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A Validated Stability-Indicating UF LC Method for Bortezomib in the Presence of Degradation Products and its Process-Related Impurities

Venkataramanna M[1,2]*, K Sudhakar Babu[2] and S.K.C.Anwar[3]

[1]*Hetero Labs Ltd., Hetero House, Sanathnagar, Hyderabad- 500078, India*
[2]*Department of Chemistry, Sri Krishna Devaraya University, Anantapur-515003, India*
[3]*Hetero Labs Ltd., Hetero House, Sanathnagar, Hyderabad- 500078, India*

Abstract

Degradation pathway for bortezomib is established as per ICH recommendations by validated and stability indicating reverse phase liquid chromatographic method. Bortezomib is subjected to stress conditions of acid, base, oxidation, thermal and photolysis. Significant degradation is observed in acid and base stress conditions. Five impurities are studied and the major degradant (RRT about 1.19) was identified by LC-MS and spectral analysis. The stress samples are assayed against a qualified reference standard and the mass balance is found close to 99.5 %. Efficient chromatographic separation is achieved on a Shim pack XR-ODS-II(100X3mmx2.2µm) stationary phase with simple mobile phase combination delivered in gradient mode and quantification is carried at 270 nm at a flow rate of 0.6 mL min^{-1}. In the developed LC method the resolution between bortezomib and five potential impurities (imp-1, imp-2, imp-3, imp-4 and imp-5) is found to be greater than 2.0. Regression analysis shows an r value (correlation coefficient) of greater than 0.999 for bortezomib and five potential impurities. This method is capable to detect the impurities of bortezomib at a level of 0.020 % with respect to test concentration of 2.0 mg mL^{-1} for an 8-µL injection volume. The developed rapid LC method is validated with respect to specificity, linearity & range, accuracy, precision and robustness for impurities determination.

Keywords: Bortezomib; RR-LC; LC-MS; Forced degradation; Validation; Stability-indicating

Introduction

Bortezomib, (R)-3-methyl-1-((S)-3-phenyl-2-(pyrazine-2-car boxamido) propanamido) butyl-boronic acid, is one of the most important members of a new class of drugs, containing a boronic acid moiety, effective on a wide group of tumors. At present, it is mainly used for the treatment of multiple myeloma, a plasma cell tumor which accounts for 10% of all blood system malignancies. Bortezomib is the first therapeutic proteasome inhibitor to be tested in humans and is a peptidomimetic compound, constituted by a modified leucine-phenylalaninedipeptide, containing a boronic acid at the C-terminal. It is able to interact with proteasome, an intracellular apparatus which breaks down damaged or unneeded proteins, inhibiting the proteolysis action. Bortezomib is commercialized by Millennium Pharmaceuticals (Mass, USA) in the US and Janssen-Cilag in Europe under the trade name Velcade, and is administered as intravenous bolus [1-5].

A few chromatographic methods have appeared in the literature for the quantification of bortezomib in using SPE–LC-MS/MS for bortezomib and its hydrolyzed metabolite in human urine and development and validation of a liquid chromatography–tandem mass spectrometric assay for Bortezomib and its hydrolyzed metabolite in human plasma [6]. One method appeared "Stability-indicating RP-HPLC method for analysis of bortezomib in a pharmaceutical dosage form" [7] (Table 4).

To the best of our knowledge, no stability-indicating rapid LC method for the quantitative estimation of bortezomib in bulk drug substance samples in the presence of degradation products and five potential impurities has been reported. The purpose of the present research work is to develop a single stability-indicating LC method 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. Accordingly the aim of the present study is to establish degradation pathway of bortezomib through stress studies under a variety of ICH recommended test conditions [12,13].

Experimental

Chemicals

Samples of bortezomib and its related impurities are received from Hetero labs limited a research foundation of the firm Hetero drugs Ltd, Hyderabad, India (Figure 1). All impurities and the bortezomib standard are of > 99% purity and as follows: bortezomib (99.1%), imp-1 (99.5%), imp-2 (95.6%), imp-3 (99.7%) imp-4 (99.5%) and imp-5(99.6%). In addition, HPLC grade acetonitrile are purchased from Merck (Darmstadt, Germany). Analytical reagent grade potassium dihydrogen phosphate, phosphoric acid is purchased from Merck. Highly pure water is prepared with the Millipore Milli-Q Plus water purification system.

