Development and Validation of Stability Indicating RP-HPLC Method for Estimation of Larotrectinib in its Formulations

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ABSTRACT

A stability indicating HPLC method for the quantification of Larotrectinib in capsule form was developed and validated as per the ICH guidelines. Separation and quantification of Larotrectinib was carried out on column Sunsil C₈ using mobile phase as KH₂PO₄ and methanol in 1:1 ratio. Larotrectinib was eluted at 3.432 minutes. Linearity was observed in between 50-150 µg/ml. LOD and LOQ were found to be 0.065 µg/ml and 0.217 µg/ml respectively. % RSD for the precision of the method was found to be 0.115. Accuracy was well within the regulated limit that is 100.13% and the recovery was found to be to 100.47%. Forced degradation analysis was carried out on Larotrectinib which established stability indicating power of the developed method.

Keywords: HPLC, Larotrectinib, Method development, ICH Guidelines.

INTRODUCTION

A medication, Vitrakvi (Larotrectinib), IUPAC term (3S)-N-{5-[(2R)-2-(2,5-difluorophenyl)-1-pyrrolidinyl]pyrazolo[1,5-a]pyrimidin-3-yl}-3-hydroxy-1-pyrrolidinecarboxamidesulphate was authorized by US Food & Drug Administration (FDA) to manage tumors with particular genetic modification regardless of cancer type. Vitrakvi (Larotrectinib) is authorized for managing adults and children having solid tumors which give positive test for NTK genes¹. Tumors with that kind of genetic modification are not prevalent but it can be seen in salivary gland cancer, pulmonary cancer and sarcoma in tissue. Tumors which have distributed or not surgically removed and have grew up during earlier medicines must be treated with Larotrectinib. Present work is aimed to develop a new, efficient and reproducible HPLC method for
the analysis of Larotrectinib. The developed method is validated according to ICH guidelines for various
c parameters specified in guidelines2,3,4. Separation
and quantification of Larotrectinib was carried on
column Sunsil C18 using mobile phase as KH2PO4
and methanol in 1:1 ratio. Larotrectinib was eluted
at 3.432 minutes. The method was validated for
parameters such as specificity, linearity, precision,
accuracy, system suitability, limit of detection , limit
of quantification and robustness.

Preparation of stock solution
100 mg of standard Larotrectinib was dissolved in 100 mL volume of mobile phase. Stock Larotrectinib solution-1000 µg/ml concentration.

Assay methodology
Larotrectinib capsules (label claim – 100 mg/capsule) were emptied. Capsule powder weight equivalent to 100 mg Larotrectinib was taken to standard flask (100 mL). 25 mL mobile phase was added and dissolved and make up the volume to 100 mL. Concentration of Larotrectinib in solution (stock capsule solution) was 1000 µg/ml. 1 mL stock capsule solution is mixed with 9 mL of diluent. Then concentration of Larotrectinib in this capsule solution was 100 µg/mL. This capsule solution was analyzed employing proposed HPLC conditions. The Larotrectinib amount in capsule was calculated with acquired peak areas.

ASSAY%:

Table 1: Instruments used

| Instrument         | Model       | Description          |
|--------------------|-------------|----------------------|
| HPLC system        | 2695 model  | Water alliance       |
| Column             | Sunsil C18  | 250 mm x 4.6 mm, 5 µm|
| Software           | Empower     | Water alliance       |
| Photodiode array   | 2998 Model  | Water alliance       |

Table 2: Drug, chemicals and solvents used

| Material                   | Source                        |
|----------------------------|-------------------------------|
| Larotrectinib             | Octapharma pvt.ltd, India     |
| Dipotassium hydrogen phosphate | Sd Fine-Chem Ltd, India     |
| Hydrochloric acid         | Sd Fine-Chem Ltd, India       |
| Sodium hydroxide          | Sd Fine-Chem Ltd, India       |
| Hydrogen peroxide         | Sd Fine-Chem Ltd, India       |
| Phosphoric acid           | Sd Fine-Chem Ltd, India       |
| Methanol                  | Merck specialties Ltd, India  |

