A Validated LC-MS/MS Method for the Estimation of Bortezomib and Bortezomib D3 (IS) in Human Plasma with Protein Precipitation and SPE Filter Cartridges

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ABSTRACT

A simple, rapid, specific and precise liquid chromatography-tandem mass spectrophotometric (LC–MS/MS) validated method was developed for quantification of Bortezomib in human plasma. Bortezomib D3 was used as internal standard, added to plasma sample prior to extraction using 0.1% formic acid in acetonitrile as a precipitating agent. The interferences due to protein denaturation were removed using captiva SPE filter cartridge of 0.45µm. Chromatographic separation was achieved on ACE 5CN column (150mmx4.6mm) with acetonitrile: 10 mM ammonium formate buffer (75:25 v/v) as an isocratic mobile phase with a flow rate of 1 ml/min. the LC eluent was split, and approximately 0.1ml/min was introduced in to Tandem mass spectrometer using turbo Ion Spray interface at 325°C. Quantitation was performed by transition of $367.3 \rightarrow 226.3$ (m/z) for Bortezomib and $370.3 \rightarrow 229.2$(m/z) for Bortezomib D3. The concentrations of ten working standards showed linearity between 2 to 1000 ng/ml ($r2 \geq 0.998$). Chromatographic separation was achieved within 3.5 min. The average extraction recoveries of three quality control concentrations were 82.71% for Bortezomib and were within the acceptance limits. The coefficient of variation was ≤15% for intra- and inter-batch assays. The assay is suitable for pharmacokinetic study samples as demonstrated by its specificity, precision, accuracy, recovery, and stability characteristics

INTRODUCTION

Bortezomib, [(1*R*)-3-methyl-1-({(2*S*)-3-phenyl-2-[(pyrazin-2-ylcarbonyl) amino] propanoyl} amino) butyl] boronic acid, is the first therapeutic proteasome inhibitor used for treating multiple myeloma. (Adams and Kauffman, 2004). Several UV-Visible (Venkat rao etal., 2012), HPLC (Appala raju *et al*., 2014; Brinda *et al* 2013; Srinivasulu *et al*., 2012; Rambabu *et al*., 2011) UFLC (Venkataramana *et al*., 2012) methods are available for quantification of bortezomib in its bulk and formulation dosage forms. Three LC-MS/MS(Liquid Chromatography coupled with Mass Spectroscopy) (Nix *et al*., 2001; Christos *et al*., 2004; Pekol *et al*., 2005) methods were reported for the estimation of bortezomib and isotope labeled

bortezomib D3 (ISTD) from human plasma in gradient mode using buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in 90:10 acetonitrile and water) and samples were prepared by precipitating plasma proteins using chilled 0.1% formic acid in acetonitrile, chromatograms obtained on C18 column. Literature survey reveals, the reported Lc-ms/ms methods were suffered in preparing clean samples by protein precipitation extraction, shows plasma protein interference.

The aim of our study is to develop a validated (FDA guidelines, 2013) LC-MS/MS method and increasing the column life by removing interferences due to plasma protein denaturation using captiva SPE (Solid Phase Extraction) 0.45µm filter cartridge in sample preparation employing protein precipitation technique. The developed method was simple. Fast, accurate, specific and economical for the estimation of Bortezomib and Bortezomib D3 as internal standard (ISTD) from human plasma.

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EXPERIMENT

Apparatus and software

The HPLC system with an auto sampler was a Shimadzu LC- 20ADvp (Shimadzu, Japan) coupled with Applied Biosystem Sciex (MDS Sciex, Canada) API 3000 Tandem mass spectrometer. The auto sampler was SIL-HTC from Shimadzu, Japan. Captive SPE filtercatridge, 0.45µm from Agilent Technologies. The solvent delivery module was LC-20AD from Shimadzu, Japan. The chromatographic integration was performed by Analyst software (version: 1.4.2; Applied Biosystems).

