LC-MS/MS Estimation Of The Anti-Cancer Agent Entrectinib Levels In Human Plasma Samples

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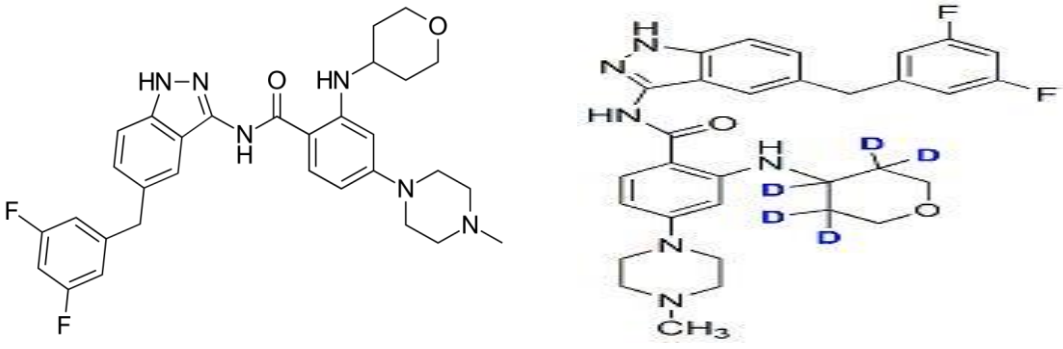
INTRODUCTION

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 ROS1and anaplastic lymphoma kinase (ALK). TRK receptors produce cell proliferation through downstream signaling through the mitogenactivated 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 Favour of apoptosis, resulting in shrinking of tumor volume [1-9].

Entrectinib is a kinase inhibitor. The molecular formula for entrectinib is C31H34F2N6O2 and the molecular weight is 560.64 Daltons. The chemical name is N-[5-(3,5-difluorobenzyl)-1H-indazol-3-yl]-4-(4Â-methylpiperazin-1-yl)-2-(tetrahydro-2H-pyran-4-ylamino) benzamide. (Figure-1) [10].



A **B**

Fig.1: Chemical structures of A) Entrectinib B) Entrectinib-D5

Literature survey revealed that there is no bio-analytical methods have been reported individually or in combination with other drugs for estimation of Entrectinib in human plasma samples by LC-MS/MS using Entrectinib-D5 as internal standard. 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 [11-16].

MATERIALS AND METHODS

Chemical Resources

Entrectinib (MedKoo Biosciences, USA) and Entrectinib-D5 (Clearsynth, Mumbai, India), methanol and acetonitrile (J.T Baker, USA), formic acid (Merck, Mumbai, India), Ultra pure water (Milli-Q system, Millipore, Bedford, MA, USA), human plasma (Doctors pathological labs, hyderabad, India). The chemicals and solvents were used in this study analytical and HPLC grade.

Instrument Resources

An API 4000 HPLC-ESI-MS/MS system (Applied Biosystems), 1200 Series HPLC system (Agilent Technologies, Waldbronn, Germany), data acquisition and processing were accomplished using Analyst® Software 1.4.1.

Methods:

Chromatographic conditions

The chromatographic separation was achieved with 0.1% formic acid in combination with methanol (25:75 v/v), gave the best peak shape and low baseline noise was observed using the Ascentis Express C_{18} (50 mm × 4.6 mm, 2.7 µm). The total analysis time was 3 min and flow rate was set to 0.6 ml/min. The temperature was set to 40°C for the column oven. The sample volume for the injection into mass spectrometry was adjusted to 10 µl for better ionization and chromatography.

Detection

The pure drug of Entrectinib and Entrectinib-D5 were prepared in methanol (10.00 ng/mL) and injected with a flow rate of 5 µL/min into positive ion mode mass spectrometer for optimization of mass parameters like source temperature, IS, heater gas, nebulizer gas, curtain gas, CAD gas (all gas channels were purged with ultra high pure nitrogen gas), EP, DP, CE, FP and CXP were optimized. Analysis was performed using MRM positive ion mode with mass transitions of m/z (amu) 560.61→475.12 and 566.64→475.12 for Entrectinib and Entrectinib-D5. The mass fragmentation pattern of parent and product ions mass spectras were depicted in figure 2 and 3.

