Simultaneous Estimation of Netarsudil and Latanoprost by Stability Indicating RP-HPLC-Pda in Pure Binary Blend and their Ophthalmic Solution

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ABSTRACT

A trouble-free, easy, specific, and extremely sensitive HPLC method was established for concurrent estimation of netarsudil and latanoprost in pure bulk and their combined ophthalmic solution. A good separation was achieved by using kromosil C18 (250 x 4.6 mm, 5µ,100 A") column, a mobile portion of Acetonitrile: buffer (0.1N KH2PO4) (50:50 v/v) with isocratic elution, a flow rate of 1-mL/min and detection wavelength of 220 nm. The drug substance was exposed to an intense stress environment like hydrolysis with acid and base, peroxide oxidation, and thermal degradation as per ICH provisions to evaluate the stability of the analytes. The netarsudil and latanoprost were eluted at 2.53 and 3.51 mintues, respectively. The anticipated method shows the linear response from 2.5 to 15 ppm and 0.625 to 3.75 ppm for netarsudil and latanoprost, respectively. The limit of detection (LoD), limit of quantitation (LoQ) were calculated as 0.023 and 0.07 ppm for netarsudil, 0.007 ppm, and 0.022 ppm latanoprost, respectively. The degradants formed by forced degradation studies were well resolved from netarsudil and latanoprost peaks. The method was extremely sensitive, accurate, economical, and stability indicating. Hence, the method has a high capacity to be used in the pharmaceutical industry for analysis of netarsudil and latanoprost in the quality control department.

Keywords: Netarsudil, Latanoprost, Stability indicating, C18column, Isocratic elution.

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INTRODUCTION

Glaucuoma is still the most common cause of permanent blindness, affecting more than 60 million people globally.1 By 2040, the global glaucoma population is expected to reach over 110 million people, necessitating the development of innovative glaucoma treatments.1,2 The US FDA has licensed netarsudil (NSL), a new topical ocular agent have therapeutic significance in hypotensive glaucoma. The drug works primarily to reduce intraocular pressure (IOP) by relaxing the trabecular meshwork and contracting the ciliary muscle, resulting in an increase in aqueous outflow.1-3 Latanoprost (LTP) is a prostaglandin F2α agonist used to treat individuals with ocular hypertension who have an increased IOP. It lowers IOP by enhancing uveoscleral outflow and boosting aqueous humor outflow.4,5 Many patients do not achieve adequate IOP reduction with a single medication. Multiple IOP-lowering medications, each with a distinct dosing schedule, might be confusing and lead to poor patient compliance.5 Multiple-agent combinations are available, but none contained a prostaglandin analog, the most often recommended first-line therapy, until recently. In 2019 US FDA authorized a once-daily, fixed-dose combination of LTP and NSL (ROCKLATAN®) to manage glaucoma5-7 effectively. The chemical structures of NSL and LTP were mentioned in Figure 1.
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MATERIALS AND METHODS

Pure drugs of NSL and LTP were procured from Sectrum Pharma, Hyderabad. All HPLC grade and analytical grade solvents were gained from a local distributor of Finar chemicals. The method was done by using WATERS HPLC coupled with a PDA detector with Empower-2 software. In addition, 1mg sensitive balance (SCALETEC-SAB224CL), a digital pH meter (SMIS-PH-7000), and water (Milli-Q) were used for the preparation of the solution and mobile system.

Chromatographic Method Conditions
Adequate and effective separation of both NSL and LTP were achieved with kromosil 150 C18(250 x 4.6mm, 5µ) column, using a mobile system composition of ACN: Buffer (0.1N KH₂PO₄) (50:50 v/v) at a flow rate of 1ml/min and wavelength of 275nm. Water and ACN in 1:1 ratio were used as diluent.

Preparation of Standard Solution
5 mg of NSL and 1.25 mg of LTP API were blended together, placed into 50 mL volumetric flask, and diluted to the required volume with diluent. 1ml of the resulting solution was further diluted 10 mL to attain 10 ppm and 2.5 ppm of NSL and LTP, respectively, considered as 100% level.

