British Journal of Pharmaceutical Research
14(4): 1-17, 2016; Article no.BJPR.30565
ISSN: 2231-2919, NLM ID: 101631759

SCIENCEDOMAIN *international*
www.sciencedomain.org

Development and Validation of Bio Analytical Method for Estimation of Bortezomib in k_3 EDTA Human Plasma Using HPLC-ESI-MS/MS and Its Application to a Bioequivalence & CME Studies

Ravi Pratap Pulla[1*], K. Vanitha Prakash[1], U. B. Abhini[1], B. Naga Maheswari[1], V. Divya[1], M. V. Shushmitha[1] and V. Ravali[1]

[1]SSJ College of Pharmacy, V. N. Pally, Gandipet, Hyderabad-75, Telangana State, India.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJPR/2016/30565
Editor(s):
(1) Rahul S. Khupse, Pharmaceutical Sciences, University of Findlay, USA.
Reviewers:
(1) Marina Quartu, University of Cagliari, Italy.
(2) Tommasina Guglielmelli, San Luigi Hospital, Italy.
Complete Peer review History: http://www.sciencedomain.org/review-history/17605

Original Research Article

Received 18th November 2016
Accepted 3rd January 2017
Published 26th January 2017

ABSTRACT

Purpose: To develop a highly selective, reproducible & precise rugged bio analytical method for estimation of Bortezomib (BTZ), "A Protease Inhibitor" in human plasma by validating the developed method in accordance to US-FDA guidelines.
Methodology Envisaged: BTZ D_3 was used as an internal standard (ISTD) for the determination of BTZ in human plasma using a rapid & specific liquid chromatographic – Electron Spray Ionization –Mass spectrometric method. The analytical method was moduled with liquid-liquid phase extraction by using annular centrifugal contactor & the samples were analyzed by HPLC, on a column - ACE 5CN (150 x 4.6 mm 5 µm), using mobile phase consisting of ammonium formate buffer: ACN (25:75 v/v), delivered at 1.0 ml/min & 90% flow spitting. Applied Bio system MDS Sciex API 3000 Triple Quadruple MS equipped with Turbo Ion Spray (TIS) as LC/MS interface was used in for MS detection. TIS with multiple reaction monitoring (MRM) were acquired by ESI mass spectra, using the transitions m/z 362.95→310.21 & m/z 172.64→146.06 to quantify BTZ & BTZ D_3 respectively.

**Corresponding author: E-mail: ravipratappulla@gmail.com;*

Results: % variability was ≤ 5.52 & ≤ 6.15 [that was ≤ 15], indicating the specificity of the method, showing no matrix interferences across the elution system. Acceptance is ranging between -8.30 to 2.83 & -4.32 to 1.00% (< 5% CV) & accuracy in the range of 92.73 – 102.20 (< 10% difference) was observed over a linear range of 2.00 – 1000 ng/mL. The mean (n=3) correlation coefficient was 0.9991 & overall mean recovery was 85.62%. Retention time for drug & ISTD is found out to be 0.08 & 0.07; % CV of area ratio is 1.91% & area ratio ≤ 2.51%, which indicated system suitability.

Interpretation and Conclusion: The intended analyte is stable below 10ºC in all the performed stability experimentation & within the acceptance limits. It can be used for investigating drug concentration in routine quality control analysis in API & its pharmaceutical dosage forms.

Keywords: Bortezomib; Bortezomib D_3; method validation; HPLC-ESI-MS/MS; human plasma; multiple reaction monitoring.

1. INTRODUCTION

Bortezomib (BTZ) is the first therapeutic proteasome inhibitor [1-3] tested in humans. Proteasomes are cellular complexes that break down proteins. Bortezomib interrupts this process and lets those proteins kill the cancer cells. It is used for treating relapsed multiple myeloma and mantle cell lymphoma [4, 5]. BTZ, originally codenamed PS-341 (Fig. 1) is chemically, [(1R)-3-methyl-1-({(2S)-3-phenyl-2-[(pyrazin-2-ylcarbonyl) amino] propanoyl} amino) butyl] boronic acid. It has a molecular formula of $C_{19}H_{25}BN_4O_4$ and a molecular weight of 384.237 g/mol.

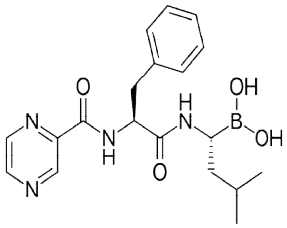

Fig. 1. Bortezomib

The drug is an N-protected dipeptide boronic acid analogue. The boron atom in BTZ binds the catalytic site of the 26S proteasome [6] with high affinity and specificity. In normal cells, the proteasome regulates protein expression and function by degradation of ubiquitylated proteins, and also cleanses the cell of abnormal or misfolded proteins. While multiple mechanisms [7-9] are likely to be involved, proteasome inhibition may prevent degradation of pro-apoptotic factors, permitting activation of programmed cell death in neoplastic cells dependent upon suppression of pro-apoptotic pathways. In PK studies, after SC administration plasma levels are ~25-50 nM and this peak is sustained for 1-2 hrs. After IV injection, peak plasma levels are ~500 nM but only for ~5 minutes [10,11]. PD studies are measured by measuring proteasome inhibition in peripheral blood mononuclear cells [12].

As evident from literature, the techniques of choice for the analysis of BTZ, and/ or its metabolites in biological samples include HPLC with UV; [13-18] UFLC method [19] and Mass spectroscopy detection, [15,20,21] have been developed. The aim of the present study was to develop a sensitive, selective and rapid HPLC-ESI-MS/MS method for the estimation of BTZ in human plasma for clinical studies. The proposed method is practically free from matrix interference and is successfully applied for bioequivalence studies in healthy subjects. From the point of view, a new quantification method of BTZ in human plasma was processed, using LLE (Liquid – Liquid Extraction phase) with Methyl-tert-Butyl Ether (MTBE) and assessed by tandem mass spectrometric detection. The method was developed and validated according to the FDA guidelines on bio analytical method validation [22]. The present study provides a best alternative with a simpler and cheaper approach for the quantification of BTZ in human plasma respectively.

2. EXPERIMENTAL MODULE

2.1 Chemicals and Materials

Bortezomib (BTZ, purity: 99.7%) was obtained from Unichem Laboratories Ltd., Mumbai, India, Bortezomib D_3 (Internal Standard -BTZ D_3, purity: 99.2%) was procured from Varda Biotech (P) Ltd., Mumbai, India and Drugs for Concomitant Medication Experiment (CME)- Acetaminophene (Samex Overseas Surat, India), Domperidone (Intra Labs Pvt., Lmited, Bangalore, India), Ibuprofen (Varda Biotech (P) Ltd, Mumbai, India)

& Cetirizine Hydrochloride (Unichem Laboratories Limited, Mumbai, India.) were also procured. HPLC-grade methanol (MeOH), Acetonitrile (ACN) and buffer capacitor (NH_4HCO_2) were procured from Merck Life Sciences Pvt., Limited Mumbai, India. LLE - annular centrifugal contactor was purchased from Techno Force Solutions (i) Pvt., Limited, Mumbai, India. MTBE was purchased from Mivon chemicals, Mumbai, India. Water was purified using Milli-Q Plotwater purification system from Millipore (Bedford, MA, USA). Blank human blood was collected with Na-Heparin as anticoagulant from healthy and drug free volunteers at St. Theresa Blood Bank, Hyderabad, India. Plasma was separated by centrifugation at 2061 g at 10ºC and stored at -70°C.

2.2 Operative Equipment

A Shimadzu-LC-20 Series HPLC system (Kyoto, Japan) with an ACE 5CN (150 x 4.6 mm, 5 µm) column from ACT Ltd., (Aberdeen, Scotland) was used for chromatographic separation of the analytes. The column temperature was maintained at 40°C. The mobile phase consisted of Ammonium formate buffer: ACN (25:75 v/v), delivered at 1.0 ml/min & 90% flow spitting. The auto sampler temperature was maintained at 5°C, injection volume was kept at 2 µL, and the pressure of the system was maintained at 440 psi. The LC system was connected to a triple quadruple mass spectrometer MDS Sciex API – 3000 (Toronto, Canada), equipped with Electro Spray Ionization (ESI) and operated in positive ionization mode.

The optimized source parameters for the analyte and internal standard (BTZ & BTZ D_3) were set as follows: Turbo ion spray interface voltage - 2500V; Curtain gas - 40 psi; [(Gas 1: 48 psi); (Gas 2: 60 psi)]; turbo heater temperature - 460°C; Collision activation dissociation - 7 psi. The sample was done by LLE, using 300 µL process volumes. The compound dependent mass parameters and multiple reaction monitoring (MRM) transitions were used for quantization of analytes and internal standard respectively. Watson LIMS Version 7.3, Analyst Version 1.4.2 was used to control all parameters of LC and MS. The data capturing system was an Apple iMAC MK142HN. Annular centrifugal contactor, the Tarson 3020 Spinix vortex shaker and the tube rotator – PTR-35 360º Vertical multi-function rotator were used. Beckman Coulter - Biomek® series auto pipettes were used for dispensing plasma and stock solutions. Polypropylene sample tubes (5 ml) from Bio Globus, New Delhi and Chromacol (Inkarp Instruments Pvt. Ltd., Hyderabad, India) 250 ml auto sampler vials were used throughout.

