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Original Article

A VALIDATED LC-MS/MS METHOD FOR THE ESTIMATION OF BOCEPREVIR AND BOCEPREVIR D6 (IS) IN HUMAN PLASMA EMPLOYING LIQUID-LIQUID EXTRACTION

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ABSTRACT

Objective: This study was aimed to develop a simple, rapid, specific and precise liquid chromatography-tandem mass spectrophotometric (LC–MS/MS) validated method for quantification of Boceprevir and internal standard (ISTD) Boceprevir D6 in human plasma.

Methods: Plasma samples were pretreated with 100 µl of 0.1N Sodium Hydroxide and are subjected for Liquid-Liquid Extraction (LLE) using 2.5 ml of ethyl acetate. Chromatographic separation was achieved on Chromolith RP18e column (100 mmx4.6 mmx5 µm) with Acetonitrile: 20 mM Ammonium formate (80:20%v/v) as an isocratic mobile phase with a flow rate of 1.2 ml/min. the LC eluent was split, and approximately 0.1 ml/min was introduced into Tandem mass spectrometer using turbo Ion Spray interface at 400 °C. Quantitation was performed by transitions of *m/z* 586.2 precursor ion to the *m/z* 422.2 for Boceprevir and *m/z* 592.2/574.20 for Boceprevir D6.

Results: The concentrations of nine working standards showed linearity between 2 to 1000 ng/ml ($r^2 \geq 0.998$). Chromatographic separation was achieved within 3 min. The limit of Quantification (LOQ) was found to be 2ng/ml. The intraday and interday precision with quality control samples was found to be 0.09 to 3.17%.

Conclusion: The assay is suitable for pharmacokinetic study samples as demonstrated by its specificity, precision, accuracy, recovery, and stability characteristics.

Keywords: Boceprevir, LC-MS/MS, Human plasma, Liquid-Liquid Extraction

INTRODUCTION

Boceprevir, (1*R* 3-{[(1R,2S,5S)-3-[(2S)-2-[(tertbutyl carbamoyl) amino] -3,3-dimethylbutanoyl]-6,6-dimethyl-3-azabicyclo [3.1.0] hexan-2-yl] formamido}-4-cyclobutyl-2-oxobutanamide, is a protease inhibitor used in treatment of hepatitis caused by hepatitis C virus (HCV) genotype 1 in combination with peginterferon and ribovirin [1-3]. One RP-HPLC Method was developed for the estimation of boceprevir from its bulk and dosage forms [4]. LC-MS/MS method was reported for characterization of human liver enzymes in the biotransformation of boceprevir [5]. Second LC-MS/MS method was developed for *in vitro* study of drug-drug interactions of boceprevir with metabolic enzymes and transporters [6]. Only two LC-MS/MS methods were reported for simultaneous estimation of boceprevir in combination with other drugs from human plasma [7-8]. Literature survey reveals, there are no HPLC and LC-MS/MS methods for quantitative estimation of boceprevir alone from Human Plasma. We, therefore, aimed to develop a simple, rapid, robust, and efficient HPLC-MS/MS validated method for the quantification of boceprevir from human plasma using boceprevir D6 as internal standard employing an economical liquid-liquid extraction technique.

MATERIALS AND METHODS

Apparatus and software

The HPLC system with an autosampler was a Shimadzu LC-20ADvp (Shimadzu, Japan) coupled with Applied Biosystem Sciex (MDS Sciex, Canada) API 3000 Tandem mass spectrometer. The autosampler was SIL-HTC from Shimadzu, Japan. 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

Boceprevir and Boceprevir D6 (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 the 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±5°C.