Preparation of Sample Solution
The ophthalmic solution volume equivalent to 5 mg of NSL and 1.25 mg of LTP API were blended together, placed into a 50 mL volumetric flask, and diluted to the required volume with diluent. 1-mL of the resulting solution further diluted 10 mL to attain 10 ppm and 2.5 ppm of NSL and LTP, respectively.

Method Validation
Validation is printed evidence that furnishes reasonable and high degree level assurance about the method or process. Analytical methods are validated in accordance with Q2 specification of the ICH guidelines.13,14

System Suitability Test
The method’s system applicability was tested by injecting a standard solution 6 times in a subsequent manner. Further examination of characteristics such as theoretical plates (N), %relative standard deviation (%RSD), and tailing factors (T) was done to confirm the suitability of the stated method.

Linearity
The linearity of an analytical procedure represents the proportionate relationship between the experiential outcomes and the indicated concentrations. It was carried out at NSL and LTP concentrations ranging from 2.5 ppm to 15 ppm and 0.625 ppm to 3.75 ppm, respectively. To compute the regression coefficient, a liner plot was made between concentration and peak area (R²).

Precision
The approach is said to be precise when a close association is established among the results from the homogenous sample on repeated replications under identical circumstances. The predicted method’s system precision was achieved by injecting the standard concentration six times, while the method precision was achieved by injecting the sample solution six times. The %RSD values of observed peak areas and retention time (RT) were calculated.

Figure 1: Chemical structures of NSL and LTP.

Figure 2: Chromatogram of the optimized method.
Accuracy
The %recovery technique may be used to determine the accuracy of analytical methods to a significant degree. A predetermined quantity of sample solution is spiked into 50, 100, and 150% levels of standard solution. At each %level, the %mean(n = 3) recovery sample concentration was calculated.

Specificity
It refers to an analytical method’s ability to identify the specific drug material despite the presence of additional constituents. Individually, 10 µL of blank solution, standard, standard treated with placebo, and forced degradation solutions were loaded into the HPLC. Some of the other peaks’ interference with the targeted analyte peak was investigated in detail.

Sensitivity
The LOD and LOQ were determined by using the standard deviation method.

\[
\text{LoD} = 3.3\sigma/S
\]
\[
\text{LoQ} = 10 \sigma/S
\]

Where \(\sigma\) is the standard deviation of the intercept \(S\) is the slope of the linear curve.

Robustness
Robustness is described as a method’s competence to achieve the same outcome when the method’s conditions are purposely changed to some extent. Method conditions, such as flow rate (± 0.1 mL/min), Temperature (± 5°C), and detection mobile phase ratio (± 1-mL), were altered to a minor extent and deliberately. Loading standard solution in six continuous repeated injections, the %RSD of peak responses at each altered condition was assessed.

Forced Degradation Studies
The drug substance was purposely subjected to high stress in the forced degradation (FD) testing than in the accelerated stability conditions. The FD studies determine the drug’s chemical stability and form a stable formulation with suitable storage requirements. Specific degradation conditions, such as acid hydrolysis and base hydrolysis, oxidative degradation, UV and thermal degradation, are specifically listed in the ICH regulatory standards.

To conduct acid, base, and oxidative degradation, separate 5 mL portions of standard stock solution were combined with 5 mL each of 2N HCl, 2N NaOH, and 20% H₂O₂, then refluxed for 30 minutes at 60°C and set aside for 24 hours. All three solutions were diluted further to produce concentrations of 10 ppm of NSL and 2.5 ppm of LTP, respectively. The standard stock solution was thermally degraded for 6 hours in a hot air oven at 105°C/70 %RH. A total of 1-mL of the solution as mentioned above was diluted to achieve a concentration of 10 ppm of NSL and 2.5 ppm of LTP, respectively. Photodegradation solution was prepared by exposing the standard stock solution to UV light (200 Watt hours/m²) in a photostability chamber for 7 days. 10 µL of each degradation solution was loaded onto the HPLC instrument, and the %degradation of NSL and LTP was computed from recorded chromatograms. According to regulatory criteria, up to 20% degradation of the drug substance is the most effective and appropriate approach for the validation of the stability-indicating HPLC method.¹⁵