Chemicals and reagents

Bortezomib and Bortezomib D3 (ISTD) were procured from Unichem Laboratories Ltd., Mumbai, India, Formic acid was procured from Merck Specialities Pvt. Ltd, Mumbai, India. Water used was collected from water purification systems (Milli Q, Milli Pore, USA) installed in laboratory. Methanol and acetonitrile were of HPLC grade and were supplied by J. T. Baker, USA. Pooled drug free expired frozen human plasma (K2-EDTA as anticoagulant) was obtained from St. Theresa Blood Bank, Hyderabad, was used during validation and study sample analysis. The plasma was stored into $-70\pm5^{o}C$.

Standards and working solutions
Calibration standard Solutions

Stock solutions of Bortezomib and Bortezomib D3 internal standard **(ISTD)** were prepared in methanol. Further dilutions were carried out in 50% methanol. Calibration standards of ten concentration levels were prepared freshly by spiking drug free plasma with Bortezomib stock solution to give the concentrations of 2.00, 4.00, 8.00, 12.0, 25.0, 50.0, 100, 200, 500, and 1000ng/ml.

Quality control standards

Lowest quality control standards, Median quality control standards and highest quality control standards were prepared by spiking drug free plasma with Bortezomib to give solution containing 6, 41and 880ng/ml respectively. They were stored at -20^{0}c till the time analyzed.

Chromatographic conditions

Chromatographic separation was performed on a Hypurity ACE 5CN (150mmx4.6mm), analytical column and the mobile phase was a mixture of 0.10% (v/v) formic acid in water to acetonitrile at a ratio of 25:75, v/v. Injection volume was 5µL. The flow rate was 1.00 mL/ min. Total analysis time of single injection was 3.00 minutes. Column oven temperature and auto sampler temperature was set to 40 °C and 5 °C, respectively.

Mass Spectrometric conditions

The LC eluent was split, and approximately 0.100 ml/min was introduced via electrospray ionization using a Turbo IonSpray interface set at 325 °C to generate positive ions [M+H] +. Ionization was assisted with nebulizer and IonSpray gas (nitrogen) at 8 (arbitrary units) and 7l/min, respectively. The IonSpray potential was maintained at 5 kV. During ms/ms analysis, the collision energies used ranged from 18 to 25 V.

Quantification was performed by multiple reaction monitoring of the protonated precursor ion and the related product ion for Bortezomib using the IS method with a peak area ratios and a linear least-squares regression curve with weighting factor of $1/x2$. The mass transitions used for Bortezomib and Bortezomib D3 were m/z $367.3\rightarrow226.3$ and m/z $370.3\rightarrow 229.2$ respectively.

Sample preparation method

To 300µl of plasma, 50µl of bortezomib D3 (5µg/ml) was added and vortexed. The drug was extracted with 2ml of 0.1% formic acid in acetonitrile, followed by centrifugation at 2000 rpm/min on a cooling centrifuge for 15min at 4^{0}c. The supernatant was withdrawn, filtered using captiva SPE filter catridge of 0.45µm and dried using lyophiliser. To the residue 150µl of mobile phase was added and respective samples were injected into column.

VALIDATION

Specificity

A solution containing 2.0ng/ml was injected on to the column under optimized chromatographic conditions to show the separation of bortezomib from impurities and plasma. The specificity of the method was checked for the interference from plasma.

Linearity

Spiked concentrations were plotted against peak area ratios of bortezomib to internal standard and the best fit line was calculated .Wide range calibration was determined by solutions containing 2ng/ml to 1000ng/ml.

Recovery studies

The % mean recoveries were determined by measuring the responses of the extracted plasma Quality control samples at High, Middle and Low (HQC, MQC and LQC) against un extracted Quality control samples at HQC, MQC and LQC.

