New RP-Chromatography Method For Bortezomib And Its Related Compounds In Large And Small Volume Injections With UP Detection

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ABSTRACT:

For the Bortezomib(BTZ) a validated, specific, stability indicating RP- liquid chromatographic method in liquid injection has been developed for quantitative analysis and its related substances. The degradation products was achieved on a X-Terra RP8, 150mm x 4.6 mm, 5µm or Equivalent As a mobile phase a mixture of water 715 v/v, Acetonitrile 285 v/v, Formic acid 1 v/v were prepared. The detection is achieved at 270nm.The Rt of BTZ is 4min. The flow rate was measured at 1.5ml/min. The method was linear. The volume Injected is 10µL. This method is validated in according to ICH guidelines USP with reference to accuracy, precision, specificity. This method can be used to determine related impurities and degradation products of BTZ.

***KEYWORDS**: RP HPLC, UV&PDA detector, BTZ, flow rate, column, ICH guideline.*

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1. Introduction

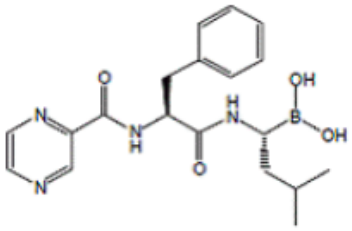


Fig: 1 Structure of BTZ

The BTZ is an anti-cancer drug with trade name (R) - 3 - methyl - 1 - ((S) - 3 - phenyl - 2 - (pyrazine - 2 - car boxamido) propanamido) butyl-boronic acid. This BTZ constitutes boronic acid moiety and effective wide group of tumors, due to this reason it is treated as most important members of a new class of drugs. For the treatment of multiple myeloma BTZ is mainly used for, a plasma cell tumor which accounts for 10% of all blood system malignancies. BTZ is the first therapeutic proteasome inhibitor to be tested in humans and is a peptidomimetic compound, constituted by a modified leucine-phenylalaninedipeptide, containing a H_3BO_3 at the C-terminal.. BTZ 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]. Some research papers are available for BTZ [6] characterization of BTZ. By using MDS sciex in human plasma metabolites are observed , At 325^{o}C using API 3000 triple quadruple LC MS turbo ion spray interface set and [7] Enhanced Delivery of cisplatin to international Ovarian Carcinomas mediated by the effects of BTZ on human copper transporter and one of it is in Human plasma using LC MS. Venkataramanna M et.al.,[8] proposed the degradation pathway for BTZ. BTZ degradation is observed in different pH conditions. By using LC-MS and spectral analysis total 05 impurities were studied and the major degradant (RRT about 1.19) was identified. The mass balance is described around 99.5%. Efficient separation is carried out by using a Shim pack -ODS-II(100X3 mmx2.2 µm). λmax is 270 nm and flow rate at 0.6 mL min. Regression analysis value is greater than 0.999 for BTZ. Bhetanabotla Chandramowli et.al.,[9] developed a LC–MS/MS for BTZ in human plasma. The interferences due to protein denaturation are separated using 0.45µm filter cartridge. At 3.5 min Chromatographic separation is identified. Column is ACE 5CN column (150mmx4.6mm) with acteonitrile: 10 mM ammonium formate buffer (75:25 v/v) as an isocratic mobile phase with 1 ml/min flow rate. 2 - 1000 ng/ml (r2 $\geq$ 0.998) is the linearity. The % recovery is at 82.71%. M.V. Basaveswara Rao et.al.,[10] proposed assay of Quetiapine in tablet dosage form by using 1.0ml/min as flow rate. Column used as C18 (250x4.6mm, 5µm in particle size). Mobile phase as methanol 90V: water 10V: O.P.A 01 V. 250 nm is the wavelength and injected sample was 20µl. Rt was 3.64 min. The % RSD for all requirements is found that less than 2%.