2.3 Preparation and Calibration of Standard and Quality Control Samples

BTZ stock solution (2 mg/mL) was prepared by dissolving accurately weighed amounts in methanol. A 50 - fold dilution of the stocks were prepared in methanol, refrigerated and protected from light for up to 45 days. Calibration Standards (CSs) and Quality Control (QC) samples were made by spiking blank plasma with appropriate volumes of working solutions prepared from intermediate stock solutions for both the analytes. The final CSs concentrations were 2, 4, 8, 12, 25, 50, 100, 200, 500, & 1000 ng/mL for BTZ respectively. The QC samples were prepared at six levels, i.e., 2.00 ng/mL (LLOQ - lower limit of quantification), 2.00 ng/mL – (LLOQ QC **-** lower limit of quantification quality control), 6.00 ng/mL (LQC – Low Quality Control), 41.0 ng/mL (MQC – Medium Quality Control), 880 ng/mL (HQC – High Quality Control) and 1000 ng/mL (ULOQ – Upper Limit of Quantification) for BTZ respectively.

Separate stock solutions for Internal standard - BTZ D_3 (2, 6, 41 & 880 ng/mL) were prepared fresh on day of analysis run by dissolving accurately weighed amounts in methanol. The combined working solution was prepared from their stock solutions in mobile phase ammonium formate buffer: ACN (25:75 v/v). Standard stock and working solutions were used for spiking and stored at 2–8°C until use, while CSs and QC samples in plasma were kept at -70°C.

2.4 Sample Extraction Procedure

Prior to extraction, all frozen subject samples, CSs and QC samples were thawed and allowed to equilibrate at normal room temperature. Fresh QC samples, bulk spiked QC samples & Calibration standards were extracted in 5 ml polypropylene tubes. Accurately 250 µL of plasma was pipetted out into prelabelled polypropylene tubes and to this 50 µL of ISTD (1 µg/mL) was added and vortexed for 20 seconds. To this 2.5 ml of MTBE was added and vortexed for 10 minutes, and the tubes were centrifuged for 5 min at 4500 rpm, and the upper organic phase was transferred to another 5 ml

polypropylene tube and evaporated to dryness under a steam of nitrogen at 60ºC (N-EVAP11155, Organomation, USA), for 25 minutes. The residue obtained, was added in 150 µL of mobile phase by vortex mixing at 3000 rpm for 3 min. The final reconstituted sample obtained was transferred to the glass auto sampler vial insert and 2 µL was injected into the chromatographic system.

2.5 Chromatographic Methodology and Mass Spectrometric Conditions

Mobile phase of NH_4HCO_2 buffer, 10 mM pH 4.5: Acetonitrile (25:75 v/v) was delivered at a rate of 1.0 mL/minute, 90% flow spitting using, Shimadzu-LC-20 Series HPLC system (Kyoto, Japan) with an ACE 5CN (150 x 4.6 mm 5 µm) column from ACT Ltd., (Aberdeen, Scotland), with a split of 200 µL to mass spectrometer and 800 µL to waste was maintained at 40°C [23]. The injection volume was of 2 µL and the injector needle was washed in methanol: ACN (50:50 v/v). Peaks of the HPLC–MS/MS chromatograms were evaluated using Watson LIMS Version 7.3, Analyst Version 1.4.2. The mass spectrometer was operated in positive ion mode with the Turbo Ion Spray (TIS) heater set at 450ºC (MDS Sciex API – 3000 (Toronto, Canada).

The samples were analyzed employing the transition of Drug: m/z 362.95→310.21 for BTZ with a dwell time of 220 milli sec. The mass transition for the ISTD was m/z 172.64→146.06, with the same dwell time. The TIS voltage was set at 5500.00 and the decluster potential was set at 82.00, 90.00 V and the collision energy at 32.00, 25.00 for BTZ & BTZ D_3 respectively. The entrance potential was set at 10.00V and the focusing potential at 400V. The nebulizer gas (nitrogen) pressure was set at 8 (arbitrary units). The curtain gas (nitrogen) was set at 15.00 (arbitrary units).

2.6 Method Validation Assessment

The validation of the method was in accordance with the United States Food and Drug Administration (USFDA) guidelines [22] were performed and the detailed procedures and their acceptance criteria are briefed in.

2.6.1 Bioequivalence and concomitant medication experimental (CME) analysis- A study design

The selective experimental study design was an open label, randomized, balanced, crossover, two- treatment, two-period and two-sequence bioequivalence design between a single dose of BTZ & BTZ D_3 (i.e.) 2 mg/vial (NATCO Bortenat-Deep Lifecare Ltd., New Delhi, India) with CME Drugs concentrations - Acetaminaphene (4.00 µg/mL), Nimesulide (9.00 µg/mL), Cetirizine (400 ng/mL), Ibuprofen (45.0 µg/mL), Domperidone (20.0 ng/mL), Ranitidine (545 ng/mL) and Diclofenac (2.00 µg/mL). The studies were accomplished following International Conference on Harmonization, E6 Good Clinical Practice guidelines [24].

3. RESULTS AND DISCUSSION

3.1 LC-MS/MS method Development/ Optimization

In order to develop a method with the desired sensitivity (0.1 ng/mL), it was necessary to use MS/MS detection, as the compound did not possess the UV absorbance or fluorescence properties which needed to achieve this limit. The inherent selectivity of MS/MS detection was also expected to be beneficial in developing a selective and sensitive method. Mass spectrometry settings were carried out in the positive electro spray ionization (ESI) mode due to the presence of N-protected dipeptide boronic acid analogue group which can be readily protonated under acidic conditions in the mobile phase. Initially, the Q1 MS spectra obtained by infusing 100 ng/mL solutions of BTZ and ISTD contained abundant protonated precursor ions at m/z 362.95→310.21 for BTZ with a dwell time of 220 ms and mass transition for the ISTD (BTZ D_3) was m/z 172.64→146.06, with the same dwell time. The source dependent and compound dependent parameters were suitably optimized to obtain a consistent and sufficient response for BTZ. A dwell time of 200 ms afforded a sufficient number of data points for the quantization of BTZ, avoiding cross talk between BTZ and ISTD with identical product ions. Under the optimized conditions, the cross talk experiment showed no detectable change in the peak area of BTZ and ISTD at their respective MRMs.

The HPLC conditions were optimised such that the retention time was kept for BTZ at 0.08 minutes and BTZ D_3 at 0.07 minutes in order to assure high throughput. Some retention of the compound on the HPLC column was employed with the eluent from the first 45 sec of the run going to waste. This limited the amount of endogenous material entering the mass

spectrometer and thereby reduced the amount of system maintenance required. The ACE 5 CN, (150x4.6 mm, 5 µm) HPLC column was chosen based on positive experience in the chromatography of acid compounds and because it demonstrates good stability at the low pH of the mobile phase. The composition of the mobile phase with Ammonium formate buffer, 10 mM pH 4.5: Acetonitrile (25:75 v/v) was chosen for its compatibility with mass spectrometric detection to get symmetric peak shape and better analyte response.

The pH of ammonium formate buffer was kept at 4.5 because it was found to be necessary in order to lower the pH to protonate the acidic BTZ and thus deliver good peak shape. The percentage of ammonium buffer was optimised to maintain this peak shape whilst being consistent with good ionisation and