Precision and accuracy

Intraday precision and accuracy was determined by analyzing quality control standards (6, 41and 880ng/ml) and LLOQ (Lower Limit of Quantification) Quality control standard (2.00 ng/mL) five times a day randomly, interday precision and accuracy was determined from the analysis of each quality control standards (6, 41and 880ng/ml) and LLOQ Quality Control standards (2.00 ng/mL) once on each of five different days.

Matrix effect

The matrix effect for the intended method was assessed by using chromatographically screened human plasma. Concentrations equivalent to LQC and HQC of bortezomib were prepared with six different lots of plasma and are injected.

Ruggedness

Ruggedness was determined by analyzing six replicates of MQC in two batches using two different columns.

Stability studies

Short term (6 hours 38 minutes at ambient temperature) and long term (7 days below 8 °C) stock solution stability was determined at concentration 1mg/ml and 400µg/ml for bortezomib and bortezomib D3 respectively. Stability was assessed by comparing against freshly prepared same stock solutions.

Freeze thaw stability of the spiked HQC and LQC samples was determined during three freeze thaw cycles stored below -20 °C. Stability was assessed by comparing against the freshly thawed quality control samples.

RESULTS & DISCUSSION

The chromatography observed during the course of validation was acceptable and representative chromatograms of standard blank, LLOQ, HQC, MQC and LQC samples are shown in (Fig 1 to 2). The method developed was validated for

specificity, accuracy & precision, linearity and stability as per USFDA guidance. The results of validating parameters are given below.

Specificity

Six different lots of plasma were analyzed to ensure that no endogenous interferences were present at the retention time of bortezomib and bortezomib D3. Six LLOQ level samples along with plasma blank from the respective plasma lots were prepared and analyzed. (Table-1) shows results of specificity. In all plasma blanks, the response at the retention time of bortezomib was less than 20% of LLOQ response and at the retention time of IS, the response was less than 5% of mean IS response in LLOQ. The typical chromatogram of plasma blank (a) and chromatogram of LLOQ (b) was shown in (Fig 1).

Linearity

The calibration curve (peak area ratio Vs Concentration) was linear over working range of 2ng/ml to 1000ng/ml with ten point calibration used for quantification by linear regression, shown in (Fig 3). The regression equation for the analysis was Y=0.708x+3.864 with coefficient of correction (r^2) = 0.998.

