



# RP-HPLC Method Development and Validation for Determination of Bortezomib in Bulk Drug Substance and Pharmaceutical Dosage Forms

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## Abstract

A new, rapid, economical and isocratic reverse phase high performance liquid chromatography (RP-HPLC) method was developed for the determination of bortezomib in bulk drug substance and pharmaceutical dosage forms. The developed method was validated as per of ICH guidelines. The chromatographic separation was achieved isocratically on RP Phenomenex Gemini C<sub>18</sub> (250 mm x 4.6 mm I.D.) with particle size 5 μm was selected. Bortezomib exhibited linearity over the concentration range of **2-14 μg/mL (r<sup>2</sup> = 0.996)** with limit of detection of **0.282 mg/mL**. **The drug content was found to be 99.608 % for BZB**. The present successfully validated method with excellent selectivity, linearity, sensitivity, precision and accuracy was applicable for the assay of Bortezomib in bulk drug substance and pharmaceutical dosage forms.

**Key-words:** Bortezomib, Validation, Tablets, Bulk, RP-HPLC

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## Introduction

There are many reasons to validate analytical procedures. Among them are regulatory requirements, good science, and quality control requirement. The Code of Federal Regulations (CFR) 311.165c explicitly states that “accuracy, sensitivity, specificity, and reproducibility of test methods employed by the firm shall be established and documented”. Of course as Scientists we would want to apply good science to demonstrate that the analytical method used had demonstrated accuracy, sensitivity, specificity and reproducibility. Finally the management methods had demonstrated uses to release its product are properly validated for its intended use so the product will be safer for human use. [1]

Bortezomib is a dipeptide boronic acid derivative and proteasome inhibitor used to treat multiple myeloma and mantle cell lymphoma.<sup>1</sup>The 26S proteasome is a protein complex that degrades ubiquitinated proteins in the ubiquitin-proteasome pathway: reversible inhibition of the 26S proteasome, leading to cell cycle arrest and apoptosis of cancer cells, is thought to be the main mechanism of action of bortezomib. However, multiple mechanisms may be involved in the anticancer activity of bortezomib. Bortezomib was first synthesized in 1995.<sup>4</sup>In May 2003, bortezomib became the first anticancer proteasome inhibitor that was approved by the FDA under the trade name VELCADE.<sup>1</sup>Phase I, II, III, and IV clinical trials are undergoing to investigate



the therapeutic efficacy of bortezomib in leukemia, myasthenia gravis, systemic lupus erythematosus, rheumatoid arthritis, and solid tumours. Bortezomib, sold under the brand name Velcade among others, is an anti-cancer medication used to treat multiple myeloma and mantle cell lymphoma. This includes multiple myeloma in those who have and have not previously received treatment. It is generally used together with other medications. It is given by injection [2-4] The aim of present works in to develop and validate the drug using RP-HPLC method in bulk and tablet formulation.

**Methodology**

**Development of RP-HPLC Method for the Determination of Bortezomib (BZB) from Bulk and Injection**

In this study, a precise, sensitive and robust gradient reversed-phase HPLC (RP-HPLC) method was developed and validated for determination of BZB in API samples. The developed method was validated based on International Conference on Harmonization (ICH) guidelines and it was proved to be accurate, precise and robust. Additionally,

the limit of detection (LOD) and limit of quantification (LOQ) were also determined.

**Selection of chromatographic mode**

The reverse phase HPLC was selected for separation because it is convenient and rugged than other forms of the liquid chromatography and is more likely to result in a satisfactory final separation.

**Selection of stationary phase**

On the basis of reversed phase HPLC mode and number of carbon present in molecule (analyte) stationary phase with C<sub>18</sub> bonded phase i.e. RP Phenomenex Gemini C<sub>18</sub> (250 mm x 4.6 mm I.D.) with particle size 5 µm was selected.

**Selection of mobile phase**

The selection was made on the basis of literature survey. After assessing the solubility of drug in different solvents as well in mobile phases; Methanol was selected as a first choice.

**Selection of detector and detection wavelength**

Photo Diode Array detector was selected, as it is reliable and easy to set at the correct wavelength. From the spectra of drug, 270 nm wavelength was selected as detection wavelength.

