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Development and validation of stability-indicating UHPLC methods for bortezomib injection solutions

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Abstract

A rapid and robust stability-indicating Ultra-High-Performance Liquid Chromatography (UHPLC) method was developed and validated for the quantitative determination of bortezomib in injectable formulations. The method employed a C18 column with gradient elution using a phosphate buffer and acetonitrile, achieving optimal separation within a short runtime. Linearity was confirmed across the concentration range of 2–10 mg/mL with $R^2 > 0.999$. The method was validated in accordance with ICH Q2(R2) guidelines, demonstrating specificity, precision, accuracy, robustness, and solution stability. Forced degradation studies under acidic, basic, oxidative, thermal, and photolytic stress confirmed that the method is capable of separating bortezomib from its degradation products with acceptable mass balance. The developed method is suitable for routine quality control and stability analysis of bortezomib injection solutions.

Keywords: Analytical method validation; bortezomib; degradation study; forced degradation; stability; UHPLC

1. Introduction

Bortezomib (chemically *N*–[(2S)-2-amino-3-methylbutanoyl]-L-phenylalanine 2,2,2-trifluoroacetate) is a proteasome inhibitor used to treat multiple myeloma and certain solid tumors, such as pancreatic, gastric, and ovarian carcinomas. It works by inhibiting the 26S proteasome, leading to an accumulation of pro-apoptotic proteins and accelerated degradation of anti-apoptotic proteins, which together trigger apoptosis in cancer cells [1,2]. Clinically, bortezomib is often combined with other agents (e.g., dexamethasone, lenalidomide, cyclophosphamide) to enhance its anticancer efficacy [3–5]. Notably, bortezomib-based regimens have achieved response rates up to 95% in patients with newly diagnosed multiple myeloma [6].

Bortezomib is a dipeptidyl boronic acid derivative. Its structure consists of two amino acid residues (phenylalanine and a modified leucine) linked to a pyrazine-based cap, with a boronic acid functional group at the C-terminus. The boron atom in bortezomib is essential for binding to the proteasome's catalytic site, a feature underpinning its inhibitory mechanism. Despite bortezomib's success in multiple myeloma, its effectiveness in solid tumors has been inconsistent [7,8]. Moreover, its safety profile is a significant concern in clinical practice. Patients commonly experience adverse effects such as peripheral neuropathy, thrombocytopenia, and gastrointestinal disturbances [9,10]. Relapse or disease progression is also observed in some cases after bortezomib treatment [2,11]. Clinicians have adopted strategies like dose adjustments and prophylactic antivirals to mitigate these toxicities and improve patient outcomes [9,12]. These challenges highlight the importance of rigorous quality control for bortezomib, particularly comprehensive evaluation of its purity and stability.

In pharmaceutical analysis, stability-indicating methods (SIMs) are crucial for ensuring the safety, efficacy, and quality of drug products. SIMs allow analysts to detect the active pharmaceutical ingredient (API) in the presence of its degradation products, impurities, and excipients, thereby revealing the drug's degradation profile under various conditions [13,14]. To develop a robust SIM, regulatory guidelines recommend subjecting the drug to a variety of stress conditions such

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as acidic or alkaline environments, oxidation, and photolysis [15,16]. These forced-degradation studies demonstrate the method's specificity by showing how the drug behaves under stress and confirming that the analytical method can distinguish the intact API from its degradation products. Furthermore, any stability-indicating method must be validated in accordance with FDA, ICH, or USP guidelines to ensure its accuracy, precision, and reliability [17,18].

Ultra-High-Performance Liquid Chromatography (UHPLC) is a powerful technique for implementing SIMs in pharmaceutical analysis. Previous studies have shown that reversed-phase UHPLC methods can provide high resolution and sensitivity, enabling the separation of bortezomib from its impurities and potential degradation products [19,20]. In this study, we adopted this reversed-phase UHPLC approach to assess its applicability and performance in the quantitative determination of bortezomib, with particular focus on its separation from impurities and degradation products.

UHPLC can detect impurities at levels as low as 0.1%, which is critical for monitoring drug stability [19,21]. Achieving such specificity and sensitivity requires careful optimization of chromatographic parameters, including the choice of stationary phase, mobile phase composition, flow rate, and detection wavelength [19,22].

Despite UHPLC's advantages, very few studies have reported stability-indicating UHPLC methods for bortezomib. In this study, we developed and validated a robust stability-indicating UHPLC assay for bortezomib (3.5 mg injectable formulation). We applied this method to evaluate bortezomib's stability profile and to identify degradation products arising from both the active drug and its excipients under various stress conditions.

Bortezomib is an antineoplastic agent belonging to the proteasome inhibitor class, widely used in the treatment of multiple myeloma and mantle cell lymphoma. Due to its clinical significance, developing accurate, sensitive, and reliable quantitative determination methods plays a critical role in ensuring the quality, efficacy, and safety of the drug [23]. In the literature, various techniques such as UV spectrophotometry, capillary electrophoresis, and different high-performance liquid chromatography (HPLC/UHPLC) methods have been reported for bortezomib determination [24]. However, some of these existing methods present limitations, including long analysis times, restricted separation efficiency, or peak co-elution with degradation products.

The stability-indicating UHPLC method developed in this study offers several advantages, including shorter analysis time, high resolution, low solvent consumption, and effective separation from degradation products. Furthermore, the method was validated in accordance with ICH Q2(R2) guidelines for robustness, accuracy, and repeatability, making it suitable for routine quality control and stability studies [25].

2. Materials and methods

The study protocol was developed according to the Analytical Method Validation Protocol, based on ICH Q2(R2) guidelines. Method specificity, accuracy, linearity, repeatability, intermediate precision, and robustness were assessed.

2.1. Materials

Mettler Toledo XP 2U Micro Balances (T-6 and T4; Switzerland), Waters Ultra High-Pressure Liquid Chromatography System (HP-6; USA), Thermo Scientific Variomag Multipoint Magnetic Mixer-MAN-1 (USA), Bandelin Sonorex RK510 Ultrasonic Bath UB-1 (Germany), and Milli-Q water purification system (Millipore, Milford, USA) were used in the study. Acetonitrile (UHPLC Grade, J.T. Baker, USA), Tetrahydrofuran (THF, Fischer Chemical, Switzerland), Ammonium Formate (UHPLC Grade, Sigma, USA), and Formic Acid (ACS Grade, Merck, Germany) were used as reagents. The specifications of the UHPLC system, active substance, finished product, and placebo are as follows:

The UHPLC system used in this study was an ultra-high performance liquid chromatography instrument equipped with a Zorbax Extend C18 column (100 × 4.6 mm, 1.8 µm). Detection was performed using a UV-VIS detector set at a wavelength of 270 nm. The injection volume was 10 µL, with a flow rate of 0.7 mL/min. The column temperature was maintained at 30 °C, while the autosampler was kept at 10 °C. The finished pharmaceutical product used in the study was bortezomib 3.5 mg powder for solution for injection (Batch No: 90617400), manufactured by Onko Pharmaceuticals. The corresponding placebo, labeled as bortezomib 3.5 mg powder for solution for injection – placebo (Batch No: 90617400-P), was also provided by the same manufacturer.

Bortezomib drug substance, reference standard, and four impurities (Impurity A: Bortezomib Amide; Impurity B:Hydroxyamide; Impurity C: Bortezomib Isomer; and Impurity D: Bortezomib Impurity Mixed Standard) were obtained from the R&D Center of Onko Pharmaceuticals.

The RC filter (regenerated cellulose, 0.45 µm) used in this study is a hydrophilic, low protein-binding membrane filter. It is produced by chemically regenerating cellulose to obtain a uniform structure with broad chemical compatibility (pH 3–12) and minimal

adsorption of proteins and small molecules. Such properties minimize analyte loss and effectively remove particulate matter from solutions, thereby protecting the UHPLC column from clogging and ensuring reproducible chromatographic performance [26].

The purity of a spectral peak is evaluated using the Peak Purity Angle (PA) and the Peak Purity Threshold (PT). PA represents the spectral deviation within the peak, while PT refers to the predetermined reference limit. A peak is considered spectrally pure when the condition PA < PT is met. However, this condition alone is not always sufficient to confirm purity, as co-eluting impurities may not be detected under certain conditions [27]. Therefore, in addition to spectral purity analysis, mass balance calculations, impurity profiling, and chromatographic resolution assessments are also used to ensure the reliability of purity analysis [28].

2.2. Preparation of standard solutions

2.2.1. Method for related substances

For the related substances analysis, Dilution Solution A (Fig.S1) was prepared by mixing 80 mL UHPLC-grade acetonitrile with 20 mL water, then degassing. This served as the primary diluent for standard and sample preparations. Dilution Solution B (Fig.S2), used for specific preparations, was obtained by mixing 30 mL acetonitrile with 70 mL water, followed by degassing.

Mobile Phase A (Fig.S3) was composed of 850 mL ammonium formate solution, 140 mL tetrahydrofuran (THF), and 10 mL acetonitrile. The mixture was filtered through a 0.2 µm membrane and sonicated for ≥10 min. Mobile Phase B (Fig.S4) consisted of 110 mL water, 140 mL THF, and 75 mL acetonitrile, also sonicated for ≥10 min.

The system suitability solution, used to verify chromatographic performance and resolution before analysis, was prepared by dissolving 1.0 mg bortezomib impurity standard in 1 mL acetonitrile in an amber vial, sonicating for 1 min, and storing at 2–8 °C for up to one week.