Assay
The %purities of NSL and LTP in the ophthalmic solution were assessed by injecting consecutive injections of standard and sample solution.¹⁶

RESULTS AND DISCUSSION

Method Optimization
The method development was initiated by ensuring the solubility of both NSL and LTP. It was found that NSL was freely soluble in water, soluble in methanol, and ACN on other side LTP was freely soluble in acetone, methanol, ethanol, and ACN but insoluble in water. Equal volumes of water and ACN were chosen as diluents based on the solubility of NSL and LTP.
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The trial and error approach was used to optimize the process. After several trials, a method with Kromosil 150 C18(250 x 4.6mm, 5µ) column, using a mobile system composition of ACN: Buffer (0.1N KH₂PO₄) (50:50 v/v) at a flow rate of 1-mL/min and wavelength of 220 nm. Water and ACN in 1:1 ratio used as diluent. The retention times of NSL and LTP were noticed at 2.54 and 3.51 minutes, respectively. The chromatogram of the optimized system is shown in Figure 2.

### Method Validation

#### System Suitability

The practical data was obtained by administering a standard solution six times, meeting the acceptability requirements for parameters including % RSD (≤ 2), resolution (> 2), tailing (≤ 2), and plate count (> 2000). Table 1 displays the results.

#### Table 1: Results of system suitability parameters of standard solution

<table>
<thead>
<tr>
<th></th>
<th>NSL</th>
<th></th>
<th></th>
<th>LTP</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>S.no</td>
<td>RT (min)</td>
<td>Peak area</td>
<td>Plate count</td>
<td>Tailing factor</td>
<td>RT (min)</td>
<td>Peak area</td>
</tr>
<tr>
<td>1</td>
<td>2.538</td>
<td>151458</td>
<td>2957</td>
<td>1.46</td>
<td>3.496</td>
<td>69033</td>
</tr>
<tr>
<td>2</td>
<td>2.54</td>
<td>150307</td>
<td>2972</td>
<td>1.53</td>
<td>3.509</td>
<td>70545</td>
</tr>
<tr>
<td>3</td>
<td>2.548</td>
<td>151296</td>
<td>3139</td>
<td>1.47</td>
<td>3.513</td>
<td>70629</td>
</tr>
<tr>
<td>4</td>
<td>2.55</td>
<td>149648</td>
<td>3041</td>
<td>1.39</td>
<td>3.513</td>
<td>70190</td>
</tr>
<tr>
<td>5</td>
<td>2.55</td>
<td>148679</td>
<td>3150</td>
<td>1.44</td>
<td>3.514</td>
<td>71859</td>
</tr>
<tr>
<td>6</td>
<td>2.551</td>
<td>150938</td>
<td>3107</td>
<td>1.42</td>
<td>3.518</td>
<td>70180</td>
</tr>
<tr>
<td>Mean</td>
<td>2.54</td>
<td>150387.7</td>
<td>3061</td>
<td>1.45</td>
<td>3.510</td>
<td>70406</td>
</tr>
<tr>
<td>SD</td>
<td>0.006</td>
<td>1072.0</td>
<td></td>
<td>0.008</td>
<td>912.4</td>
<td></td>
</tr>
<tr>
<td>%RSD</td>
<td>0.22</td>
<td>0.72</td>
<td></td>
<td>0.21</td>
<td>1.3</td>
<td></td>
</tr>
</tbody>
</table>

