STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF NINTEDANIB ESYLATE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT
Nintedanib is a tiny molecule which exhibits natural antagonist activity against tyrosine kinase receptor, inhibits collagen formation and is also used to treat the idiopathic pulmonary fibrosis. This method reports the development and validation of stability indicating RP-HPLC method for Nintedanib Esylate and its impurities. Nintedanib Esylate was subjected to force degradation under different conditions recommended by the International Conference on Harmonization to detect the degradation products. Chromatographic separation of Nintedanib Esylate was achieved using a gradient program at a flow rate of 1.0 ml/min on a Supelco Ascentis Baker bond C18 (150mm x 4.6 ID, Particle size: 5 micron) maintained at 30°C. The mobile phase consists of water pH 3.0 with Methanol: Water (90:10). UV detection was carried out at wavelength 390 nm. The developed RP-HPLC method was validated as per ICH guidelines with respect to accuracy, specificity, precision, linearity, ruggedness, robustness, LOD and LOQ.

KEYWORDS: Nintedanib Esylate, Stability Study, Validation.

INTRODUCTION
Nintedanib, an intracellular tyrosine kinase inhibitor was one of the first drug approved for use in idiopathic pulmonary fibrosis and is currently approved for use in other chronic
fibrosing interstitial lung diseases (ILD) with a progressive phenotype and systemic sclerosis-associated ILD (SScILD).\(^1\) Idiopathic pulmonary fibrosis (IPF) is a disease arising predominantly in older adults, characterized by chronic loss of pulmonary capacity and poor prognosis. It is a specific form of chronic fibrosing interstitial pneumonia limited to the lung and related with the pathological pattern of typical interstitial pneumonia.\(^2,3,4\)

Chemically Nintedanib Esylate is Methyl (3Z)-3-\{[(4-\{methyl\[(4-methylpiperazin-1-yl)acetyl\] amino\}henyl)amino\} (phenyl) methylidene\}-2-oxo-2,3-dihydro-1H-indole-6-carboxylate corresponding to molecular formula C\(_{31}\)H\(_{33}\)N\(_5\)O\(_4\). It has relative mass 539.636 g·mol\(^{-1}\).\(^5,6\) The drug was approved in United States in 2020 to treat the chronic fibrosing (scarring) interstitial lung diseases (ILD) with a progressive phenotype (trait) and received an U. S. Food and Drug administration approval in 2014 for the use in idiopathic pulmonary fibrosis. Nintedanib Esylate (marketed under trade name Ofev) is approved based on several studies that demonstrated its efficacy in reducing the progressive terminal lung disease.\(^7,8,9\)

Lung function declines, dyspnea and cough worsen, exercise capacity is reduced and health-related quality of life deteriorates as the IPF progresses. Decline in Forced Vital Capacity (FVC) and reduce the walking distance over 6 minutes that are predictors of mortality in patients with IPF. IPF is categorised by the presence of a Usual Interstitial Pneumonia (UIP) pattern on High-Resolution Computed Tomography (HRCT).\(^10\) In the Phase II TOMORROW trial and the two Phase III INPULSIS trials, Nintedanib Esylate 150 mg bid considerably reduced the annual rate of decline in forced vital capacity (FVC) in patients with IPF.\(^11,12,13\)

Literature review suggest that various methods involving UPLC, LC-MS, HPTLC, UV are already reported for the estimation of Nintedanib Esylate in bulk drug, formulation, rat plasma & human plasma. There is no single pharmacopeial monograph available for this drug substance or drug product. The current research paper is an attempt to report a validated stability indicating RP-HPLC method as per ICH Q2(R1) guidelines for estimation of Nintedanib Esylate.\(^14-21\)

This study was designed to develop a simple, rapid, precise & accurate RP-HPLC method for determination of Nintedanib Esylate in bulk drug and to validate as per ICH guidelines.
MATERIALS AND METHODS

1. Materials: A pure sample of Nintedanib esylate was provided by MacLeod's Pharmaceuticals, India as a gift sample. HPLC-grade solvents and chemicals were used. In this study, Sun Pharmaceuticals' Ninteda-100 100 mg capsules were used as the marketed dosage form.

2. Instruments: Chromatographic measurements were performed on HPLC by ANALYTICAL TECHNOLOGIES LTD. Infrared spectroscopy was performed by FTIR (Bruker, Japan).