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**Table 1: Initial chromatographic conditions**

Chromatographic mode	Chromatographic condition
Standard solution	100 µg/mL of BZB in methanol
HPLC System	Shimadzu HPLC system
Pump	LC-10AT VP solvent delivery system
Detector	SPDM-10AVP photo diode array detector
Data processor	Class-M 10 data station
Stationary phase	Phenomenex Gemini C <sub>18</sub> column(250mm x4.6mm,5µ)
Mobile phase	Methanol : water (80:20,v/v)
Detection wavelength	270 nm
Flow rate	1 mL/min
Sample size	10 µL
Column temperature	35 °C

**Preparation of standard stock solution**

Standard stock solution was prepared by dissolving

10 mg of BZB in 100 mL methanol and sonicated to complete dissolve, which gives concentration of



100 µg/mL of BZB.

**Optimization of chromatographic parameters**

Optimization in HPLC is the process of finding a set of conditions that adequately separate and enable the quantification of the analytes from the endogenous material with acceptable accuracy, precision, sensitivity, specificity, cost, ease and speed.

**Optimization of mobile phase strength**

The mobile phase was chosen after several trials with methanol and water in various proportions. A mobile phase consisted of methanol: water: (80:20, v/v) was selected to achieve symmetrical peak and sensitivity. The effects of flow rates in the ranges of 0.9 to 1.1 mL/min were examined. A

flow rate of 1 mL/min gave good sensitivity, system suitability parameter and reasonable retention time; using reversed phase C<sub>18</sub> column, the retention times of BZB was observed 14.48 min at 270 nm wavelength. The total time of analysis was less than 20 min.

**Optimization of detection wavelength**

PDA detector was used, as it is reliable and easy to set at the correct wavelength. A fixed concentration of analyte was analyzed at different wavelengths. As per the response of analyte, 270 nm wavelength was selected. A spectrum of BZB was shown in Figure.

Finalized chromatographic conditions for BZB

**Table 2: Final chromatographic conditions for BZB**

Chromatographic mode	Chromatographic condition
Standard solution	100 µg/mL of SLN in methanol
HPLC System	Shimadzu HPLC system
Pump	LC-10AT VP solvent delivery system
Detector	SPDM-10AVP photodiode array detector
Data processor	Class-M10 data station
Stationary phase	Phenomenex Gemini C <sub>18</sub> Column (250mmx4.6mm,5µ)
Mobile phase	Methanol : water (80:20,v/v)
Detection wavelength	270 nm
Flow rate	1 mL/min
Sample size	20 µL
Column temperature	35 °C

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**Linearity studies for BZB**

From stock solution aliquots of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 mL were taken in 10 mL volumetric flasks and diluted up to the mark with methanol such that the final concentration of BZB in the range 2-14 µg/mL. Volume of 10 µL of each sample was injected with the help of syringe. All measurements were repeated five times for each concentration and calibration curve was constructed by plotting the peak area vs the drug

concentration.

**Application of the proposed method to bulk sample of BZB**

Accurately weighed quantity 10 mg (BZB) was transferred to 100 mL volumetric flask. It was dissolved in methanol by sonication and volume was adjusted to mark and sonicated. The solution was further diluted to get concentration 5 µg/mL was subjected to proposed method and amount of BZB was determined. The procedure was repeated



for six times.

#### **Application of proposed method to injection formulation of BZB**

To determine the content of BZB in conventional injection (Label claim 2 mg Bortezomib per Vial) The twenty vials were weighed, their average weight determined and powder equivalent 2 mg BZB was transferred into a 10 mL volumetric flask containing 5 mL methanol, sonicated for 30 min and diluted to 10 mL with methanol. The resulting solution was filtered, using 0.45  $\mu\text{m}$  filter (Millifilter, Milford, MA). Excipients were separated by filtration. The solution was further diluted to get final concentration of 5  $\mu\text{g}/\text{mL}$  was analyzed by proposed method and amount of BZB was determined. The assay procedure was repeated for six times.

#### **Validation proposed of RP-HPLC method for the determination of bortezomib (BZB) from bulk and formulation**

The proposed method was validated as per ICH guidelines. The drug solutions were prepared as per the earlier adopted procedure given in the experiment.

#### **Accuracy**

It was done by recovery study using standard addition method at 80, 100 and 120 % level; known amount of standard BZB was added to pre-analyzed sample (5  $\mu\text{g}/\text{mL}$  of BZB) and analyzed by the proposed HPLC method.

#### **Precision**

Precision is the measure of how close the data values are to each other for a number of measurements under the same analytical conditions.

#### **Repeatability**

It is measured by multiple injections of a homogenous sample of 5  $\mu\text{g}/\text{mL}$  of BZB that indicates the performance of the HPLC instrument under chromatographic conditions.

