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Research Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF ENTRECTINIB IN RAT PLASMA BY LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY

PRAVALLIKA KE*, PRAMEELA RANI A, RATNA KUMAR M

Department of pharmaceutical analysis, University College of Pharmaceutical Sciences, Acharya Nagarjuna University, Andhra Pradesh, India. Email: elvina2108@gmail.com

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ABSTRACT

Objective: The objective of the study was to develop and validate the bioanalytical liquid chromatography–mass spectrometry (LCMS/MS) method for the estimation of entrectinib in bulk and pharmaceutical drugs in rat plasma.

Methods: Chromatographic separation of entrectinib with D_4-entrectinib as internal standard (IS) was achieved using Waters Alliance high-performance liquid chromatography system, quaternary gradient pump of e2695 using Luna, 250×4.6 mm, 5 µm column and the mobile phase containing 0.1% formic acid and acetonitrile (ACN) within the ratio of 70:30% v/v. The flow was 1.0 ml/min; detection was carried out by absorption at 294 nm using a photodiode array detector at ambient temperature.

Results: The peak of entrectinib was eluted at retention times of 5.225 min. The multiple reaction monitoring was 560.6/475.1 (m/z) for entrectinib and 580.6/496.3 (m/z) for IS entrectinib (D_4). The linearity range was 1–20 ng/ml with a regression coefficient of 0.999. % relative standard deviation of peak areas of all measurements was found to be <2.0 which complies with acceptance criteria.

Conclusion: The method was successfully validated and it had been found to be within limits for accuracy, precision, and linearity and it is stable under analytical conditions used.

Keywords: Liquid chromatography–mass spectrometry, Entrectinib, D_4 - entrectinib.

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INTRODUCTION

Bioanalytical methods are used for the qualitative and quantitative analysis of drug substances in biological fluids (mainly plasma, serum, and urine) or tissue [1]. Bioanalytical methods are essential for bioavailability and bioequivalence studies. IUPAC name of entrectinib *N*-[5-(3,5-Difluorobenzyl)-1*H*-indazol-3-yl]-4-(4-methyl-1-piperazinyl)-2- weight is 560.64 g/mol and the molecular formula is $C_{31}H_{34}F_2N_6O_2$. Entrectinib (INN, trade name Rozlytrek previously known as RXDX-101 and NMS-E628) is an anti-cancer drug used to treat ROS1-positive non-small cell lung cancer and NTRK fusion-positive solid tumors [2,3].

Entrectinib is a tyrosine kinase inhibitor; hence, it acts on several receptors. It acts as an adenosine triphosphate competitor and inhibits tropomyosin receptor tyrosine kinases (TRK) TRKA, TRKB, and TRKC, and also as proto-oncogene tyrosine-protein kinase ROS1 and anaplastic lymphoma kinase (ALK). TRK receptors produce cell proliferation through downstream signaling through the mitogen-activated protein kinase, phosphoinositide 3-kinase, and phospholipase C-γ. ALK produces similar signaling with the addition of downstream JAK/STAT activation. Inhibition of those pathways suppresses neoplastic cell proliferation and shifts the balance in favor of apoptosis, resulting in shrinking of tumor volume. Literature survey revealed that there is no analytical methods have been reported individually or in combination with other drugs. This study describes that a validated liquid chromatography–mass spectrometry (LC-MS)/MS method was developed for entrectinib in rat plasma along with stability studies.

METHODS

Chemicals and reagents

Entrectinib and internal standard (IS) (entrectinib D_4) was procured from Gland Pharma PVT LTD, Hyderabad, India. Acetonitrile (ACN) of LCMS grade was purchased from Rankem. Methanol and formic acid of LCMS grade from MERK. Water was from Milli Q System and plasma from local suppliers.

Instrumentation

Chromatographic separation of entrectinib was achieved on Waters Alliance-e2695 using Luna, 250×4.6 mm, 5 µm column and the mobile phase containing 0.1% formic acid and ACN in the ratio of 70:30% v/v. The flow rate was 1.0 ml/min. An injection volume was 10 µl and the column temperature was 30°C. The runtime was 10.0 min. The LC-MS/MS consists of SCIEX QTRAP 5500 triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) with an automatic sample injector. The mass spectrometer was operated in positive ESI mode. The drying gas temperature was 120–150°C and the Dwell time was 1 s. Quantification was performed using multiple reaction monitoring (MRM) of the transitions.

Preparation of standards and quality control (QC) samples

The stock solution of entrectinib used during LCMS method development stage was prepared by dissolving the accurately weighted standard compound in ACN. Concentration of entrectinib standard solution was 0.5 mg/ml, appropriate dilutions with the mobile phase were made from the stock solution to prepare the working standard solutions for method development, calibration curve, and QC samples. Working standard solution of entrectinib and IS (D_4) was prepared in diluents (mobile phase) to get both have a concentration of 10 ng/ml. Calibration curve was prepared by spiking appropriate amounts of working solution into the blank plasma to get final concentrations of 1, 2.5, 5, 7.5, 10, 12.5, 15, and 20 ng/ml for the entrectinib. The calibration curve was prepared by plotting the peak area ratio of the transition pair of entrectinib to that of IS against the nominal concentration of calibration standards.

The purpose of QC standards (QC) are to assess the performance of the assay procedure. It also covers the whole range of the calibration line. It must also cover the whole range of the calibration line. Low QC (LQC), that is, 3 times of lower limit of QC (LLOQ), mid QC (MQC), that is, 100% or near about of highest calibration point, high QC (HQC), that is, 150% or near about of highest calibration point.