The placebo solution (Fig.S5), serving as a blank matrix in specificity studies, was prepared by dissolving two vials of bortezomib 3.5 mg powder for injection – placebo – in 1 mL water each, rinsing the vials with Dilution Solution B, and diluting to volume in a 20 mL amber flask. The solution was filtered through a 0.45 µm regenerated cellulose (RC) filter, discarding the first 1 mL of filtrate.

2.2.2. Preparation of stock and standard solutions for related substances analysis

An accurately weighed 17.5 mg bortezomib working standard was diluted with Dilution Solutions A and B to obtain stock (0.07 mg/mL) and standard (0.00175 mg/mL) solutions, followed by filtration through a 0.45 µm RC filter.

2.2.3. Preparation of solutions for assay method

Stock (0.7 mg/mL) and standard (0.14 mg/mL) solutions were prepared using similar procedures. Two independent standard solutions were prepared for quality assurance.

2.2.4. Validation parameters and stability-indicating forced degradation studies of bortezomib injection

The UHPLC method was validated in accordance with ICH Q2(R2) guidelines for reproducibility, selectivity, precision, and accuracy. Parameters evaluated included system suitability, accuracy, linearity, limit of detection (LOD), and limit of quantification (LOQ).

2.2.5. System suitability testing

Following baseline and pressure stabilization, system suitability and diluent solutions were analyzed. Acceptance criteria were Relative Standard Deviation (%RSD) of bortezomib peak areas <5.0% (related substances) and <2.0% (assay); theoretical plate number >40,000; tailing factor <2.0; and resolution ≥4.0 (bortezomib vs. isomer) and ≥1.2 (isomer vs. hydroxyamide).

2.2.6. Specificity and mass balance study

Specificity was assessed per ICH Q2(R2) using four main impurities (bortezomib amide, hydroxyamide, bortezomib isomer, and a mixed impurity standard) at relevant levels. Stress testing under acidic, alkaline, oxidative, thermal, photolytic, and hydrolytic conditions was performed. Stressed samples were neutralized and diluted before analysis. Peak purity was verified by PDA detection, and mass balance was calculated as the sum of bortezomib, impurities, and degradation products.

2.2.7. Linearity

Linearity was evaluated across five concentrations: 60–160% of specification level (assay) and LOQ–160% (related substances). Data were analyzed by least-squares linear regression.

2.2.8. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined at signal-to-noise ratios of 3:1 and 10:1, respectively. Precision at LOQ was assessed with six replicate injections; accuracy was verified by spiking impurities at LOQ concentration in triplicate.

2.2.9. Accuracy

Accuracy was determined at 80%, 100%, and 120% of the target concentration with triplicate injections at each level. Recoveries were calculated against known concentrations.

2.2.10. Precision

Repeatability was assessed using six independent injections of a standard solution; intermediate precision included different operators and days. %RSD was required to be <2.0% for the assay.

2.2.11. Robustness

Robustness was evaluated via a central composite design (CCD), varying flow rate (0.6–0.8 mL/min), column temperature (29–31 °C), and column type (two Zorbax Extend-C18 columns, 100 × 4.6 mm, 1.8 µm).

2.3. Chromatographic conditions and optimization

A stability-indicating UHPLC method was systematically developed to enable the accurate quantification of bortezomib in the presence of its related substances and potential degradation products. The development strategy was based on sequential optimization of mobile phase composition, pH, column selection, temperature, flow rate, and detection wavelength, with the goal of achieving high resolution, acceptable run time, and robust analytical performance.

In the initial stage, various combinations of organic modifiers (acetonitrile and methanol) and aqueous buffers (ammonium acetate and phosphate buffers) were evaluated across a pH range of 3.0–6.0. Acetonitrile was favored over methanol due to its lower backpressure and sharper peak profiles. Among buffer systems, ammonium formate at pH 4.0 provided the best compromise between selectivity and peak symmetry, especially for resolving bortezomib from its structurally similar isomer and hydroxyamide impurity.

The choice of stationary phase was critical. Several C18 columns with varying surface chemistries and particle sizes were tested. The selected column demonstrated superior performance with high theoretical plate counts, minimal peak tailing, and baseline separation between closely eluting peaks.

Temperature and flow rate were optimized in the ranges of 29–31 °C and 0.6–0.8 mL/min, respectively. The final conditions—30 °C column temperature and 0.7 mL/min flow rate—offered the best balance of resolution, retention time, and robustness, without compromising peak shape or system suitability.

The detection wavelength of 270 nm was selected based on UV absorption spectra, providing optimal sensitivity for bortezomib while ensuring adequate detection of all relevant impurities.

Overall, the finalized UHPLC method delivered reproducible and selective separation, laying the foundation for its validation and application in routine analysis of bortezomib injection formulations.

3. Results and discussions

3.1. Specificity, mass balance, and degradation conditions

The specificity of the developed UHPLC method was evaluated by subjecting Bortezomib injection solutions to various stress conditions in accordance with ICH Q1A(R2) guidelines [14,18,27]. Stress conditions included acid (Table S1 and Table S2), alkali (Table S3 and Table S4), oxidative (Table S5 and Table S6), thermal (Table S7 and Table S8), photolytic (Table S9 and Table S10) and hydrolytic (Table S11 and Table S12) degradation. These studies aimed to assess the method's ability to distinctly detect Bortezomib in the presence of its degradation products, excipients, and potential impurities.

In each case, chromatograms demonstrated well-resolved peaks without co-elution, confirming the method's specificity. Bortezomib peak purity was assessed using diode array detection (DAD), and the purity angle was consistently less than the purity threshold, indicating that the Bortezomib peak was spectrally homogeneous [25].

Mass balance calculations were also performed to evaluate the extent of degradation and recovery under each stress condition. Mass balance values ranged between 96.5% and 99.5%, supporting the conclusion that degradation products were adequately accounted for and that no significant loss of analyte occurred unaccounted for by degradation [12,18] (Table 1).

Forced degradation conditions were optimized to induce approximately 10–20% degradation to establish the stability-indicating nature of the method. Acidic and basic hydrolysis were achieved using 0.1 N HCl and 0.1 N NaOH at 70 °C for 10 minutes and 80 °C for 5 hours, respectively, yielding degradation products with well-resolved peaks. Oxidative stress using 0.5% H_2O_2 for 10 minutes and photolytic stress (1.2 million lux hours) resulted in moderate degradation. Thermal stress at 105°C for 72 hours showed that Bortezomib was comparatively more stable under heat, with minimal degradation observed [16,18,26].

The degradation products identified under these conditions did not interfere with the quantification of the main analyte peak, reaffirming the method's robustness and suitability as a stability-indicating assay [10,14,17].

The degradation products identified—particularly hydroxyamide—have been reported in the literature to potentially exhibit altered pharmacological or toxicological profiles [29,30].

Table 1. Mass balance study

Stress Condition	Time	Assay of Active Substance (% w/w)	Total Impurities (% w/w)	Mass Balance (Assay + Total Impurities) (% w/w)	Remarks
Thermal degradation (105 °C)	72 h	96.2	1.1	98.1	Impurity-B and hydroxyamide were major degradation products
Photolytic degradation	48 h	90.1	8.5	97.8	Hydroxyamide, Bortezomib Isomer were major degradation products
Acid degradation (5.0 N HCl, 70 °C)	10 min	85.7	14.0	99.4	Bortezomib Amide, Bortezomib Isomer, Hydroxyamide were major degradation products
Alkali degradation (0.1 N NaOH, 80 °C)	5 h	81.1	14.6	96.5	Bortezomib Isomer was the major degradation product
Oxidative degradation (0.5% H_2O_2)	10 min	91.7	9.5	98.1	Hydroxyamide was the major degradation product
Hyrolytic degradation (Room Temperature)	72 h	98.1	0.5	99.5	Minor impurities detected

While these were present in low concentrations, their presence under oxidative and UV stress underscores the importance of protective packaging and controlled storage conditions for bortezomib. Further studies evaluating the in vivo relevance of these degradation products are warranted.

3.2. Validation parameters

The validation parameters are summarized in Table 2, showing that the method met all acceptance criteria with %RSD values below 2.0, accuracy between 98.5% and 101.2%, and linearity (R^2) of 0.9995. The LOD and LOQ were determined as 0.05 µg/mL and 0.15 µg/mL, respectively.

Table 2. Summary of validation parameters

Validation Parameter	Result	Acceptance Criteria
System Suitability (%RSD)	< 2.0	< 2.0
Accuracy (Recovery, %)	98.5 - 101.2	98 - 102
Linearity (R^2)	0.9995	> 0.990
LOD	0.05 µg/mL	-
LOQ	0.15 µg/mL	-

3.3. System suitability

All acceptance criteria were met, as evidenced by the %RSD values, theoretical plate numbers, tailing factors, and resolution values, confirming the reliability and robustness of the UHPLC method [31,32]. %RSD values were <5.0% for Related Substances and <2.0% for the assay. The theoretical plate number exceeded 40,000, the tailing factor was <2.0, and the resolution criteria (≥4.0 between bortezomib and its isomer, ≥1.2 between the isomer and hydroxyamide) were met.

Stress conditions produced distinct degradation profiles under photolytic, thermal, acidic, basic, oxidative, and hydrolytic conditions. Peak purity testing confirmed no co-eluting impurities at the bortezomib retention time. Mass balance values in all stress conditions were close to 100%, confirming the stability-indicating nature of the method.