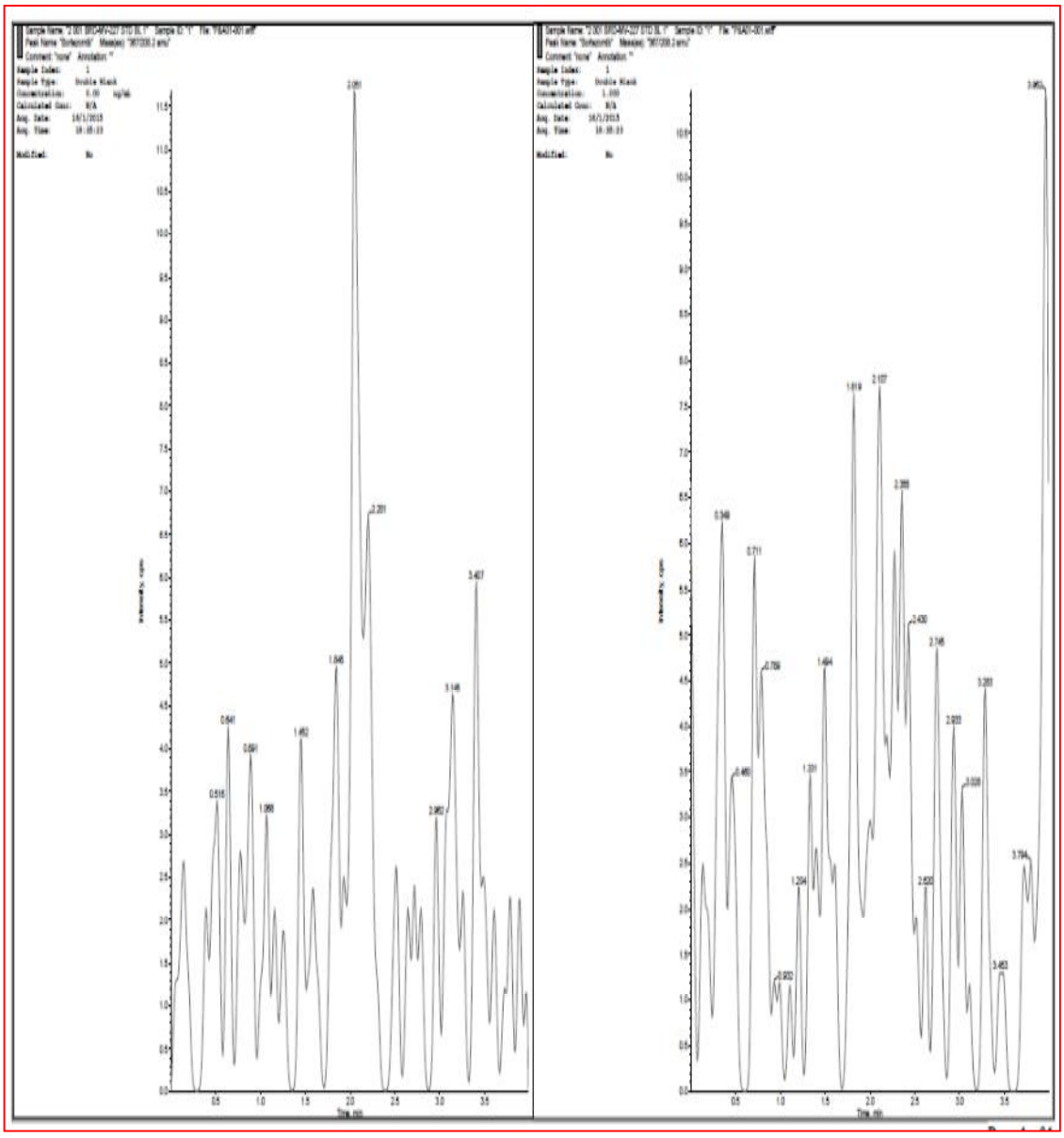

Fig. 2. A Representative chromatogram of standard blank

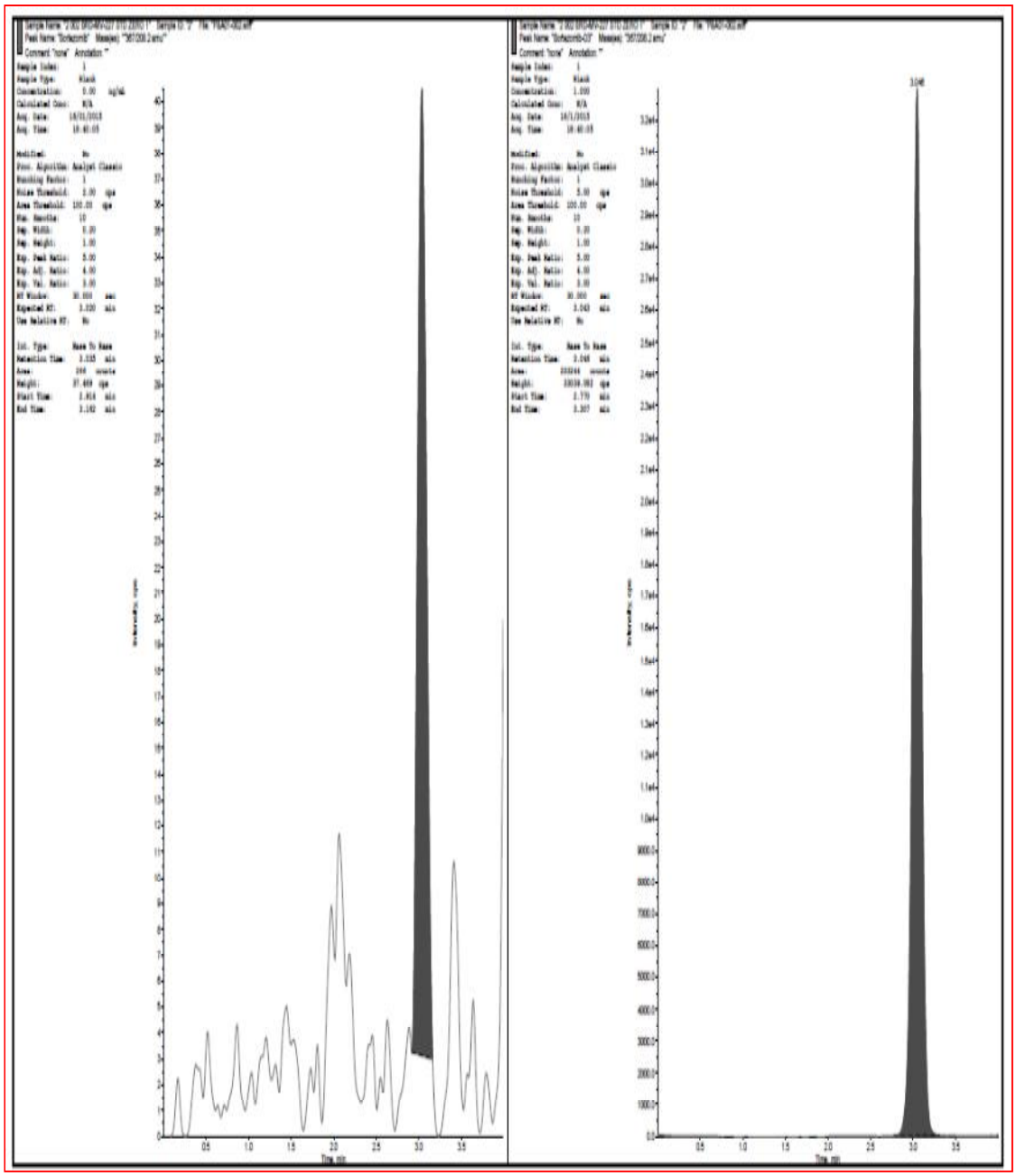

Fig. 3. A Representative chromatogram of standard zero

fragmentation in the mass spectrometer. Additionally, the flow rate was varied from 0.6-1.0 ml/min to minimize any possible interference at the retention time of BTZ. Chromatographic conditions were optimized to achieve a short runtime, adequate retention, acceptable peak shapes and baseline separation of the drugs. The typical chromatograms of Standard Blank (Fig. 2), Standard Zero (Fig. 3) & Matrix Specificity of Blank Samples - without BTZ and BTZ D_3 (Fig. 4) were shown below.

3.2 Method Validation Results

3.2.1 Specificity, selectivity, carryover and matrix effects

The standard curve in biological fluids was compared with standard in buffer to detect matrix

effects. Besides, parallelism of diluted study samples were evaluated with diluted standards to detect matrix effects. The results showed that precision, selectivity, and sensitivity were not compromised. The specificity/selectivity of the method was investigated by screening several separate human plasma samples and looking for endogenous peaks which accounted for more than 20% of the peak area of BTZ or the ISTD in the LLOQ of calibration samples and significantly no endogenous substances were detected. Pre-dose samples analyzed from preliminary clinical studies have confirmed that there were no other endogenous plasma components, which would have led to significant interference in the assay. The carryover evaluation was performed before and after each analytical run to ensure that it does not influence the accuracy and the precision of the proposed method. The column and auto-sampler carryover evaluation showed negligible carryover in extracted & un-extracted samples as outlined in the given below Table 1.

3.3 Linearity, Precision, Accuracy and Limit of Quantification

Regression analysis was carried out to assess the linearity between the peak area ratio's of BTZ & BTZ D_3 concentration. The standard curve fitted to a 1/*c* weighted linear regression which was calculated by the quantitative module of Analyst software. All calibration curves were linear (r^2 = 0.9991±0.008) through the studied