Extraction procedure

Simple liquid extraction is done. To a glass tube containing200 µl of blank plasma to this add 300 µl of ACN, add 500 µl of entrectinib of 10 ng/ml and IS of 10 ng/ml. Finally, add 500 of diluent. The solution was mixed on a vortex mixer for approximately 5 min then centrifuges it for 20 min at 5000 rpm. Collect the 2 ml supernatantant, these were directly injected into LC-MS/MS column.

Assay validation

The LCMS method was validated to satisfy the acceptance criteria of industrial guidance for the bioanalytical method validation [4], Food and Drug Administration of the United States, 2001 [5].

LOD and LOQ

LOD and LOQ were separately determined by the calibration curve method. LOD and LOQ of the compound were determined by injecting progressively lower concentrations of standard solutions using the developed LCMS method. The LOD concentrations for entrectinib are 0.10 µg/ml and their s/n values are 5. The LOQ concentration for entrectinib is 1.0 µg/ml; their s/n values are 26. The results are shown in Table 1.

Validation of developed bioanalytical LCMS method for entrectinib

System suitability

It is used to indicate whether the instrument in use is functioning properly or not and to give the green light to proceed with the assaying of the next batch of samples. System suitability samples were included at the start, middle, and end of each batch of samples. The final concentration of the system suitability samples was made up to contain 10 ng/ml entrectinib and 10 ng/ml IS in mobile phase. Relative standard deviation (RSD) % of peak area and retention time (RT) for entrectinib and IS for six consecutive injections were checked to see whether they were below 2% and 5%, respectively. The results are shown in Table 2.

Stability of stock solution

An aqueous stock solution containing 10 ng/ml entrectinib and 10 ng/ml IS was prepared in diluent. The solution was divided into three containers, the first one stored at room temperature, the second one stored at deep freezer, and the last one stored at −20°C (assumed stable as a freshly prepared solution). The solutions of drug and IS from each storage conditions taken out at predetermined time intervals (0, 12, and 24 h) and were injected onto the LCMS. The peak area from the chromatogram of each sample was compared with that of freshly prepared samples. The results are shown in Table 2

Calibration curve

An 8-point calibration curve was prepared by spiking appropriate amounts of working solution into the blank plasma to get final concentrations of 1, 2.5, 5, 7.5, 10, 12.5, 15, and 20 ng/ml for the entrectinib. The calibration curve represented in Fig. 2 was prepared by plotting the peak area ratio of the transition pair of entrectinib to that of IS against the nominal concentration of calibration standards. The results were fitted to linear regression analysis

Table 1: LOD and LOQ data for entrectinib

Name	LOD		LOQ	
	Concentration (ng/ml)	s/n	Concentration (ng/ml)	s/n
Entrectinib	0.01	5	0.1	26

LOD: Limit of detection, LOQ: Limit of quantification, s/n: Signal to noise ratio

RESULTS AND DISCUSSION

In the present study, LC-MS/MS assay was developed for positive ionization which was evaluated, and therefore, the full scan mass spectrum of entrectinib and IS in the positive MRM is presented in Figs. 3 and 4. Finally, the reliability of the method was assessed on the basis of linearity, accuracy, precision, sensitivity, selectivity, and recovery studies.

Accuracy and precision

Accuracy and precision should be assessed by analyzing a minimum of three validation batches, including both intra- and inter-day runs. Both within and between run accuracy and precision should be assessed. Each validation batch must comprise a minimum of six to eight non-zero calibration standards, one standard blank (matrix blank) and standard zero (matrix blank with IS) and six replicates of QC standards at each limit of quantification (LOQ) (LOQQC), low (LQC), middle (MQC), and high (HQC) levels [6-8]. Acceptance criteria should be between and within batch CV for low, middle, and HQC levels should be ≤15% and for the LOQQC level should be ≤20%. The results are shown in Table 3.

Specificity and selectivity

Selectivity or specificity should be evaluated to assess the interference at the RT of the analyte and IS with method conditions shown in Figs. 5-7 [9]. At least six lots of blank matrix should be processed and after analysis, spike six LOQ samples in the least interference blank and analyzed. For all the chromatographic assays, the peak response related to blank matrix at the RT of analyte should be not more than 20% of the mean response of the LOQ samples and the peak response at the RT of the IS should be no more than 5% of the mean peak response of the IS of the LOQ.

Linearity

The standard curves were linear over the concentration range of 1.0–20.00 ng/ml of entrectinib Fig. 1. The mean correlation coefficient was 0.999. Samples were quantified using the ratio of peak area of the analyte to that of IS. Peak area ratios were plotted against plasma concentrations. The results are shown in Table 4 and the chromatograms shown in Figs. 8-15.

Development of LCMS method for entrectinib

Acceptance criteria

The criteria for the acceptability of the data include accuracy within 85–115% from the actual values. No interfering peaks were found

Table 2: System suitability results of entrectinib

Sample name	Analyte area	Analyte RT (min)	IS area	IS RT (min)	Area ratio
MQC	3.428×10^5	5.226	3.485×10^5	5.222	0.9836
MQC	3.462×10^5	5.221	3.481×10^5	5.236	0.9941
MQC	3.479×10^5	5.223	3.476×10^5	5.227	1.0009
MQC	3.466×10^5	5.227	3.449×10^5	5.231	1.0049
MQC	3.458×10^5	5.229	3.478×10^5	5.235	0.9942
MQC	3.487×10^5	5.226	3.461×10^5	5.230	1.0075
Mean	3.463×10^5	5.225	3.474×10^5	5.230	0.9980
SD	0.02045	0.00288	0.0198	0.00519	0.00874
%RSD	0.59	0.06	0.62	0.10	0.88

Analyte RT (min): Analyte retention time in minutes, IS area: Internal standard area, IS RT (min): Internal standard retention time in minutes

Table 3: Accuracy and precision of data of the entrectinib (n=6)