Recovery

The % mean recovery for bortezomib in LQC, MQC and HQC was 82.02%, 81.75% and 85.36% respectively (Table-2).
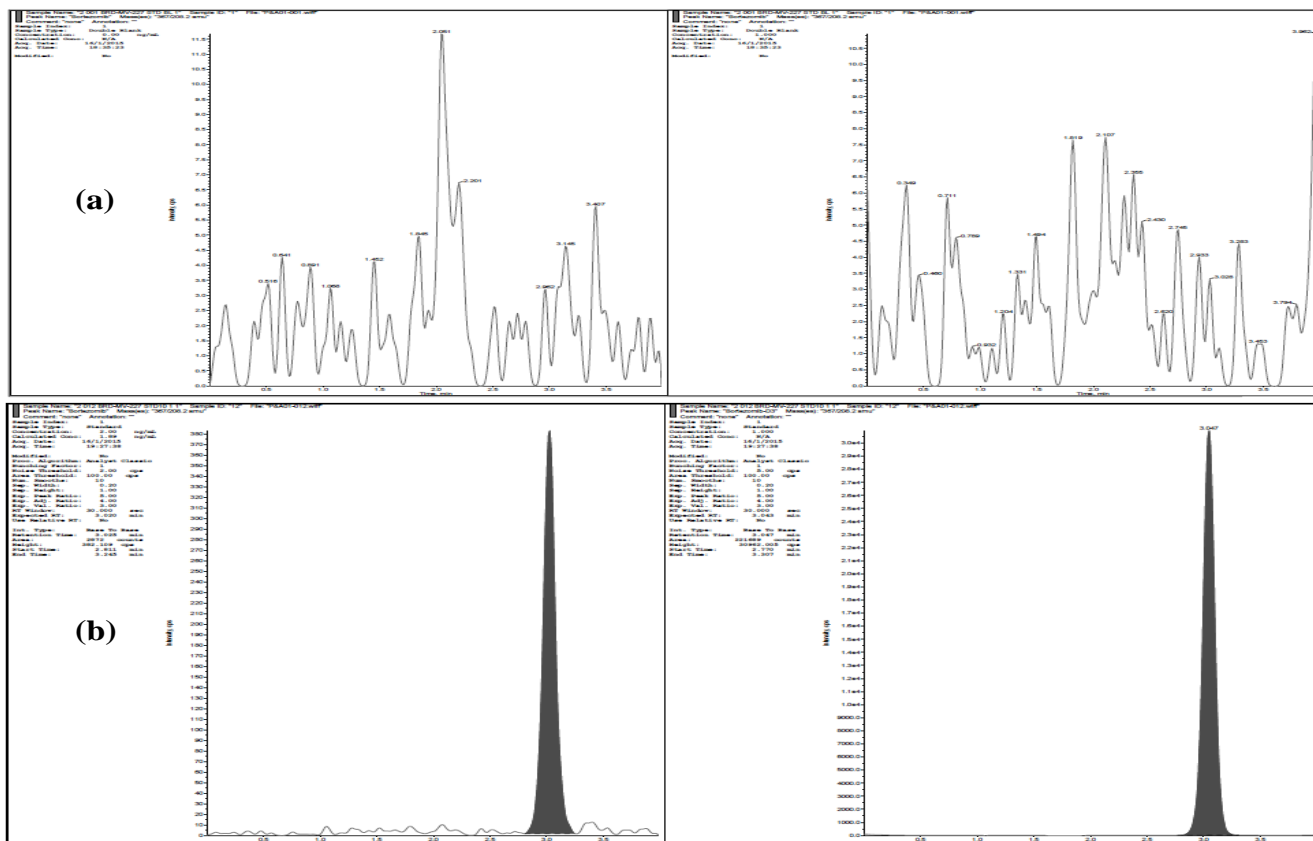