#### **Intra-day and Inter-day precision**

Intra-day precision was determined by analyzing, the three different concentrations 6  $\mu\text{g}/\text{mL}$ , 8  $\mu\text{g}/\text{mL}$  and 10  $\mu\text{g}/\text{mL}$  of BZB, for three times in the same day. Day to day variability was assessed using above mentioned three concentrations analyzed

on three different days, over a period of one week. This result shows reproducibility of the assay.

#### **Robustness**

To evaluate robustness few parameters were deliberately varied. The parameters include variation of flow rate, percentage of methanol using 5  $\mu\text{g}/\text{mL}$  solution of BZB.

#### **Sensitivity**

Sensitivity of the proposed method was estimated in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ).  $\text{LOD} = 0.282 \text{ SD}/S$  and  $\text{LOQ} = 0.896 \text{ SD}/S$ , where SD is the residual standard deviation and S is the slope of the line. LOD and LOQ were found to be 0.282  $\mu\text{g}$  and 0.896  $\mu\text{g}$  for SLN, respectively.

#### **Specificity and Selectivity**

The analytes should have no interference from other extraneous components and be well resolved from them. Specificity is a procedure to detect quantitatively the analyte in presence of component that may be expected to be present in the sample matrix, while selectivity is the procedure to detect qualitatively the analyte in presence of components that may be expected to be present in the sample matrix. The method is quite selective. There was no other interfering peak around the retention time of BZB; also the base line did not show any significant noise.

#### **Ruggedness**

From the stock solution, sample solution of BZB (5  $\mu\text{g}/\text{mL}$ ) was prepared and analyzed by two different analysts using similar operational and environmental conditions. Peak area was measured for same concentration solutions, six times.

#### **System suitability test**

System suitability testing is essential for the assurance of the quality performance of the chromatographic system. Earlier prepared solutions for chromatographic conditions were tested for system suitability testing.

### **Results and Discussion**

#### **Development of RP-HPLC Method for Determination of Bortezomib (BZB) from Bulk and Tablets**

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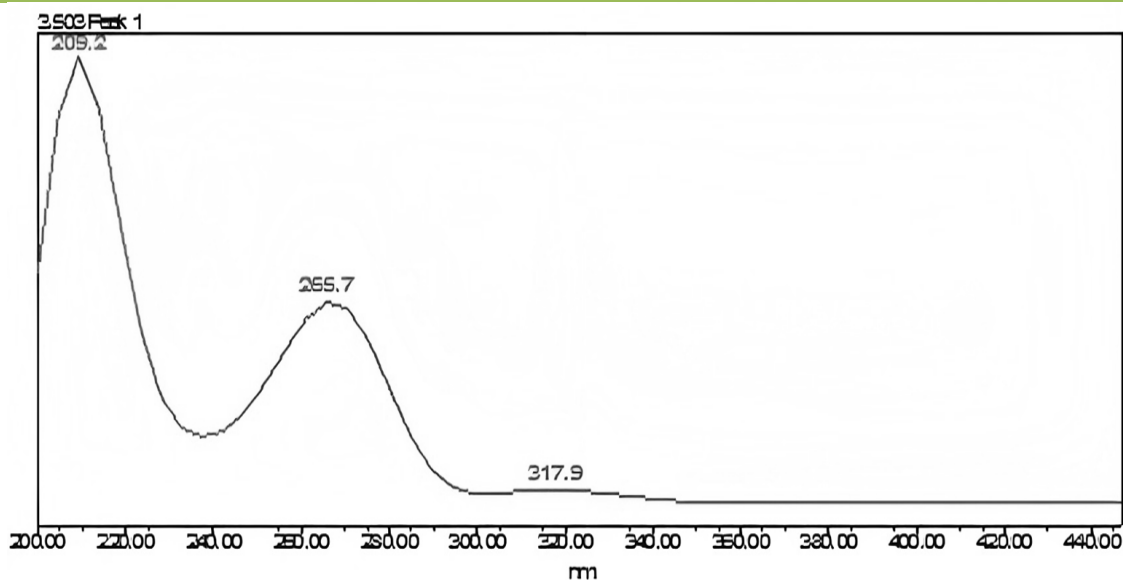
The HPLC analysis was performed on the Phenomenex Gemini C<sub>18</sub> (250 mm × 4.60 mm), 5µm particle size in isocratic mode, at 35 °C temperature using a mobile phase consisting of methanol: water (80:20, v/v) at a flow rate of 1.0 mL/min. Table represents the different concentration of mobile phase along with retention time in minutes of BZB. The detection was carried out at 270 nm. The spectra of BZB are depicted in Figure. Table represents the final chromatographic conditions employed for the detection of BZB in bulk and in formulation. Linearity was observed in the concentration range from 2-14 µg/mL (r<sup>2</sup> = 0.996) as shown in the Table. Figure depicted the Linearity of SLN with Correlation Coefficient = 0.996, Slope = 74207.52,