Quality control sample	Spiked concentration (ng/ml)	Mean (ng/ml)	SD	Accuracy (%)	RSD (%)
Intra-day					
LLOQ	0.3865×10^5	0.3841×10^5	0.0157	96.32	0.82
LQC	1.6724×10^5	1.6711×10^5	0.0326	98.28	0.16
MQC	3.4625×10^5	3.4628×10^5	0.0458	100.05	0.33
HQC	5.0637×10^5	5.0664×10^5	0.0269	99.89	0.08
Inter-day					
LLOQ	0.3851×10^5	0.3836×10^5	0.0126	95.63	0.76
LQC	1.6739×10^5	1.6759×10^5	0.0364	97.46	0.27
MQC	3.4696×10^5	3.4665×10^5	0.0428	99.58	0.26
HQC	5.061×10^5	5.0643×10^5	0.0238	97.42	0.14

SD: Standard deviation, RSD: Relative standard deviation, LLOQ: Lower limit of quality control, LQC: Low-quality control, MQC: Mid quality control, HQC: High-quality control

Table 4: linearity data of entrectinib

Linearity	Plasma (µl)	ACN (µl)	Std stock (µl)	IS (µl)	MP added (µl)	Entrectinib concentration (ng/ml)	Entrectinib response	Area res ratio
Linearity-1	200	300	50	500	1450	1.00	0.388	0.111
Linearity-2	200	300	125	500	1375	2.50	0.849	0.244
Linearity-3	200	300	250	500	1250	5.00	1.688	0.486
Linearity-4	200	300	375	500	1125	7.50	2.463	0.714
Linearity-5	200	300	500	500	1000	10.00	3.429	0.986
Linearity-6	200	300	625	500	875	12.50	4.163	1.203
Linearity-7	200	300	750	500	750	15.00	5.058	1.463
Linearity-8	200	300	1000	500	500	20.00	6.529	1.877
Slope						0.0940		
Intercept						0.01637		
r^2						0.99935		

ACN: Acetonitrile, std stock: Standard stock, IS: Internal standard, MP added: Mobile phase added

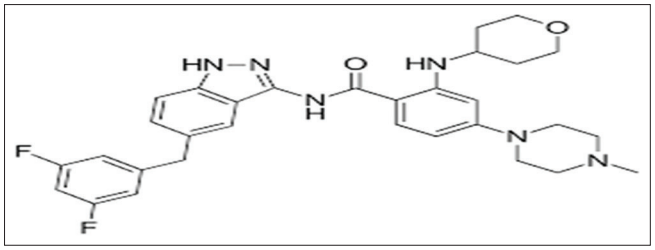

Fig. 1: Structure for entrectinib

in six different random blank rat plasma samples at the RTs of either entrectinib or IS.

Specificity and selectivity

No interfering peaks were found in six different random blank rat plasma samples at the RTs of either entrectinib or IS.

As observed from the above chromatogram, total run time was 10 min and the RT of drugs and IS was about 5.211 and 5.214 min, respectively. For blank plasma chromatogram, there were no interfering peaks near the peaks for entrectinib and IS. Same is observed in the case of the chromatogram of blank plasma spiked with IS.

Acceptance criteria

The Linearity Regression coefficient should be $R^2 = 0.999$

Recovery or extraction efficiency

Recovery studies are often determined by comparing the detector response of the analyte or IS from an extracted sample to the unextracted samples. Unextracted sample might be a neat drug solution of equivalent concentration. A minimum of six samples at each QC level should be injected [10]. Recovery deemed acceptable if %CV is 15% for %mean recovery between low, middle, and HQC levels [11]. The results are shown in Table 5.

Sensitivity

The lowest standard (LOQ) is always accepted as the LOQ of the method. Sensitivity should be evaluated using at least five replicates of the samples at the LOQ. The compliance limits for LOQ should be ±20% for accuracy and ≤20% for precision [12]. In addition, signal to noise ratio (S/N) should be at least 5:1. The results are shown in Table 6.

Matrix effect

Matrix factor is a way of assessing the matrix effect. Since ionization of analyte is going to be suffering from the presence of endogenous components in the biological matrix, it could be either suppression or enhancement [13].

According to the method peak, response could be peak area, peak height, and peak area ratio or peak height ratio. Matrix factor equal to 1 indicates no matrix effect, matrix factor <1 indicates suppression, and >1 indicates enhancement [14]. The IS normalized matrix factor (ratio of analyte and IS matrix factor) using stable isotope-labeled IS is generally usually close to unity for the bioanalytical samples. It is recommended that matrix factor or IS normalized matrix factor being determined in six different lots of matrices. The variability in matrix factors as measured by the coefficient of variation (%CV) should be <15%. The results are shown in Table 7.

Acceptance criteria

The %RSD of recovery at each QC level and for ISTD should be ≤15.00%. The overall mean recovery %RSD for all QC levels should be ≤ 20.00

Acceptance criteria

At least 67 % (4 of 6) of samples should be within 80.00–120.00.

% Mean accuracy should be within 80.00–120.00%. %RSD accuracy should be ≤20.00%.