This analysis confirms that bortezomib requires careful storage and formulation considerations to minimize degradation, especially protection from strong acids, bases, oxidation, and light exposure. These results collectively suggest that the method accurately captures degradation pathways and maintains mass balance close to 100%.

3.3.1. Linearity

Linearity was established over a concentration range of 0.084 mg/mL to 0.223 mg/mL. The correlation coefficient (R^2) was determined to be 1.0000, indicating excellent linearity (Fig. 1). Calibration curves for bortezomib and impurities demonstrated linearity across the tested range, with correlation coefficients above 0.999. This high degree of correlation indicates that the method provides a proportional response across the tested range, fulfilling the ICH guideline requirements for quantitative analytical procedures.

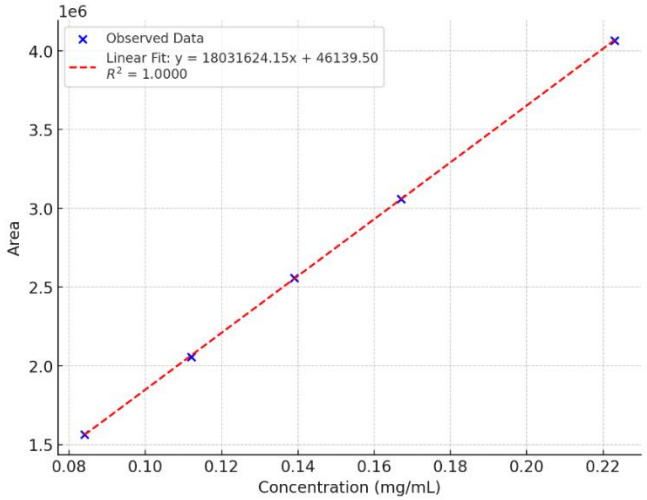

Figure 1. Calibration curve with regression

The regression equation for the linearity curve was given in Eq. 1.

$$y = 18031624.15x + 46139.5 \quad (1)$$

Table 3. Regression data

Parameter	Bortezomib	Impurity A	Impurity B	Impurity C	Impurity D
LOD (µg/mL)	0.039	0.014	0.027	0.041	0.045
LOQ (µg/mL)	0.162	0.053	0.108	0.167	0.182
Regression Equation (y)	$y = 14567x - 389$	$y = 21891x - 312$	$y = 19873x - 278$	$y = 11567x + 98$	$y = 13432x + 52$
Slope (b)	14567	21891	19873	11567	13432
Intercept (a)	-389	-312	-278	98	52
Correlation Coefficient (R^2)	0.9996	0.9995	0.9997	0.9993	0.9994

Note: To ensure consistency, all regression equations and calibration parameters were expressed using concentration units in µg/mL. The high slope values observed in the graphical calibration equation (Equation 1) are attributed to the use of mg/mL units during plotting, while Table 3 reports slope values based on µg/mL. After unit normalization, the data remained consistent, confirming the excellent linearity and accuracy of the method.

3.3.2. *Limits of detection (LOD) and quantification (LOQ)*

According to the LOD and LOQ results, the Y-intercept was found to be 2.22%, meeting the acceptance criteria [32]. The correlation coefficients (R^2 >0.999) indicate a strong linear relationship for bortezomib and its impurities. The LOD (0.039 µg/mL) and LOQ (0.162 µg/mL) for bortezomib indicate high sensitivity, while impurities show slight variations in detection limits. The validated method supports a LOQ–150% range for impurities and a 50–150% range for bortezomib. Precision at LOQ showed %RSD below 2%, and recovery studies at LOQ confirmed method accuracy.

3.3.3. *Accuracy*

Table 4 presents the recovery results for bortezomib spiked with its four known impurities at three concentration levels: 80%, 100%, and 120% of the target concentration. Each level was tested in triplicate. The recovery values for all samples are tightly clustered between 101.51% and 101.73%, indicating a high degree of accuracy in the analytical method. The reproducibility across the triplicates at each level is excellent, with minimal variation in both found concentrations and recovery percentages. These results demonstrate that the method accurately quantifies bortezomib and its impurities across a range of concentrations, even when impurities are present. Overall, the consistently high recovery rates validate the method's accuracy and reliability for quantifying bortezomib in the presence of its impurities.

Table 4. Recovery results of bortezomib spiked with its four impurities

Level (%)	Mean Recovery (%)	SD	n
80	101.51	0.29	3
100	101.65	0.20	3
120	101.73	0.27	3

To assess the accuracy of the developed method, known amounts of bortezomib reference standard were spiked into the placebo matrix at three concentration levels corresponding to 80%, 100%, and 120% of the nominal assay concentration (0.143 mg/mL). Each level was analyzed in triplicate. The calculated percent recoveries were within the acceptable range of 98–102%, with %RSD values below 0.5%, confirming the method's accuracy and repeatability (Table 5).

Table 5. Accuracy study

Nominal Concentration Level (%)	Spiked Concentration (mg/mL)	Mean % Recovery (n = 3)	%RSD
80	0.114	101.5	0.30
100	0.143	101.7	0.20
120	0.172	101.7	0.27

3.3.4. *Precision*

Table 6 presents the assay results for six replicate injections (N-1 to N-6) of a bortezomib sample at the 100% target concentration (7.00 mg/mL). The peak areas ranged from approximately 2,600,467 to 2,622,256, with only minor variation between replicates. The calculated assay values ranged from 102.01% to 102.86%, with a mean of 102.50% and a standard deviation (SD) of 0.31%.

These results indicate that the assay method provides excellent repeatability and accuracy, consistently measuring slightly above the nominal concentration. The %RSD value for the six replicates was below 2.0%, which meets the acceptance criteria for system precision in accordance with ICH guidelines.

Overall, the data confirm that the method is precise, robust, and suitable for the quantitative analysis of bortezomib at the 100% target concentration level.

Table 6. System precision results

Parameter	Mean (%)	SD	n
Assay (%)	102.50	0.31	6

3.3.5. *Robustness*

The Robustness of the method was evaluated to assess the reliability of the UHPLC method under slight variations in experimental conditions. A Box–Behnken experimental design was applied using Design-Expert® software (Version 13.0, Stat-Ease Inc., Minneapolis, USA) to investigate the influence of small but deliberate changes in the analytical parameters on the retention time and peak area of Bortezomib.

The column type (Phenomenex Kinetex C18 and Waters XBridge C18) was evaluated independently to

check method reproducibility but was not included in the statistical design.

A total of 15 experimental runs were carried out as per the BBD matrix. The results were statistically analyzed to determine the main effects, interactions, and quadratic effects of the factors on critical response variables such as retention time, peak area, and theoretical plate number of Bortezomib. The obtained polynomial models showed good predictive power with $R^2 > 0.98$. The selected factors were the flow rate (0.9, 1.0, and 1.1 mL/min), column temperature (28, 30, and 32 °C), and mobile phase pH (2.8, 3.0, and 3.2). Equation 2 demonstrates the regression model derived from the experimental design:

$$\text{Retention Time} = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 \quad (2)$$

where A: flow rate, B: column temperature, and C: pH of mobile phase.

Table 7 summarizes the experimental robustness results for Bortezomib under varied chromatographic conditions. The method proved to be robust, as small variations in the studied parameters did not significantly affect the assay performance.

Table 7. Robustness study results of bortezomib under varying conditions

Flow Rate (mL/min)	Column Temp (°C)	Mobile Phase pH	Retention Time (min)	Peak Area
1.0	30	3.0	4.515	2,614,354
1.0	28	3.0	4.637	2,579,061
0.9	30	2.8	4.788	2,523,745
1.1	30	3.2	4.221	2,681,201
1.0	32	3.0	4.395	2,640,651

The forced degradation studies provided critical insights into the stability profile of the bortezomib injection solution. Under acidic conditions, moderate degradation was observed, with minor formation of bortezomib amide, isomer, and hydroxyamide, while the mass balance remained above 99%, confirming that the degradation pathways were effectively captured by the method. Alkaline conditions caused a more pronounced decrease in assay values and a significant increase in the bortezomib isomer level. Despite these changes, our UHPLC method still yielded acceptable peak purity, resolution, and reproducibility.

In contrast, oxidative stress led to a marked formation of hydroxyamide, resulting in a drop in assay values that highlights the drug's sensitivity to oxidation. Thermal and hydrolytic conditions, on the other hand, produced only minor changes in both assay and impurity profiles, suggesting that bortezomib remains relatively stable under these stresses. Exposure to UV light was particularly harmful, causing substantial degradation and increased impurity formation; this underscores the need for proper light protection during storage and handling (Fig. 2).