Standard calibration and quality control samples preparation

Stock solutions of Entrectinib (1000.00 µg/ml) and Entrectinib-D5 (1000.00 µg/ml) were prepared in methanol. The internal standard (Entrectinib-D5) spiking solution (500.00 ng/ml) was prepared in 75% methanol from Entrectinib-D5 stock solution. Stock solutions of Entrectinib, Entrectinib-D5 and intermediate spiking solutions were stored in refrigerated conditions (2-8°C) until analysis.

Calibration standards (5.00, 10.00, 50.00, 100.00, 500.00, 1000.00, 2000.00, 4000.00, 6000.00, 8000.00 and 10000.00 pg/ml), quality control samples of lower limit QC, low QC, mid QC, high QC (5.00, 15.00, 3000.00, 7000.00 pg/ml) were used by spiking the appropriate amount of standard solution in the drug free plasma and stored at -30 °C till analysis.

Sample extraction

The protein precipitation method was applied to extract Entrectinib and Entrectinib-D5. To each labelled polypropylene tube 50 µl of Entrectinib-D5 (500.00 ng/ml) was mixed with the 100 µl plasma sample, then 0.25 ml of acetonitrile were added, vortexed for 5 min and centrifuged at 4000 rpm for 10 min at 20°C. The organic phase was transferred to auto sampler vials containing 100 µl of 0.1% formic acid and injected into the HPLC-ESI-MS/MS for analysis.

Method Validation

The developed method was validated over a linear concentration range of 5.0–10000.0 ng/ml. The validation parameters include selectivity and specificity, LOQ, Linearity, precision and accuracy, matrix effect, recovery, stability (freeze–thaw, auto sampler, bench top, long term) was evaluated under validation section [12-16].

Selectivity and Specificity

Ten lots of blank plasma samples were analyzed out of which six lots free from interference were selected for assessing the selectivity and specificity. The endogenous/potential interfering peak areas for blank samples must be less than 20% of the LLOQ peak area of Entrectinib retention time and less than 5% for Entrectinib-D5 retention time.

Limit of Quantification (LOQ)

Six LLOQ standards were prepared in screened plasma lot along with IS (500.00 ng/ml) and signal to noise ratio (S/N) was calculated using analyst software.

Linearity

Calibration standards were prepared to obtain linearity range of 5.00, 10.00, 50.00, 100.00, 500.00, 1000.00, 2000.00, 4000.00, 6000.00, 8000.00 and 10000.00 pg/ml and assayed in five replicates on five different days.

Precision & Accuracy

One set of calibration standards and one set contains four different concentrations of quality control standards of Lower limit QC (5.00 pg/ml), Low QC (15.00 pg/ml), Mid QC (3000.00 pg/ml) and High QC (7000.00 pg/ml) concentrations were prepared in screened plasma and analyzed each quality control (QC) standards in six replicates on the same day (Intra day) and five different days (Inter day).

Matrix Effect

Six extracted blank plasma samples in three replicates were spiked with the un-extracted concentration of mid QC (3000.00 pg/ml) and compared with un-extracted standards of the same concentration.

Recovery

The recovery of samples was performed by protein precipitation method.The extraction recovery was determined in sextuplicate by comparing the extracted QC standards with un-extracted QC standards at three different concentrations of low (15.00 pg/ml), medium (300.00 pg/ml), high (7000.00 pg/ml).

Stability studies

Bench top Stability (Room Temperature Stability, 24 h)

Six replicates of spiked low and high concentrations (BT stability samples) were set aside at ambient temperature up to 24 h. Samples were processed and compared with newly prepared low and high concentrations (comparison samples).

Freeze and thaw stability (after 3rd cycle at -30˚C)

Six replicates of low and high concentrations (FT stability samples) were frozen at -30°C and subjected to three freeze-thaw cycles of 24, 36 and 48 h (-30°C to room temperature) and compared with newly prepared low and high concentrations (comparison samples).

Autosampler stability (2-8˚C, 65 h)

Six replicates of low and high concentrations (AS stability samples) were stored in auto-sampler up to 65 h at 2-8°C. Stability samples were compared with newly prepared low and high concentrations (comparison samples).