2. EXPERIMENTAL

2.1 Instrumentation:

By using X-Terra RP8, 150mm x 4.6 mm, 5µm or Equivalent, the chromatographic analysis was performed. With the help of a Loba ultrasonic bath sonicator degassing of the mobile phase is performed. For weighing the drugs and other materials a Denwar Analytical balance is used.

2.2 Chemicals and Solvents:

The reference sample of BTZ is collected. Formic acid is AR grade and Acetonitrile and water used are HPLC grade and purchased from Ranken Merck Specialities Private Limited, Mumbai, India.

2.3 The buffer solution

A mixture of 250 volumes of water and 750 volumes of Acetonitrile, are mixed well. By using 0.45µ nylon filter the solution is subjected to filtration.

2.4 The mobile phase:

As a mobile phase mixture of water 715 v/v, Acetonitrile 285 v/v and Formic acid 1 v/v were prepared.

2.5 Preparation of Solutions:

Diluent:

250 volumes of water and 750 volumes of Acetonitrile are prepared, mixed the contents thoroughly. Finally the contents are subjected to filtration.

Standard Solution:

2.80 mg of BTZ is weighed and transferred into 20.0 ml volumetric flask. To this 5.0 ml of diluent was added and dissolved and diluted to the required volume with the help of diluent which indicates that the prepared solution is equivalent to 0.140 mg/ml.

Sample preparation:

In the 25 ml volumetric flask 5 vials of sample was taken and reconstitute each with 3.5 ml of diluents which is diluted to the volume with diluent. After that 2.0 mL of this sample solution is transferred in to a 10.0 mL volumetric flask. To this solution again added 5.0 mL of diluent. Sonicated and diluted to the volume with diluent.

Sample preparation:

Take 1 vial and reconstitute with 5 ml of diluent. Further transfer 2.0 mL of this sample solution in to a 10.0 mL volumetric flask. To the above constituents added 5.0 mL of diluent

and diluted to volume with the diluent. Separately inject Blank (diluent) (one injection) and standard solution (five injections) into chromatograph and check the system suitability.

3. Method development

For the investigation of this method[10-18], used the consistent methodology. By varying the parameters the various factors effect were taken into the consideration at a time by keeping remaining conditions as constant.

3.1 Detection wavelength:

For10ppm of solution the peak of maximum absorbance wavelength 270nm was detected.

3.2 Choice of stationary phase and Mobile Phase:

By using X-Terra RP8, 150mm x 4.6 mm, 5µm or Equivalent column the expected separation and peak shapes are obtained. Water 715 v/v, Acetonitrile 285 v/v, Formic acid 1v/v are used as mobile phase. This shows that better defined and resolved and almost free from tailing.

3.3 Flow rate:

At last it is observed from the experiment that 1.5 mL/min flow rate was ideal for the successful elution of the analyte.

4. Requirements for Validation of Proposed Method

4.1 System Suitability

To verify that the analytical system is working properly and can give accurate and precise results, the system suitability parameters are to be set. Injected Blank (1 injection), Standard preparation (5 injections) into HPLC and recorded the chromatograms. The Tailing factor for Bortezomib peak is 0.1. The % Relative standard deviation (RSD) for 5 injections of standard solution is 1.4. Column Efficiency for BTZ peak of Standard solution is observed that 3075. From the below results, it is concluded that the system is suitable for analytical method validation. The results are tabulated in Table 1.

Injection No.	Area Response
1	1205220
2	1206806
3	1206404
4	1208424
5	1205295
Mean	1206430
%RSD	**0.1**

Table 1: System suitability results

4.2 Specificity:

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. Separately injected Blank (Diluent),Placebo Preparation, Specified impurity 1,Specified Impurity 2,Specified Impurity 3, Standard Preparation & Sample Preparation into the HPLC system to examined; BTZ Peak is not affected by Diluent. Recorded the retention times of Diluent, Placebo, Specified Impurity-I, Specified Impurity-2, Specified Impurity-3, Standard Preparation & Sample preparation. The peaks of diluent, placebo & impurity peaks should not interfere with BTZ Peak. From the results, it is concluded that the Diluent, Placebo & Impurity peaks are not interfere with Bortezomib peak and each other. The results are tabulated in Table:2