Table 3: HPLC method conditions

| Column with temperature | Ambient         |
|-------------------------|-----------------|
| pH units                | 4.3 units       |
| Injection vol sample   | 10 µl           |
| Column rate of flow    | 1 ml/min        |
| Run time               | 5 minutes       |
| wave length of detection | 228 nm         |

Preparation of mobile phase
KH2PO4 with strength 0.1 M and methanol mixed in 50:50 v/v ratios and the pH was fixed to
4.3 with the aid of phosphoric acid. As both mobile phase and diluent, this solvent mix was used.

ASSAY%:  

Table 4: Optimized chromatographic conditions

| Mobile Phase                   | Na2HPO4 (50 mL and methanol (50 mL) |
|--------------------------------|-------------------------------------|
| pH of mobile phase            | 4.3                                 |
| Chromatographic column        | Phenomenex, C18, length – 250 mm, Identification -4.6 mm, particle -5 µm |
| Flow Rate                     | 1.0 ml/min                          |
| Injection Volume              | 10 µl                               |
| Temperature of column         | 25°C                                |
| Detection wavelength          | 228 nm                              |
| Time of run                   | 5 minutes                           |

ASSAY%:  

Table 4: Optimized chromatographic conditions

Fig. 1. Larotrectinib structure

Fig. 2. Chromatogram with optimized conditions
Assay of formulation

Standard and sample solutions were injected separately into the system and chromatograms were recorded. The drug present in sample was calculated using mentioned formula.

Table 5: Assay of formulation

| S.No | % Assay |
|------|---------|
| 1    | 99      |
| 2    | 99      |
| 3    | 99      |
| 4    | 99      |
| 5    | 99      |
| 6    | 100     |

Average assay: 99
Standard deviation: 0.11
%RSD: 0.12

Method validation

Selectivity

Interference of blank diluent, placebo and excipient in capsule solution was assessed. Analysis was done on blank diluent, placebo and excipient in capsule solution and compared with Larotrectinib standard (100 µg/mL). Interference peaks were not noticed at the retention time of Larotrectinib in chromatograms of blank diluent, placebo and capsule solution. This clearly showed ability of method to selectively analyze Larotrectinib.

System suitability

To test system effectiveness 10 µl of Larotrectinib standard (100 µg/mL) injected five times. Result of system suitability (Plate count, RSD of peak area, retention time and tailing factor) were computed. The results were well within the limits of ICH prescribed.

Prescribed limits

- More than 2000-Plate count
- Less than or equal to 2%- Peak area RSD
- Retention time- reliably less
- Less than or equal to 2% - Tailing factor

Table 6: Results for system suitability

| S.No | Sample Name | Rt  | Area    | USP Plate Count | USP Tailing |
|------|-------------|-----|---------|-----------------|-------------|
| 1    | Sample 1    | 3.436 | 5547055 | 12095           | 1.29        |
| 2    | Sample 2    | 3.425 | 5549323 | 12105           | 1.29        |
| 3    | Sample 3    | 3.434 | 5538730 | 12289           | 1.29        |
| 4    | Sample 4    | 3.436 | 5546059 | 12179           | 1.29        |
| 5    | Sample 5    | 3.435 | 5536725 | 12211           | 1.29        |
| Mean |             |      | 5543578 | 2.28            | 1.29        |
| %RSD |             |      |         | 0.1             |             |

Linearity

Five calibration samples of Larotrectinib were made (50 µg/mL, 75 µg/mL, 100 µg/mL, 125 µg/mL and 150 µg/mL) and injected into chromatographic system. Plot the graph of measured Peak area Vs. concentration and calculated the regression coefficient. Good linear relationship is observed with correlation coefficient of 0.9998.