Long-term Stability (-30˚C, 45 Days)

After completion of the stability period stored at -30 °C (45 days) six replicates of low and high concentrations (LT stability samples) were compared with newly prepared low and high concentrations (comparison samples).

RESULTS AND DISCUSSION

Method development

On the way to develop a simple and easy applicable method for determination of Entrectinib in human plasma, HPLC-MS/MS was selected as the method of choice. During method development process chromatographic (mobile phase composition, column, flow rate, injection volume, sample volume), mass spectrometric, sample extraction and internal standard parameters were optimized in logical and sequential manner to achieve the best results.

Separation of the Entrectinib was performed with different branded RP-HPLC C_{18} columns. Initial separation was performed with isocratic elution of 10mM ammonium formate and acetonitrile was selected as a mobile phase in varying combinations were tried, but a low response was observed. A mobile phase consisting of 0.1% acetic acid: acetonitrile (20:80 v/v) and 0.1%acetic acid: methanol (20:80 v/v) gave the best response, but poor peak shape was observed.

After a series of trials a mobile phase consisting of 0.1% formic acid in combination with methanol and acetonitrile in varying combinations were tried. Using a mobile phase containing 0.1% formic acid in combination with methanol (25:75 v/v), gave the best signal along with a marked improvement in the peak shape and low baseline noise was observed using the Ascentis Express C_{18} (50 mm × 4.6 mm, 2.7 µm) analytical column with a flow rate of 0.6 ml/min and reduced runtime to 3 min. The column oven temperature was kept at a constant temperature of about 40 °C and temperature of auto sampler was maintained at 4°C. Injection volume of 10 µl sample was adjusted for better ionization and chromatography. For selection of internal standard, Afatinib Dimaleate, Imatinib Mesylate and Lenvatinib Mesylate were tried with optimized mobile phase and column conditions. Finally Entrectinib-D5 was selected as internal standard in terms of better chromatography and extractability.

The retention times of analyte (Entrectinib) and internal standard (Entrectinib-D5) were eluted at 1.42 ± 0.2 min and 1.44 ± 0.2 min respectively with 3 min total runtime. Different procedures like PPT (Protein precipitation), SPE (solid phase extraction) and LLE (liquid-liquid extraction) methods were optimized. Out of all, it was observed that the PPT was suitable due to simple extraction, high recovery and the less ion suppression effect on drug and internal standard.
Electro spray ionization (ESI) provided a maximum response over atmospheric pressure chemical ionization (APCI) mode, and was chosen for this method. The instrument was optimized to obtain sensitivity and signal stability during infusion of the analyte in the continuous flow of mobile phase to electrospray ion source operated at a flow rate of 20 µl/min. Entrectinib gave more response in positive ion mode as compare to the negative ion mode.
To get high intense productions source dependent parameters were optimized like nebulizer gas flow 30 psi, CAD gas and curtain gas flow 25 psi, ion spray voltage 5500 V, and temperature 500°C. The compound dependent parameters such as the declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), cell exit potential (CXP) were optimized during tuning as 35, 25, 10, 20, 12 eV for Entrectinib and Entrectinib-D5, respectively. The collision activated dissociation (CAD) gas was set at 4 psi using nitrogen gas. Quadrupole-1 and quadrupole-3 were both maintained at a unit resolution and dwell time was set at 200 ms for Entrectinib and Entrectinib-D5.
The predominant peaks in the primary ESI spectra of Entrectinib and Entrectinib-D5 correspond to the MH^+ ions at m/z 560.61 and 566.64 respectively. Productions of Entrectinib and Entrectinib-D5 scanned in quadrupole-3 after a collision with nitrogen in quadrupole-2 had a m/z of 475.12 for both respectively. The parent and productions mass spectrums of Entrectinib and Entrectinib-D5 were shown in Figure 2 & 3.