Equipment

The LC system used for method development, forced degradation studies and method validation consisted of a Shimadzu UFLC (LC20ADXR) binary pump with an auto sampler and a photo diode

***Corresponding author:** Venkataramanna M, Hetero Labs Ltd., Hetero House, Sanathnagar, Hyderabad- 500078, India, E-mail: venky75@gmail.com

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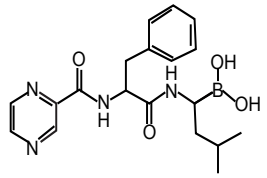
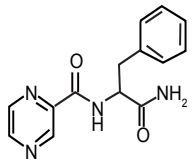
array detector (PDA). The output signal is monitored and processed using LC solution software on a Pentium computer (Digital equipment Co.).Photo stability studies are carried out in a photo stability chamber (Atlas Suntest CPS+). Thermal stability studies are carried out in a dry hot air oven (Cintex precision hot air oven).

Chromatographic conditions

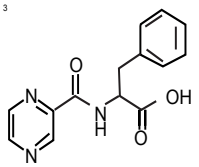
A Shimpack XR-ODS-II (100 mm X 3mm X 2.2µm) is used with a mobile phase containing a gradient of solvents A and B. The buffer is composed of 0.1 M potassium dihydrogen phosphate, with its pH adjusted to 3.0 with orthophosphoric acid. The flow rate of the mobile phase is 0.6 ml/min with a gradient program of 0.00/20, 2/30, 5/50, 6/70, 8/20 and 10/20 (time (min) / %B). The column temperature is maintained at 30˚C and the detection wavelength is set at 270 nm. The injection volume is 8 µl.



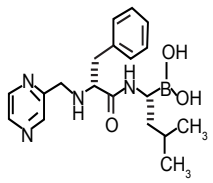
Impurity-1: N-((S)-1-carbamoyl-2-phenylethyl) pyrazine-2-carboxamide *(Molecular weight 270.29)* $C_{14}H_{14}N_4O_2$



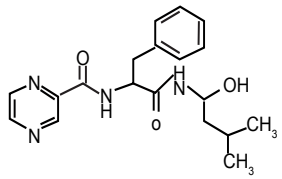
Impurity-2: N-(pyrazine-2-ylcarbonyl) –L-phenylalanine *(molecular weight 271.27)* $C_{14}H_{13}N_3O$



Impurity-3: [(1R)-3-methyl-1-[[(2R)-1-oxo-3-phenyl-2-(pyrazinylcarbonyl) amino] propyl]amino] butyl]boronic acid *(molecular weight 384.24)*. $C_{19}H_{25}BN_4O_4$



Impurity-4: N(S)-(1-(1(R)-hydroxy-3-methyl butyl amino)-1-oxo-3-phenyl propane-2-yl) pyrazine-2- carboxamide *(molecular weight 356.42)* $C_{19}H_{24}N_4O_3$



Impurity-5: N(S)-(1-(1(S)-hydroxy-3-methyl butyl amino)-1-oxo-3-phenyl propane-2-yl) pyrazine-2- carboxamide *(molecular weight 356.42)* $C_{19}H_{24}N_4O_3$

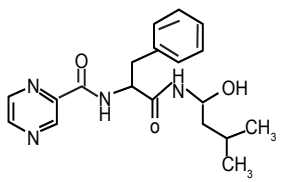


Figure 1: Chemical structures and names of bortezomib and its impurities. Bortezomib (molecular weight 384.2) $C_{19}H_{25}BN_4O_4$.

LC-MS conditions

The LC-MS system (Agilent 2010 EV series liquid chromatography system triple quadrapole mass spectrometer) is used for the identification of unknown compounds formed during forced degradation. A symmetry shield RP 18 100 x 4.6 mm, 3.5-µm column was used as the stationary phase. Acetonitrile is used as mobile phase for gradient mode.0.01M ammonium formate and the pH is adjusted to 3.0 using formic acid and are used as buffer. The flow rate was 0.6 ml/min. The injection volume is 20 µl. The analysis was performed in positive and negative electrospray ionization modes. The capillary and cone voltages were 4.5 kV and 5 V, respectively. The source and dissolvation temperatures are 250 ºC and 200 ºC, respectively, and the dissolvation gas flow is 1.2 min^{-1}.