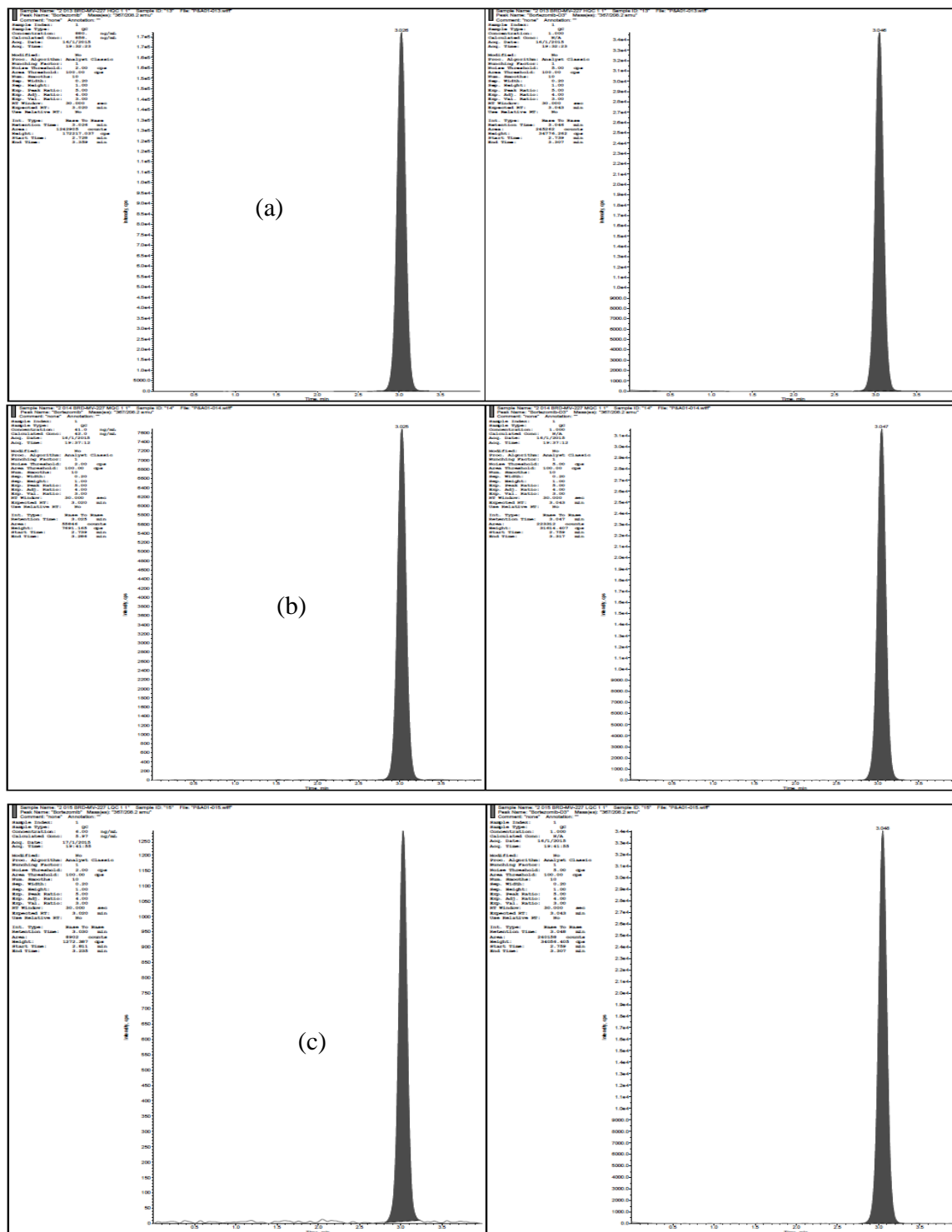**Fig. 1**: Showing chromatograms of blank plasma (a) and spiked LLOQC (b) samples.

Fig. 2: Showing chromatograms of spiked HQC (a), MQC (b) and LOQC (c) samples

Table 1: Showing results of specificity for bortezomib and bortezomib D3(ISTD).

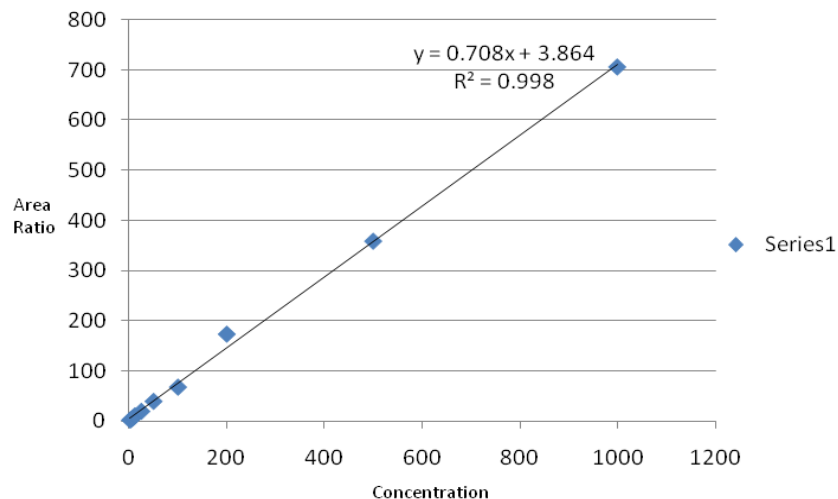| S.No.	Area of STD BL	LLOQ Area	LLOQ RT	% Interference	Area of STD BL	LLOQ Area	LLOQ RT	% Interference
		Drug Response				**ISTD Response**		
1	0	4821	3.024	0	0	354857	3.042	0
2	0	5000	3.023	0	0	369547	3.042	0
3	0	5099	3.023	0	0	385361	3.046	0
4	0	4681	3.02	0	0	348594	3.045	0
5	0	4856	3.016	0	0	360828	3.045	0
6	0	4801	3.019	0	0	356700	3.043	0

Fig. 3: Spiked concentrations (2ng/ml to1000ng/ml) were plotted against peak area ratio Vs Concentration with ten point calibration used for quantification by linear regression.