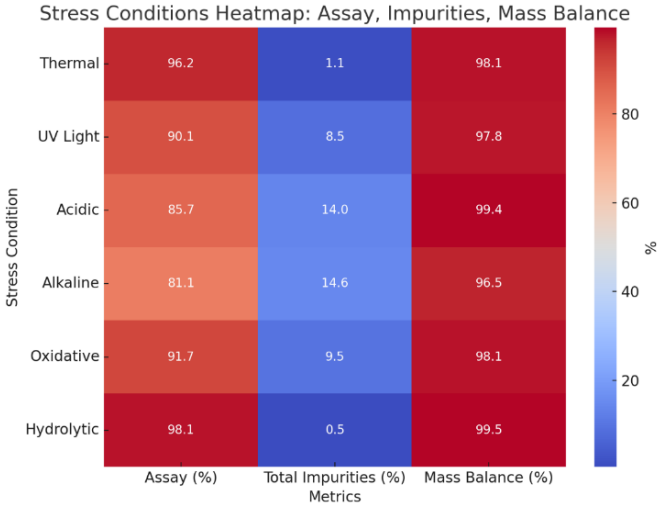

Figure 2. Stress conditions heatmap: assay, impurities, mass balance

4. Conclusions

In this study, a robust, accurate, and stability-indicating UHPLC method was successfully developed and validated for the quantification of bortezomib in injectable formulations. The method demonstrated excellent specificity, sensitivity, linearity, precision, and accuracy in accordance with ICH Q2(R2) guidelines. Forced degradation studies under various stress conditions (acidic, basic, oxidative, thermal, photolytic, and hydrolytic) confirmed the method's ability to distinctly separate bortezomib from its known impurities and degradation products, with mass balance values consistently above 96%.

The method's high sensitivity was reflected in low LOD and LOQ values for both bortezomib and its impurities, and the calibration curves exhibited excellent linearity with correlation coefficients (R^2) above 0.999. Accuracy and precision assessments confirmed the method's reliability across different concentration levels, with %RSD values below 2.0%. Moreover, recovery studies showed consistent results across spiked levels (80%, 100%, 120%), further supporting the method's reproducibility and suitability for routine analysis.

Given its comprehensive validation and strong performance in stress-testing scenarios, the proposed UHPLC method is well-suited for use in:

- Routine quality control during manufacturing,
- Stability testing in product shelf-life assessment,
- Regulatory submissions requiring validated analytical data for drug substance and drug product evaluation.

The ability to detect and quantify degradation products, such as hydroxyamide and bortezomib isomer, which may exhibit altered pharmacological or toxicological profiles, highlights the method's utility in ensuring patient safety and product integrity. Therefore, this validated method provides a reliable and practical tool for the pharmaceutical industry in the analytical control of bortezomib-containing formulations.

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Data availability statement:

The data presented in this study are available on request from the corresponding author.

Conflict of interest statement:

The author declared no conflict of interest.

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Supporting Information

Appendix A.

Development and validation of stability-indicating UHPLC methods for bortezomib injection solutions

Table S1. Results of acid degradation

Components		**Initial (%)**	**Acid Degradation (%)**	**Initial**		**Acid Degradation**	
				Purity1 Angle	**Purity1 Threshold**	**Purity1 Angle**	**Purity1 Threshold**
Blank Solution		-	-	-	-	-	-
Placebo Solution		-	-	-	-	-	-
	Bortezomib Amide	0.1	0.03	5.952	12.450	6.527	10.38
	Bortezomib Isomer	0.01	0.02	44.475	90.000	13.223	19.478
Sample	Hydroxyamide	0.2	0.2	7.767	9.727	7.143	9.375
Solution	Highest Unknown Impurity	0.04	13.7	10.940	17.044	0.271	7.044
	Total Impurity	0.4	14.0				
	Bortezomib	98.8 (assay result)	85.7 (assay result)	0.287	1.032	0.399	1.031
Acceptance Criteria		Mass balance should be achieved.		The peak purity angle must be less than the peak purity threshold.			

Table S2. Mass balance for results of acid degradation

Components	Result of Assay (%)	Total Impurities %	Mass Balance
Protected	98.8 %	0.4 %	-
Acid Degradation	85.7 %	14.0 %	99.4%

Table S3. Results of alkali degradation

Components		Initial (%)	Alkali Degradation (%)	Initial		Alkali Degradation	
				Purity1 Angle	Purity1 Threshold	Purity1 Angle	Purity1 Threshold
Blank Solution		-	-	-	-	-	-
Placebo Solution		-	-	-	-	-	-
Sample Solution	Bortezomib Amide	0.1	0.5	5.952	12.450	1.352	7.938
	Bortezomib Isomer	0.01	5.8	44.475	90.000	1.487	7.186
	Hydroxyamide	0.2	0.02	7.767	9.727	17.218	28.448
	Highest Unknown Impurity	0.04	8.2	10.940	17.044	0.422	7.100
	Total Impurity	0.4	14.6				
	Bortezomib	98.8 (assay result)	81.1 (assay result)	0.287	1.032	0.237	1.041
Acceptance Criteria		Mass balance should be achieved.		The peak purity angle must be less than the peak purity threshold			

Table S4. Mass balance for results of alkali degradation

Components	**Result of Assay (%)**	**Total Impurities %**	**Mass Balance**
Protected	98.8 %	0.4 %	
Acid Degradation	81.1 %	14.6 %	96.5%

Table S5. Results of oxidative degradation.

Components		Initial (%)	Oxidation Degradation (%)	Initial		Oxidative Degradation	
				Purity1 Angle	Purity1 Threshold	Purity1 Angle	Purity1 Threshold
Blank Solution		-	-	-	-	-	-
Placebo Solution		-	-	-	-	-	-
Sample Solution	Bortezomib Amide	0.2	0.2	3.483	9.654	1.353	10.592
	Bortezomib Isomer	0.03	N.D.	19.981	35.558	n/a	n/a
	Hydroxyamide	0.2	9.3	8.109	10.104	0.141	7.116
	Highest Unknown Impurity	0.05	0.06	10.900	17.980	5.829	19.488
	Total Impurity	0.5	9.5				
	Bortezomib	98.8 (assay result)	91.7 (assay result)	0.069	7.040	0.101	7.043
Acceptance Criteria		Mass balance should be achieved.		The peak purity angle must be less than the peak purity threshold.			

Table S6. Results of oxidative degradation.

Components	Result of Assay (%)	Total Impurities %	Mass Balance
Protected	98.8 %	0.5 %	
Acid Degradation	91.7 %	9.5 %	98.1%

Table S7. Heat degradation analysis results.

Components		Initial (%)	Heat Degradation (%)	Initial		Heat Degradation	
				Purity1 Angle	Purity1 Threshold	Purity1 Angle	Purity1 Threshold
Blank Solution		-	-	-	-	-	-
Placebo Solution		-	-	-	-	-	-
Sample Solution	Bortezomib Amide	0.1	0.1	5.952	12.450	0.932	5.681
	Bortezomib Isomer	0.01	0.03	44,475	90,000	11.999	18.285
	Hydroxyamide	0.2	0.1	7.767	9.727	6.954	8.909
	Highest Unknown Impurity	0.04	0.3	10.940	17.044	2.378	5.695
	Total Impurity	0.4	1.1				
	Bortezomib	98.8 (assay result)	96.2 (assay result)	0.287	1.032	0.199	1.033
Acceptance Criteria		Mass balance should be achieved.		The peak purity angle must be less than the peak purity threshold			

Table S8. Mass balance for results of heat degradation

Components	Result of Assay (%)	Total Impurities %	Mass Balance
Protected	98.8 %	0.4 %	
Acid Degradation	96.2%	1.1 %	98.1%

Table S9. Photolytic degradation analysis results.

Components		Initial (%)	UV Light Degradation (%)	Initial		UV Light Degradation	
				Purity1 Angle	Purity1 Threshold	Purity1 Angle	Purity1 Threshold
Blank Solution		-	-	-	-	-	-
Placebo Solution		-	-	-	-	-	-
Sample Solution	Bortezomib Amide	0.2	0.5	3.483	9.654	2.073	7.961
	Bortezomib Isomer	0.03	0.1	19.981	35.558	7.875	12.039
	Hydroxyamide	0.2	4.3	8.109	10.104	1.171	7.150
	Highest Unknown Impurity	0.05	0.55	10.900	17.980	3.973	8.145
	Total Impurity	0.5	8.5				
	Bortezomib	100.3 (assay result)	90.1 (assay result)	0.069	7.040	0.076	7.036
Acceptance Criteria		Mass balance should be achieved.		The peak purity angle must be less than the peak purity threshold.			

Table S10. Balance for results of Photolytic degradation.

Components	Result of Assay (%)	Total Impurities %	Mass Balance
Protected	100.3 %	0.5 %	
Acid Degradation	90.1%	8.5 %	97.8 %

Table S11. Hydrolysis degradation analysis results.

Components		Initial (%)	Hydrolysis Degradation (%)	Initial		Hydrolysis Degradation	
				Purity1 Angle	Purity1 Threshold	Purity1 Angle	Purity1 Threshold
Blank Solution		-	-	-	-	-	-
Placebo Solution		-	-	-	-	-	-
Sample Solution	Bortezomib Amide	0.1	0.1	5.952	12.450	7.813	13.055
	Bortezomib Isomer	0.01	0.002	44.475	90.000	73.494	90.000
	Hydroxyamide	0.2	0.3	7.767	9.727	7.224	9.824
	Highest Unknown Impurity	0.04	0.1	10.940	17.044	9.642	16.033
	Total Impurity	0.4	0.6				
	Bortezomib	98.8 (assay result)	98.1 (assay result)	0.287	1.032	0.134	8.266
Acceptance Criteria		Mass balance should be achieved.		Peak purity angel must be less than peak purity threshold			

Table S12. Mass balance for results of hydrolysis degradation.