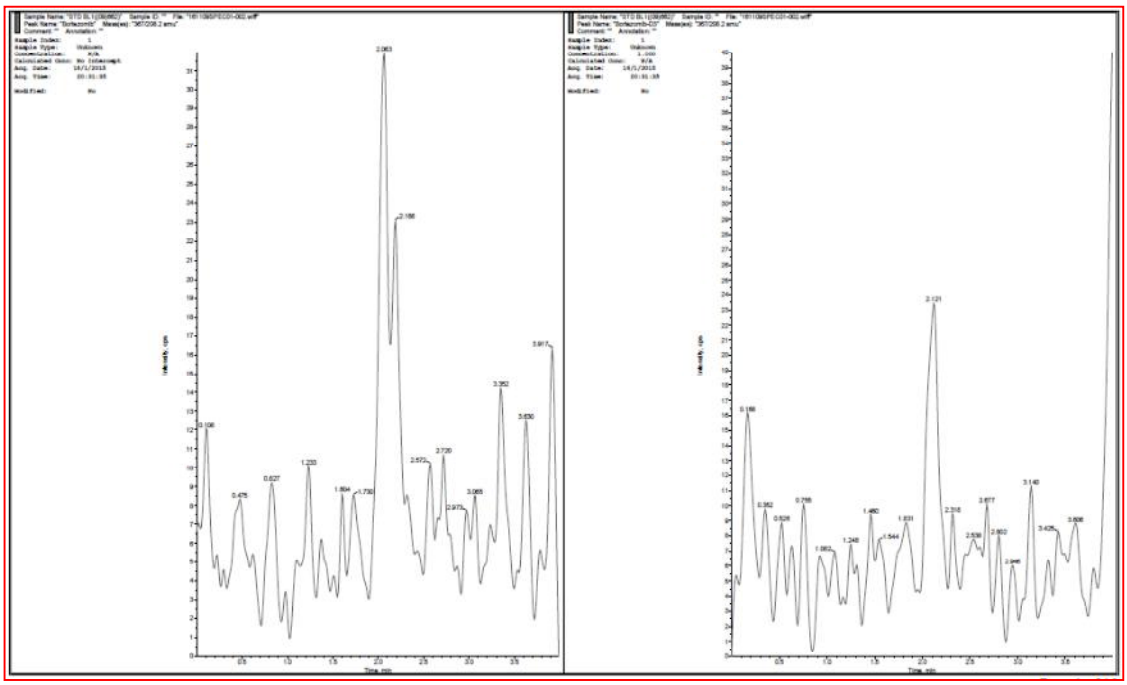

Fig. 4. Chromatogram of matrix specificity of blank samples

Table 1. Specificity studies of biological matrix

Specificity	:	10 out of 10 Human Plasma Lots Passed
Matrix Effect	:	07 out of 07 Human Plasma Lots Passed
Matrix Factor for drug	:	ISTD Normalized Matrix Factor – 1.0 to 1.1
Precision (% CV) of ISTD Normalized Matrix Factor	:	6.9%
Concomitant Selectivity for drug and internal standard	:	06 out of 06 Human Plasma Lots Passed
Concomitant Medication Experiment (CME)	:	Acetaminophene - 4.00 µg/mL concentration
		Nimesulide - 9.00 µg/mL concentration
		Domperidone - 20.0 ng/mL concentration
		Diclofenac - 2.00 µg/mL concentration
		Ibuprofen - 45.0 µg/mL concentration
		Ranitidine - 545 ng/mL concentration
		Cetirizine - 400 ng/mL concentration

concentration range of 2.00 to 1000 ng/mL of BTZ. The mean linear equation for calibration curve concentrations was y = (0.0027±0.0001) x + (0.0027±0.0007) by $1/x^2$ weighting factor. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 92.73 to 102.2% for BTZ. The limit of quantification of the method was 1.0 ng/mL and 2.00 ng/mL (%CV) ranged from 1.81-2.14%.

Intra-batch inaccuracy and imprecision were assessed by running a single batch of samples containing a calibration curve and six replicates of test samples at each of the four concentrations (2, 6, 41 and 880 ngmL−1). For inter-batch inaccuracy and imprecision three batches of samples were analyzed. Each batch contained a calibration curve and duplicate test samples at each of the four concentrations. The inter and intra-batch CV and accuracy of the method, as measured by the performance of the test samples for BTZ at all four levels of concentration as shown in Table 2. The imprecision and inaccuracy were within the pre specified acceptable limits, across the calibration range.

3.4 Extraction Recovery and Matrix Factor/Effect

The extraction recovery and matrix effect results for BTZ and ISTD are presented in Tables. 3 and 4 respectively. The overall % recovery extraction for BTZ and ISTD were 83.04% & 76.39% respectively across QC levels. The potential impact of matrix effect on the quantification of BTZ was expressed as Matrix Factor (MF). MFs can be determined from the peak area response

Table 2. Intra- inter assay precision and accuracy for drug

Run date	LLOQ QC 2.00 ng/mL	% Bias	LQC 6.00 ng/mL	% Bias	MQC 41.0 ng/mL	% Bias	HQC 880 ng/mL	% Bias
	1.97	-1.50	5.97	-0.50	42.0	2.44	858	-2.50
	1.81	-9.50	6.09	1.50	43.7	6.59	842	-4.32
Day-1	1.85	-7.50	6.07	1.17	42.1	2.68	855	-2.84
	1.84	-8.00	6.34	5.67	42.0	2.44	852	-3.18
	1.94	-3.00	6.15	2.50	40.0	-2.44	853	-3.07
Intrarun Mean	1.88	-	6.12	-	42.0	-	852	-
Intrarun SD	0.0691	-	0.137	-	1.31	-	6.04	-
Intrarun% CV	3.68	-	2.24	-	3.12	-	0.71	-
Intrarun% Bias	-6.00	-	2.00	-	2.44	-	-3.18	-
n	5	-	5	-	5	-	5	-
	2.14	7.00	5.94	-1.00	38.5	-6.10	831	-5.57
	2.02	1.00	5.72	-4.67	40.5	-1.22	799	-9.20
Day-2	2.09	4.50	5.65	-5.83	38.8	-5.37	786	-10.68
	1.90	-5.00	5.81	-3.17	39.8	-2.93	820	-6.82
	1.85	-7.50	6.37	6.17	39.9	-2.68	800	-9.09
Intrarun Mean	2.00	-	5.90		39.5	-	807	-
Intrarun SD	0.123	-	0.285		0.828	-	18.0	-
Intrarun% CV	6.15	-	4.83		2.10	-	2.23	-
Intrarun% Bias	0.00	-	-1.67		-3.66	-	-8.30	-
n	5	-	5	-	5	-	5	-
	1.95	-2.50	6.01	0.17	40.8	-0.49	876	-0.45
	1.74	-13.00	6.20	3.33	41.1	0.24	868	-1.36
Day-3	1.94	-3.00	6.29	4.83	39.7	-3.17	869	-1.25
	1.86	-7.00	6.25	4.17	40.7	-0.73	859	-2.39
	1.89	-5.50	6.09	1.50	41.0	0.00	869	-1.25
Intrarun Mean	1.88	-	6.17	-	40.7	-	868	-
Intrarun SD	0.0844	-	0.116	-	0.559	-	6.06	-
Intrarun% CV	4.49	-	1.88	-	1.37	-	0.70	-
Intrarun% Bias	-6.00	-	2.83	-	-0.73	-	-1.36	-
n	5	-	5	-	5	-	5	-
Mean concentration found (ng/mL)	1.92	-	6.06	-	40.7	-	842	-
Inter-run SD	0.106	-	0.218	-	1.36	-	28.8	-
Inter-run% CV	5.52	-	3.60	-	3.34	-	3.42	-
Inter-run% Bias	-4.00	-	1.00	-	-0.73	-	-4.32	-
n	15	-	15	-	15	-	15	-

of BTZ and ISTD separately, while the ratio of the two factors gives the ISTD normalized MF. The ISTD normalized MFs using stable-isotope-labelled ISTD should be close to unity because of the similarities in the chemical properties and elution times.

Table 3. Recovery of drug and ISTD

Replicate no.	HQC		MQC (Drug)		MQC (ISTD)		LQC	
	Extracted peak area ratio	Un-extracted peak area ratio	Extracted peak area ratio	Un-extracted peak area ratio	Extracted peak area ratio	Un-extracted peak area ratio	Extracted peak area ratio	Un-extracted peak area ratio
1	6.212	7.300	0.310	0.376	3.230	4.290	0.053	0.059
2	6.301	7.284	0.304	0.385	3.290	4.120	0.046	0.059
3	6.252	7.029	0.299	0.366	3.340	4.570	0.048	0.061
4	5.992	7.300	0.304	0.361	3.290	4.240	0.044	0.057
5	6.185	7.227	0.301	0.366	3.290	4.340	0.049	0.058
Mean	6.1884	7.2281	0.3037	0.3710	3.2940	4.3120	0.0479	0.0587
SD	0.11821	0.11524	0.00392	0.00967	0.04159	0.16574	0.00324	0.00124
%CV	1.91	1.59	1.29	2.61	1.26	3.84	6.76	2.11
% Mean recovery	85.62		81.86		76.39		81.63	
% Overall recovery	83.04							
% Overall CV	2.69							

Wet extract stability: 27 Hours at 5±3℃ in Mobile Phase, Dry extract stability: 24 Hours at -20±5℃

Table 4. Matrix factor for drug and internal standard

Replicate No.	HQC			MQC			LQC		
	Post spiked area ratio	AQ area ratio	Matrix factor	Post spiked area ratio	AQ area ratio	Matrix factor	Post spiked area ratio	AQ area ratio	Matrix factor
1	1679225	1958630	0.9	89553	111142	0.8	13281	16914	0.8
2	1609235	1869216		92150	106391		13624	16650	
3	1620942	1911512		92124	105611		13132	16324	
Mean	1636467.3	1913119.3		91275.7	107714.7		13345.7	16629.3	
Mean matrix factor								0.84	
SD								0.028	
%CV								3.41	