Standards and working solutions

Calibration standard solutions

Stock solutions of Boceprevir and Boceprevir D6 internal standard (IS) were prepared in methanol. Further dilutions were carried out in Formic acid in Methanol (0.1%v/v): Water 50:50 v/v. Calibration standards of nine concentration levels were prepared freshly by spiking drug-free plasma with Boceprevir stock solution to give the concentrations of 2.00, 4.00, 10.00, 25.0, 50.0, 125, 250, 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 Boceprevir to give a solution containing 6, 400and 800ng/ml respectively. They were stored at-20 °c till the time analyzed.

Chromatographic conditions

Chromatographic separation was performed on a Chromolith RP18e (100 mmx4.6 mmx5 µm), analytical column and the mobile phase was Acetonitrile: 20 mM Ammonium formate (80:20% v/v) as an isocratic mobile phase with a flow rate of 1.2 ml/min. Injection volume was 10µl. Total analysis time of single injection was 5.00 min. Column oven temperature and autosampler 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, and Quantitation was achieved with MS/MS detection in

positive ion mode for the analytes and IS using a MDS Sciex API-4000 mass spectrometer (Foster City, CA, USA) equipped with Turboion spray™ interface at 400 °C. The ion spray voltage was set at 5500 V. The source parameters viz., the nebulizer gas, curtain gas, CAD gas were set at 40, 30 and 5 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) for MT and MT-d_6 were similar and are 105, 32, 10, 14 V. For FF and FF-d_{10} the DP, CE, EP and CXP were 100, 36, 10 and 15 V. A Turbo ion spray interface (TIS) operated in positive ionization mode was used for the detection. Detection of the ions was carried out in the multiple-reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 586.2 precursor ion to the *m/z* 422.2 for Boceprevir and *m/z* 592.2/574.20 for Boceprevir-d_6. Quadrupoles Q1 and Q3 were set on unit resolution.

Sample preparation method

To 200 µl of plasma, 50 µl of Boceprevir D6 (5µg/ml) was added and vortexed. The sample was pretreated with 100 µl of 0.1N Sodium Hydroxide then extracted with 2.5 ml of ethyl acetate followed by centrifugation at 2000 rpm/min on a cooling centrifuge for 15 min at 4 °c. The 2 ml of supernatant was withdrawn and dried using lyophiliser. The residue was reconstituted in 300 µL of the mobile phase, and a 4 µL was injected into the column.

Validation

Specificity

A solution containing 2.0ng/ml was injected onto the column under optimized chromatographic conditions to show the separation of Boceprevir 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 Boceprevir to the 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 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, 400and 800ng/ml) and LLOQ 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, 400and 800ng/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 Boceprevir 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.

RESULTS AND DISCUSSION

The chromatography observed during the course of validation was acceptable and representative chromatograms of standard blank, HQC, MQC and LQC samples are shown in (fig. 1 to 4).

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 Boceprevir and Boceprevir D6. 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 Boceprevir 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.

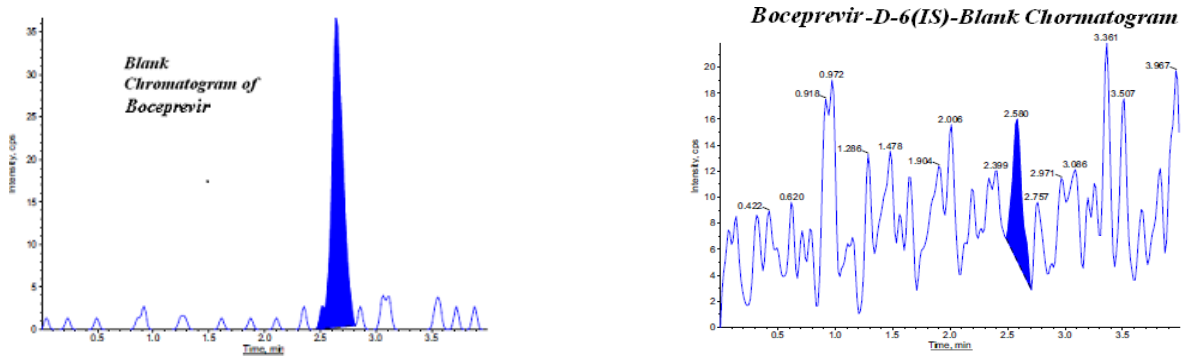

Fig. 1: Representative blank chromatograms of boceprevir and IS in blank plasma

Table 1: Showing results of specificity for Boceprevir and Boceprevir D6 (ISTD)