Table 7: Results of linearity

| % Concentration with respect to target conc. | Larotrectinib area | Larotrectinib conc.(µg/ml) |
|--------------------------------------------|--------------------|---------------------------|
| 50                                         | 2767034            | 50                        |
| 75                                         | 4153769            | 75                        |
| 100                                        | 5539444            | 100                       |
| 125                                        | 6921686            | 125                       |
| 150                                        | 8318378            | 150                       |
LOD and LOQ

The concentration of Larotrectinib with signal to noise ratio 3:1 is taken as LOD and 10:1 as LOQ.

Table 8: Signal to noise details in LOD and LOQ

| S.No | Sample name | Rt  | Area     | S/N ratio |
|------|-------------|-----|----------|-----------|
| 1    | LOD         | 3.531| 31853    | 3.96      |
| 2    | LOQ         | 3.537| 29391    | 10.28     |

Precision

Standard Larotrectinib solution is injected (n= 6 times) in the system. Measured mean area and RSD for 6 injections. The RSD for area of 6 injections is lower than 2%, which shows good precision.

Table 9: Results of precision

| Larotrectinib-100 mg |
|----------------------|
| S.No | Area |
|------|------|
| 1    | 5529736 |
| 2    | 5528264 |
| 3    | 5523125 |
| 4    | 5523255 |
| 5    | 5530039 |
| 6    | 5539924 |
| Average area | 5528803 |
| STD    | 0.114 |
| %RSD   | 0.115 |

Accuracy

Accuracy was determined through analysis (n = 3) for different three concentrations (49.5 µg/mL - 50% level; 99 µg/mL - 100% level; 148.5 µg/mL-150% level) of Larotrectinib spiked to already analyzed capsule solution. Mean recovery at different three concentrations were computed. The values are nearby 100%, which shows good accuracy.

Robustness

Robustness was checked by determining parameters for system suitability by making small but deliberate variations in assay conditions as given.

- Flow 1: 0.9 mL/min
• Flow 2: 1.1 ml/min
• Temperature 1: 23°C
• Temperature 2: 27°C

Table 10: Results of accuracy evaluation

| Level added | Larotrectinib area µg/ml | Larotrectinib added µg/ml | Larotrectinib found µg/ml | %Larotrectinib recover | Mean |
|-------------|---------------------------|---------------------------|---------------------------|------------------------|------|
| 50%         | 2755478                   | 49.500                    | 49.56                     | 100.11                 | 100.13 |
| 50%         | 2756510                   | 49.500                    | 49.58                     | 100.15                 | 100.13 |
| 50%         | 2755948                   | 49.500                    | 49.57                     | 100.13                 | 100.13 |
| 100%        | 5525880                   | 99.000                    | 99.38                     | 100.39                 | 100.36 |
| 100%        | 5520131                   | 99.000                    | 99.28                     | 100.28                 | 100.28 |
| 100%        | 5527423                   | 99.000                    | 99.41                     | 100.41                 | 100.41 |
| 150%        | 8295365                   | 148.500                   | 149.19                    | 100.46                 | 100.47 |
| 150%        | 8292770                   | 148.500                   | 149.14                    | 100.43                 | 100.43 |
| 150%        | 8298628                   | 148.500                   | 149.25                    | 100.50                 | 100.50 |

Table 11: Results of robustness evaluation

| Sample name | Rt   | Area     | USP Tailing | USP plate count |
|-------------|------|----------|-------------|-----------------|
| Flow-1      | 2.937| 4687232  | 1.26        | 11621           |
| Flow-2      | 3.235| 5215096  | 1.27        | 11925           |
| Temp-1      | 4.026| 6541959  | 1.29        | 12290           |
| Temp-2      | 4.618| 7286964  | 1.28        | 13298           |
| Comp-1      | 2.937| 4687232  | 1.26        | 11621           |
| Comp-2      | 4.026| 6541959  | 1.29        | 12290           |
| pH-1        | 3.433| 5539736  | 1.28        | 12220           |
| pH-2        | 3.435| 5545264  | 1.28        | 12237           |

There were no substantial changes to the values. This proves the robustness of the method.