Table 2: **Specificity results**

Solution	Retention time (in Min)
Blank	---
Placebo	---
Standard	4.337
Sample	4.371
Specified Impurity-1	3.295
Specified Impurity-2	5.986
Specified Impurity-3	2.188

FORCED DEGRADATION STUDIES:

These studies are carried out to confirm that during stability study or through its shelf life, any degradation product if found will not interfere with the BTZ peak. For these studies different solutions have been prepared such as Sample, Placebo, Acid Stressed sample, Alkali Stressed sample, 3.0%w/v Hydrogen Peroxide Stressed sample, Neutral Stressed sample, UV light exposed sample, Photo stability exposed sample, Sunlight exposed sample, Alkali Stressed sample, 3.0%w/v Hydrogen Peroxide Stressed sample, 1.0%w/v Hydrogen Peroxide Stressed sample, 0.1%w/v Hydrogen Peroxide Stressed sample and Thermal Stressed (Dry heat) sample and the results are summarized as follows. Finally it is concluded that in the Alkali condition the sample (1.0N NaOH) is degraded more and in the 0.1%w/v H_2O_2, Photo stability stressed condition sample is degraded more. In 0.1N NaOH condition the sample is slightly degraded. No Degradation is observed In Thermal condition and Acidic conditionFinally, Unknown impurities, known impurities and degradation products are well separated from BTZ Peak. Hence, the method is indicating selectivity, specific and stability indicating. The results are tabulated in the table:3.

Condition	Purity Angle	Purity Threshold	% Assay
Sample as such	0.313	0.536	99.9
1N HCl	0.309	0.535	99.1
0.1N HCl	0.334	0.557	97.8
1N NaOH	0.272	0.499	47.3
0.1 N NaOH	0.240	0.459	80.6
3.0%w/v H_20_2	14.104	16.373	0.5
Neutral	0.297	0.521	93.1
UV Light	0.309	0.537	96.8
Sun Light	0.371	0.601	81.7
Thermal	0.301	0.528	100.2
Photostability	0.423	0.641	71.5

Table 3: Force degradation studies results

4.3 Precision:

The precision of analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of series of measurement.

4.3.1 System precision

The Rt and area of 6 determinations are measured and % RSD was calculated. Injected Standard solution into the HPLC. From Standard preparation the % RSD for Retention time and area response of BTZ is recorded. It is observed from the below data that the Rt and area responses are consistent i.e., < 1.0% and < 2.0% respectively. Hence, it is concluded that the system precision parameter meets the requirement of method validation. The results are tabulated in the table 4.

Injection No.	Retention Time (min.)	Area Response
1	4.557	1205220
2	4.557	1206806
3	4.558	1206404
4	4.558	1208424
5	4.557	1205295
6	4.558	1206567
Mean	4.558	1206453
%RSD	0.0	0.1

Table 4: System Precision results

4.3.2 METHOD PRECISION:

A homogenous sample of a single batch was analyzed 6 times. As per analytical procedure the Sample of BTZ for injection 10 units of a single batch was analyzed. Calculated the content uniformity of BTZ in the sample preparation and calculated the % of BTZ. For

Assay of BTZ for injection 3.5mg/Vial the %RSD was found that 0.2. From the results, it can be concluded that the method is precise.

4.3.3 INTERMEDIATE PRECISION:

Calculated the % of impurities compared the results obtained in method precision and Intermediate Precision, calculated the % RSD From the obtained results, it can be concluded that method is rugged. The results are tabulated in the table 5,6 and table 7.

