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Development and validation of HPLC-UV method for the estimation of Bortezomib in human plasma

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ABSTRACT

A Simple, rapid, selective and sensitive HPLC method was developed and validated for the determination of Bortezomib from human plasma. The drug was extracted with ethyl acetate. Bortezomib was measured in plasma using a validated a HPLC method with UV detector at 270nm chromatographic peaks were separated on 5µm intensil, C18 column (4.6x250mmx5µm) using a mixture of water-acetonitrile-formic acid (71: 28: 1, v/v/v) as mobile phase at a flow rate of 1 ml/min, pH 6 was adjusted with triethyllamime. The chromatograms showed good resolution and no interference from plasma. The retention time of Bortezomib and internal standard were approximately 5.9±0.05 min and 10.19± 0.03 min respectively. The mean recovery from human plasma was found to be above 88%. The method was linear over the concentration range of 0.3 to 20 µg/ml with coefficient of correlation (r^2) 0.998. Both intraday and interday accuracy and precision data showed good reproducibility. This method was successfully applied to pharmacokinetics studies.

Keywords: Bortezomib, HPLC, Validation, Atazanavir

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), [10] HPLC (Appala raju et al., 2014; Brinda et al 2013; Srinivasulu et al., 2012; Rambabu et al., 2011) [2] UFLC(Venkataramana et al., 2012) methods are available for quantification of bortezomib in its bulk and formulation dosage forms. Three LC-MS/MS (Nix et al., 2001; Christos et al., 2004; Pekol et al., 2005) [4, 5, 9] methods were reported for the estimation of bortezomib and 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) isocratic HPLC method and increasing the column life by removing interferences due to plasma proteins using LLE (liquid-liquid extraction) technique. The developed method was simple. Fast, accurate, specific and economical for the estimation of Bortezomib and atazanavir (ISTD) from human plasma.

MATERIALS AND METHODS

Bortezomib and atazanavir (IS) 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 Red Cross blood bank, Hyderabad, was used during validation and study sample analysis. The plasma was stored into -70±5^{o}C.

Chromatographic Conditions

The HPLC system consisted of Alliance waters 2695 with dual λ Absorbance UV detector. The wavelength of detection as set at 270nm. Separation was carried out on inertsil C18 column (4.6x250mmx5µm) using a mixture of water-acetonitrile-formic acid (71: 28: 1, v/v/v) as mobile phase at a flow rate of 1 ml/min, pH 6 was adjusted with triethyllamime. The mobile phase filtered through nylon milli pore (0.2µm) membrane filter, purchased from pall life sciences, Mumbai and degassed with Ultrasonicator prior to use. Chromatography was carried out at room temperature 25^{0}c and maintains the column temperature at 32^{0}c.

Preparation of Standard Solutions

Stock solutions of Bortezomib (0.5mg/ml) and atazanavir (1mg/ml) internal standard were prepared in methanol. Further dilutions were carried out in methanol. Calibration standards were prepared freshly by spiking drug free plasma with Bortezomib stock solution to give the concentrations of 0.3, 0.6, 2, 3, 5, 10, 15 and 20 µg/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 0.9, 10 and 18 µg/ml respectively. They were stored at -20^{0}c till the time analyzed.

Sample Preparation Method

To 500µl of plasma 50µl of atazanavir (50µg/ml) was added and vortexes. The drug was extracted with 2.5ml of ethyl acetate followed by centrifugation at 2000 rpm/min on a cooling centrifuge for 15min at 4^{0}c. The organic phase was withdrawn and dried using lyophiliser. To the residue 300µl of mobile phase was added and respective samples were injected into column.

VALIDATION

Specificity

A solution containing 0.3µg/ml was injected on to the column under optimized chromatographic conditions to show the separation of Bortezomib from the impurities from the 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 stds and the best fit line was calculated .Wide range calibration was determined by solutions containing 0.3µg/ml to 20 µg/ml

Recovery studies

The % mean recoveries were determined by measuring the responses of the extracted plasma Quality control samples at HQC, MQC and LQC against unextracted Quality control samples at HQC, MQC and LQC.

Limit of quantification

To estimate the LOQ, a drug free blank plasma sample was extracted and injected ten times and analyzed as described under optimized chromatographic conditions .The noise level was then determined, the limit of quantification for Bortezomib was determined.(signal to noise ratio=10).

Precision and accuracy

Intraday precision and accuracy was determined by analyzing quality control standards (0.9, 10,18 µg/ml) and LLOQ Quality control standards (0.3µg/ml) five times a day randomly, interday precision and accuracy was determined from the analysis of each quality control stds (0.9,10,18 µg/ml) and LLOQC standards(0.3µg/ml) once on each of five different days.