Acceptance criteria

At least 67 % (2 of 3) of samples at each level should be within 85.00–115.00 %. At least 80 % (5 of 6) of the matrix lot should be within

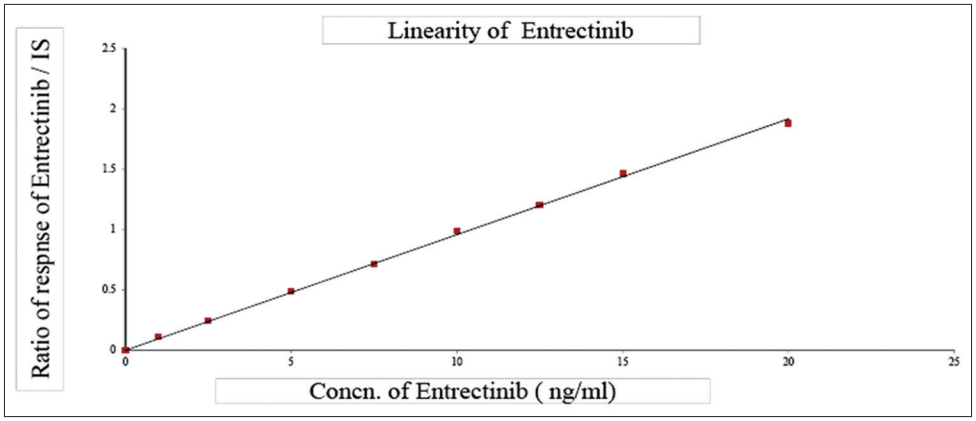

Fig. 2: Calibration curve for entrectinib

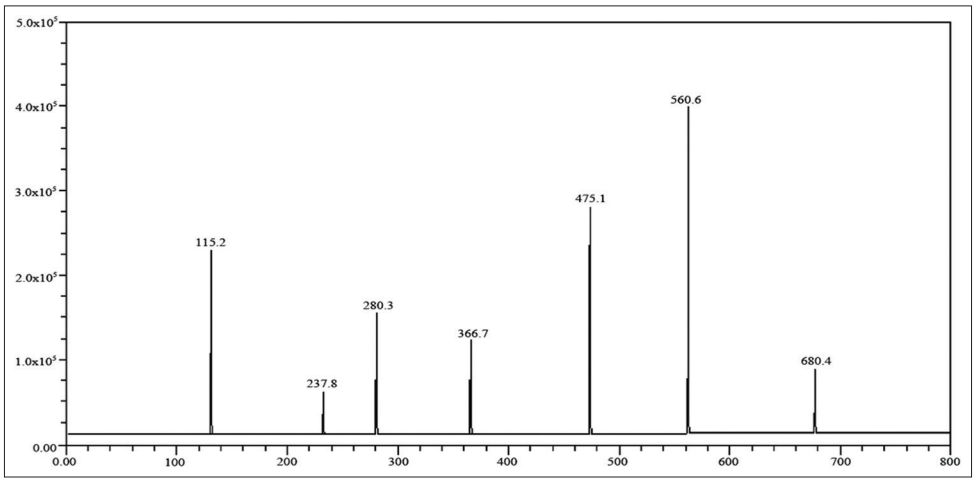

Fig. 3: Mass spectrometry spectra of entrectinib

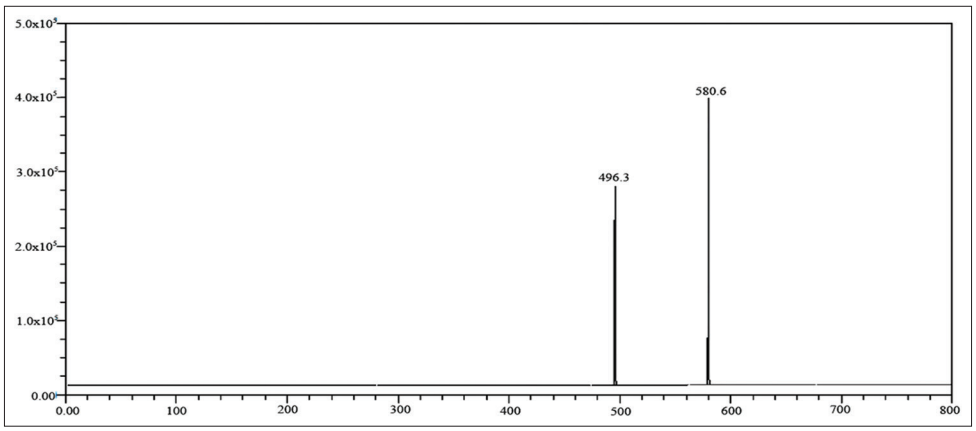

Fig. 4: Mass spectrometry spectra of D4-entrectinib

the acceptance criteria. The % mean accuracy of back-calculated concentration of LQC and HQC samples prepared from different biological matrix lots should be within 85.00–115.00 %.

Stability experiments

The stability study was evaluated as part of the method validation. To assess the decomposition of the entrectinib that may occur due to different reasons, the following stability test was prepared. The stability tests should reflect the situations likely to be encountered during routine sample handling and analysis [15]. The following stability test was performed.

Freeze-thaw stability

Six replicates of each (LQC, MQC, and HQC) that were stored at −20°C were thawed completely thawing at room temperature and refrozen immediately to −20°C. This process was repeated twice and the samples were extracted for injection into LCMS. The results are shown in Table 8.

Benchtop stability

For benchtop stability experiment, stability of entrectinib in the rat plasma after 8 h exposure on benchtop was determined at three concentrations (LQC, MQC, and HQC) in six replicates. The results are shown in Table 9.