SD-Standard deviation; %RSD-Relative Standard Deviation

#### Table 2: Results of percentage recovery.

<table>
<thead>
<tr>
<th></th>
<th>NSL</th>
<th></th>
<th></th>
<th>LTP</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>% Level</td>
<td>Amount added (ppm)</td>
<td>Amount recovered (ppm)</td>
<td>% Recovery</td>
<td>% mean recovery</td>
<td>Amount added (ppm)</td>
<td>Amount recovered (ppm)</td>
</tr>
<tr>
<td>50</td>
<td>1.25</td>
<td>1.24</td>
<td>99.80</td>
<td>99.56</td>
<td>5</td>
<td>4.94</td>
</tr>
<tr>
<td>125</td>
<td>1.25</td>
<td>1.24</td>
<td>99.89</td>
<td>99.56</td>
<td>5</td>
<td>5.04</td>
</tr>
<tr>
<td>2.5</td>
<td>2.51</td>
<td>100.59</td>
<td>100.57</td>
<td>100.57</td>
<td>10</td>
<td>9.97</td>
</tr>
<tr>
<td>100</td>
<td>2.5</td>
<td>2.53</td>
<td>101.50</td>
<td>101.50</td>
<td>5</td>
<td>10.00</td>
</tr>
<tr>
<td>3.125</td>
<td>3.16</td>
<td>101.14</td>
<td>100.93</td>
<td>100.93</td>
<td>15</td>
<td>15.11</td>
</tr>
<tr>
<td>150</td>
<td>3.125</td>
<td>3.13</td>
<td>100.19</td>
<td>100.19</td>
<td>15</td>
<td>15.04</td>
</tr>
</tbody>
</table>

The %recovery at each percentage level within the acceptable limit

#### Table 3: Precision results of NSL and LTP 100% level solution

<table>
<thead>
<tr>
<th>Precision Parameter</th>
<th>NSL</th>
<th>LTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>System precision (Peak area)</td>
<td>Mean (n=6) 150387.7</td>
<td>70406</td>
</tr>
<tr>
<td></td>
<td>SD 1072.0</td>
<td>912.48</td>
</tr>
<tr>
<td></td>
<td>%RSD 0.72</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Mean(n=6) 100.1</td>
<td>100.54</td>
</tr>
<tr>
<td>Method precision (% Assay)</td>
<td>SD 0.47</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>%RSD 0.47</td>
<td>0.54</td>
</tr>
</tbody>
</table>

The trial and error approach was used to optimize the process. After several trials, a method with Kromosil 150 C18(250 x 4.6mm, 5µ) column, using a mobile system composition of ACN: Buffer (0.1N KH₂PO₄) (50:50 v/v) at a flow rate of 1-mL/min and wavelength of 220 nm. Water and ACN in 1:1 ratio used as diluent. The retention times of NSL and LTP were noticed at 2.54 and 3.51 minutes, respectively. The chromatogram of the optimized system is shown in Figure 2.

**Method Validation**

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The practical data was obtained by administering a standard solution six times, meeting the acceptability requirements for parameters including % RSD (≤ 2), resolution (> 2), tailing (≤ 2), and plate count (> 2000). Table 1 displays the results.
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Table 4: Results of the robustness of the standard solution

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Flow rate (1 ± 0.1 mL/min)</th>
<th>Mobile phase (Organic phase, ACN: Buffer) (50:1)</th>
<th>Temperature (30 ±5°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.9 mL/min</td>
<td>1.1 mL/min</td>
<td>49:51</td>
</tr>
<tr>
<td>NSL</td>
<td>Mean (n=6)</td>
<td>168965</td>
<td>128429</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>2429.8</td>
<td>764.2</td>
</tr>
<tr>
<td></td>
<td>%RSD</td>
<td>1.4</td>
<td>0.6</td>
</tr>
<tr>
<td>LTP</td>
<td>Mean (n=6)</td>
<td>89907</td>
<td>67966</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>443.4</td>
<td>984.5</td>
</tr>
<tr>
<td></td>
<td>%RSD</td>
<td>0.5</td>
<td>1.4</td>
</tr>
</tbody>
</table>