Components	Result of Assay (%)	Total Impurities %	Mass Balance
Protected	98.8 %	0.4 %	
Acid Degradation	98.1%	0.5 %	99.5 %

Appendix B

Development and validation of stability-indicating UHPLC methods for bortezomib injection solutions

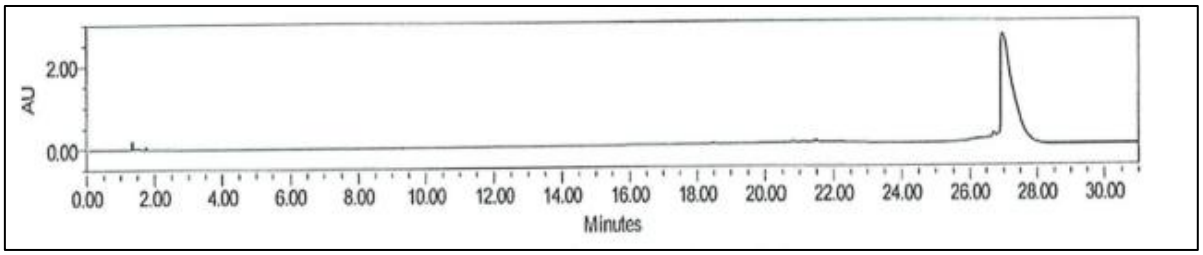


Figure S1. Diluent solution A chromatogram.

Diluent Solution A was prepared by mixing 80 mL of UHPLC-grade acetonitrile with 20 mL of water, followed by degassing after mixing. The Figure should illustrate the mixing process, container type, and degassing step.

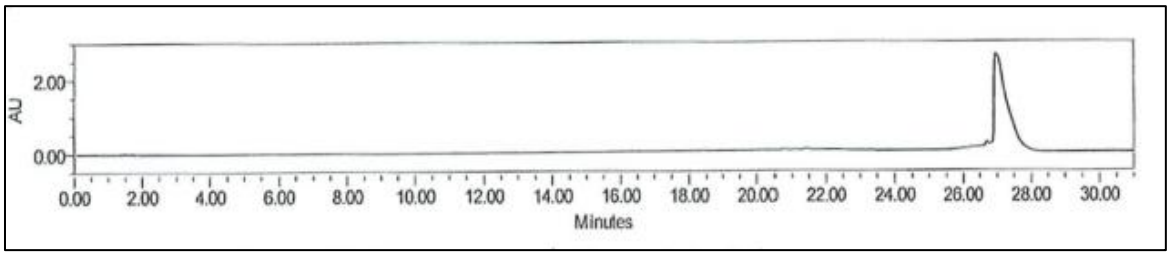


Figure S2. Diluent solution B chromatogram.

Diluent Solution B was prepared by mixing 30 mL of acetonitrile with 70 mL of water and degassing before use. The Figure should show the preparation setup and degassing step.

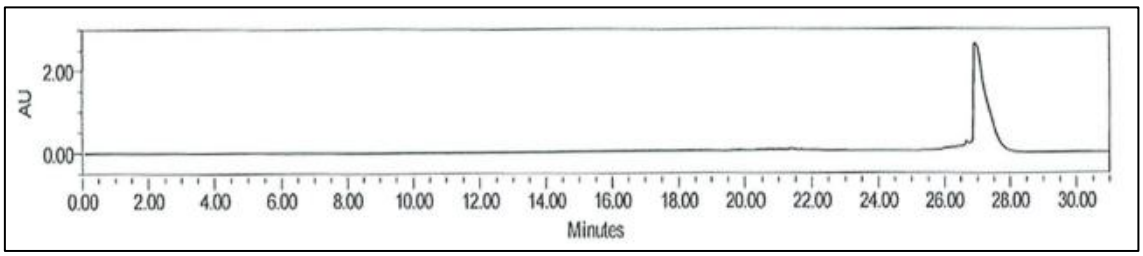


Figure S3. Mobile phase A chromatogram.

Phase A consisted of 850 mL ammonium formate solution, 140 mL tetrahydrofuran (THF), and 10 mL acetonitrile. The mixture was filtered through a 0.2 μm membrane and sonicated for at least 10 minutes before use. The Figure should depict the solution preparation, filtration, and sonication process.

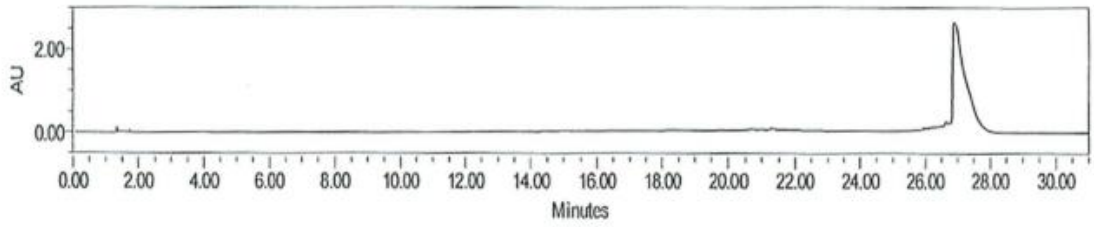


Figure S4. Mobile phase B chromatogram.

Mobile Phase B was prepared by combining 110 mL of water, 140 mL of THF, and 75 mL of acetonitrile, followed by sonication for a minimum of 10 minutes. The Figure should show the mixing and sonication steps.

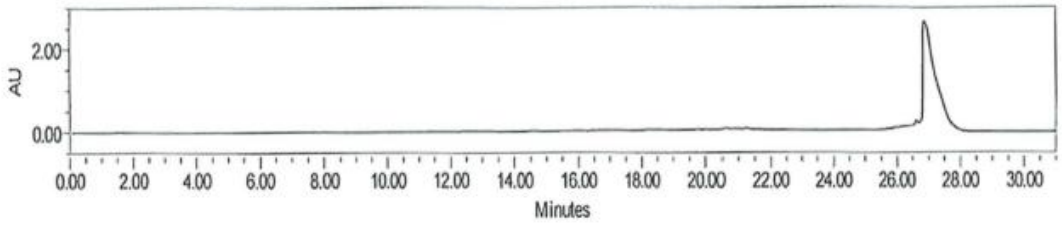
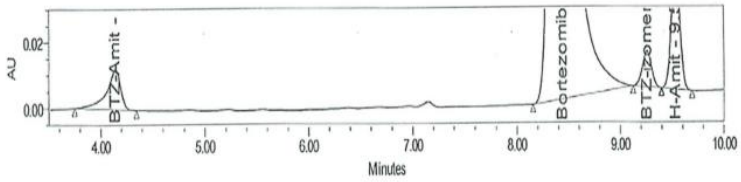
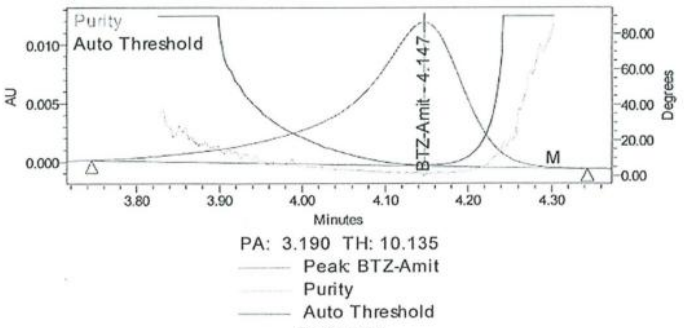


Figure S5. Plasebo chromatogram.

The placebo solution was prepared by dissolving two vials of bortezomib 3.5 mg powder for injection – placebo with 1 mL of water each, rinsing the vials with Dilution Solution B, and diluting to volume in a 20 mL amber flask. The solution was then filtered through a 0.45 µm regenerated cellulose (RC) filter, discarding the first 1 mL of filtrate. The Figure should illustrate each preparation step, including filtration.



	Peak Name	RT	Area	% Area	Height	Purity1 Angle	Purity1 Threshold
1	BTZ-Amit	4.147	116498	0.73	12277	3.190	10.135
2	Bortezomib	8.444	15506874	97.50	2117836	0.696	1.255
3	BTZ-izomer	9.262	66249	0.42	10828	2.905	9.101
4	H-Amit	9.539	214434	1.35	38348	0.866	2.831



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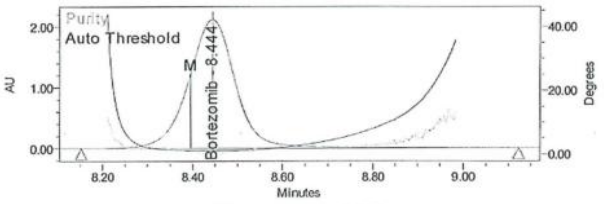
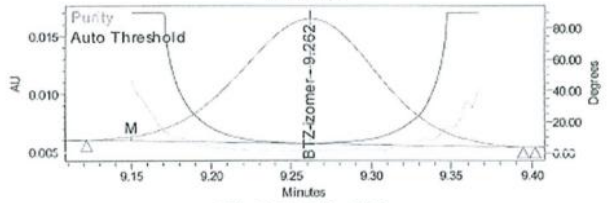
Peak BTZ-Amit
Purity
Auto Threshold

Purity Plot



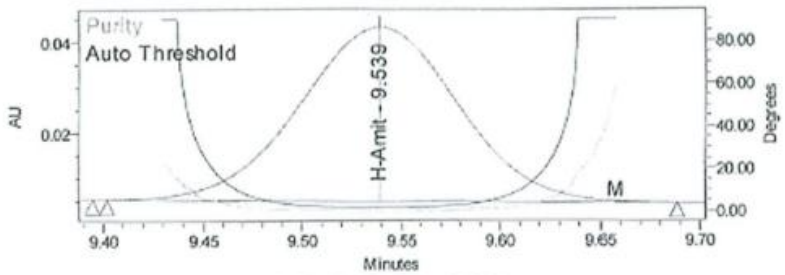
PA: 0.696 TH: 1.255
Peak Bortezomib
Purity
Auto Threshold