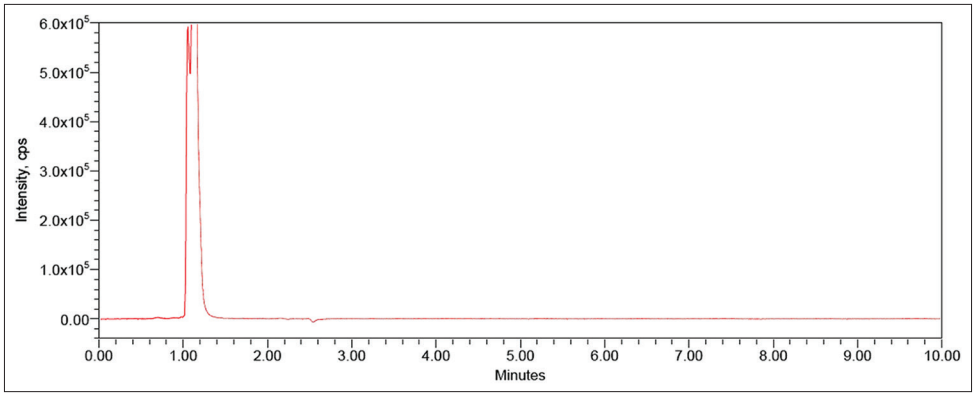

Fig. 5: Blank rat plasma

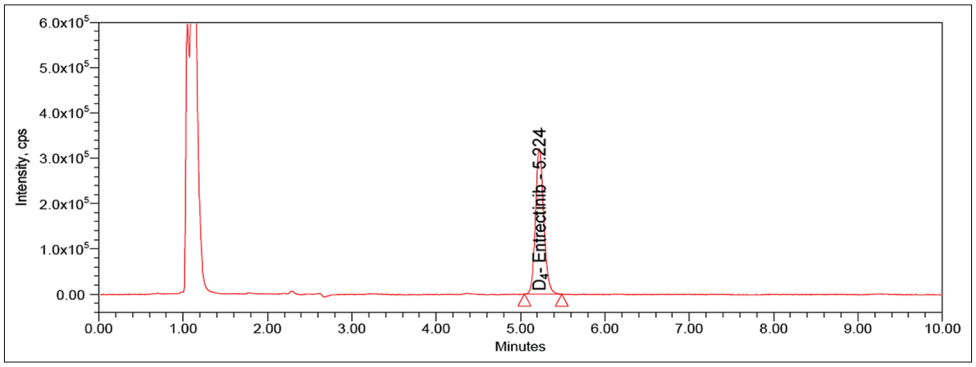

Fig. 6: Blank rat plasma spiked with internal standard

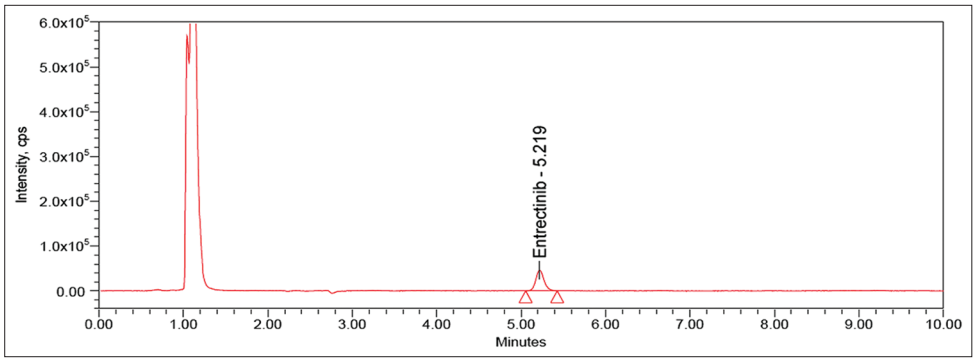

Fig. 7: Blank rat plasma spiked with analyte at a lower limit of quality control and internal standard

Table 5: Recovery of the analyte of entrectinib

Replicate number	HQC		MQC		LQC	
	Extracted response	Unextracted response	Extracted response	Unextracted response	Extracted response	Unextracted response
1	5.056×10^5	5.642×10^5	3.325×10^5	3.859×10^5	1.684×10^5	2.159×10^5
2	5.064×10^5	5.638×10^5	3.319×10^5	3.847×10^5	1.623×10^5	2.135×10^5
3	5.068×10^5	5.614×10^5	3.367×10^5	3.863×10^5	1.647×10^5	2.147×10^5
4	5.055×10^5	5.632×10^5	3.342×10^5	3.824×10^5	1.619×10^5	2.152×10^5
5	5.047×10^5	5.628×10^5	3.335×10^5	3.855×10^5	1.665×10^5	2.133×10^5
6	5.062×10^5	5.629×10^5	3.371×10^5	3.829×10^5	1.634×10^5	2.124×10^5
n	6	6	6	6	6	6
Mean	5.059×10^5	5.631×10^5	3.343×10^5	3.846×10^5	1.645×10^5	2.142×10^5
SD	0.00753	0.00971	0.02156	0.01620	0.02532	0.01317
%RSD	0.15	0.17	0.63	0.42	1.54	0.61
%Mean Recovery	96.72	101.22%	95.87%	101.47%	94.35%	100.01%
Overall % Mean Recovery	98.27%					
Overall SD	3.0167					
Overall %RSD	3.07					

SD: Standard deviation, RSD: Relative standard deviation, LQC: Low-quality control, MQC: Mid quality control, HQC: High-quality control, SD: Standard deviation, RSD: Relative standard deviation,

