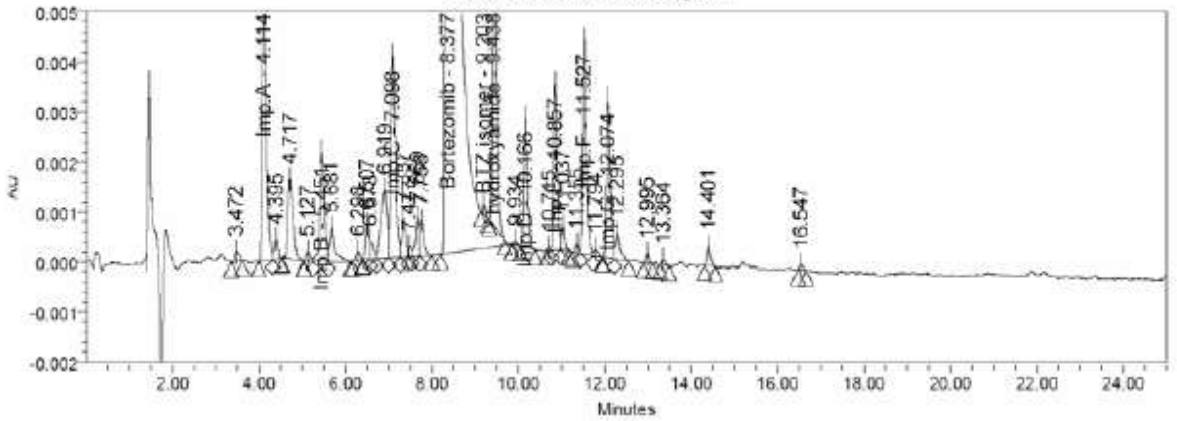
f) Chromatograms of bortezomib and impurities under heat stress condition



g) Chromatograms of bortezomib and impurities under UV stress condition



h) Chromatograms of bortezomib and impurities UV stress condition



Forced degradation studies: Then bortezomib stressed samples were subjected to forced degradation studies using light and heat. significant degradation of the drug substance and its products was detected with thermal, acid and oxidation, which leading to the formation of one major un known degradation product with RRT of 1.19 (Fig. 2 (C, D, E, F, G, H)). Peak purity test results derived from the PDA detector confirmed that the bortezomib peak and the degraded peaks were homogeneous and pure in all of the analyzed stress samples. The degradation studies were carried out for the stress samples at 100>g/ml against a qualified reference standard of bortezomib. The mass balance of the stressed samples was close to 99.5%. These results indicate that assay of bortezomib was unaffected by the presence of bortezomib and Impurities A, B, C, D, E, F and G, bortezomib isomer, hydroxyamide, and bortezomib ester and its degradation products,

confirming the stability-indicating power of the developed method (Table 10).

Table 10: Forced degradation bortezomib

S. No.	Conc. of solution	mL add stress agent	Temperature (°C)	Time in Hours	Mass balance (% assay + % impurities + % degradation products)	%Total Impurities	Remarks
1	650	1 mL Coc.HCl	35°C	1	93.1	2.5	Prominent Degradation is observed
2	650	0.5 mL of 24% NH4OH	35°C	1	80.2	15.5	Prominent Degradation is observed
3	650	100uL of 3% H2O2	35°C	1	86.1	10.5	Prominent Degradation is observed
4	650	100 W Hg-lamp (~ 1m distance)	35°C	1	85.7	12.0	Prominent Degradation is observed
5	650	Heating in normal atm.	60°C	24	95.2	2.5	No Prominent Degradation is observed
6	1000	100 W Hg-lamp (~ 1m distance)	35°C	72	90	7.5	Prominent Degradation is observed
7	1000	Heating in normal atm.	100°C	5	88.6	8.5	Prominent Degradation is observed
8	650	Unstressed			99.3	0.25	

Conclusion

The degradation pathway of bortezomib was established as per ICH recommendations[17-21]. The gradient LC method was developed and used for stress studies also fit for quantitative impurities of bortezomib drug product. The behavior of bortezomib under various stress conditions was studied. The thermal and all of the degradation products and process impurities were well separated from the bortezomib and related impurities demonstrates the stability-indicating power of the method. This method is sensitive and can be detected up to 0.002% impurities. This method was precise, accurate and stability indicative. The method was validated as per ICH recommendations. The developed method can be used to determine the impurities in bortezomib injection in the routine and stability sample analysis[17-21].

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