Stability studies

The stability of Bortezomib was determined by measuring concentration change in control sample overtime. The plasma control samples were stored in eppendrorff tubes at -20^{0}c. Stability was tested by subjecting the plasma controls to three freeze thaw cycles and stored for 24hrs at room temperature.

RESULTS & DISCUSSION

Under the chromatographic conditions employed, the sample showed sharp peaks of drug & internal standard with good resolution. The retention time of the drug was found to be 5.9 +0.05 min and the retention time of internal standard was 10.19±0.03 min (figure-2). The method developed was validated for specificity, accuracy & precision, linearity and stability as per USFDA guidance[9]. The results of validating parameters are given below.

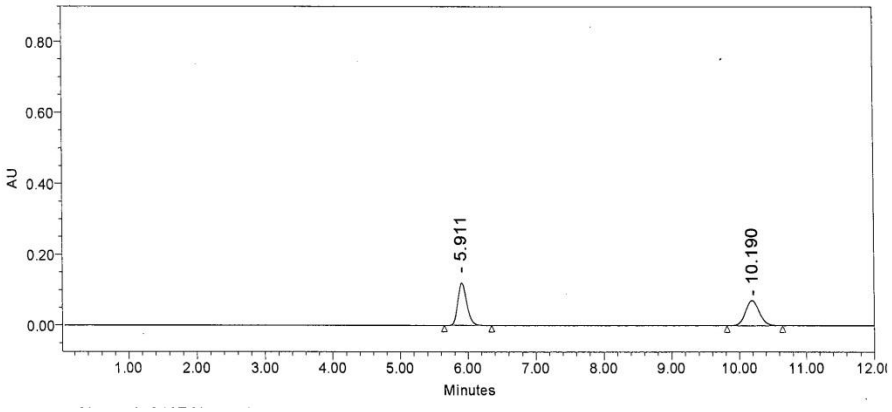

Fig-2: Retention times of Aqueous Mixture consists of Bortezomib (5µg/ml) and Atazanavir (5µg/ml)

Specificity of the method was proven by the absence of the peaks near the reaction time of drug as well as the internal standard (figure-3).

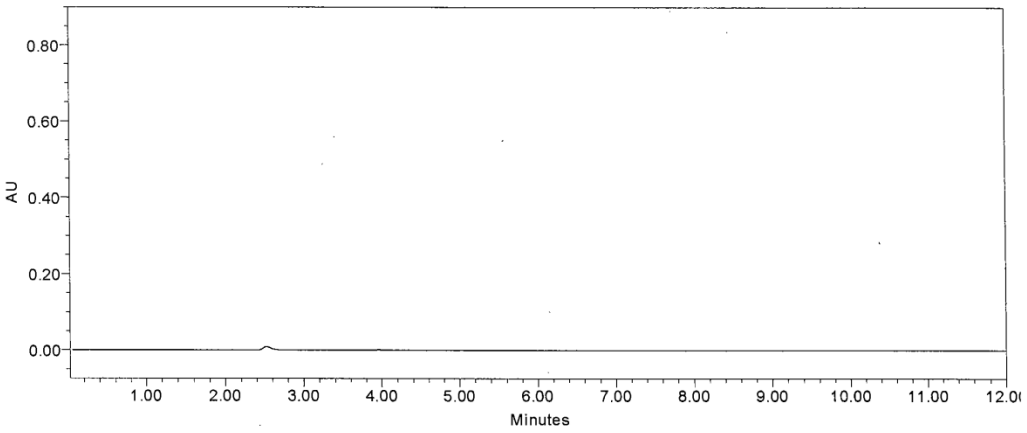

Figure-3: Blank plasma sample showing no interference at the RT of Bortezomib and atazanavir.

The calibration function (peak area ratio Vs Concentration) was linear over working range of 0.3 to 20 µg/ml with eight point calibration used for quantification by linear regression. The regression equation for the analysis was Y=5.32e-0.05x-4.82e-0.003 with coefficient of correction (r^2) = 0.998. (figure-4).