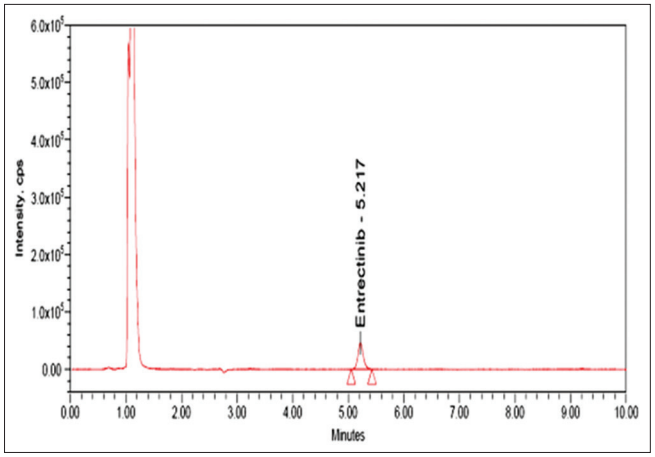

Fig. 8: Chromatogram for linearity-1

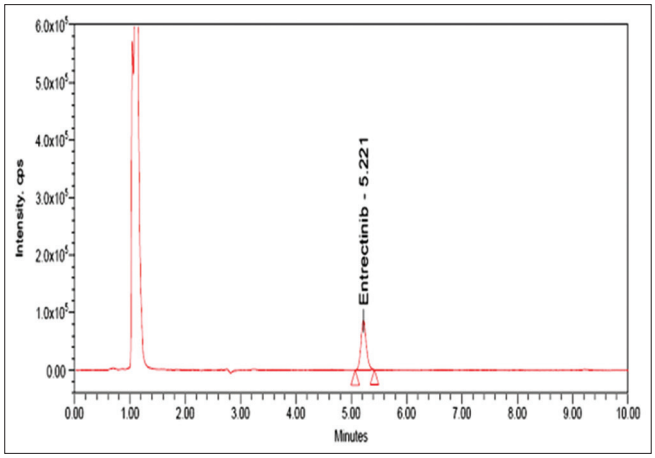

Fig. 9: Chromatogram for linearity-2

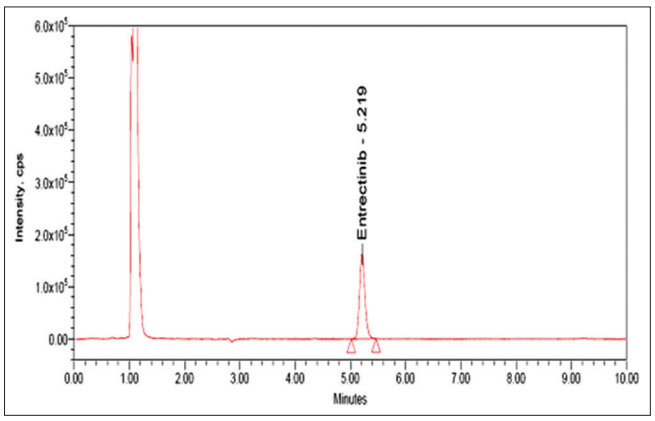

Fig. 10: Chromatogram for linearity-3

Wet extract stability

Freezer stability of entrectinib in plasma was assessed by analyzing LQC, MQC, and HQC samples in six replicates stored at −20°C for 24 h for the stability study. All samples compared with the fresh prepare samples of three different QC in six replicates. Samples were considered to be stable if assay values were in compliance with the acceptable limits of accuracy (i.e., ±15% SD) and precision (i.e., ±15% RSD; Food and Drug Administration of the United States, 2001). The results are shown in Table 10.

Auto sampler stability

Samples of entrectinib in plasma were assessed by analyzing LQC, MQC, and HQC samples are injected every 1 h up to 24 h for the stability

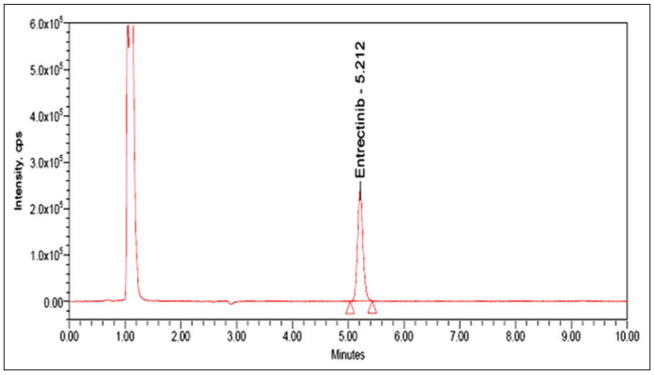

Fig. 11: Chromatogram for linearity-4

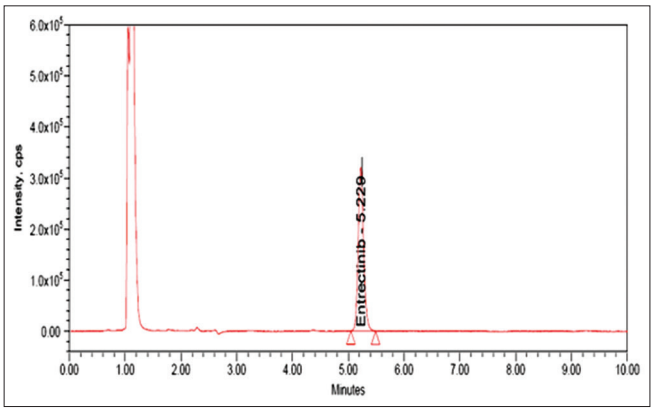

Fig. 12: Chromatogram for linearity-5

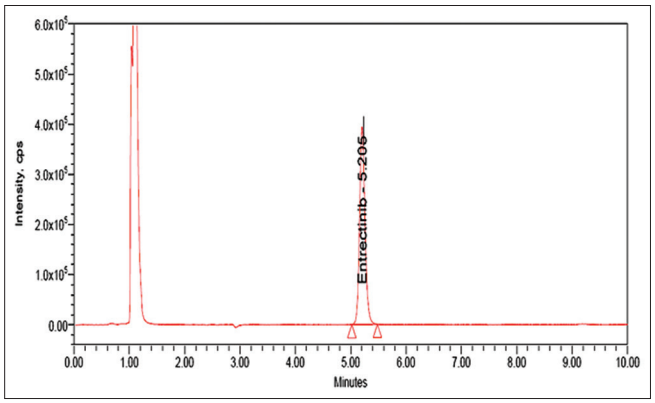

Fig. 13: Chromatogram for linearity-6

study. All samples compared with the fresh prepare samples of 0 Hr of different QC in six replicates. Samples were considered to be stable if assay values meet the compliance with the acceptable limits of accuracy (i.e., ±15% SD) and precision (i.e., ±15% RSD; Food and Drug Administration of the United States, 2001). The results are shown in Table 11.