3. Chromatographic method: The analytical method development and validation was performed on High Performance Liquid Chromatography (Make & Model: Analytical Technologies.; Detector: UV-3000 - M) equipped with binary solvent delivery pump, degasser, auto-sampler and column thermostat using HPLC WorkStation. Chromatographic separation was achieved on Baker bond C18 (150mm x 4.6ID, Particle size: 5 micron) using a gradient program at a flow rate of 1.0 mL/min and an injection volume of 20 µL with wavelength detection at 390 nm. Column oven temperature was set at 30°C. The mobile phase consists of water pH 3.0 with Methanol: Water (90:10).

   i. Preparation of standard stock solutions
   An accurately weighed 100.0 mg of nintedanib was transferred to a 100.0 mL volumetric flask. 50.0 ml of mobile phase was added and dissolved, and the drug solution was diluted with the mobile phase to the marked line to obtain a nintedanib esylate stock solution of 1000 µg/ml. Working standard solutions of these drugs were obtained by appropriately diluting their respective mobile phase stock solutions

   ii. Preparation of Diluent
   Dilute with a mixture of methanol and water (90:10 v/v), mix well, and sonicate to degas.

   iii. Selection of mobile phase
   Prepare the mobile phase by taking methanol and water in different ratios of water:methanol (10:90). The mobile phase was filtered through a 0.45 µm membrane filter and degassed by sonication for 20 minutes.
iv. Assay of marketed formulation

Five capsules of Nintedanib Esylate marketed formulation (Ninteda 100) were taken, and dissolved in 500 ml solvent. Sonicate for 10 min with occasional swirling. Withdraw 0.1 ml and transferred to 10 mL volumetric flask & diluent was added to make up the volume. Remove 0.15 ml again, transfer to a 10 ml volumetric flask and add diluent to adjust volume. Sonicate for 10 min with occasional swirling. The above solution was filtered through a 0.45 µm membrane filter and the prepared stock solution is 15 µg/ml.

4. VALIDATION OF METHOD

Linearity

Chromatographic conditions were set according to the optimized parameters and the mobile phase was equilibrated with the stationary phase as indicated by a constant baseline. Different concentrations of test solutions were injected separately and chromatograms were recorded. A series of test formulations (Table No. 1) ranging from 5 to 25 µg/ml of nintedanib were prepared in the mobile phase. A volume of 20 µl of each concentration was injected in triplicate under optimized chromatographic conditions in HPLC.

Accuracy

Samples are typically prepared to cover 50% to 150% of the nominal sample preparation concentration. Analyze these samples and calculate the yield for each. Accuracy results are shown in Table 1.2.

Precision Study

Intraday and interlay precision: Intraday precision studies were performed by preparing test solutions of the same concentration and analyzing them at two different times of the day. To determine inter-day precision, the same procedure was performed on two different days. Results were reported as % RSD (Table Nos. 3 and 4). Precision results showed good reproducibility with a percentage relative standard deviation of less than 2.

Robustness

The effect of small deliberate changes in the optimized method was studied by robustness evaluation. To evaluate the robustness of the developed method, the parameter was deliberately varied. These parameters included a variation in flow rate and variation wavelength. The results of robustness are shown in Table no. 5.
Ruggenedness
Ruggenedness is the study to determine the effect of external parameters on the method. To evaluate the ruggenedness of the developed method, the parameter was deliberately varied. These parameters included a variation of the system, different analysts, and Atmospheric changes. The results of ruggenedness are shown in Table no. 6

Limit Of Detection (LOD) And Limit of Quantification (LOQ)
The limit of detection (Lod) determines the lower limit that can be detected by the detector regardless of its quantification. The limit of quantification(Loq) determines the lowest concentration with acceptable accuracy and precision. Lod was 0.01 µg/ml and loq was 0.05 µg/ml.

Specificity
Excipients and impurities did not interact with the standard drug. So the method is specific. Specificity results are shown in Table no.8

System Suitability
System suitability parameters were measured to validate system, method, and column performance. A standard solution of nintedanib was injected into the system six times to check system suitability parameters. System suitability results are shown in Table no. 9

5. Forced Degradation Study

Acid induced-degradation
Nintedanib (10.0 mg) was transferred to a 100.0 mL volumetric flask, to which 50.0 mL of mobile phase was added, sonicated for 20.0 minutes, and then the volume was adjusted to the required level using the mobile phase. Take 14 ml of the solution from this stock, add 10 ml of mobile phase, and 1 ml of 1N HCl, and then stress the samples under the conditions listed in Table No. 10 in a water bath. Cool the samples at room temperature, then neutralize the acid with a base of the same concentration and volume. Mobile phase and mixing are used to make up the volume. Use the filtrate after passing the solution through a membrane filter with a 0.45-inch inner diameter. Following that, these filtrates were chromatographed using an optimized condition.
Base induced-degradation

10.0 mg of nintedanib was transferred to a 100.0 mL volumetric flask, 50.0 mL of mobile phase was added, sonicated for 20.0 minutes, and the volume was made up to the mark with the mobile phase. From this stock solution, pour 14 mL of solution into a 20 mL volumetric flask, then add 10 mL of mobile phase, then add 1 mL of 1N NaOH, and run the sample under the conditions described in Table No 10. Filter. Cool at room temperature and neutralize the base with the same concentration and volume of acid. Make volume with mobile phase and mix. Filter the solution through a 0.45 µm membrane filter and use the filtrate. These filtrates were then chromatographed under an optimized chromatography system.