S. No.	Drug response				ISTD response			
	Area of STD blank	LLOQ Area	LLOQ RT	% Interference	Area of STD blank	LLOQ Area	LLOQ RT	% Interference
01	41	2169	2.652	1.89	68	470648	2.624	0.01
02	4	2314	2.651	0.17	17	492693	2.615	0.00
03	8	1691	2.652	0.47	6	413426	2.635	0.00
04	8	2016	2.650	0.40	9	478343	2.623	0.00
05	10	2430	2.653	0.41	9	519770	2.633	0.00
06	6	1751	2.652	0.34	65	482317	2.627	0.01

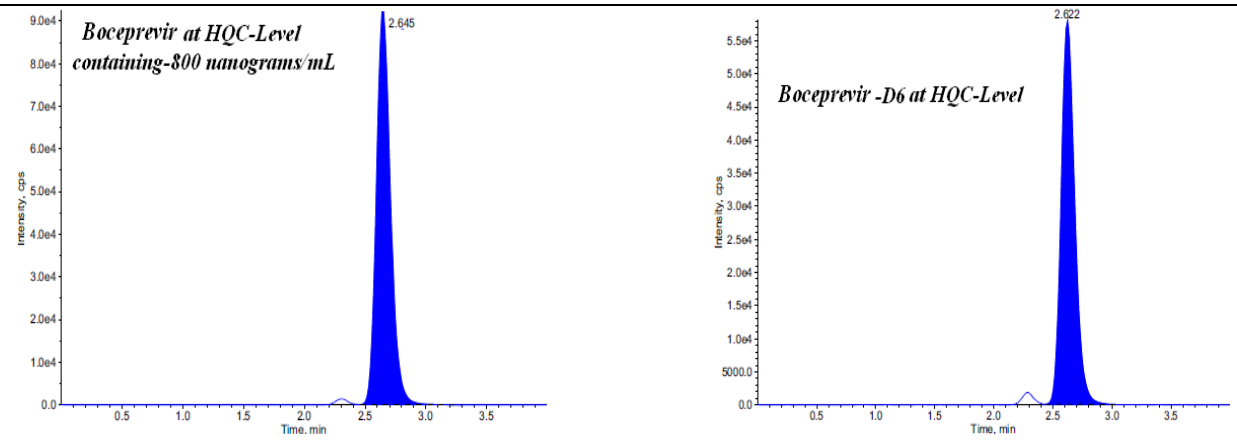

Fig. 2: Representative HQC-chromatograms of Boceprevir in Plasma with internal standard