Assay of BTZ for injection		
Method precision3.5mg/Vial		**Intermediate precision3.5mg/Vial**
Sample Set No.	**% of Assay**	**% of Assay**
1	103.0	100.0
2	102.6	101.1
3	102.5	101.2
4	102.7	101.4
5	102.8	100.7
6	102.5	100.8
Mean	101.8	
%RSD of 12 Determination	1.0	
Absolute difference	1.7	

Table 5: Comparsion between the method precision and intermediate precision:

Assay of BTZfor injection		
Method precision 3.5mg/Vial		**Intermediate Precision 3.5mg/Vial**
Sample Set No.	**% of Assay**	**% of Assay**
1	99.6	102.6
2	100.2	99.2
3	100.5	100.4
4	100.9	101.6
5	101.5	102.2
6	100.7	101.0
7	101.5	100.0
8	99.4	100.9
9	102.4	103.1
10	101.5	101.5

Mean	101.0
%RSD of 20 Determination	1.1
Absolute difference	**-0.4**

Table 6: Comparsion between the method precision and intermediate precision for content uniformity

S.No	Lower Level	Higher Level	BTZ Conc. (ppm)	Area Response
1	603993	1897963	**0.0000**	**0**
2	601840	1897520	66.1144	603979
3	604664	1896872	113.7167	1012247
4	604477	1898457	126.9396	1124445
5	604516	1897489	140.1625	1255631
6	604384	1898551	153.3854	1367367
			169.2528	1507830
			211.5660	1897809
			Slope	8903.857
			Intercept	4771.429
			Intercept %	0.38
Mean	603979	1897809	Correlation Coefficient	1.000
%RSD	**0.2**	**0.0**	Regression Coefficient	1.000

Table7: Precision at Lowest and higher level of BTZ

STABILITY IN ANALYTICAL SOLUTION:

By injecting different solutions at regular intervals the stability was evaluated. From the results it is concluded that the standard solution is stable for 30 hrs (% difference is 1.7) at Room Temperature 25°C. Sample solution 3.5mg/vial is stable for 33 hrs at 25°C (% difference is -1.0). The results are tabulated in the table 8.

Standard Solution Stability at 25°C			**Sample Solution (3.5mg/vial) Stability at 25°C**	
Time (Hrs.)	**Area**	**% Difference**	**Area**	**% Difference**
Initial	1205220	-	1337395	-
2	1203814	-0.1	1331655	-0.4
3	1205956	0.1	1328439	-0.7
4	1204779	0.0	1328739	-0.6
6	1208604	0.3	1327839	-0.7
9	1214408	0.8	1325630	-0.9
12	1221207	1.3	1324388	-1.0
15	1225460	1.7	1327070	-0.8
18	1223158	1.5	1324601	-1.0

21	1223843	1.5	1323624	-1.0
24	1225570	1.7	1324088	-1.0
27	1225974	1.7	1323443	-1.0
30	1225885	1.7	1324186	-1.0

Table 8: Standard and sample solution stability results

4.4. Linearity:

Performed the linearity with BTZ standard in the range of 50% to 150% of working concentration and covered minimum five levels from 80% to 120%. It is clear that the response of BTZ is linear between 50% - 150%. The correlation and regression coefficient were more than 0.998. The P-Value is >0.05 then the intercept is statistically equal to zero for BTZ, P-value of BTZ is 0.7 Hence, it is statistically equal to Zero. Moreover, the value of intercept is with in ±2.0% of the area response at 100%Level.

Preparation of Linearity Standard Stock Solution:

Weighed and transferred about 5.718mg of BTZ standard into 20ml volumetric flask. Dissolved and diluted to volume with diluent. Further dilutions are in the range of 1.25,2.15,2.40,2.65,2.90,3.20 and the volume is made up with diluents in the range of 5.0ml. The results are tabulated in the table 9, and the graphs are shown in the fig. 2,3 and the chromatogram shown in the fig.4.