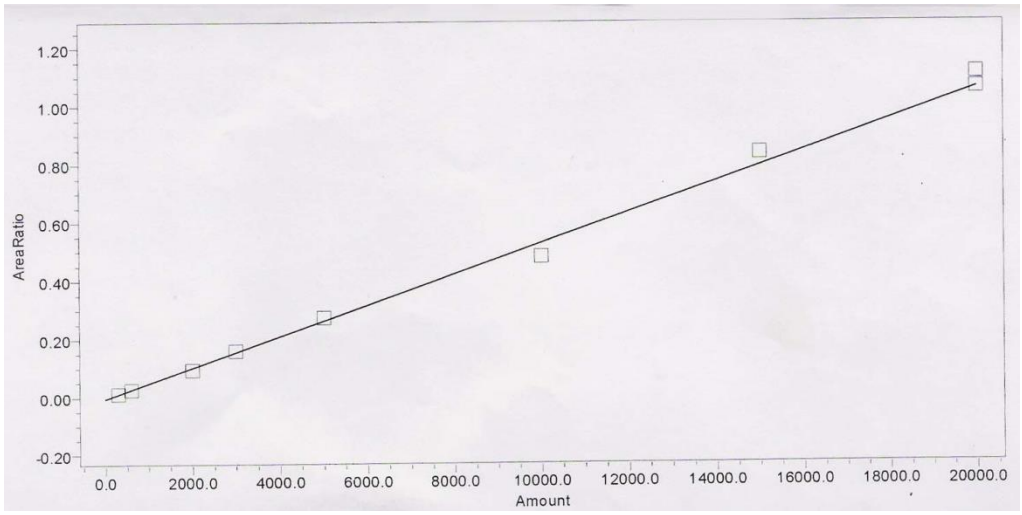

Fig-4: Spiked concentrations (0.3 to 20 µg/ml) were plotted against peak area ratio Vs Concentration with eight point calibration used for quantification by linear regression.

The % mean recovery for Bortezomib in LQC, MQC and HQC was 85.69%, 80.56% and 81.58% respectively (Table-1).

TABLE-1: Recovery Studies - Bortezomib

	LQC			MQC			HQC		
ID	**Unextracted**	**Extracted**	**% Recover**	**Unextracted**	**Extracted**	**% Recovery**	**Unextracte**	**Extracted**	**%Recove**
	(area ratio)	(area ratio)		(area ratio)	(area ratio)		(area ratio)	(area ratio)	
1	0.117	0.093	79.49	1.5	1.314	87.60	2.644	2.173	82.19
2	0.116	0.097	83.62	1.494	1.193	79.85	2.628	2.143	81.54
3	0.123	0.109	88.62	1.48	1.135	76.69	2.619	2.139	81.67
4	0.124	0.106	85.48	1.486	1.162	78.20	2.627	2.144	81.61
5	0.122	0.107	87.70	1.475	1.182	80.14	2.634	2.132	80.94
6	0.121	0.108	89.26	1.478	1.196	80.92	2.627	2.142	81.54
Mean	0.121	0.103	85.695	1.486	1.197	80.566	2.630	2.146	81.583
±SD	0.003	0.007		0.010	0.062		0.008	0.014	
%CV	2.71	6.44		0.66	5.15		0.32	0.66	

The intraday and interday precision and Accuracy of the method was found to be 0.90 to 1.53% and 87.84 to 100.21% respectively for the quality control samples. (Table-2).

TABLE-2: Precision & Accuracy

Batch ID	QC ID	LQC	MQC	HQC	Batch ID	QC ID	LQC	MQC	HQC
Intraday	**Actual conc.(µg/ml)**	**0.9**	**10**	**18**	**Interday**	**Actual conc.(µg/ml**	**0.9**	**10**	**18**
	1	0.844	9.983	18.165		1	0.781	9.796	18.126
	2	0.866	9.914	17.842		2	0.787	9.626	17.794
	3	0.854	9.98	17.731		3	0.801	9.846	17.719
	4	0.842	9.751	18.068		4	0.785	9.801	18.077
	5	0.844	9.795	18.385		5	0.796	9.706	18.405
	Mean	0.85	9.88	18.04		Mean	0.79	9.76	18.02
	± SD	0.010	0.107	0.260		± SD	0.008	0.088	0.276
	% CV	1.19	1.08	1.44		% CV	1.04	0.90	1.53
	% Accuracy	94.4	98.8	100.2		% Accuracy	87.8	97.6	100.1

Stability was assessed by comparing against the freshly thawed quality control samples. The %mean stability for HQC and LQC were 103.29 and 99.65 respectively, Plasma Quality control samples of Bortezomib were found to be stable for at least one month (Table-3).

TABLE-3: Freeze-thaw Stability

Freeze – thaw III Cycles		
QC ID	**LQC**	**HQC**
Actual conc.(µg/ml	**0.9**	**18**
1	0.91	19.51
2	0.91	18.58
3	0.89	18.55
4	0.92	18.24
5	0.87	18.15
6	0.88	18.52
Mean	0.90	18.59
± SD	0.020	0.485
% CV	2.214	2.611
% Accuracy	99.65	103.29

Bortezomib is soluble in methanol. Hence standard solutions were prepared in methanol. The extraction of Bortezomib was based on liquid-liquid extraction technique. Various solvent systems were tried for recovery studies. The maximum recovery was obtained with ethyl acetate. Five drugs were attempted for selection as internal standard. The other drugs tried were found to be overlapping with reaction time of Bortezomib under the optimized chromatographic conditions.

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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