Long-term stability studies

Long-term stability was also performed at day 1, day 7, day 14, day 21, and day 28. The percentage mean accuracy was within limits (85–115%). These values indicating that entrectinib is stable for 28 days.

Freeze thaw at −80°C

The %RSD and mean accuracy for entrectinib were found to be 0.28%, 96.60% and 0.70%, 94.25% and 0.30%, and 98.72%. Hence it passed the Freeze-thaw at −80°C.

Table 6: Sensitivity results of entrectinib

Replicate number	LLOQ
	Nominal concentration (ng/ml)
	1.154
	Nominal concentration range (ng/ml)
	(1.023–1.241)
	Area Of Analyte
1	0.342×10^5
2	0.336×10^5
3	0.357×105
4	0.312×105
5	0.328×105
6	0.364×105
N	6
Mean	0.340×105
SD	0.01904
%RSD	5.6
% mean accuracy	98.18%

LLOQ: Lower limit of quality control, SD: Standard deviation, RSD: Relative standard deviation

Table 7: Matrix effect results of entrectinib

S. no.	Plasma lot no.	HQC	LQC
		Nominal concentration (ng/ml)	
		15.341	5.369
		Nominal concentration range (ng/ml)	
		(15.269–15.517)	(5.206–5.578)
		Calculated concentration (ng/ml)	
1.	Lot 1	5.056×10^5	1.683×10^5
		5.047×10^5	1.657×10^5
		5.052×10^5	1.658×10^5
2.	Lot 2	5.055×10^5	1.625×10^5
		5.026×10^5	1.556×10^5
		5.047×10^5	1.586×10^5
3.	Lot 3	5.033×10^5	1.574×10^5
		5.029×10^5	1.536×10^5
		5.026×10^5	1.527×10^5
4.	Lot 4	5.038×10^5	1.529×10^5
		5.047×10^5	1.533×10^5
		5.022×10^5	1.547×10^5
5.	Lot 5	5.057×10^5	1.549×10^5
		5.053×10^5	1.558×10^5
		5.022×10^5	1.542×10^5
6.	Lot 6	5.036×10^5	1.531×10^5
		5.018×10^5	1.574×10^5
		5.045×10^5	1.529×10^5
N		18	18
Mean		5.039×10^5	1.572×10^5
SD		0.01320	0.05011
%CV		0.26	3.19
% mean accuracy		97.01%	90.81%
No. of QC failed		0	0

HQC: High-quality control, LQC: Low-quality control, N: Number of samples, SD: Standard deviation, CV: Coefficient of variation

Benchtop stability

The %CV of HQC, LQC, and MQC mean accuracy for entrectinib was found to be 0.24%, 0.67%, and 0.23%. Hence, it passed the benchtop stability.

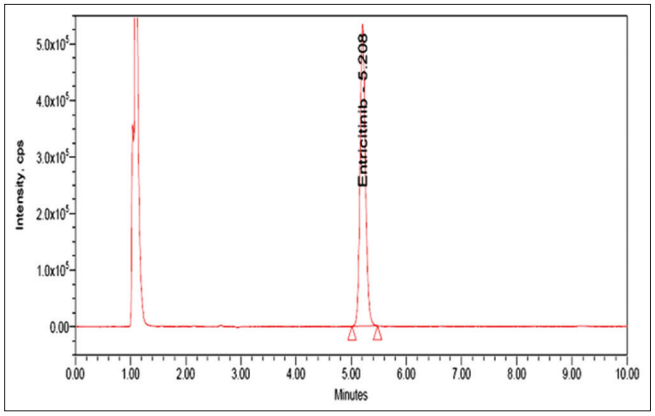

Fig. 14: Chromatogram for linearity-7

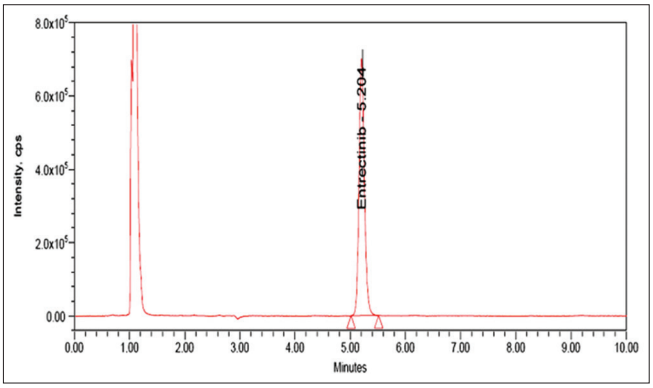

Fig. 15: Chromatogram for linearity-8

Table 8: Freeze-thaw at −80°C of entrectinib

Replicate no.	HQC	LQC	MQC
	Nominal concentration (ng/ml)		
	15.269	5.127	10.154
	Nominal concentration range (ng/ml)		
	(15.2128–15.369)	(5.028–5.260)	(10.022–10.254)
	Analyte peak area		
1	5.029×10^5	1.635×10^5	3.415×10^5
2	5.005×10^5	1.628×10^5	3.417×10^5
3	5.014×10^5	1.642×10^5	3.425×10^5
4	5.003×10^5	1.611×10^5	3.436×10^5
5	5.017×10^5	1.641×10^5	3.412×10^5
6	5.039×10^5	1.632×10^5	3.408×10^5
N	6	6	6
Mean	5.018×10^5	1.632×10^5	3.419×10^5
SD	0.01395	0.01136	0.01015
%CV	0.28	0.70	0.30
% mean accuracy	96.60%	94.25%	98.72%

LQC: Low-quality control, MQC: Mid quality control, HQC: High-quality control, N: Number of samples, SD: Standard deviation, CV: Coefficient of variation

Wet extract

The %RSD and mean accuracy for entrectinib were found to be 0.34%, 97.14% and 1.33%, 95.58% and 0.59%, and 99.97%. Hence, it passed the wet extract at −28°C.