Intercept = 17598.80. The average retention time for BZB was found to be 14.48 min as shown in Figure . The limit of detection and quantitation of BZB was 0.282 µg and 0.896 µg, respectively. The method has been successively applied for the determination of BZB in bulk (Table). The method has been successively applied for the determination of BZB in injectables. There was no interference from the excipients commonly present in the injections. The drug content was found to be 99.608 % for BZB (Table). Figure represents the Chromatogram of BZB Tablet solution (5 µg/mL) can be interpreted that the retention time does not affected by the excipients of the formulation.

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**Table 4: Optimization of mobile phase strength for BZB**

Sr. No.	Mobile Phase Strength [Methanol: Water v/v]	Flow rate [mL/min]	R <sub>T</sub> of BZB [min]
1	80:20	1	14.48
2	90:10	1	18.67
3	100	1	19.28



**Figure 1: Spectra of BZB**

**Table 5: Final chromatographic conditions for BZB**



Chromatographic mode	Chromatographic condition
• Standard solution	100 µg/mL of BZB in methanol
• HPLC System	Shimadzu HPLC system
• Pump	LC-10 AT VP solvent delivery system
• Detector	SPD M-10AVP photo diode array detector
• Data processor	Class-M 10 data station
• Stationary phase	Phenomenex Gemini C <sub>18</sub> column (250 mm x 4.6mm, 5 µ)
• Mobile phase	Methanol: water (80:20, v/v)
• Detection wavelength	270 nm
• Flow rate	1 mL/min
• Sample size	20 µL
• Column temperature	35 °C

**Table 6: Linearity study of BZB**

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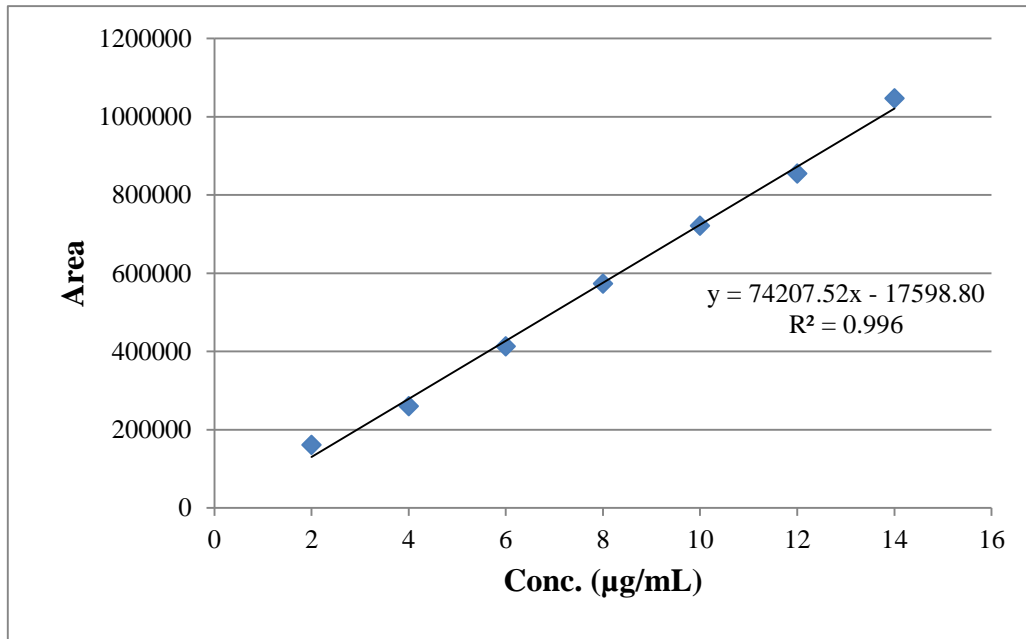
Sr. No.	Concentration of BZB [µg/mL]	Mean peak area [n=5]	%RSD
1	2	161239.28	2.72
2	4	260622.40	2.53
3	6	413051.65	1.96
4	8	573437.38	2.24
5	10	721537.13	2.32
6	12	855435.82	2.50
7	14	1047056.88	2.64