Replicate No.	HQC			MQC			LQC		
	Post spiked area ratio	AQ area ratio	Matrix factor	Post spiked area ratio	AQ area ratio	Matrix factor	Post spiked area ratio	AQ area ratio	Matrix factor
1	310130	370635	0.8	330916	417994	0.8	334029	416410	0.8
2	295366	347014		334005	417952		329494	421514	
3	302311	351327		328409	415354		327057	398348	
Mean	302602.3	356325.3		331110.0	417100.0		330193.3	412090.7	
Mean matrix factor				0.81					
SD				0.030					
%CV				3.69					

Sample ID	Mean matrix factor for Boretezomib	Mean matrix factor for ISTD-1	ISTD normalized matrix factor
HQC	0.9	0.8	1.1
MQC	0.8	0.8	1.0
LQC	0.8	0.8	1.0
MEAN			1.04
STD DEV			0.072
%CV			6.9

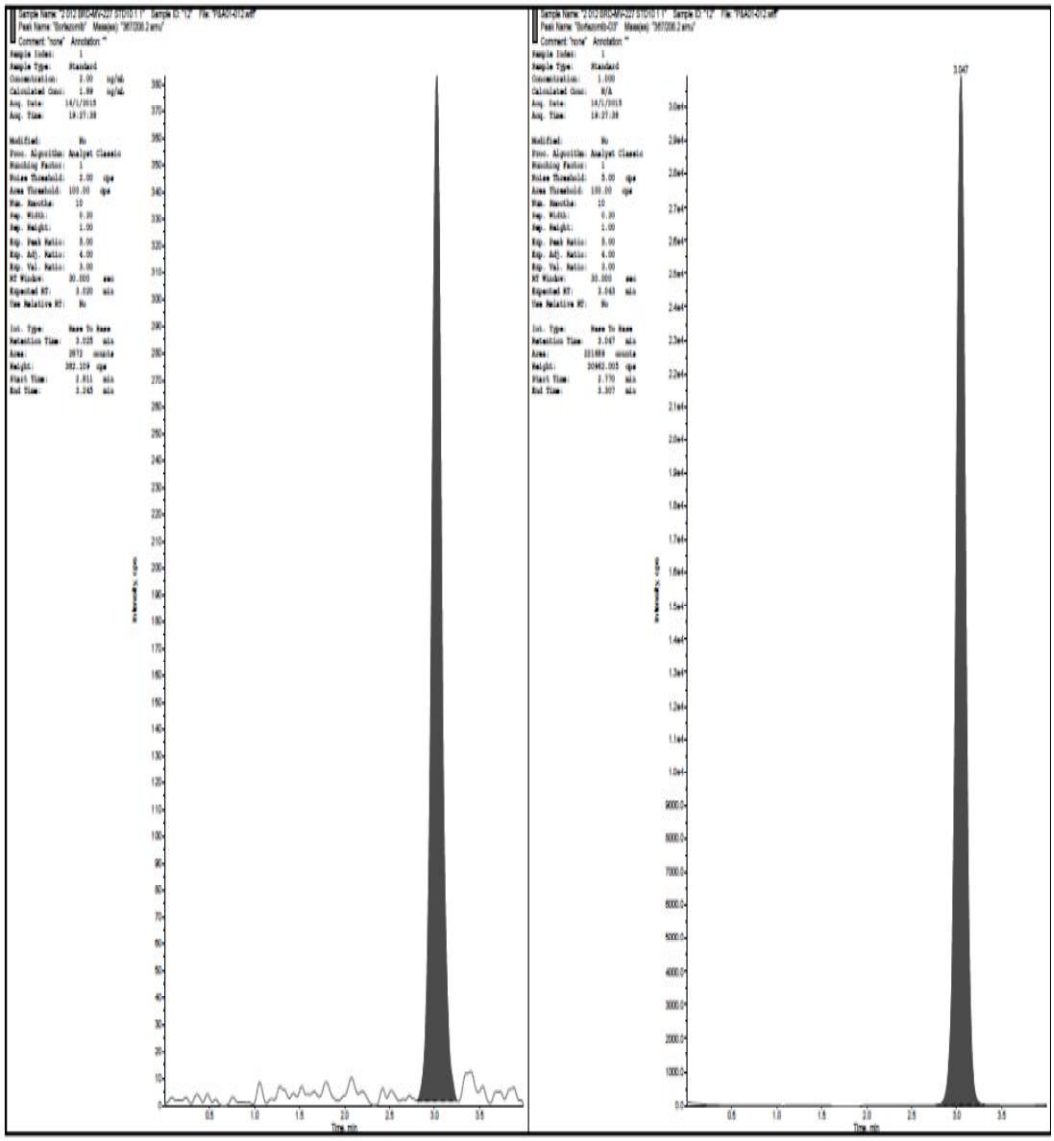

Fig. 5. A representative chromatogram of LLOQ standard (2.00 ng/mL)

The ISTD-normalized MFs ranged within 1.00 - 1.1. Further, matrix effect needs to be checked in lipemic and haemolysed plasma samples in addition to normal K_3EDTA plasma. The coefficient of variation (% CV) of the slopes of calibration lines RT, ISTD RT & area ratio for relative matrix effect in eight different plasma lots was 2.51%, which is within the acceptance criteria of 3.0% [25]. These results indicated that the sample procedure of ion pair liquid–liquid extraction with MTBE was efficient for the extraction of trace BTZ in plasma. The assay has been proven to be robust in high throughput bio analysis.

3.5 Stability, Dilution Reliability and Method Ruggedness

Samples kept for short term and long term stock and working solution stability remained unaffected up to 18 hours and 57 days respectively for BTZ and ISTD. Bench top stability of BTZ in plasma was established up to 8 h and for a minimum of five freeze and thaw cycles at -20±5°C and -78±8°C**.** Auto sampler stability (wet extract) of the spiked quality control samples maintained at 5±3°C in mobile phase was determined up to 27 h without significant loss of BTZ. Spiked plasma samples stored at–

20°C-70°C, for long term stability experiment were found stable for a minimum period of 7 d at 5±3°C. Dry extract stability was observed at 24 h at -20±5°C. For method ruggedness, the precision (%CV) and accuracy values for different columns and analysts ranged from 0.58 to 8.39% and 89.66 to 102.20% respectively for BTZ at four QC levels.

Similarly, four different concentrations of spiked plasma ((2, 6, 41 & 880 ngmL−1) were analyzed at fresh preparing and stored at −20◦C, then subjected to three freeze and thaw (12 h) cycles to investigate freeze and thaw stability. The concentrations found were within the allowed limit ±15% of nominal concentration, revealing no significant substance loss during repeated freezing and thawing. The plasma samples remained stable after freezing and thawing for at least three times. The experimental details are outlaid in the following Tables 5 - 7 and Fig. 5 – 10.