Table 2: Showing % Mean Recovery of bortezomib for LQC, MQC and HQC.

S.N0	LQC			MQC			HQC		
	Un-Extracted (area ratio)	Extracted (area ratio)	%Recovery	Un-Extracted (area ratio)	Extracted (area ratio)	%Recovery	Un-Extracted (area ratio)	Extracted (area ratio)	%Recovery
1	0.059	0.053	89.83	0.376	0.31	82.45	7.215	6.212	86.10
2	0.059	0.046	77.97	0.385	0.304	78.96	7.284	6.301	86.50
3	0.061	0.048	78.69	0.366	0.299	81.69	7.189	6.252	86.97
4	0.057	0.044	77.19	0.361	0.304	84.21	7.289	5.992	82.21
5	0.058	0.049	84.48	0.366	0.301	82.24	7.227	6.185	85.58
6	0.059	0.046	77.97	0.378	0.306	80.95	7.282	6.176	84.81
Mean	0.059	0.048	81.021	0.372	0.304	81.751	7.248	6.186	85.362
±SD	0.001	0.003		0.009	0.004		0.043	0.106	
%CV	2.26	6.59		2.45	1.27		0.59	1.71	

Table 3: Showing intraday and inter day Precision & Accuracy of Quality Control Standards.

Batch	QC ID	LQC	MQC	HQC	Batch	QC ID	LQC	MQC	HQC
	Actual conc.(µg/mL)	6	41	880		Actual conc.(µg/mL)	6	41	880
	1	5.97	42	858		1	5.94	38.5	831
	2	6.09	43.7	842		2	5.72	40.5	799
	3	6.07	42.1	855		3	5.65	38.8	786
Intra day	4	6.34	42	852	Inter	4	5.81	39.8	820
	5	6.15	40	853	day	5	6.37	39.9	800
	6	6.13	42.4	852		6	5.88	39.9	834
	Mean	6.107	42.033	852.0		Mean	5.89	39.56	811.6
	± SD	0.123	1.188	5.404		± SD	0.255	0.75	19.48
	% CV	2.01	2.83	0.63		% CV	4.33	1.92	2.40
	% Accuracy	101.79	102.52	96.81		% Accuracy	98.25	96.50	92.23

Intraday and Inter-day Precision

The intraday and inter-day precision and Accuracy of the method was found to be 0.63 to 4.33% and 92.23 to 102.52 respectively for the quality control samples. This is within the acceptance limits of precision is 15% and accuracy is 85 to 115% (Table-3). The limit of Quantification was found to be 2ng/ml. at such concentration the inter day precision was 3.64 and the accuracy was 95.5%. Which are within the acceptance limits of precision is 20% and accuracy is 80 to 120% (Table-4)

Table 4: Precision & Accuracy of LLOQC Standard.

Actual conc.(ng/mL)	LLOQ 2
1	1.97
2	1.81
3	1.85
4	1.84
5	1.94
6	1.96
Mean	1.910
± SD	0.069
% CV	3.64
% Accuracy	95.50

Matrix effect

The % CV for HQC and LQC samples was observed 0.72 and 1.7% respectively (Table-5), whichare within 15% as per the acceptance criteria.