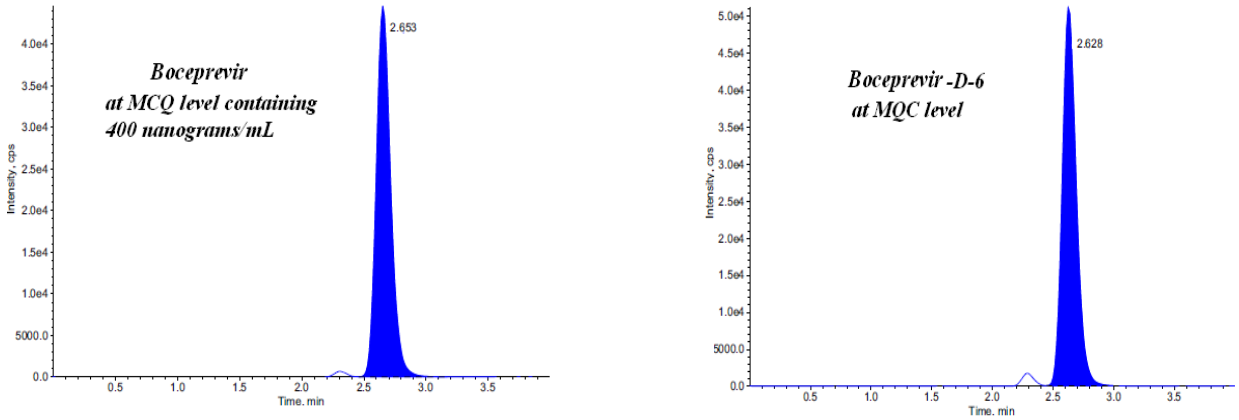

Fig. 3: Representative MQC-chromatograms of boceprevir and its internal standard

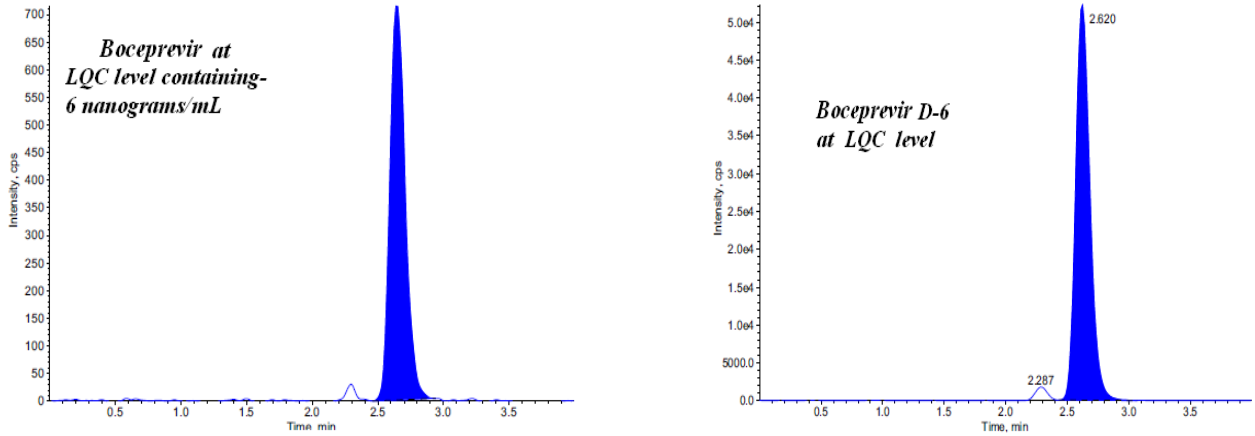

Fig. 4: Representative chromatograms of boceprevir and its internal standard at LQC Level

Linearity

The calibration curve (peak area ratio Vs Concentration) was linear over working a range of 2ng/ml to 1000ng/ml with ten point calibration used for quantification by linear regression, shown in (fig. 5).

The regression equation for the analysis was Y=0.710-2.318 with a coefficient of correction (r^2) = 0.999.

Recovery

The % mean recovery for Boceprevir in LQC, MQC and HQC was 66%, 74.7% and 62.8% respectively (table 2).

Intraday and interday precision

The intraday and interday precision of the method was found to be 0.09 to 3.17% for the quality control samples. This is within the acceptance limits of precision is 15%. The limit of Quantification was found to be 2ng/ml. at such concentration, the interday and intraday precision were found to be 7.54% and 3.52% respectively. Which are within the acceptance limits of precision is 20%. (table 3)

Matrix effect

The % CV for HQC and LQC samples was observed 1.15% and 4.36% respectively (Table 4), which are within 15% as per the acceptance criteria.