Degradation/Stability test for Larotrectinib
Stability check/degradation study of Larotrectinib was carried out using ICH criterion with capsule solution of 1000 µg/ml concentration.

Acid degradation8-12
1 mL of stock Larotrectinib solution is mixed with 1 mL 0.1 N HCl followed by sonication for nearly 30 min at 25±2°C temperature. The mixture was made to 10 mL volume by diluent (100 µg/mL). This degraded capsule solution was analyzed employing proposed HPLC conditions. The Larotrectinib amount degraded and remained in capsule was calculated with acquired peak areas.

Alkali degradation13,14
1 mL of stock Larotrectinib solution is mixed with 1 mL 0.1 N NaOH followed by sonication for nearly 30 min at 25±2°C temperature. The mixture was made to 10 mL volume by diluent (theoretical Larotrectinib concentration - 100 µg/mL). This degraded capsule solution was analyzed employing proposed HPLC conditions. The Larotrectinib amount degraded and remained in capsule was calculated with acquired peak areas.

Thermal degradation17-19
Capsule powder weight similar to 100 mg Larotrectinib was placed in petri plate and exposed to 100°C for nearly 6 hours. Cool the sample to 25±2°C temperature and transfer to standard flask (100 mL). To which 25 mL of mobile phase added and dissolved and make up the volume to 100 mL.
1 mL prepared solution is mixed with 9 mL diluent (theoretical Larotrectinib concentration - 100 µg/mL). This capsule solution was analyzed employing proposed HPLC conditions. The Larotrectinib amount in capsule was calculated with acquired peak areas.

**Photo degradation**

Capsule powder weight equivalent to 100 mg Larotrectinib was placed in petri plate and exposed to sunlight for nearly 6 hours. Cool the sample to 25±2°C temperature and transfer to standard flask (100 mL). 25 mL mobile phase added and dissolve the drug through sonication. Mobile phase volume of 75 mL is added and properly mixed. 1 mL of prepared solution is mixed with 9 mL of diluent (theoretical Larotrectinib concentration - 100 µg/mL). This capsule solution was analyzed employing proposed HPLC conditions. The Larotrectinib amount in capsule was calculated with acquired peak areas.

**Table 12: Results of Larotrectinib stability evaluation**

| Condition   | Larotrectinib area after degradation | % remained after degradation | % remained after stress applied |
|-------------|-------------------------------------|----------------------------|--------------------------------|
| Acid        | 4902388                             | 88.17                      | 11.83                          |
| Alkali      | 5211566                             | 93.73                      | 6.27                           |
| Peroxide    | 5388047                             | 96.9                       | 3.1                            |
| Thermal     | 5020477                             | 90.29                      | 9.71                           |
| Photolytic  | 5296807                             | 95.26                      | 4.74                           |

The generation of separate peaks with distinct retention times with the peak of Larotrectinib showed its degradation. The retention time of additional peaks was completely different from retention time of Larotrectinib which proved specificity and stability indicating power.

**DISCUSSION**

The method development and validation of Larotrectinib was performed and the results were within the guidelines as mentioned in the standards i.e. the relative standard deviation was found to be not more than 2%, method precision was found to be not more than 2.0%, accuracy was found to be between 98% - 102%, robustness was found to be within the regulated limits.

**CONCLUSION**

In the present investigation a simple, sensitive and accurate RP-HPLC procedure was developed for evaluation of Larotrectinib in capsule dosage form. Degradation analysis was done and concluded that Larotrectinib is more stable in peroxide and less stable in acid form. From the above studies it was concluded that the proposed RP-HPLC method can be successfully used for the estimation of Larotrectinib in capsule form. This method can be used for the routine analysis in research institutions, QC departments of the pharmaceutical industries.

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Conflicts of Interests
The authors declare that they have no conflict of interest.

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