Preparation of standard solutions and sample solutions

A stock solution of bortezomib (0.02 mg/ml) is prepared by dissolving the appropriate amount of bortezomib solid in the diluent. Working solutions containing 0.15 % of impurities are prepared from the stock solution for the determinations of related substances. A stock solution of impurities (mixture of imp-1, imp-2, imp-3, imp-4 and imp-5) at 0.03 mg/ml is also prepared in the diluent. The drug substance powder equivalent to 20 mg of sample is transferred into a 10-ml volumetric flask, and diluent is added. The flask is attached to a rotary shaker and shaken for 2 min to disperse the powder completely. The mixture is sonicated for 2 min and then diluted to the appropriate volume with diluent to make a solution containing 0.03 mg/ml of impurities. The solution is then filtered through a 0.45-µ nylon 66 membrane filter.

Stress studies / Specificity

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities [10-11]. The specificity of the developed LC method for bortezomib is determined in the presence of its related impurities (namely imp-1, imp-2, imp-3, imp-4 and imp-5) and degradation products. Forced degradation studies are also performed on bortezomib to provide an indication of the stability-indicating property and specificity of the proposed method [12-17]. The stress conditions employed for the degradation study included light (carried out as per ICH Q1B), heat (105˚C), acid hydrolysis (0.2 M HCl), base hydrolysis (0.2 M NaOH) and oxidation (25% H_2O_2). For heat and light studies, the samples are exposed for 24 hrs, whereas the samples are treated for 2 h for acid, base hydrolysis and for oxidation. The peak purity of the bortezomib stressed samples is also checked by using a Shimadzu UFLC photo diode array detector (PDA). The purity angle is within the purity threshold limit in all of the stressed samples, demonstrating the homogeneity of the analyte peak. The contents of impurities are calculated for the stress samples against a qualified reference standard. The mass balance (% assay + % of impurities + % of degradation products) is calculated for all of the samples.

Method validation

The proposed method was validated per ICH guidelines [14-16].

Precision: The precision of the related substance method is investigated by injecting six individual preparations of (0.02 mg/ml) bortezomib spiked with 0.03% each of imp-1, imp-2, imp-3, imp-4 and imp-5. The %RSD of the areas of imp-1, imp-2, imp-3, imp-4 and imp-5 is calculated.

The intermediate precision of the method is evaluated using a different analyst and instrument located within the same laboratory.

The precision of the method is evaluated by carrying out six independent analysis of a test sample of bortezomib against a qualified reference standard. The %RSD of six obtained values is calculated.

Limit of detection (LOD) and limit of quantification (LOQ): The LOD and LOQ for imp-1, imp-2, imp 3, imp-4 and imp-5 are estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. The precision study is also carried out at the LOQ level by injecting six individual preparations of imp-1, imp-2, imp-3, imp-4 and imp-5 and calculated the %RSD of the areas.

Linearity: Linearity test solutions for the assay method are prepared from a stock solution at six concentration levels from 50 to 150% of the analyte concentration (50(0.075%), 75(0.120%), 100(0.150%), 125(0.180%) and 150(0.225%).The peak area versus concentration details analyzed with least-squares linear regression. Linearity test solutions for the related substance method are prepared by diluting the impurity stock solution (2.5) to the required concentrations. The solutions are prepared at six concentration levels from the LOQ to 150% (LOQ, 0.075%, 0.12%, 0.15%, 0.18% and 0.225%).The slope and y-intercept of the calibration curve are reported. The peak area versus concentration data is analyzed with least-squares linear regression. Linearity test solutions for the related substance method are prepared by diluting the impurity stock solution (2.5) to the required concentrations. The slope and y-intercept of the calibration curve are reported (Figure 4).

Accuracy: Accuracy of the assay method is evaluated in triplicate at three concentration levels 50(0.075%), 100(0.15%) and 150(0.225%), and the percentage recoveries are also calculated. Bortezomib did not show the presence of imp-2, imp-3 and imp-4, but contained 0.03% of imp-1, 0.02% of imp-5. Standard addition and recovery experiments are conducted to determine the accuracy of the related substance method for the quantification of all five impurities (imp-1, imp-2, imp-3, imp-4 and imp-5) in the drug substance. The study is carried out in triplicate at 0.075%, 0.15% and 0.225% of the analyte concentration (2.0µg/ml). The percentage of recoveries for imp-1, imp-2, imp-3, imp-4 and imp-5 are calculated.