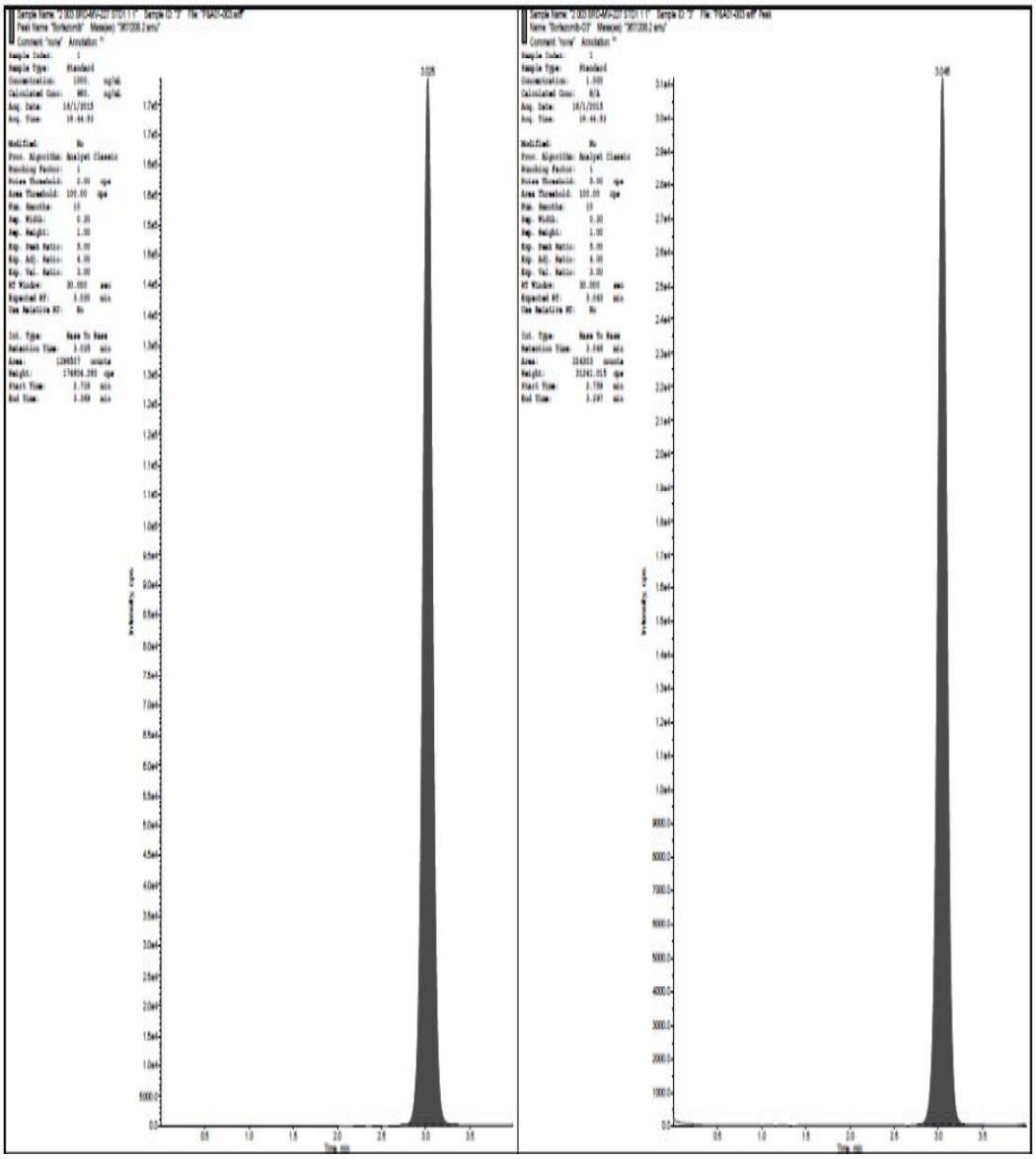

Fig. 6. A representative chromatogram of ULOQ standard (1000 ng/mL)

Table 5. Freeze thaw stability and bench top stability drug at LQC level

Run	BT LQC 6.00 ng/mL	% Bias	LQC 6.00 ng/mL	% Bias	LQC (-20±5°C) 6.00 ng/mL	% Bias	LQC (-78±8°C) 6.00 ng/mL	% Bias
	6.60	10.00	6.15	2.50	5.61	-6.50	6.05	0.83
	6.21	3.50	5.77	-3.83	6.29	4.83	6.14	2.33
	6.04	0.67			5.86	-2.33	6.25	4.17
Mean	6.28		5.96		5.92		6.15	
S.D.	0.287		0.269		0.344		0.100	
% CV	4.57		4.51		5.81		1.63	
% Accuracy	104.67		99.33		98.67		102.50	
% Bias	4.67		-0.67		-1.33		2.50	
n	3		2		3		3	
Overall % CV	2.80							

Freeze thaw stability: 3 cycles at 20±5°C & 78±8°C Bench top stability: 8 hours at ambient temperature

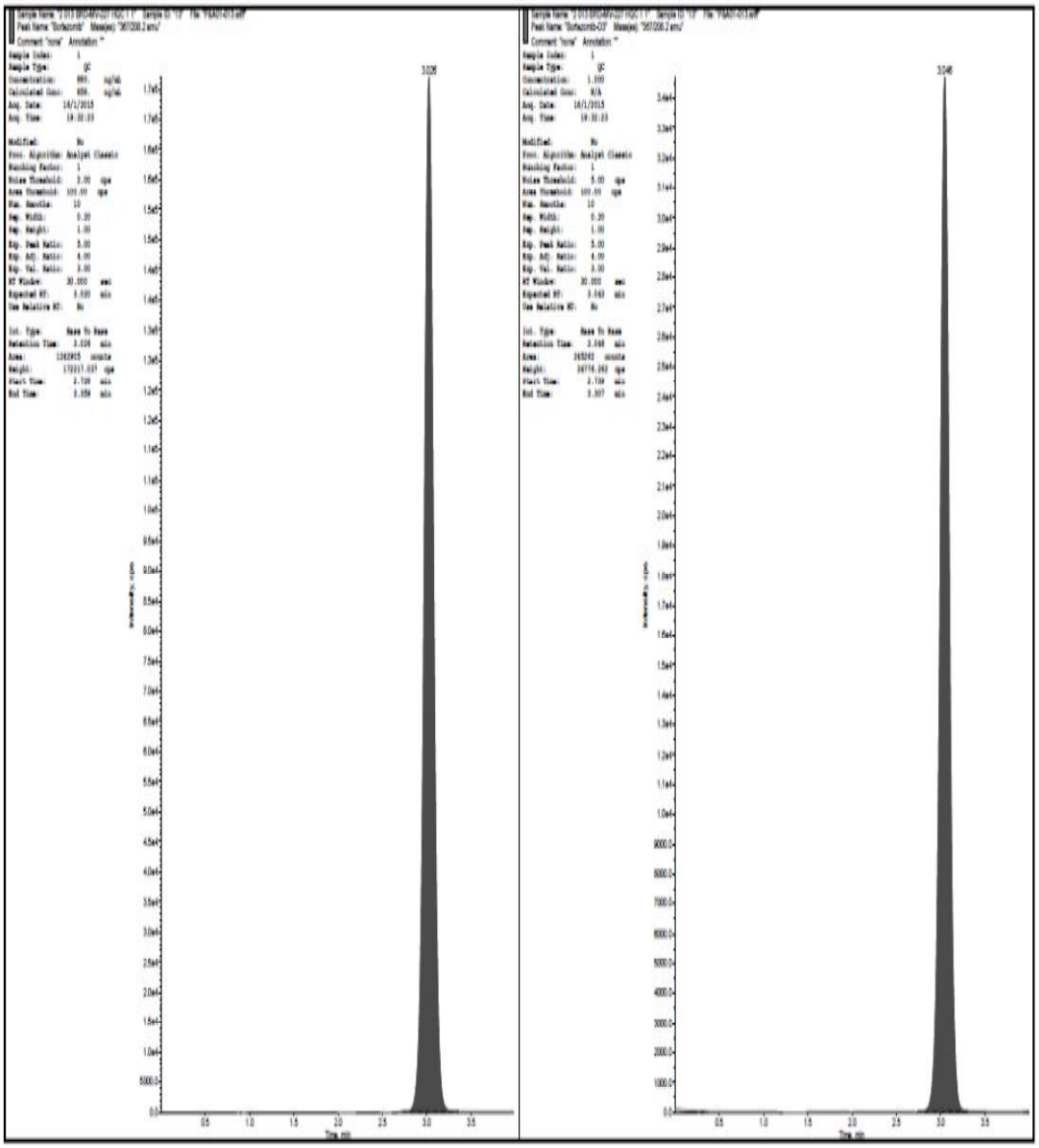

Fig. 7. A representative chromatogram of HQC sample (880 ng/mL)