**Table 7: Analysis of BZB in bulk sample**

Component	Amount taken [µg/mL]	Amount Found [µg/mL]	Amount found [%]
BZB	5	5.02	100.36
	5	4.98	99.68
	5	5.01	100.26
	5	4.96	99.16



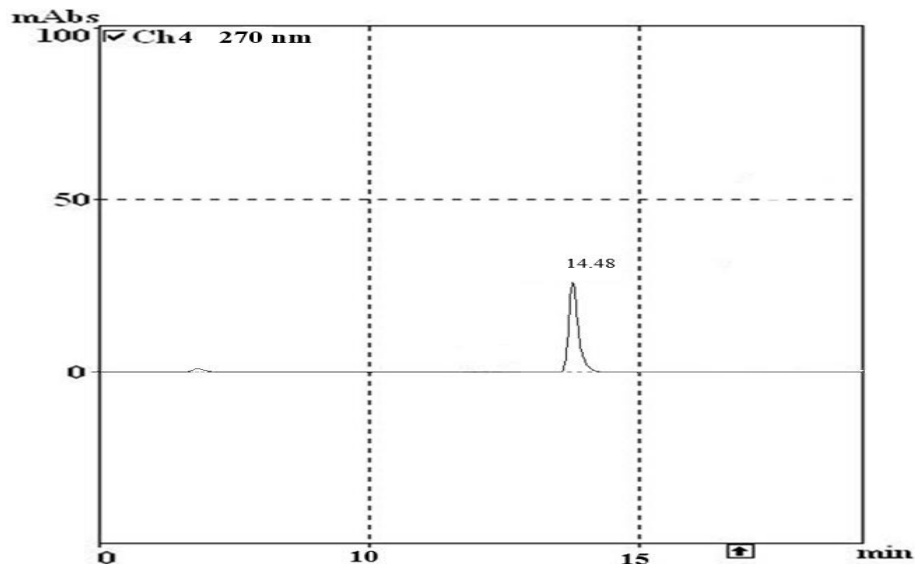
	5	4.98	99.62
	5	5.02	100.48
	Mean ± SD	4.996 ± 0.026	99.927 ± 0.519
	% RSD	0.520	0.520



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**Figure 2: Linearity of BZB**

**Correlation Coefficient = 0.996, Slope = 74207.52, Intercept = 17598.80**



**Figure 2: Chromatogram of standard BZB (5 µg/mL)**



**Table 8: Assay of BZB Injection**

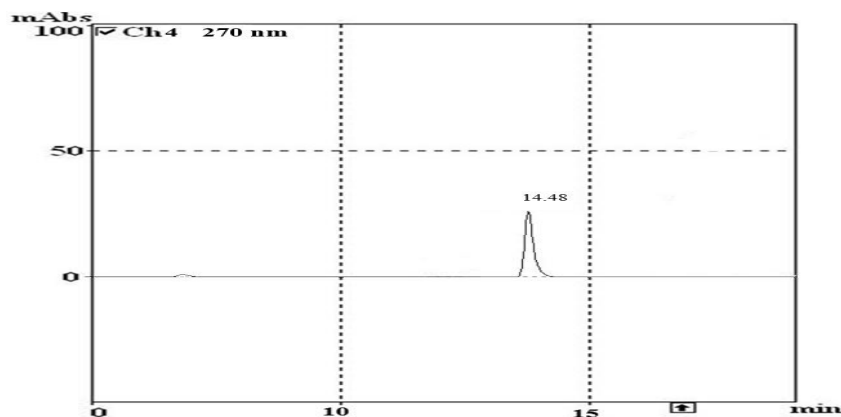
**Brand name: BORTECAD** (Oncocare A Division of Cadila Pharmaceuticals Ltd., Gujarat)

**Batch no. BBZL22052C Mfg.: 06/22 Exp.: 05/24**

**Average wt = 249.6 mg**

Drugs	Label claim [mg]	Amount found [mg]	Amount found [%]
BZB	2	1.989	99.45
	2	1.993	99.65
	2	2.023	100.95
	2	2.028	101.40
	2	1.983	99.15
	2	1.937	96.85
	Mean $\pm$ SD	1.992 $\pm$ 0.033	99.608 $\pm$ 1.639
	%RSD	1.645	1.645

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**Figure 3: Chromatogram of SBZB Injection solution (5 µg/mL)**