Purity Plot



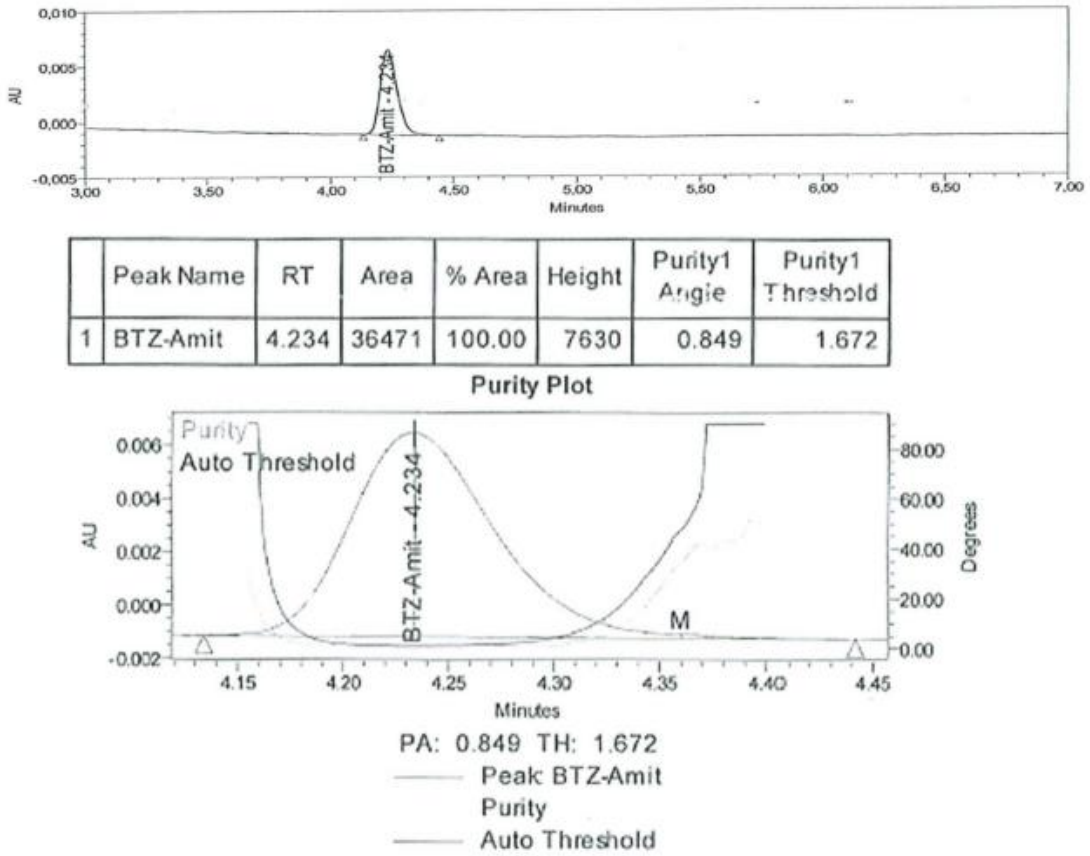
PA: 2.905 TH: 9.101
Peak BTZ-izomer
Purity
Auto Threshold

Purity Plot



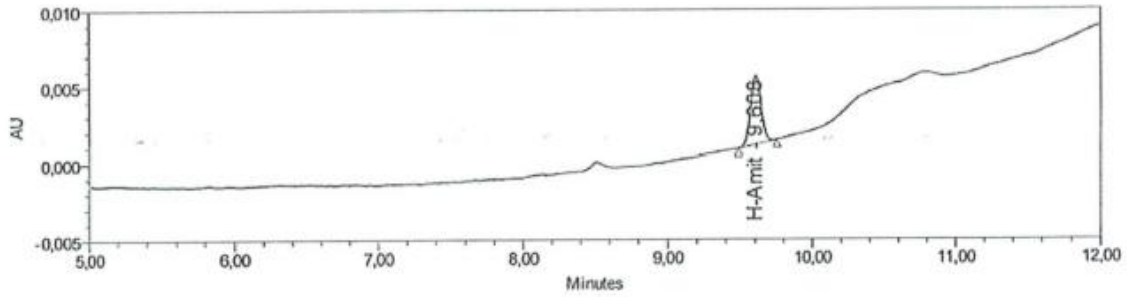
PA: 0.866 TH: 2.831
Peak H-Amit
Purity
Auto Threshold

Figure S6. System Suitability Chromatogram.



	Peak Name	RT	Area	% Area	Height	Purity1 Angle	Purity1 Threshold
1	BTZ-Amit	4.234	36471	100.00	7630	0.849	1.672

Figure S7. Limit Level Bortezomib Amit Solution Chromatogram.



	Peak Name	RT	Area	% Area	Height	Purity1 Angle	Purity1 Threshold
1	H-Amit	9.608	22500	100.00	4511	1.613	3.048

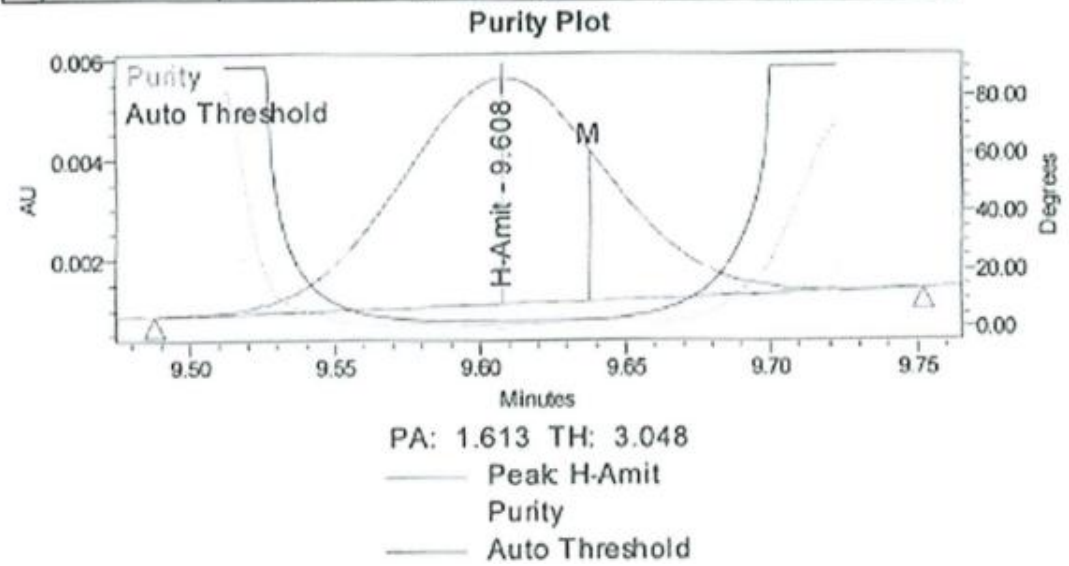
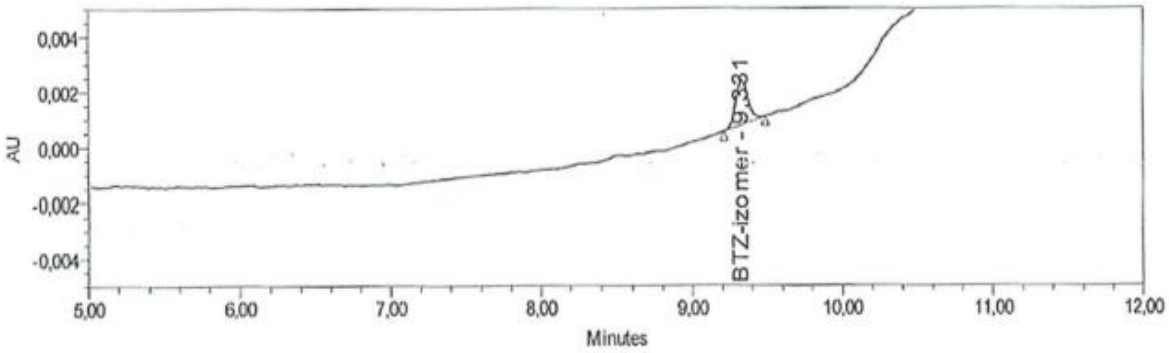


Figure S8. Limit Level Hydroxyamide Solution Chromatogram.