Table 5: showing results of matrix effect obtained by preparing LQC and HQC with six different lots of plasma

QC ID	LQC	HQC
Actual conc.(µg/mL)	6	880
1	6.01	876
2	6.2	868
3	6.29	869
4	6.25	859
5	6.09	869
6	6.21	876
Mean	6.175	869.500
± SD	0.105	6.285
% CV	1.70	0.72
% Accuracy	102.92	98.81

Ruggedness

The ruggedness for two batches of MQC (n=6) was obtained using two different columns. The % mean ruggedness for batch-1 MQC and batch-2 MQC were found 96 and 87 respectively (Table-6), which is within acceptance limits of 85 to 115%.

Table 6: Showing the results of Ruggedness

	MQC (41ng/ml)	
S.NO	Column 1 (area ratio)	Column 2 (area ratio)
1	40.4	39.4
2	41.7	39.5
3	40.9	39.4
4	41.7	39.9
5	41.1	40.7
6	41.7	40.1
Mean	41.250	39.833
± SD	0.543	0.513
% CV	1.32	1.29

% Accuracy	100.61	97.15

Stability studies

The % mean stability for 6 hours 38 minutes at ambient temperature was found as 100.11 and 93.59 % for drug and ISTD respectively (Table-7) and the % mean stability for 7days at below 8ºC was found as 100.07 and 101.15 % for drug and ISTD respectively (Table-8), which is within the acceptance limits of 90 to 110%.

The % mean stability for HQC and LQC in Freeze Thaw Stability were 105 and 103 respectively **(Table-9)**, which is within the acceptance limits of 85 to 115%.

Table 7: Showing Short Term stock solution stability for bortezomib and bortezomib D3

	Bortezomib		Bortezomib-D3 (ISTD)	
	Nominal Concentration (mg/mL)		Nominal Concentration (µg/mL)	
Replicate No.	1.00	1.00	400	400
	Area Ratio		Area Ratio	
	Comparison Samples	Stability Samples	Comparison Samples	Stability Samples
01	6.293	6.237	0.185	0.173
02	6.322	6.254	0.186	0.176
03	6.137	6.142	0.189	0.178
04	6.233	6.381	0.183	0.174
05	6.233	6.255	0.192	0.174
06	6.215	6.204	0.188	0.176
Mean	6.2388	6.2455	0.1872	0.1752
SD	0.06459	0.07878	0.00319	0.00183
% CV	1.04	1.26	1.70	1.05
% Mean Stability	100.11		93.59	

Table 8: Showing long Term stock solution stability for bortezomib and bortezomib D3

	Bortezomib		Bortezomib-D3 (ISTD)	
	Nominal Concentration (mg/mL)		Nominal Concentration (µg/mL)	
Replicate No.	1.00	1.00	400	400
	Area Ratio		Area Ratio	
	Comparison Samples	Stability Samples	Compariso n Samples	Stability Samples
01	6.271	6.300	0.159	0.158
02	6.203	6.182	0.161	0.160
03	6.263	6.255	0.160	0.163
04	6.372	6.134	0.157	0.165
05	6.212	6.304	0.161	0.162
06	6.251	6.424	0.160	0.161
Mean	6.2620	6.2665	0.1597	0.1615
SD	0.06047	0.10214	0.00151	0.00243
% CV	0.97	1.63	0.94	1.50
% Mean Stability	100.07		101.15	

Table 9: Showing mean of three freeze thaw stability cycles of LQC and HQC

QC ID	LQC	HQC
Actual conc.(µg/mL)	6	880
1	5.86	843
2	5.95	845
3	5.67	847
4	5.92	850
5	5.51	837
6	5.53	845
Mean	5.740	844.500
± SD	0.196	4.370
% CV	3.42	0.52
% Accuracy	95.67	95.97

CONCLUSION

The analytical method developed and validated for the quantitative determination of Bortezomib from plasma was simple, rapid, specific, sensitive, accurate and precise. Hence, the method is quite suitable to detect the drug from plasma samples of human volunteers.

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Conflict of Interests: There are no conflicts of interest.

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