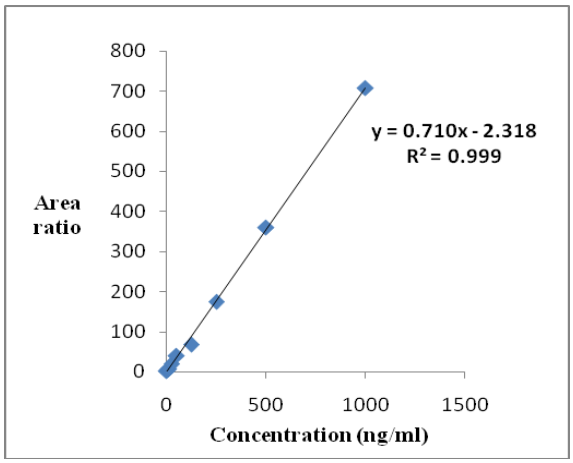

Fig. 5: Spiked concentrations (2ng/ml to1000ng/ml) were plotted against peak area ratio Vs Concentration with nine point calibration used for quantification by linear regression

Table 2: Showing % mean recovery of boceprevir for LQC, MQC and HQC

Replicate number	Extraction recovery studies of boceprevir (analyte-1)					
	HQC (800 ng/ml)		MQC (400 ng/ml)		LQC (6 ng/ml)	
	extracted peak area	Un-extracted peak area	Extracted peak area	Un-extracted peak area	Extracted peak area	Un-extracted peak area
1	779111	1117607	54310	68298	5935	8185
2	736269	1127551	52789	68719	5220	8918
3	721212	1245455	51784	70905	5060	8560
4	764700	1223297	52137	68785	5420	8409
5	770962	1232020	50933	69041	6541	9082
6	743630	1239998	51334	73870	6683	9686
Mean	752647.3	1197654.5	52214.5	69936.3	5809.8	8806.7
SD	22444.43	58717.10	1210.79	2130.98	689.18	541.64
% CV	2.98	4.90	2.32	3.05	11.86	6.15
% Mean recovery	62.8		74.7		66.0	
% Overall Recovery	68.70					
% Overall CV	7.71					

Table 3: Summery of intra-and inter-batch precision and accuracy study

QC	Boceprevir (ng/ml)			
Intra-batch	LLOQ QC(2)	LQC (6)	MQC (400)	HQC (800)
Mean	2.07	6.35	386	820
SD	0.0703	0.201	4.20	13.2
%CV	3.52	3.17	1.09	1.61
% Bias	0.00	5.83	-3.50	2.50
N	6	6	6	6
Inter-batch	LLOQ QC(2)	LQC (6)	MQC (400)	HQC (800)
Mean	2.01	5.95	404	767
SD	0.156	0.109	5.18	7.59
%CV	7.54	1.83	1.28	0.99
% Bias	3.50	-0.83	1.00	-4.13
N	6	6	6	6

Table 4: showing results of matrix effect obtained by preparing LQC and HQC with six different lots of plasma

QC ID	LQC	HQC
Actual conc. (ng/ml)	6	800
1	5885	769161
2	5523	771264
3	5290	751619
4	5520	766100
5	5941	766962
6	5683	751610
Mean	5640.333	762786
±SD	246.1964	8837.913
% CV	4.364926	1.158636

Table 5: Showing the results of ruggedness

QC ID	MQC	MQC
S. No.	Column-1	Column-2
1	64310	65298
2	62789	66719
3	61784	67905
4	62137	68485
5	60933	67041
6	61334	63870
Mean	62214.5	66553
±SD	1210.787	1708.786
% CV	1.94615	2.567556

Ruggedness

The ruggedness for two batches of MQC (n=6) was obtained using two different columns. The % CV for batch-1 MQC and batch-2 MQC were found 1.9 and 2.5 respectively (table 5), which is within acceptance limits of 15%

CONCLUSION

The analytical method developed and validated for the quantitative determination of boceprevir 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 and for pharmacokinetic studies.

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CONFLICT OF INTERESTS

Declared none

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