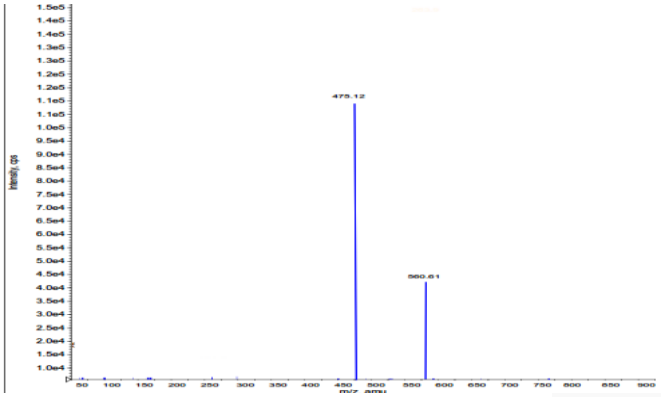


Figure.2: Parent ion mass spectra (Q1) and (Q3) of Entrectinib

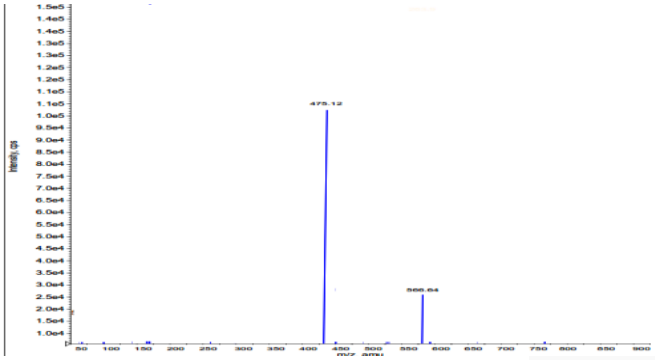


Figure.3: Parent ion mass spectra (Q1) and (Q3) of Entrectinib-D5

Method validation

Selectivity and Specificity, Limit of Quantification (LOQ)

No significant response was observed at retention times of Entrectinib and Entrectinib-D5 in blank plasma as compared to LLOQ and blank with IS samples. The limit of quantification for this method was proven as the lowest concentration of the calibration curve which was proven as 5.0 ng/ml. Represent chromatograms were shown in Figure 4.

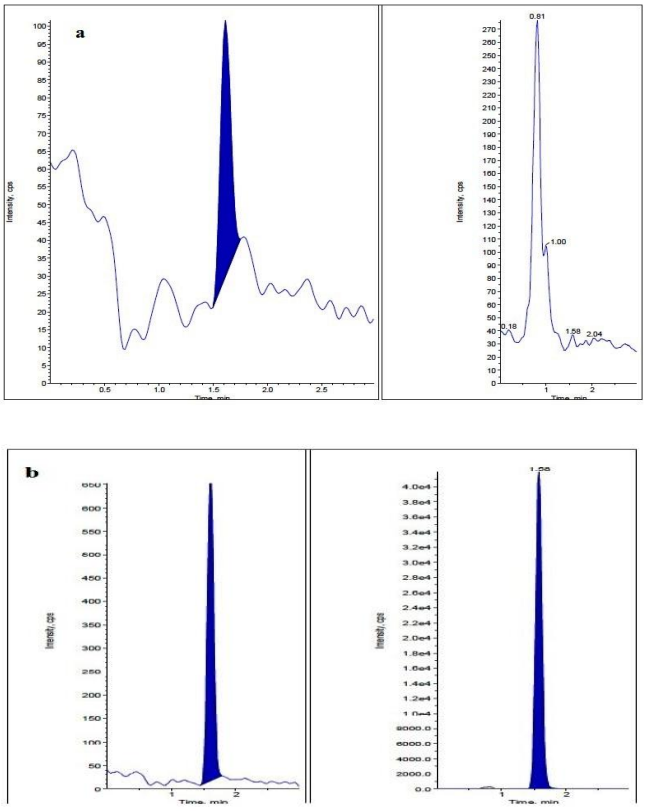


Fig. 4 - Representative chromatograms of Entrectinib in plasma samples a) Blank plasma chromatogram for interference free Entrectinib and Entrectinib-D5 Chromatogram of LLOQ sample (Entrectinib and Entrectinib-D5).