	Peak Name	RT	Area	% Area	Height	Purity1 Angle	Purity1 Threshold
1	BTZ-izomer	9.331	9228	100.00	1625	1.768	6.786

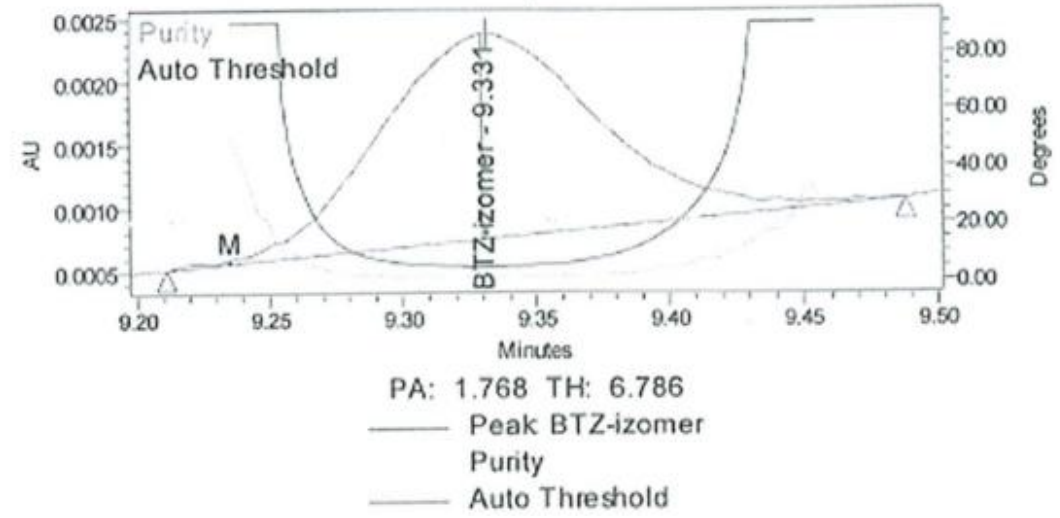
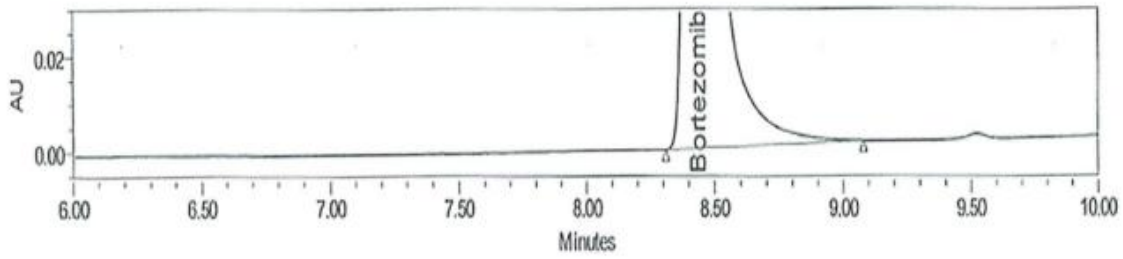


Figure S9. Limit Level Bortezomib Isomer Chromatogram.



	Peak Name	RT	Area	% Area	Height	Purity1 Angle	Purity1 Threshold
1	Bortezomib	8.442	2611183	100.00	453967	0.070	0.265

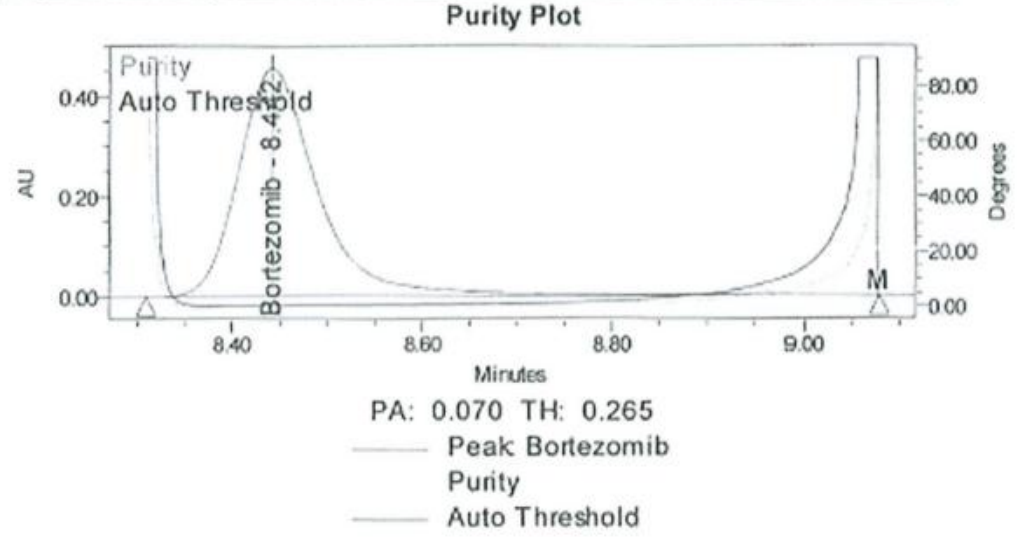
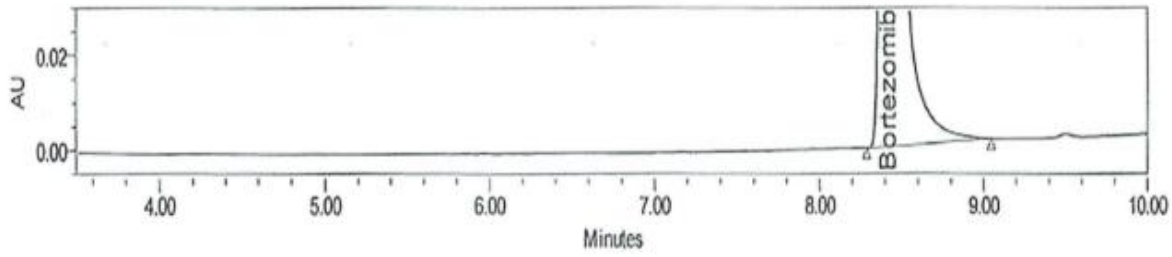


Figure S10. Standard Solution Chromatogram.



	Peak Name	RT	Area	% Area	Height	Purity1 Angle	Purity1 Threshold
1	Bortezomib	8.425	2649701	100.00	445219	0.068	0.264

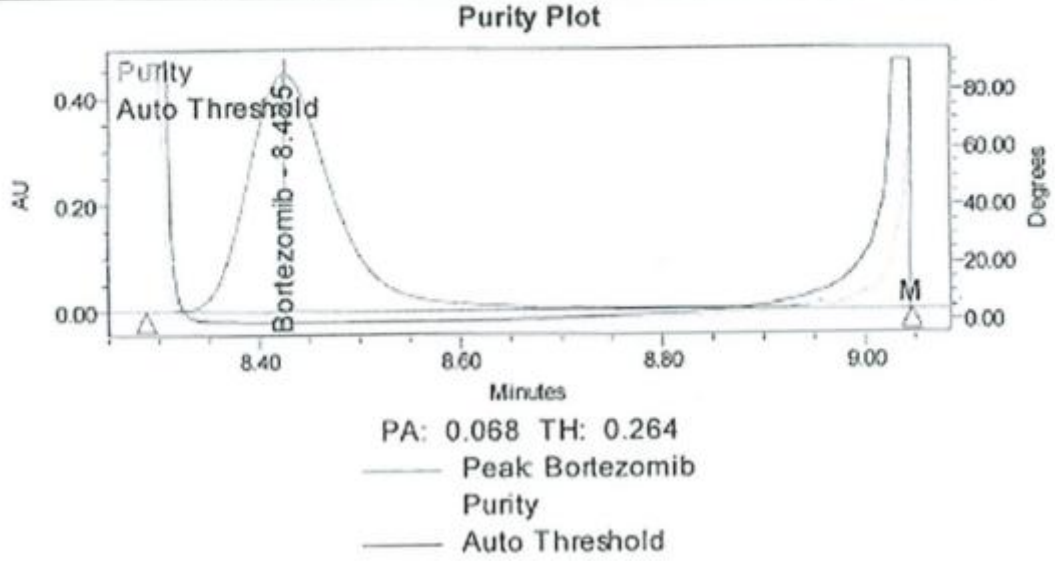
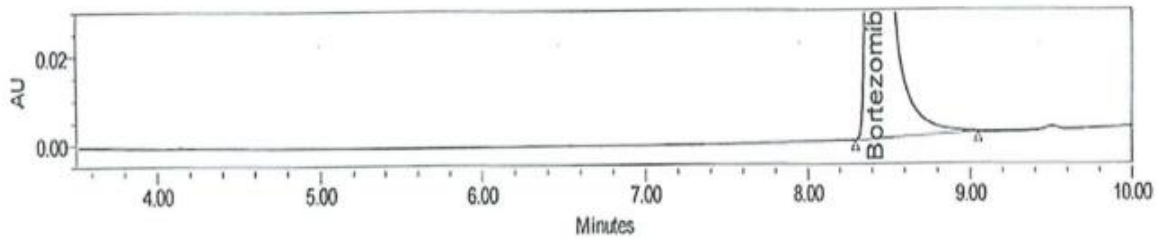


Figure S11. Raw Material Solution Chromatogram.