S.No	Name	Rt	Purity 1 Angle	Purity 1 Threshold	Area (µV*Sec)	% Area
1	BTZ	---	4.371	0.536	1220496	100.00
2	Specified Impurity 1	3.295	0.164	0.374	1275556	100.00
3	Specified Impurity 2	5.986	0.181	0.403	1150561	100.00
4	Specified Impurity 3	2.188	0.884	1.234	1578622	100.00

Table 9

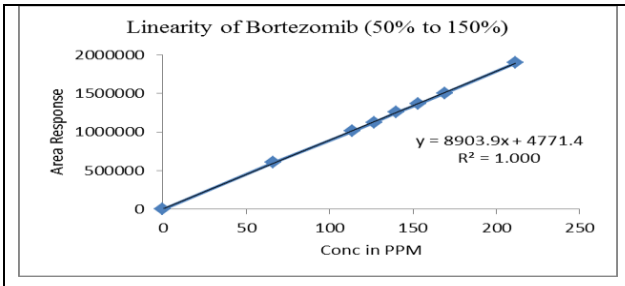


Fig.2: Linearity graph

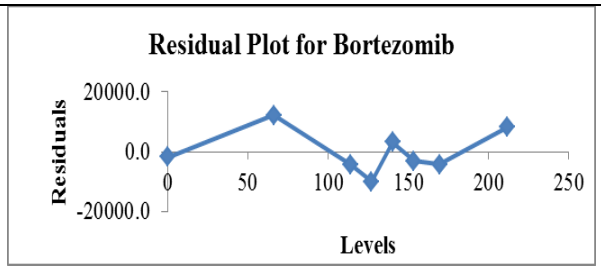


Fig.3: Residual plot

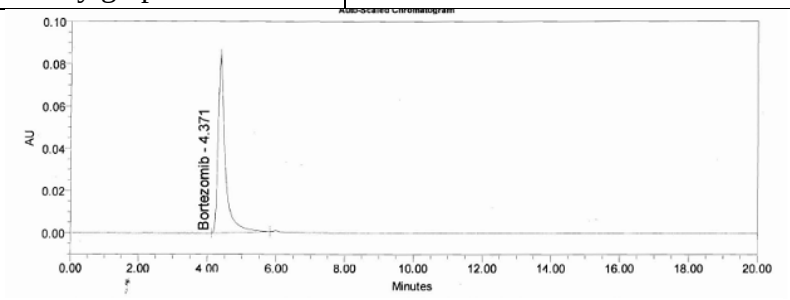


Fig.4: Chromatogram for BTZ

4.5. ACCURACY:

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Spiked known quantity of BTZ at 50%, 100% and 150% of working concentration into the placebo. Analyzed samples in triplicate for each level. Calculated the % recovery & RSD. From the results, it can be concluded that the recovery is well within the limit and recovery at LOQ level with in the acceptance criteria. Hence, the method is accurate and the results are tabulated in table 10.

Set	Levels (About)	Area Response	mg added	mg Added (Actual)	mg recovered	% recovery	Mean % Recovery	% RSD
1	50 %	613461	3.704	3.4255	3.4252	100.0	99.5	0.6
2	50 %	614441	3.721	3.4412	3.4302	99.7		
3	50 %	614437	3.751	3.4689	3.4297	98.9		
1	100 %	1254231	7.601	7.0294	7.0039	99.6	100.1	0.5
2	100 %	1257579	7.584	7.0137	7.0225	100.1		
3	100 %	1263914	7.590	7.0192	7.0579	100.6		
1	150 %	1924234	11.600	10.7277	10.7453	100.2	100.1	0.7
2	150 %	1912442	11.614	10.7406	10.6794	99.4		
3	150 %	1925098	11.541	10.6731	10.7501	100.7		