Robustness: To determine the robustness of the developed method, the experimental conditions are altered and the resolution between bortezomib and imp-1, imp-2, imp-3, imp-4 and imp-5 is evaluated. The flow rate of the mobile phase is 0.6 ml/min. To study the effect of the flow rate on the resolution, the flow rate is changed by 0.1 units (to 0.5 and 0.7 ml/min). The effect of pH on the resolution of the impurities is studied by varying the pH by ± 0.2 units (buffer pH of 2.8 and 3.2). The effect of the column temperature on the resolution is studied at 28˚C and 32ºC instead of 30 ºC. In all these varied conditions, the components of the mobile phase remained constant, as outlined in Section 2.3.

Solution stability and mobile phase stability: The solution stability of bortezomib in the method is carried out by leaving both the sample and reference standard solutions in tightly capped volumetric flasks at room temperature for 24 h. The same sample solutions are assayed for in 6-h intervals over the study period. The mobile phase stability is also examined by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions for 6 h intervals up to 48 h.

The prepared mobile phase remained constant during the study period. The %RSD of the bortezomib impurities is calculated for the mobile phase and solution stability experiments. The content of imp-1, imp-2, imp-3, imp-4 and imp-5 is determined at 6 h intervals up to the study period. The mobile phase stability is also investigated for 48 h by injecting the freshly prepared sample solutions for every 6 h interval. The content of imp-1, imp-2, imp-3, imp-4 and imp-5 is determined in the test solutions. The prepared mobile phase remained constant during the study period.

Results and Discussion

Method development and optimization

The main objective of the chromatographic method is to separate imp-1, imp-2, imp-3, imp-4, imp-5, bortezomib and the generated degradation products from the analyte peak during stress studies. Impurities and degradation products are co-eluted by using different stationary phases, such Zorbax eclipse 100X3mmx1.8µm with various mobile phases with buffers, such as phosphate, sulphate and acetate with different pH 6.5, and organic modifiers, including acetonitrile and methanol, in the mobile phase.0.1M ammonium acetate buffer with a pH value of 6.5 and methanol (50:50, v/v) at a flow rate of 1.0 ml/ min is chosen for the initial trails. When an impurity-spiked solution is injected, the peak shapes are merged and no separation is achieved for the impurities (Figure 3). To improve the resolution between the impurities and analyte, acetonitrile concentration reduced using Shimpack XR-ODS-II (100X3mmx2.2µm) as a stationery phase in the mobile phase and flow rate was slightly changed and injected into the impurity-spiked solution and observed the resolution between the impurities and analyte is not achieved. To optimize the resolution between the impurities and the retention time of the impurities, trails are carried out with different mobile phase ratios using buffer and acetonitrile. Isocratic trials are not successful in achieving a favorable resolution the between impurity and analyte peaks and the elution of

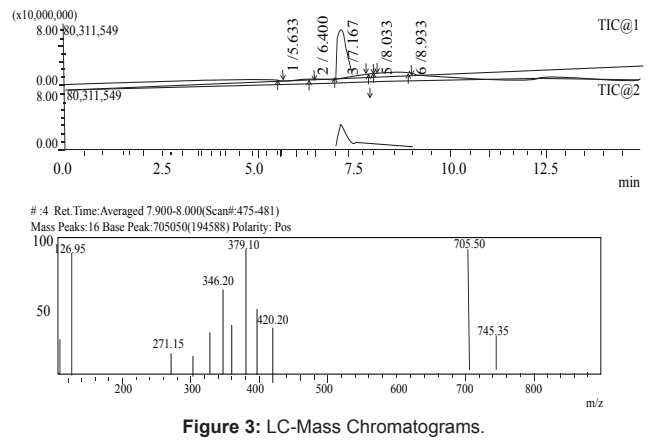


Figure 3: LC-Mass Chromatograms.

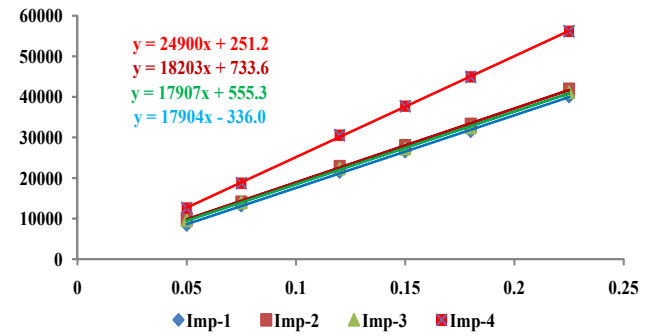


Figure 4: Linearity chart for bortezomib impurities.