Peroxide degradation

10.0 mg of nintedanib was transferred to a 100.0 mL volumetric flask, 50.0 mL of mobile phase was added, sonicated for 20.0 min, and volume was adjusted with the mobile phase. From this stock solution, transfer 14 mL of the solution to a 20 mL volumetric flask, add 10 mL of mobile phase, then 1 mL of 3% w/v H₂O₂, and purify the sample under the conditions specified in Table No 10. Filter. Cool the water bath to room temperature and neutralize the acid with the same concentration and amount of base. Make volume with mobile phase and mix. Filter the solution through a 0.45 µm membrane filter and use the filtrate. These filtrates were then chromatographed under an optimized chromatography system.

Thermal degradation

A sample of nintedanib was placed in a Petri dish and kept in an oven at 50 °C for 24 hours. 10 mg of the above sample was dissolved in methanol and diluted to a volume of 10 ml. Appropriate dilutions were prepared from this solution using mobile phase and injected under stable chromatographic conditions.

Photolytic degradation

Nintedanib samples were exposed to both cool white fluorescent and UV lamps in a light-stabilized chamber providing over 1.2 million lux hours of illumination. A 10 milligram sample was dissolved in methanol and brought to a volume of 10 ml. Appropriate dilutions were prepared from this solution using mobile phase and injected under stable chromatographic conditions.
RESULTS AND DISCUSSION

The standardisation of Nintedanib was confirmed from the organoleptic and FTIR results. λ\text{max} of the spectra was found at 390 nm. The chromatographic conditions were developed using Methanol : water (90:10), flow rate 1.0 ml/min detection wavelength 390 nm, retention time of Nintedanib Esylate was at 4.5 min and is shown in Fig.1.

![Chromatogram of Nintedanib Esylate.](image)

The optimised chromatographic condition produced a well-retained, sharp, and symmetrical peak at 4.516 minutes, which was deemed adequate. The linear detector response was demonstrated by the linearity study findings throughout the concentration range of 5-25 g/ml, with a correlation value of 0.999 and a regression equation of y = 315944x + 196110. (Table 1 and Figure 1 and 2).

With each additional concentration, the classic drug addiction method produced good recovery of the spiked substance, proving the accuracy of the approach. The observed percent recovery ranged from 98.56 to 101.44%, indicating the method's accuracy (Table 2).

Nintedanib Esylate in capsules was analysed using the suggested approach to determine the intermediate precision research, and it was shown to be highly repeatable with a low % RSD (Table 3-4).

The technique proved sufficiently reliable for the fluctuations in chromatographic parameters like wavelength, temperature, and flow rate that are often anticipated (Table 5).

The specificity analysis shows that the peak found at working concentration in the standard and sample chromatograms is entirely due to the medication. There is no peak in the blank
and excipient solutions at Nintedanib Esylate's retention period, demonstrating that the blank and excipient peaks did not interact with one another. As a result, the proposed RP-HPLC technique can estimate precisely the drug components when there are blank and other excipients peaks.

**Table 1: Data for linearity of Nintedanib Esylate.**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Conc. (μg/ml)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>1745102</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>3451841</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>4884685</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>6450214</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>8144520</td>
</tr>
</tbody>
</table>

**Fig. 2: Calibration curve of Nintedanib Esylate.**

**Table 2: Data of Accuracy for recovery study of Nintedanib.**

<table>
<thead>
<tr>
<th>Level of addition</th>
<th>% Mean recovery*</th>
<th>SD</th>
<th>% RSD</th>
<th>Level of addition</th>
<th>% Mean recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%</td>
<td>99.07</td>
<td>0.59</td>
<td>0.59</td>
<td>50%</td>
<td>99.07</td>
</tr>
<tr>
<td>100%</td>
<td>100.07</td>
<td>0.75</td>
<td>0.75</td>
<td>100%</td>
<td>100.07</td>
</tr>
<tr>
<td>150%</td>
<td>100.48</td>
<td>0.83</td>
<td>0.82</td>
<td>150%</td>
<td>100.48</td>
</tr>
</tbody>
</table>

*Average of three determination

**Table 3: Data for Intraday precision of Nintedanib Esylate.**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Conc. (μg/mL)</th>
<th>Mean*</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>1761830.33</td>
<td>18543.54</td>
<td>1.05</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>4848598.67</td>
<td>30970.39</td>
<td>0.64</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>8101645.33</td>
<td>77075.29</td>
<td>0.95</td>
</tr>
</tbody>
</table>