	Peak Name	RT	Area	% Area	Height	Purity1 Angle	Purity1 Threshold
1	Bortezomib	8.426	2619769	100.00	456037	0.070	0.263

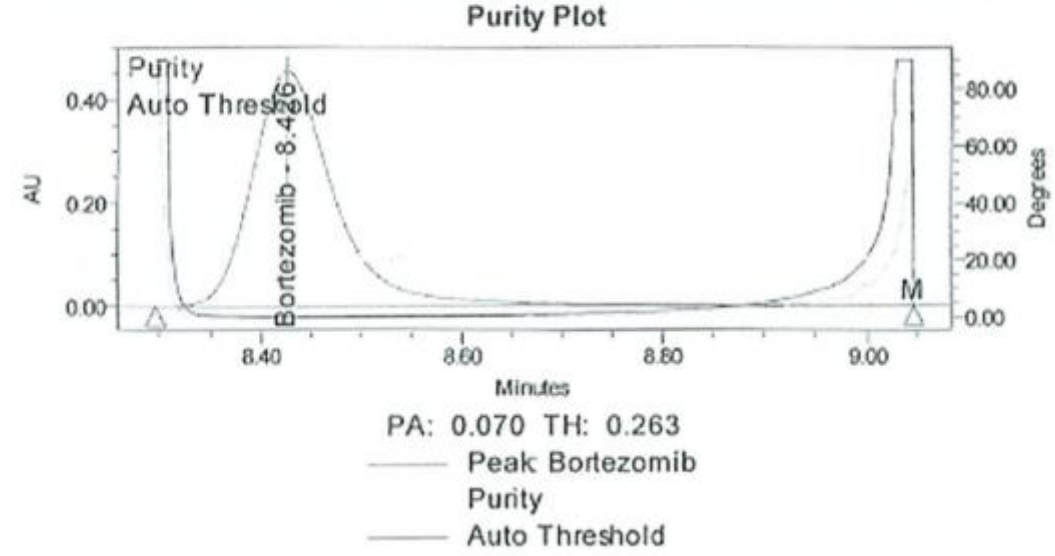
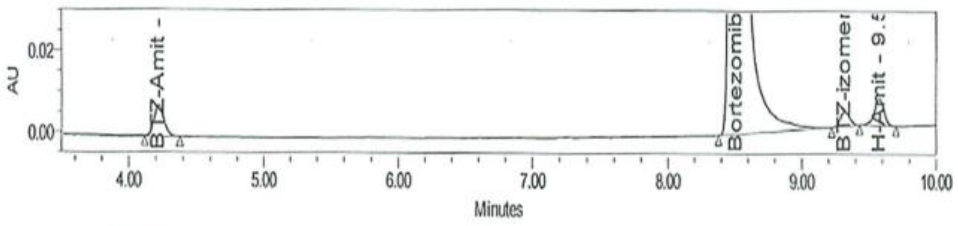
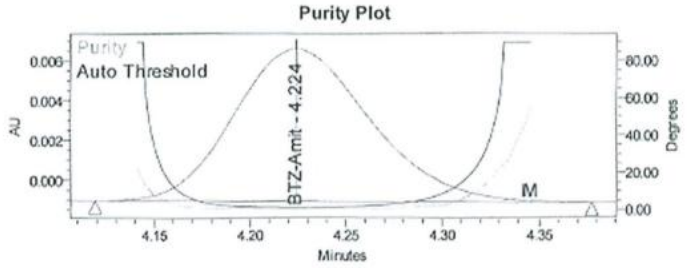


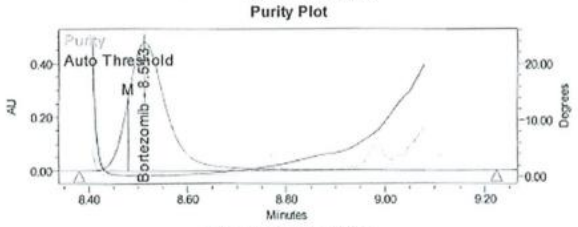
Figure S12. Raw Material + Placebo Solution Chromatogram.



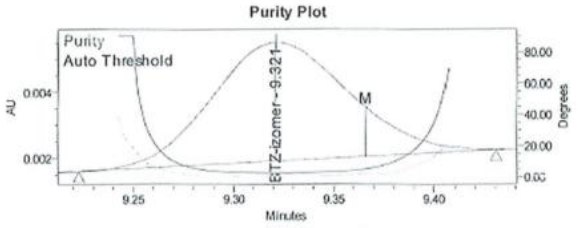
	Peak Name	RT	Area	% Area	Height	Purity1 Angle	Purity1 Threshold
1	BTZ-Amit	4.224	37928	1.40	7657	0.730	1.942
2	Bortezomib	8.513	2627750	96.74	480614	0.074	0.290
3	BTZ-izomer	9.321	17109	0.63	3613	1.243	3.580
4	H-Amit	9.580	33528	1.23	6644	0.607	2.249



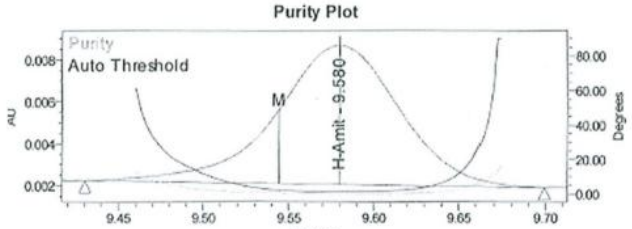
PA: 0.730 TH: 1.942
Peak BTZ-Amit
Purity
Auto Threshold



PA: 0.074 TH: 0.290
Peak Bortezomib
Purity
Auto Threshold

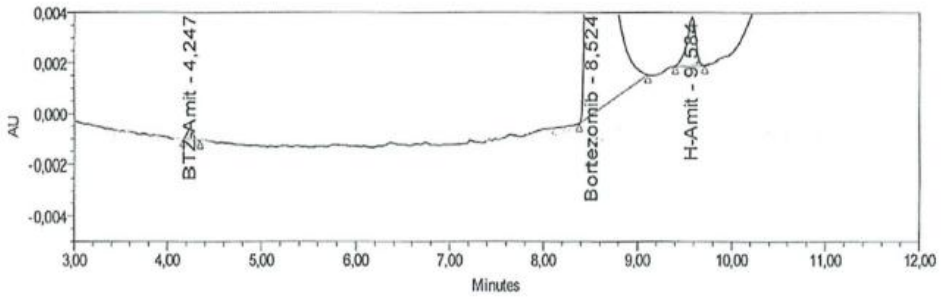


PA: 1.243 TH: 3.580
Peak BTZ-izomer
Purity
Auto Threshold

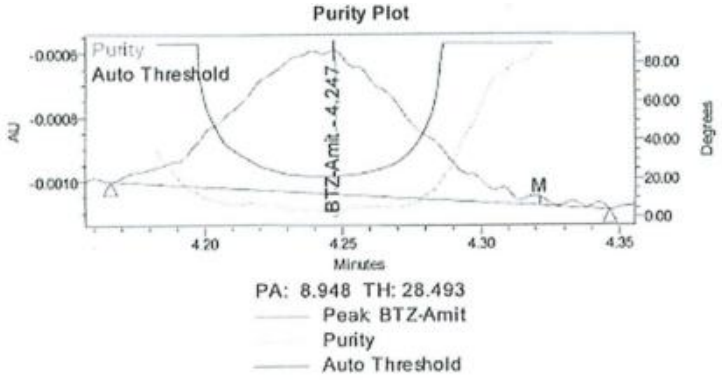


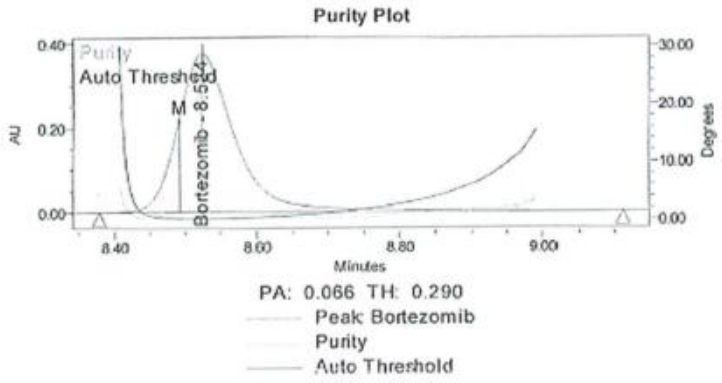
PA: 0.607 TH: 2.249
Peak H-Amit
Purity
Auto Threshold

Figure S13. Raw Material + Placebo + Impurities Solution Chromatogram.



	Peak Name	RT	Area	% Area	Height	Purity1 Angle	Purity1 Threshold
1	BTZ-Amit	4.247	1967	0.09	449	8.948	28.493
2	Bortezomib	8.524	2100359	99.33	375642	0.066	0.290
3	H-Amit	9.584	12244	0.58	1928	2.368	7.130





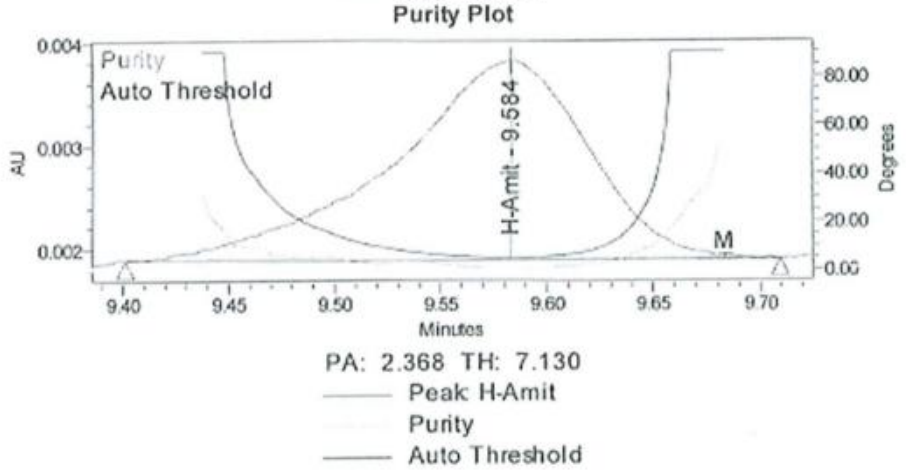


Figure S14. Sample Solution Chromatogram.