Table 9: Benchtop stability of entrectinib

Replicate no.	HQC	LQC	MQC
	Nominal concentration (ng/ml)		
	15.364	5.287	10.157
	Nominal concentration range (ng/ml)		
	(15158–15.462)	(5.036–5.369)	(10.017–10.239)
	Analyte peak area		
1	5.016×10^5	1.647×10^5	3.415×10^5
2	5.019×10^5	1.628×10^5	3.418×10^5
3	5.021×10^5	1.637×10^5	3.422×10^5
4	5.048×10^5	1.629×10^5	3.407×10^5
5	5.017×10^5	1.645×10^5	3.402×10^5
6	5.029×10^5	1.656×10^5	3.406×10^5
N	6	6	6
Mean	5.025×10^5	1.64×10^5	3.412×10^5
SD	0.01218	0.01098	0.00781
%CV	0.24	0.67	0.23
% mean accuracy	96.73%	94.71%	98.53%

LQC: Low-quality control, MQC: Mid quality control, HQC: High-quality control, N: Number of samples, SD: Standard deviation, CV: Coefficient of variation

Table 10: Wet extract stability of entrectinib

Replicate no.	HQC	LQC	MQC
	Nominal concentration (ng/ml)		
	15.247	5.298	10.356
	Nominal concentration range (ng/ml)		
	(15.142–15.336)	(5.167–5.374)	(10.247–10.464)
	Analyte peak area		
1	5.029×10^5	1.654×10^5	3.465×10^5
2	5.067×10^5	1.654×10^5	3.487×10^5
3	5.027×10^5	1.629×10^5	3.462×10^5
4	5.064×10^5	1.634×10^5	3.477×10^5
5	5.038×10^5	1.678×10^5	3.429×10^5
6	5.049×10^5	1.683×10^5	3.451×10^5
N	6	6	6
Mean	5.046×10^5	1.655×10^5	3.462×10^5
SD	0.01725	0.02205	0.02034
%CV	0.34	1.33	o.59
% Mean Accuracy	97.14%	95.58%	99.97%

LQC: Low-quality control, MQC: Mid quality control, HQC: High-quality control, N: Number of samples, SD: Standard deviation, CV: Coefficient of variation

Auto sampler stability

The %RSD and mean accuracy for entrectinib were found to be 0.42%, 0.78%, and 1.40. Hence, it passed the autosampler stability.

CONCLUSION

A bioanalytical LC-MS/MS method for the entrectinib was developed and validated with entrectinib D_4 as IS. The method has excellent accuracy, precision, and recovery compared with existed methods for the analysis of drug in rat plasma. The methods developed in our laboratory are very simple, utilizing liquid-liquid extraction procedure, which makes the method high throughput for analysis. Entrectinib was eluted within 6 min using RP-high-performance liquid chromatography Luna, 250×4.6 mm, 5 µm column and the mobile phase containing 0.1% formic acid and ACN in the ratio of 70:30% v/v and flow rate was 1.0 ml/min. All the validation data were met the range acceptance criteria of the USFDA guideline.

Table 11: Autosampler stability results of entrectinib

Replicate no.	HQC	MQC	LQC
	Nominal concentration (ng/ml)		
	15.315	10.452	5.526
	Nominal concentration range (ng/ml)		
	(15.205–15.468)	(10.312–10.629)	(5.387–5.748)
	Area of analyte		
1	5.026×10^5	3.487×10^5	1.652×10^5
2	5.039×10^5	3.425×10^5	1.635×10^5
3	5.027×10^5	3.469×10^5	1.642×10^5
4	5.018×10^5	3.427×10^5	1.685×10^5
5	5.029×10^5	3.482×10^5	1.625×10^5
6	5.044×10^5	3.415×10^5	1.641×10^5
7	5.052×10^5	3.469×10^5	1.687×10^5
8	5.058×10^5	3.451×10^5	1.633×10^5
9	5.067×10^5	3.496×10^5	1.685×10^5
10	5.062×10^5	3.421×10^5	1.624×10^5
11	5.039×10^5	3.428×10^5	1.642×10^5
12	5.027×10^5	3.496×10^5	1.625×10^5
13	5.068×10^5	3.471×10^5	1.654×10^5
14	5.047×10^5	3.485×10^5	1.624×10^5
15	5.085×10^5	3.462×10^5	1.635×10^5
16	5.069×10^5	3.451×10^5	1.639×10^5
17	5.067×10^5	3.463×10^5	1.626×10^5
18	5.064×10^5	3.481×10^5	1.643×10^5
19	5.074×10^5	3.496×10^5	1.675×10^5
20	5.078×10^5	3.465×10^5	1.684×10^5
21	5.095×10^5	3.472×10^5	1.692×10^5
22	5.082×10^5	3.436×10^5	1.637×10^5
23	5.061×10^5	3.428×10^5	1.629×10^5
24	5.072×10^5	3.419×10^5	1.651×10^5
N	24	24	24
Mean	5.056×10^5	3.458×10^5	1.649×10^5
SD	0.02122	0.02709	0.02307
%RSD	0.42	0.78	1.40
% mean accuracy	97.3%	99.9%	95.23%

LQC: Low-quality control, MQC: Mid quality control, HQC: High-quality control, n: Number of samples, SD: Standard deviation, RSD: Relative standard deviation

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