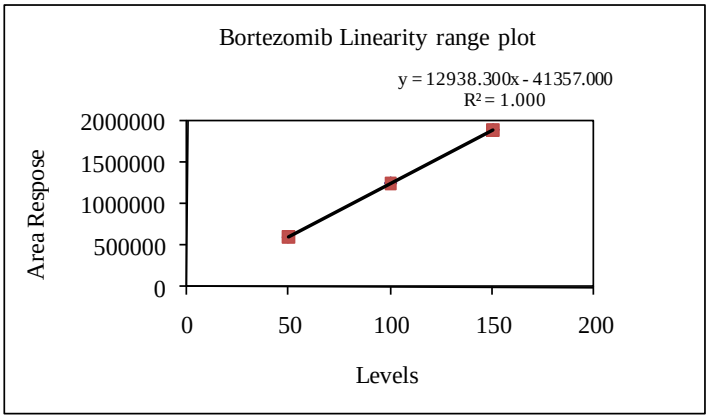
Table 10: Accuracy Results

4.6 **Range:**

The range of analytical method is the interval between the upper and lower levels of the analyte that has been demonstrated to be determined with a suitable accuracy, precision and linearity. From the below results, it can be concluded that the method is precise, linear and accurate between 50% and 150% levels of target concentration. The results are tabulated in the table 11 and Linearity range and accuracy range plots are shown in the Fig. 5 & 6.

S.No	**Linearity Range of BTZ**	**Accuracy Range of BTZ**
Level (About)	Mean Area of 3 sets for Accuracy	Mean Area ratio of 3 sets for Accuracy
50	603979	613947
100	1255631	1258574
150	1897809	1920591
Correlation coefficient	1.000	1.000
RSD	-	0.7

Table 11: Range Results



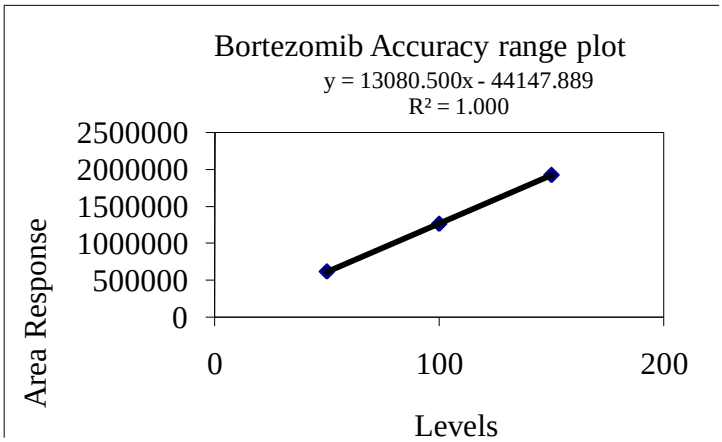


Fig. 6: Accuracy range plot

4.7. Robustness:

All known impurities should be separated from each other and from BTZ Peak in Sample spiked with impurities. Data shows that, this Assay method for BTZ for injection is robust towards small variations in method parameters. The results are shown in the table 12.

Change in Flow Rate	Initial	-0.2 ml/min	+0.20 ml/min
Flow Rate (ml/min)	1.5ml/min	1.3 ml/min	1.7 ml/min
The tailing factor is NMT 2.0	1.7	1.8	1.7
The RSD for replicate 5 injections	0.2	0.2	0.2
Column Efficiency for BTZ	3075	2357	2226
Change in Temperature	1.7	1.7	1.7
	0.2		
	3075	2194	2380
Equivalent column(Symmetry shield C18 column)	1.7	1.7	
	0.2	0.5	
	3075	3876	
Organic phase Change	1.7	1.8	1.7
	0.2	0.2	0.2
	3075	2258	2214

Table 12. Robustness results

5. Results

A satisfactory separation and good peak symmetry was found in a mixture of water, Acetonitrile, Formic acid in 715:285:1v/v ratio. Flow rate 1.5 ml / minute and wavelength detected at 270nm. The parameters considered for the Analytical Method validation of BTZ for injection assay and content uniformity 3.5mg/vial. For system suitability the Tailing factor is 1.4. The %RSD for 5 injections of standard preparation is 0.1.

6. Discussion

For Method precision the results are within specification limit. The %RSD calculated for Assay values of BTZ for 6 determinations is 0.2, for intermediate precision is 0.3 and for Content uniformity of BTZ for 10 determinations is 1.2. %RSD obtained for all the accuracy level determinations is 0.7. The Proposed HPLC Method of Assay for BTZ in the drug product BTZ for injection was validated as per analytical method validation. The method was found to be precise and robust.

7. Acknowledgement:

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