Linearity

Linearity was plotted as a peak area ratio (Entrectinib peak area / Entrectinib-D5 peak area) on the y-axis against Entrectinib concentration (pg/ml) on the x-axis. Calibration curves were found to be consistently accurate and precise for Entrectinib over a linearity range of 5 to 10000.00 pg/ml. The correlation coefficient was greater than 0.9980 for Entrectinib. The %CV was less than 15% and mean %accuracy was ranged between 99.40 - 102.67%. Results were presented in Table 1.

Table. 1 - Calibration curve details of Entrectinib

Spiked plasma Concentration (pg/ml)	Concentration measured (pg/ml) (Mean±S.D)	%CV (n=5)	%Accuracy
5.00	4.99±0.01	1.4	99.9
10.00	10.24±0.02	3.6	101.7
50.00	49.89±0.15	2.7	101.3
100.00	100.24±0.22	2.5	100.1
500.00	501.6±0.27	3.8	100.1
1000.00	1004.22±0.21	2.6	101.7
2000.00	1999.18±1.02	3.1	99.4
4000.00	4001.35±1.10	3.4	101.7
6000.00	6003.76±1.11	1.7	102.6
8000.00	8001.12±1.96	3.8	101.5
10000.00	10000.07±1.23	2.5	100.5

Precision & Accuracy

Intra and inter batch %accuracy for Entrectinib was ranged between 94.17-96.00 and 91.66 to 99.34. %CV is 2.16 to 5.64 and 1.64% - 8.00%. Results are presented in Table 2.

Table.2-Precision and accuracy (Analysis with spiked samples at three different concentrations) of Entrectinib

Spiked Plasma Concentration (pg/ml)	Within-run (Intra-day)			Between-run (Inter-Day)		
	Concentration measured (n=6;pg/ml;mean±S.D)	%CV	%Accuracy	Concentration measured (n=6;pg/ml;mean±S.D)	%CV	%Accuracy
15.00	14.8±0.07	5.6	98.9	14.9±0.08	3.2	99.93
3000.00	3002.34±1.23	2.1	102.4	2999.78±2.56	1.6	99.45
7000.00	6999.47±2.45	3.7	99.8	7004.33±3.61	2.4	103.45

Recovery

The mean %recovery for LQC, MQC, HQC samples of Entrectinib were 99.85%, 95.30% and 93.54% respectively. The overall mean %recovery and %CV of Entrectinib across QC levels is 96.23% and 3.38%. For the Entrectinib-D5 (internal standard) the mean % recovery and %CV is 91.68% and 7.09%.

Matrix Effect

No significant matrix effect found in different sources of rat plasma tested for Entrectinib, Entrectinib-D5. The %CV was found to be 3.71.

Stability (freeze-thaw, auto sampler, bench top, long term)

Quantification of the Entrectinib in plasma subjected to three freeze–thaw cycles (−30°C to room temperature), autosampler (processed), room temperature (Benchtop), long-term stability details were shown in Table 3.

Table. 3 - Stability studies of Entrectinib in spiked plasma samples

Spiked Plasma concentration (pg/ml)	Room temperature Stability		Processed sample Stability		Long term stability		Freeze and thaw stability	
	24h		65h		45 days		Cycle (48h)	
	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)	Concentration measured (n=6;pg/ml; mean±S.D)	%CV (n=6)
15.00	14.9±0.12	7.8	15.5±2.16	5.3	15.2±1.54	8.8	14.8±0.12	5.8
7000.00	7005.3±0.14	8.9	7001.3±1.23	9.5	6999.563±0.12	9.4	7001.4±2.55	2.7

CONCLUSION

The method described in this manuscript has been developed and validated over the concentration range of 5.0–10000.0 pg/ml in human plasma. The intra and inter-batch precision (%CV) was less than 6.0% and %accuracy ranged from 98.9%–102.4%. The overall %recovery for Entrectinib, Entrectinib-D5 was greater than 90%. The selectivity, sensitivity, precision and accuracy obtained with this method make it suitable for the purpose of the present study. In conclusion, the method used in the present study is easy and fast to perform; it is also characterized with an adequate

accuracy, precision, selectivity and stability. The simplicity of the method, and using rapid protein precipitation extraction with less run time of 3.0 min per sample, make it an attractive procedure in high-throughput bioanalysis of Entrectinib.

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CONFLICT OF INTEREST: Authors declare that, there is no conflict of interest.

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