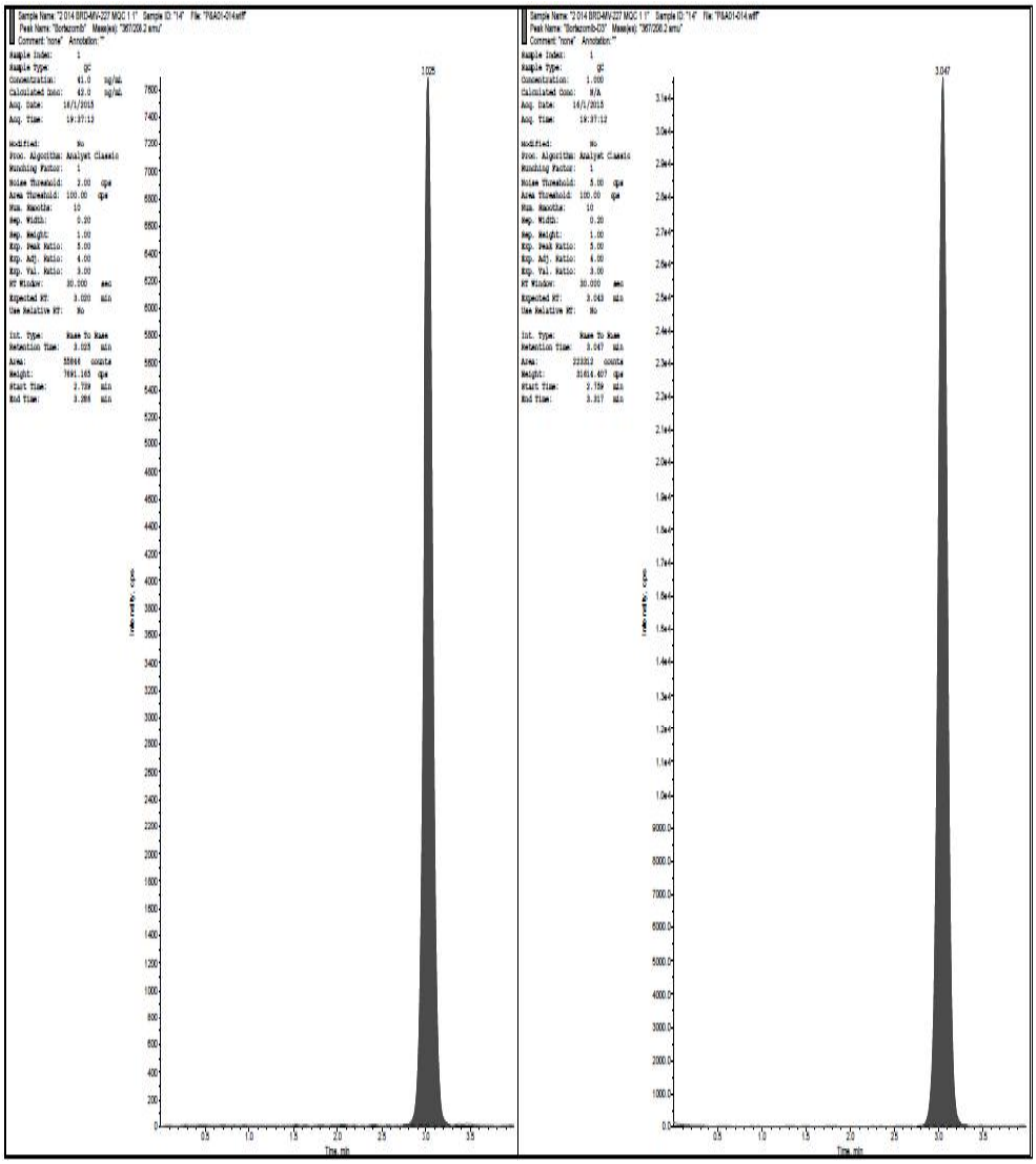

Fig. 8. A representative chromatogram of MCQ sample (41 ng/mL

Table 6. Freeze thaw stability and bench top stability drug at MQC and HQC-level

Run	MQC 41.0 ng/mL	% Bias	BT HQC 880 ng/mL	% Bias	HQC 880 ng/mL	% Bias	HQC (-20±5°C) 880 ng/mL	% Bias	HQC (-78±8°C) 880 ng/mL	% Bias
	42.0	2.44	875	-0.57	857	-2.61	816	-7.27	828	-5.91
	41.8	1.95	848	-3.64	889	1.02	866	-1.59	845	-3.98
			850	-3.41			836	-5.00	837	-4.89
Mean	41.9		858		873		839		837	
S.D.	0.141		15.0		22.6		25.2		8.50	
% CV	0.34		1.75		2.59		3.00		1.02	
% Accuracy	102.20		97.50		99.20		95.34		95.11	
% Bias	2.20		-2.50		-0.80		-4.66		-4.89	
n	2		3		2		3		3	
Overall % CV		2.80								

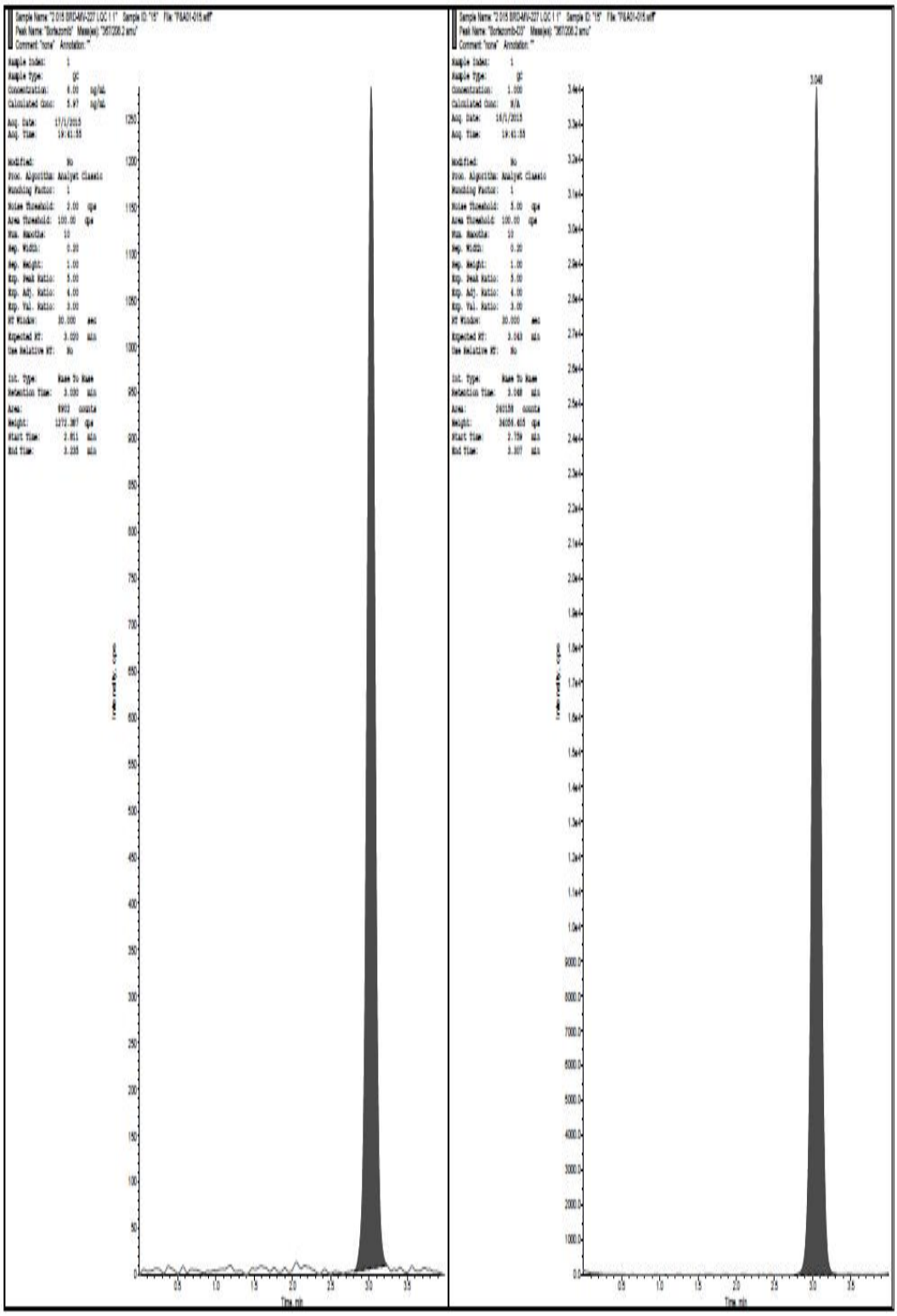

Fig. 9. A representative chromatogram of LQC sample (6.00 ng/mL)

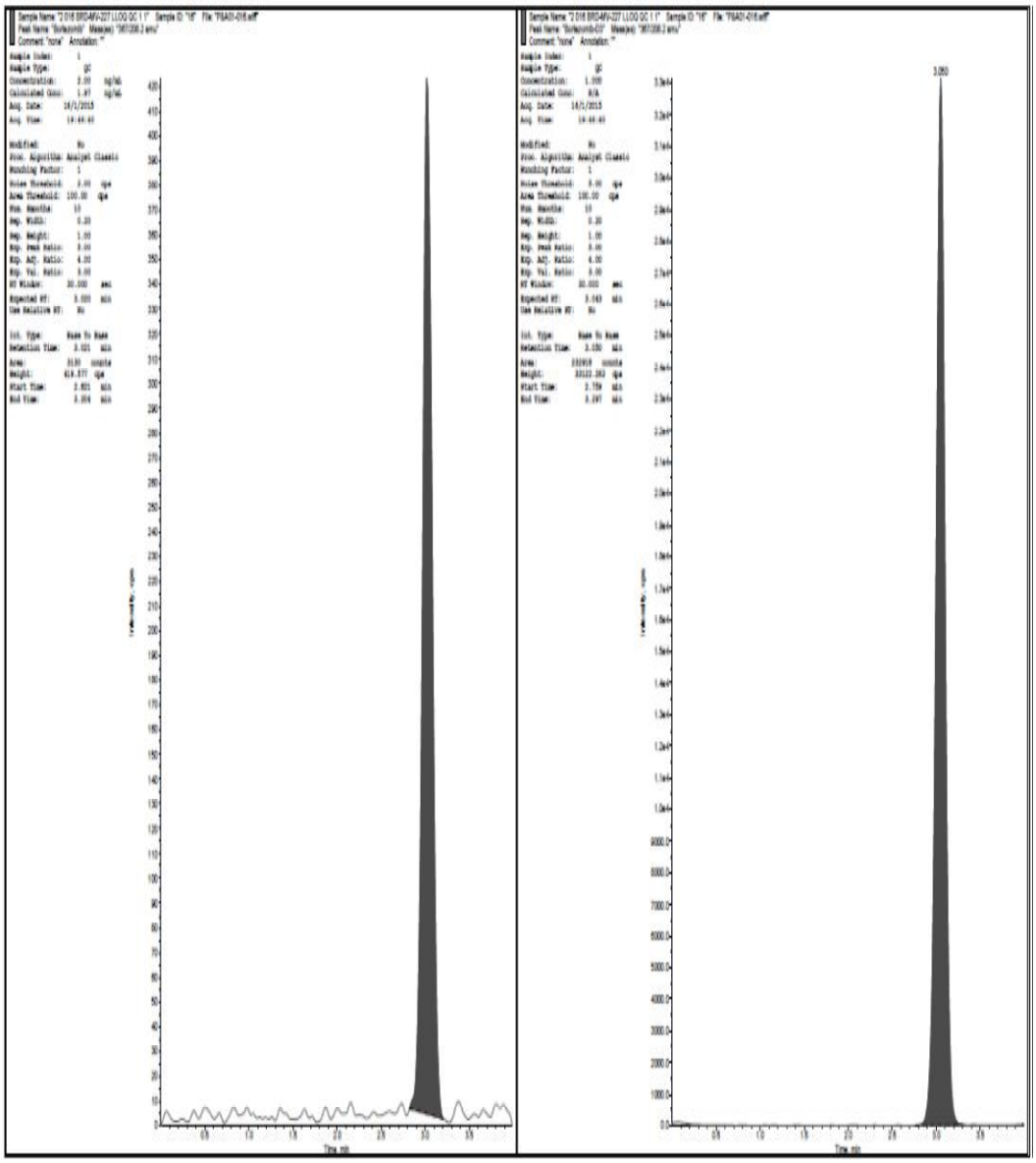

Fig. 10. A representative chromatogram of LLOQ QC sample (2 ng/mL)