**Validation of Proposed RP-HPLC Method for Determination of Bortezomib (BZB) from Bulk and Injection**

Accuracy of the method was studied by the recovery studies at three different levels 80 %, 100 % and 120 % level. The results of recovery studies are presented in Table . The % recovery was found to be within the limits of the acceptance criteria with average recovery of 99.583-100.067. The mean average recovery was found to be 99.840 %. The % RSD below 2.0 shows the high precision of proposed method. According to USP (621), system suitability tests are an integral part of

chromatographic methods. They are used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solutions. Results of precision studies carried out intraday and interday by using different concentrations of BZB 6, 8 and 10 µg/mL and showed % RSD in range of 0.683-0.897 and 0.194-1.313 respectively. The mean percentage recovery was found to be 99.315 and 98.867 intraday and interday respectively by using different concentrations of BZB, the detailed results are summarized in table. Robustness evaluation of the



HPLC method was determined by different chromatographic conditions i.e. varying in flow rate and change in concentration of mobile phase. The study was performed in triplicate. The results

obtained in study are reported in table. Whereas, the table represents the results of ruggedness.

**Table 9: Results of recovery studies of BZB**

Drug	Initial amount [µg/mL]	Amount added [µg/mL]	Amount recovered ± SD [µg/mL, n = 3]	% Recovery	% RSD	SEM	Variance
BZB	5	0	5.003 ± 0.038	100.067	0.757	0.022	0.001
	5	4	3.983 ± 0.025	99.583	0.632	0.015	0.001
	5	5	4.997 ± 0.021	99.933	0.417	0.012	0.000
	5	6	5.987 ± 0.031	99.778	0.510	0.018	0.001

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**Table 5.10: Results of repeatability for BZB**

Sr. No.	Concentration [µg/mL]	Peak area
1	5	354981
2	5	328072
3	5	367338
4	5	336837
5	5	312867
6	5	360768
Mean ± SD		343477.167 ± 21066.769
% RSD		6.133

**Table 5.11: Results of precision studies of BZB (Intra-day and inter-day)**

Drug	Conc. [µg/mL]	Intra-day Amount found [µg/mL]		Inter day Amount found [µg/mL]	
		Mean ± SD	% RSD[n=3]	Mean ± SD	% RSD[n=3]
BZB	6	5.967 ± 0.047	0.792	5.920 ± 0.046	0.774
	8	7.907 ± 0.071	0.897	7.893 ± 0.015	0.194
	10	9.967 ± 0.068	0.683	9.943 ± 0.131	1.313



**Table 5.12: Robustness evaluation of the HPLC method for BZB**

Chromatographic conditions	R <sub>T</sub>	K'	T
A: Flow rate (mL/min)			
0.90	15.73	0.86	1.29
1.00	14.48	0.76	1.48
1.10	15.13	0.82	1.41
Mean ± SD	15.113 ± 0.625	0.813 ± 0.050	1.393 ± 0.096
B: Percentage methanol immobile phase (v/v)			
75	15.38	0.73	1.66
80	14.88	0.69	1.41
85	15.46	0.76	1.83
Mean ± SD	15.240 ± 0.314	0.727 ± 0.035	1.633 ± 0.211

**Table 5.13: Results of ruggedness of BZB**

Analyst	Amount found of BZB [%]	% RSD [n=3]
I	99.26	0.575
II	98.78	0.632

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**Table 5.14: System suitability test of BZB**

System suitability parameters	Proposed method
Retention time (T <sub>R</sub> )	14.48
Capacity factor (K')	0.813
Theoretical plate (N)	1827
Tailing factor (T)	1.513

**Table 5.15: Summary of validation parameters of BZB**

Parameters	Observation
Linearity range (µg/mL)	2 - 14
Regression equation	y = 74207.52x - 17598.80
LOD (µg)	0.282

LOQ ( $\mu\text{g}$ )	0.896
Recovery (%)	99.840
<b>Precision (% RSD)</b>	
Intra- day (n = 3)	0.683 – 0.897
Inter-day (n = 3)	0.194 – 1.313
Repeatability (n = 5)	0.57
<b>Ruggedness (% RSD)</b>	
Analyst I (n = 6)	0.575
Analyst II (n = 6)	0.632
Robustness	Robust
Specificity	Specific

### Conclusion

From the present study it was concluded that and confirmed the suitability of the method for quantifying BZB in their Pharmaceutical dosage form. Also, the RP-HPLC method was developed and validated.

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