Compound	Concentration of LOQ Solution in % (With respect to sample Concentration)	Signal to noise ratio
Impurity-1	0.03	10.9
Impurity-2	0.03	12.2
Bortezomib	**0.02**	**10.6**
Impurity-3	**0.03**	**14.1**
Impurity-4	**0.03**	**13.1**
Impurity-5	**0.03**	**11.9**

Table 1: Limit of detection and quantification.

Compound	USP Resolution (R_S)	USP Tailing factor	No. of theoretical plates (USP tangent method)
Impurity-1	--	1.2	19262
Impurity-2	8.4	1.3	26407
Bortezomib	5.9	1.3	14667
Impurity-3	2.2	1.3	25578
Impurity-4	2.7	1.2	62743
Impurity-5	3.8	1.2	71445

Table 2: System suitability report.

the process impurities. Therefore, a gradient method is selected using buffer and acetonitrile as mobile phase A and B. Different gradient programs are investigated and satisfactory results are obtained when a gradient program of 0.00/20, 2/30, 5/50, 6/70, 8/20 and 10/20 (time (min) / %B) with a stationery phase Shimpack XR-ODS-II (100 mm X 3mm X 2.2µm) with a flow rate of 0.6 ml/min is used. The column temperature is maintained at 30°C and the detection wavelength is set at 270 nm with an injection volume injection volume of 8 µl (Table 2).

Method validation:

Precision: The %RSD of bortezomib during the method precision study is within 3.0% and the %RSD values of the area of imp-1, imp-2, imp-3, imp-4 and imp-5 in the related substance method precision study are within 10.0 %. The %RSD of the results obtained in the intermediate precision study was within 3.0% and the %RSD of the areas of imp-1, imp-2, imp-3,imp-4 and imp-5 are well within 5 %, revealing the high precision of the method (Table 3a,b).

Limit of detection and limit of quantification: The limits of detection and quantification of bortezomib, imp-1, imp-2, imp-3, imp-4 and imp-5 for a 8-µl injection volume are given in (Table 3a,b). The precision at the LOQ concentration for imp-1, imp-2, imp-3, imp-4 and imp-5 is below 10.0 % (Table1).

Linearity: The linear calibration plot for the method is obtained over the tested calibration range (50%- 150% level) and the obtained correlation coefficient is greater than 0.999. The results revealed an excellent correlation between the peak area and analyte concentration. The linear calibration plot for the related substance method is determined over the calibration range (LOQ to 0.225% w r to analyte concentration) for imp-1, imp-2, imp-3, imp-4 and imp-5, a correlation coefficient of greater than 0.999 is obtained. The linearity is checked for the related substance method over the same concentration range for three consecutive days. The %RSD values of the slope and y-intercept of the calibration curves are within 10%. These results showed an excellent correlation between the peak areas and concentrations of imp-1, imp-2, imp-3, imp-4 and imp-5 (Table 3a,b) Residuals are within ± 10% scattered with respect to 100% concentration response. Sensitivities are scattered within ± 10% with respect to 100% concentration sensitivity.

Accuracy: The percentage recovery of bortezomib impurities in the drug substances i.e. imp-1, imp-2, imp-3, imp-4 and imp-5 ranged from 96.85 to 102.10, 96.29 to 101.33, 97.88 to 101.11, 97.70 to 101.88 and from 96.54 to 102.44 respectively. The HPLC chromatograms of spiked samples at the 0.15% level of all four impurities in the bortezomib drug substance sample are shown in (Figure 2 (included as supplementary data)).

Robustness: In all of the deliberately varied chromatographic conditions carried out as described in Section 2.7.5 (flow rate, pH and column temperature), the resolution between the closely eluting impurities, namely imp-2, imp-3 and imp4, is greater than 2.0, illustrating the robustness of the method. The variability of bortezomib and the impurities area response is within ±2% and within ±3%, respectively.