Table 7. Ruggedness for drug

Run /Day	LLOQ QC 2.00 ng/mL	LQC 6.00 ng/mL	MQC 41.0 ng/mL	HQC 880 ng/mL
Day 1: Intrarun Mean	1.90	6.20	41.2	850
Intrarun SD	0.132	0.122	0.555	16.7
Intrarun % CV	6.95	1.97	1.35	1.96
Intrarun % Bias	-5.00	3.33	0.49	-3.41
Day 2: Intrarun Mean	1.86	6.00	39.8	829
Intrarun SD	0.156	0.304	0.554	18.6
Intrarun % CV	8.39	5.07	1.39	2.24
Intrarun % Bias	-7.00	0.00	-2.93	-5.80
Day 3: Intrarun Mean	1.91	5.78	40.0	844

Run /Day	LLOQ QC 2.00 ng/mL	LQC 6.00 ng/mL	MQC 41.0 ng/mL	HQC 880 ng/mL
Intrarun SD	0.0907	0.187	0.555	4.88
Intrarun % CV	4.75	3.24	1.39	0.58
Intrarun % Bias	-4.50	-3.67	-2.44	-4.09
Mean Concentration Found (ng/mL)	1.89	5.99	40.3	841
Inter-run SD	0.121	0.268	0.816	16.4
Inter-run % CV	6.40	4.47	2.02	1.95
Inter-run % Bias	-5.50	-0.17	-1.71	-4.43

4. CONCLUSION

The Method Validation is accepted as per acceptance criteria stated in the method validation protocol. The method is considered valid for the extraction and analysis of Bortezomib and its internal standard Bortezomib-D_3 in K_3 EDTA human plasma samples within the investigated concentration range of 2.00 to 1000 ng/mL, using 0.3mL of processing volume. The present work, successfully demonstrates a sensitive, rapid and a precise method for determination of BTZ from endogenous plasma matrix. The selectively of this bio analytical method enhances its utility for clinical pharmacokinetic studies by using deuterated ISTD , which minimizes inter-individual variability in the recovery of BTZ, followed by efficient LLE approach under acidic conditions. Finally, the method shows a good accuracy and precision as evident from the results of all validation parameters.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Adams J, Stein R. Novel inhibitors of the proteasome and their therapeutic use in inflammation. Annual Reports in Medicinal Chemistry. 1996;31:279–288.
2. Adams J. The proteasome: A suitable Antineoplastic target, Nature Reviews Cancer. 2004;4(5):349–360.
3. Richardson PG, Hideshima T, Anderson KC. Bortezomib (PS-341): A novel, first-in-class proteasome inhibitor for the treatment of multiple myeloma and other cancers. Cancer Control. 2003;10(5):361–369.
4. Takimoto CH, Calvo E. Principles of Oncologic Pharmacotherapy in Pazdur R, Wagman LD, Camphausen KA, Hoskins WJ (Eds) Cancer Management: A Multidisciplinary Approach. 11th Ed; 2008.
5. House Douglas W, FDA clears Velcade label expansion Seeking Alpha; 2014.
6. Bonvini P, Zorzi E, Basso G, Rosolen A, Bortezomib-mediated 26S proteasome inhibition causes cell-cycle arrest and induces apoptosis in CD-30+ Anaplastic large cell lymphoma. Leukaemia. 2007;21(4):838–842.
DOI: 1038/sj.leu.2404528. PMID 17268529
7. Adams J, Kauffman M, Development of the Proteasome Inhibitor - Velcade (Bortezomib). Cancer Invest. 2004;22(2): 304–311.
DOI: 10.1081/CNV-120030218
PMID15199612
8. US Department of Health and Human Services. FDA.Gov; 2008.
9. Millennium: The Takeda Oncology Company, Millennium.Com; 2014.
10. Reece DE, Sullivan D, Lonial S, Mohrbacher AF, et al. Pharmacokinetic and pharmacodynamic study of two doses of Bortezomib in patients with relapsed multiple myeloma. Cancer Chemother Pharmacol. 2011;67:57–67.
DOI: 10.1007/s00280-010-1283-3
11. Voorhees PM, Dees EC, O'Neil B, Orlowski RZ, The proteasome as a target for cancer therapy. Clin Cancer Res. 2003;9(17):6316–6325.
PMID 14695130
12. Moreau P, Pylypenko H, Grosicki S et al, Subcutaneous versus intravenous administration of Bortezomib in patients with relapsed multiple myeloma: A randomised, phase 3, non-inferiority study. The Lancet. 2011;12:431–440.
DOI: 10.1016/s1470-2045(11)70081-x
13. Brinda MN, Krishna Reddy V, Sasi Kiran Goud E, Kadarla Vinay Kumar,

Development and validation of RP-HPLC method For determination of related substances of bortezomib in injection, World Journal of Pharmacy and Pharmaceutical Sciences. 2014;3(2): 2521-2529.

14. Akshay Kumar Bisht, Brij Bhushan, Vandana Dhiman, Dhawan RK, Baghel US, Anil Kumar Gupta. A novel gradient RP-HPLC method development for bortezomib in parenteral Dosage. Journal of Biomedical and Pharmaceutical Research. 2013;2(2):10-15.
15. Stephen R. Byrn, Patrick A. Tishmack, Mark J. Milton, Helgi van de Velde. Analysis of two commercially available bortezomib products: Differences in assay of active agent and impurity profile. AAPS Pharm Sci. Tech. 2011;12(2):461–467.
16. Ram Babu C, Venkat Rao S, Ramu G, Ganesh M. Estimation of bortezomib in bulk and its pharmaceutical dosage forms by using a novel validated accurate reverse phase high performance liquid chromatography. Int J Pharm Pharm Sci. 2011;3(3):303-305.
17. Utage M, Swamy BMV. Analytical method development and validation of related substance method for bortezomib for injection 3.5 mg/vial by RP-HPLC method. International Journal for Pharmaceutical Research Scholars. 2013;2(2):27–32.
18. Kasa Srinivasulu, Mopidevi Narasimha Naidu, Kadaboina Rajasekhar, Murki Veerender, Mulukutla Venkata Suryanarayana. Development and validation of a stability indicating LC method for the assay and related substances determination of a proteasome inhibitor Bortezomib. Chromatography Research International. 2012;1–13.
19. Venkataramanna M, Sudhakar Babu K, Anwar Sulaiman KC. A validated stability-indicating UFLC method for bortezomib in the presence Of degradation products and its process-related impurities. International Journal of Life Science and Pharma Research. 2012;2(1):135–146.
20. Clemens J, Longo M, Seckinger A, Hose D, Haefeli WE, Weiss J, Burhenne J. Stability of the proteasome inhibitor Bortezomib in cell based assays determined by ultra-high performance liquid chromatography coupled to tandem mass spectrometry. J Chromatogr A. 2014; 1345:128-38.
21. Teresa Pekol J. Scott Daniels, Jason Labutti, Ian Parsons, Darrell Nix, Elizabeth Baronas, Frank Hsieh, Liang-Shang Gan, Gerald Miwa. Human metabolism of the proteasome inhibitor Bortezomib: Identification of circulating metabolites. Drug Metabolism and Disposition. 2005; 33(6):771-777.
22. Guidance for Industry: Bio analytical Method Validation, US. Department of health and human services, food and drug administration, centre for drug evaluation and research (CDER), Centre for Veterinary Medicine (CV); 2001.
    Available:http://www/fda.gov/cder/guidance/index.htm
23. Hull CK, Penman AD, Smith CK, Martin PD. J. Chromatogr B. 2002;(772):219–228.
24. Guidance for Industry: ICH E6 Good Clinical Practice, US. Department of health and human services, food and drug administration, centre for drug evaluation and research (CDER), Centre for Biologics Evaluation and Research (CBER); 1996.
25. Matuszewski BK. Standard line slopes as a measure of relative matrix effect in quantitative HPLC-MS bio analysis. J Chromatogr B. 2006;(830):293-300.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/17605