%RSD- Relative Standard Deviation; SD-Standard deviation

Table 5: Results of forced degradation studies

<table>
<thead>
<tr>
<th>Type of degradation</th>
<th>Percentage Degradation (NSL)</th>
<th>Percentage Degradation (LTP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid degradation</td>
<td>6.03</td>
<td>6.36</td>
</tr>
<tr>
<td>Base degradation</td>
<td>5.81</td>
<td>3.73</td>
</tr>
<tr>
<td>Oxidative degradation</td>
<td>4.63</td>
<td>3.98</td>
</tr>
<tr>
<td>Thermal degradation</td>
<td>2.40</td>
<td>2.58</td>
</tr>
<tr>
<td>Photo degradation</td>
<td>1.95</td>
<td>3.55</td>
</tr>
</tbody>
</table>

Percentage degradation was less than 20% in most of the different stress conditions.

Accuracy

The average %recovery of the added NSL and LTP from various levels of spiked solutions were determined to be 100% ± 2, which reveals that the method has appropriate accuracy according to Q2 prerequisites of ICH guidelines. The computed results were mentioned in Table 2.

Precision

The %RSD values of the peak responses and % assay thus attained by injecting standard solution and sample solution in six consecutive replicates, respectively, were ≤ 2. The %RSD of the NCL and LTP was found to be 0.72 and 1.3, respectively, for system precision and 0.47 and 0.54 for method precision, respectively (Table 1).

Sensitivity

The LoD and LoQ values were assessed as 0.023 ppm and 0.07 μg/mL for NSL, 0.007 ppm and 0.022 ppm for LTP, respectively.
Robustness

Intentional changes in flow rate, column temperature and mobile phase ratio of the proposed method to a slight extent could not affect the system suitability, and the %RSD of attained peak responses obeyed the acceptance criteria (Table 4), which indicates that the method has considerable robustness.

Forced Degradation

In the majority of the stability-indicating analytical methods, up to 20% degradation in the drug substance is considered by scientists. The %degradation of NSL and LTP was evaluated by comparing the peak areas of chromatograms from freshly prepared standard solution and degradation solution. Table 5 summarizes the observed and computed data, which include the purity threshold, peak purity angle, and %degradation. Figure 4 depicts the recorded chromatograms of a few stressful situations samples. The fact that the purity threshold was larger than the purity angle of all generated peaks revealing the purity of drug compounds and degradants indicates the stability indicating a characteristic of the described approach. Both pharmaceuticals were significantly degraded at acidic pH environments, showing that NSL and LTP are very susceptible to acidic media.

Assay

The %purity of the NSL and LTP in ophthalmic formulation (ROCKLATAN- Netarsudil (0.02%) and Latanoprost (0.005%)) were determined as 99.2 and 98.7% for NSL and LTP, respectively accepted as per ICH limit 100% ± 2.

The current RP-HPLC method has advantages over priori reported method in terms of good sensitivity and shorter RT with a mobile economic system. The RT attained for NSL and LTP were shorter than the previously described method, which is recommended as economical, as it minimizes the elution time and utilization of the mobile phase. Hence sample analysis time was lowered, and more samples could be tested in the stipulated time.

CONCLUSION

To assess NSL and LTP concurrently in bulk blended powder and their combined ophthalmic solution, an easy, precise, sensitive, accurate, robust, specific, and isocratic RP HPLC approach was established. The method’s stability-indicating property is represented by the application of purposefully forced degradation conditions to the analytes. The suggested approach sufficiently separated NSL, LTP, and their degradants with high sensitivity and outstanding resolution. The newly established approach offers excellent stability, specificity, and sensitivity. As a result, the suggested approach is widely used in the analytical research division of the pharmaceutical industry.

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AUTHORS’ CONTRIBUTIONS

All the authors contributed equally to the design and frame of the work, acquisition, interpretation of data, and manuscript preparation; all authors have read the prepared manuscript and approved it for publication.

CONFLICT OF INTEREST

No conflict of interest from all the authors.

REFERENCES