*Average of three determination

**Table 4: Data for Interday precision of Nintedanib Esylate.**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Conc. (μg/mL)</th>
<th>Mean*</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>1746628.67</td>
<td>1698.14</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>4856490.33</td>
<td>34638.64</td>
<td>0.71</td>
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</table>
Table 5: Data for Robustness study of Nintedanib Esylate.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Conc. (µg/mL)</th>
<th>Mean*</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Change in Flow rate (0.9, 1.1 ml/min)</td>
<td>4829057.667</td>
<td>37303.52442</td>
<td>0.77</td>
</tr>
<tr>
<td>2</td>
<td>Change in Wavelength (388, 390, 392 nm)</td>
<td>4844509.667</td>
<td>46547.95656</td>
<td>0.96</td>
</tr>
</tbody>
</table>

*Average of three determination

Table 6: Data for ruggedness study of Nintedanib Esylate.

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Analyst</th>
<th>Conc. (µg/ml)</th>
<th>Mean area*</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Analyst-I</td>
<td>15</td>
<td>4867973.667</td>
<td>13729.92</td>
<td>0.28</td>
</tr>
<tr>
<td>2</td>
<td>Analyst- II</td>
<td>15</td>
<td>4861403.333</td>
<td>41810.79</td>
<td>0.86</td>
</tr>
</tbody>
</table>

*Average of three determination

% Assay of Marketed formulation

The % Assay of Ninteda 100 marketed formulation was calculated and given in table No. 7

Table 7: % Assay of Marketed Formulation.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Marketed Formulation</th>
<th>Area Obtained*</th>
<th>Area of Standard</th>
<th>% Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ninteda 100</td>
<td>4814254</td>
<td>4884685</td>
<td>98.56</td>
</tr>
</tbody>
</table>

Table 8: Data for specificity study of Nintedanib Esylate.

<table>
<thead>
<tr>
<th>Drug conc. (µg/ml)</th>
<th>Excipients (µg/ml)</th>
<th>Total conc. (µg/ml)</th>
<th>Mean</th>
<th>SD</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>10</td>
<td>15</td>
<td>1744107.667</td>
<td>19490.26</td>
<td>1.12</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>20</td>
<td>3439705.333</td>
<td>51441.64</td>
<td>1.50</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>25</td>
<td>4858915.667</td>
<td>42411.66</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Table 9: Data for System suitability study.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>conc. (µg/ml)</th>
<th>Retention Time (min)</th>
<th>Theoretic al plates</th>
<th>Asymmetry Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td></td>
<td>4.50</td>
<td>8495.00</td>
<td>1.15</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td>0.01</td>
<td>329.14</td>
<td>0.01</td>
</tr>
<tr>
<td>% RSD</td>
<td></td>
<td>0.27</td>
<td>3.87</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*Average of six determination

FORCE DEGRADATION STUDY

Force degradation studies were conducted under five conditions. i.e. Acidic, alkaline, oxidative, thermal, and photolytic degradation. The degradation products generated during
stability studies were well separated from the pure drug, signifying the specificity of the developed method.

**Fig. 3:** Chromatogram of Acid Stressed Standard Nintedanib Esylate.

**Fig. 4:** Chromatogram of Alkali Stressed Standard Nintedanib Esylate.
Table 10: Conditions for Force degradation study.

<table>
<thead>
<tr>
<th>Test Condition</th>
<th>Acid stress</th>
<th>Alkali stress</th>
<th>Peroxide stress</th>
<th>Thermal stress</th>
<th>Photolytic stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nintedanib</td>
<td>1N HCl, 1 hr at 60°C</td>
<td>1N NaOH, 1 hr at 60°C</td>
<td>3% H₂O₂, 24hrs</td>
<td>Thermal stress for 24 hrs</td>
<td>Photolytic stress for 24 hrs</td>
</tr>
</tbody>
</table>

Fig. 5: Chromatogram of Peroxide Stressed Standard Nintedanib Esylate.

Fig. 6: Chromatogram of Thermal Stressed Standard Nintedanib Esylate.

Fig. 7: Chromatogram of Photolytic Stressed Standard Nintedanib Esylate.
Degradation studies were performed on solutions containing 15µg/ml of Nintedanib Esylate. Results of the forced degradation studies are summarized in **Table No.11**. The major degradation of drug was found to be in acidic and peroxide stress condition.

**CONCLUSION**

The developed RP-HPLC technique is specific, accurate, precise and stability-indicating. Validation of this method demonstrated that the method is suitable for the analysis of Nintedanib in capsules without any intrusion from common excipients or potential degradation product of Nintedanib and excipients. The developed method can be used for routine analysis of Nintedanib in various dosage forms.

**REFERENCES**