Solution stability and mobile phase stability: The %RSD of assaying bortezomib during the solution stability and mobile phase stability experiments is within 1%. No significant changes are observed in the content of imp-1, imp-2, imp-3, imp-4 and imp-5 during the solution stability and mobile phase stability experiments when performed using the related substances method. The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the related substance determinations are stable up to 48h. Mobile phase is proved to be stable up to five days.

Results of forced degradation studies

Degradation was not observed in bortezomib stressed samples subjected to light and heat. Significant degradation of the drug substance and product is detected under thermal, acid and oxidation, leading to the formation of one major unknown degradation product at 1.19RT (Figure 2(included as supplementary data)). Peak purity test results derived from the PDA detector confirmed that the bortezomib peak and the degraded peaks are homogeneous and pure in all of the analyzed stress samples. Degradation studies are carried out for the stress samples (at 100 µg/ml) against a qualified reference standard of bortezomib.

The mass balance of the stressed samples was close to 99.5%. The assay of bortezomib is unaffected by the presence of imp-1, imp-2,

Stress condition	%Total Impurities	Study Time	% Assay of active substance	Mass balance (% assay + % impurities + % degradation products)	Remarks
Acid hydrolysis (0.2 M HCl)	**21.0%**	**2 h**	**91.4**	**99.8**	**Prominent Degradation observed**
Water degradation (at 80°C)	**5.6%**	**2 h**	**93.6**	**99.6**	**Prominent Degradation observed**
Base hydrolysis (0.2 M NaOH)	2.8%	1 h	**97.6**	**99.5**	No Prominent Degradation observed
Oxidation ($25H_20_2$)	15.5	2 h	**84.8**	**98.6**	**Prominent Degradation observed**
Thermal (105°C)	**26.0%**	**24 h**	**73.9**	**98.5**	**One major degradation product was formed RRT about xxx and identified the mass number at "705"**
Light(photolytic degradation)	**5.5%**	**1200 KLUX/ Hr**	**99.1**	**99.5**	**Prominent Degradation observed**

Table 3(a): Summary of forced degradation results.

Parameter	Bortezomib	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5
LOD (%)	0.006	0.010	0.009	0.010	0.009	0.009
LOQ (%)	0.02	0.03	0.03	0.03	0.03	0.03
Slope (m)	128913	154578	136181	76737	90394	108964
Intercept (C)	84.91	-84.978	-24.5868	-109.83	89.05988	-24.9701
Correlation coefficient	0.99976	0.9993	0.99988	0.99995	0.99960	0.99982
Precision (%RSD)[a]	2.9	4.0	2.5	3.6	4.6	3.7

Linearity range was LOQ-150% w.r.t 2.0 mg/ml bortezomib for impurities; Linearity range was 50-150% of bortezomib.
[a]Six determinations using LOQ solutions for impurities and bortezomib.

Table 3(b): Regression and precision data.

Lot No.	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Max. Single Unknown Imp.	Total impurities	Assay by HPLC
001	0.01	ND	0.04	0.06	0.01	0.04	0.17	99.9
002	0.02	ND	0.06	0.08	0.02	0.03	020	99.8
003	0.01	ND	0.04	0.07	0.02	0.01	0.17	99.7

Table 4: Batch analysis data.

imp-3, imp-4 and its degradation products, confirming the stability-indicating power of the developed method.

Identification of major degradation product (RRT 0.31) formed in base hydrolysis (stress conditions)

A LC-MS study was carried to determine the m/z value of the major degradation product formed under acid and base hydrolysis using an Agilent-2010EV (LC-MS) series triple Quadra pole liquid chromatography system coupled with triple quadrapole mass spectrometer. Acetonitrile is used as mobile phase for gradient mode. 0.01M ammonium formate and the pH is adjusted to 3.0 using formic acid and are used as buffer and the conditions were described in section 2.4.The m/z value obtained for the degradation product resolving at 1.19 RRT in ESI positive mode is 705 (M+1).

Conclusion

The degradation pathway of bortezomib is established as per ICH recommendations. The gradient LC method developed and used for stress studies also fit for quantitative, related substance and assay determination of bortezomib. The behavior of bortezomib under various stress conditions is studied, and the thermal degradant is observed at mass number "704" by LC MS and presented. All of the degradation products and process impurities are well separated from the drug substance demonstrates the stability-indicating power of the method. The method is validated as per ICH recommendations. The developed method is stability indicating which can be used or the impurity testing and assay determination in routine analysis of production samples and also to analyze stability samples.

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