Entrectinib and internal standard (IS) (entrectinib D₄) were 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 MERCK. Water was from Milli Q System and plasma from local suppliers.

**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)-1H-indazol-3-yl]-4-(4-methyl-1-piperazinyl)-2- weight is 560.64 g/mol and the molecular formula is C₆₀H₄₁F₂N₂O₁₂. Entrectinib (INN, trade name Rozytrek previously known as RDX-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 or 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 D₄) 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 MERCK. Water was from Milli Q System and plasma from local suppliers.
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 containing 200 µl of blank plasma add this 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

**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**

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

Table 1: LOD and LOQ data for entrectinib

| Name     | LOD Concentration (ng/ml) | s/n | LOQ 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

Table 2: System suitability results of entrectinib

| Sample name | Analyte area | Analyte RT (min) | IS area | IS RT (min) | Area ratio |
|-------------|--------------|------------------|---------|-------------|------------|
| MQC         | 3.428×10²    | 5.226            | 3.485×10² | 5.222       | 0.9836     |
| MQC         | 3.462×10²    | 5.221            | 3.481×10² | 5.236       | 0.9941     |
| MQC         | 3.479×10²    | 5.223            | 3.476×10² | 5.227       | 1.0009     |
| MQC         | 3.466×10²    | 5.227            | 3.449×10² | 5.231       | 1.0049     |
| MQC         | 3.458×10²    | 5.229            | 3.478×10² | 5.235       | 0.9942     |
| MQC         | 3.487×10²    | 5.226            | 3.461×10² | 5.230       | 1.0075     |
| Mean        | 3.463×10²    | 5.225            | 3.474×10² | 5.230       | 0.9989     |
| 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         |
| Skope          |             |          |                |         |               | 0.0940                           | 0.01637              |               |
| Intercept      |             |          |                |         |               | 0.99935                          |                      |               |

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

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
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 85.00–115.00 %.
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.
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                | 5.056×10⁵            | 5.642×10⁵            | 3.325×10⁵            | 3.859×10⁵            | 1.684×10⁵            | 2.159×10⁵            |
| 2                | 5.064×10⁵            | 5.638×10⁵            | 3.319×10⁵            | 3.847×10⁵            | 1.623×10⁵            | 2.135×10⁵            |
| 3                | 5.068×10⁵            | 5.614×10⁵            | 3.367×10⁵            | 3.863×10⁵            | 1.647×10⁵            | 2.147×10⁵            |
| 4                | 5.055×10⁵            | 5.632×10⁵            | 3.342×10⁵            | 3.824×10⁵            | 1.619×10⁵            | 2.152×10⁵            |
| 5                | 5.047×10⁵            | 5.620×10⁵            | 3.335×10⁵            | 3.855×10⁵            | 1.665×10⁵            | 2.133×10⁵            |
| 6                | 5.062×10⁵            | 5.629×10⁵            | 3.371×10⁵            | 3.829×10⁵            | 1.634×10⁵            | 2.124×10⁵            |
| n                | 6                    | 6                    | 6                    | 6                    | 6                    | 6                    |
| Mean             | 5.059×10⁵            | 5.631×10⁵            | 3.343×10⁵            | 3.846×10⁵            | 1.645×10⁵            | 2.142×10⁵            |
| 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%              |

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,
Wet extract stability
Freeze 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 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

| S. no. | Plasma lot no. | HQC | LQC |
|--------|----------------|-----|-----|
|        |                | Nominal concentration (ng/ml) |
|        |                | 15.341 |
|        |                | 5.369 |
|        | Nominal concentration range (ng/ml) |
|        | (15.269–15.517) |
|        | (5.026–5.578) |

| Calculated concentration (ng/ml) |
|---------------------------------|
| 1. Lot 1 | 5.056×10^5 | 1.683×10^5 |
| 2. Lot 2 | 5.052×10^5 | 1.658×10^5 |
| 3. Lot 3 | 5.052×10^5 | 1.536×10^5 |
| 4. Lot 4 | 5.052×10^5 | 1.527×10^5 |
| 5. Lot 5 | 5.052×10^5 | 1.547×10^5 |
| 6. Lot 6 | 5.052×10^5 | 1.574×10^5 |
| N       | 6             | 6             |

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

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) |
|        | (5.026–5.578) |

| Calculated concentration (ng/ml) |
|---------------------------------|
| 1. Lot 1 | 5.056×10^5 | 1.683×10^5 |
| 2. Lot 2 | 5.052×10^5 | 1.658×10^5 |
| 3. Lot 3 | 5.052×10^5 | 1.536×10^5 |
| 4. Lot 4 | 5.052×10^5 | 1.527×10^5 |
| 5. Lot 5 | 5.052×10^5 | 1.547×10^5 |
| 6. Lot 6 | 5.052×10^5 | 1.574×10^5 |
| N       | 6             | 6             |

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.

Wet extract
The %RSD and mean accuracy for entrectinib were found to be 0.34%, 97.14% and 1.33%, 95.58% and 0.5%, and 99.97%. Hence, it passed the wet extract at −28°C.
A bioanalytical LC-MS/MS method for the entrectinib was developed and validated with entrectinib D₃ 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.

## CONCLUSION

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

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